

1 Review of Drug Metabolism in Drug Discovery and Development

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1.1 Summary	1
1.2 Introduction	2
1.3 The phenomenon of drug metabolism	3
1.4 The drug discovery and development process	12
1.5 The significance and importance of drug metabolism	14
1.6 The biochemical process of drug metabolism	19
1.7 The chemical characterization of drug metabolism	30
1.8 Conclusion	33
Abbreviations	34
References	34

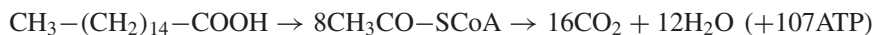
1.1 SUMMARY

Drug metabolism is a physiological phenomenon in which xenobiotic compounds are chemically transformed into metabolites of the parent drug. Drug metabolism comprises a diverse set of chemical reactions within four general categories: oxidation, reduction, conjugation, and hydrolysis. These general categories of chemical reactions correspond to general categories of enzymes, which are responsible for catalyzing the reactions. The object of drug metabolism is to clear the xenobiotics from the body, so that the metabolites tend to be more polar and soluble than the parent drug, making them easier to excrete. Transporters are now recognized as a necessary component of drug metabolism, since they facilitate penetration of the parent drug into metabolizing organs and passage of ionic metabolites across cell membranes into the excreta. Drug metabolism is important in the clinical action of drugs because it is often the main means by which drugs are cleared from the body, so the rate of metabolism is one determinant of the elimination half-life of the drug. Drug metabolism is additionally important because the metabolites may have pharmacological or toxicological properties, which are superimposed on the clinical profile of the parent drug. For these reasons, the drug discovery process aims to design molecules with rates of metabolism appropriate for clinical use and pathways of metabolism which minimize side effects or toxicities attributable to metabolites. In clinical development, characterization of the metabolic

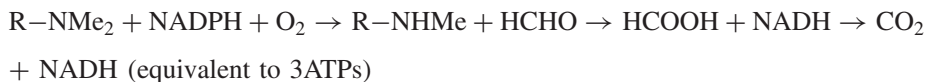
pathways, the major circulating metabolites, and the enzymes that produce these metabolites is necessary for a full understanding of the clinical profile of a new drug. Accordingly, several types of clinical studies of metabolism are mandated for new drug registration, including identification and quantitation of major circulating metabolites, determination of the major pathways of clearance (CL) and their associated metabolic enzymes, characterization of drug–drug interactions based on metabolic phenomena, and assessment of the extent of excretion of drug-derived materials from the body.

1.2 INTRODUCTION

When an organic compound enters the human body, it is normally (i) utilized as a nutrient, (ii) directly excreted, or (iii) chemically modified and then excreted. In the case of a nutrient, the molecules enter specific biochemical pathways that either split them into small units followed by complete oxidation to generate energy (*catabolism*) or utilize them as precursors for constructing physiological molecules such as nucleic acids, polysaccharides, proteins, and triglycerides (*anabolism*). The overall process of utilization of nutrients is called *intermediary metabolism*. An example is the splitting of dietary fatty acids such as palmitic acid into two-carbon acetyl CoA units that can be oxidized in the mitochondria to produce energy (as ATP).



In cases of nonnutrient compounds (*xenobiotics*), however, pathways for significant energy production seldom exist, although in some cases the body is able to partially metabolize an organic compound for its energy content, as for instance with N-demethylation of drugs (Section 1.3).



The methyl group is released as formaldehyde, which is further oxidized to formate and finally carbon dioxide, generating 2 mol of NADH. However, since 1 mol of NADPH must be invested for the metabolic demethylation, then the net energy production is 1 mol of NADH, equivalent to 3 mols of ATP. Most such reactions of xenobiotics are either energy-neutral (e.g., hydrolyses) or actually energy-consumptive (e.g., hydroxylations), since they produce no energy equivalents, but may use cofactors such as NADPH, PAPS, SAM (*S*-adenosine-L-methionine), or UDPGA, which require cellular ATP equivalents for their synthesis.

With the majority of xenobiotics, only a limited set of nonspecific chemical modifications is possible. This process is called *drug metabolism*, although it occurs with all absorbed foreign compounds, and not just drugs. To avoid confusion with intermediary metabolism, drug metabolism is sometimes called *biotransformation*. However, in fact, there is rarely any serious confusion between these two terms, and the term *biotransformation* is not really descriptive enough to convey a clear meaning in any event. So, most scientists working in this field simply call it *drug metabolism*. For the purposes of this chapter, we make no distinction between xenobiotic chemical

compounds that are unintentionally introduced into the body (e.g., natural plant alkaloids or environmental chemicals) and those that are intentionally dosed (e.g., medicinal drugs). The same CL mechanisms operate on all xenobiotics, and we use the term *drug metabolism* to describe the chemical modification of any nonphysiological compound. Drug metabolism occurs in all species, from bacteria to humans, but our primary focus in this chapter is the human phenomenon, only with reference to other species, as they are relevant to the process of discovery and development of new drugs. An increasing proportion of new drugs are proteins and nucleic acids (i.e., *biologics*), but the scope of this chapter is limited to the discussion of traditional small-molecule organic compounds.

The recognition that foreign substances may be metabolized in the body goes back almost two centuries, and an interesting history of the early discoveries is available in the form of a journal article [1] or website [2]. About 60 years ago, biochemists began to recognize drug metabolism as a distinct field of study. Soon, scientists in academia, pharmaceutical companies, and regulatory agencies realized that characterization of the metabolic fate of drugs was an important component in understanding their clinical profiles. Initially, it was sufficient to merely demonstrate that a dosed drug and/or its metabolites were eliminated from the body in a reasonable amount of time. Next, in the evolution of drug metabolism, there was a need to determine the chemical form of the major drug-related materials in the excreta. Today, the potential role of circulating metabolites in therapeutic action as well as toxicity has become apparent, and a sophisticated quantitative chemical, biochemical, pharmacological, and toxicological description of metabolism is required for the registration of new drugs.

Finally, we can ask “What is the object of drug metabolism?” As can be seen in subsequent sections, drug metabolism is more than just an attempt by the body to “eat” ingested foreign compounds. The existence of a complex, regulated, and interacting set of barriers and CL mechanisms suggests that the object is to chemically and physically limit the entry of these compounds to the body and facilitate their removal from the body. Those compounds that are not clearable by direct excretion in urine or feces are subject to sequential rounds of metabolism, which change their chemical and physical properties until they *can* be excreted. With these thoughts in mind, let us discuss in detail exactly what drug metabolism is and why it is important to the discovery and development of new drugs.

1.3 THE PHENOMENON OF DRUG METABOLISM

Drug metabolism comprises such a rich variety of chemical modifications of organic compounds that it is rare to find a drug that is not subject to some type of metabolic process. Of course, there is a kinetic component of the drug-metabolism process as well, so in some cases the metabolism occurs only slowly. For example, amiodarone is cleared from the body exclusively by metabolism [3], but because the metabolic process is very slow, this drug has a 55-day terminal half-life [4]. In other cases, the direct excretion process is much faster than metabolism and dominates CL. So we find that amoxicillin, for example, is mainly excreted as the intact parent drug in urine [5]. Nonetheless, most drugs are rapidly metabolized as the major route of CL from the body. Although the diverse manifold of possible reactions presents a complex array of

possibilities, we can recognize some patterns, so that it is possible to use relatively few terms to describe virtually all the transformations. In fact, there are only four major categories: oxidations, reductions, conjugations, and hydrolyses.

These four categories of chemical transformation arise from four broad classes of enzymes, which actually accomplish the transformation for a particular drug. It is common, if not universal, for a particular drug to be subject to more than one metabolic conversion. For example, dextromethorphan has two major metabolites, one resulting from N-demethylation and the other from O-demethylation (Fig. 1.1).

As one would expect, some metabolites are the product of sequential operation of two or more of these basic transformations, as shown for loratadine, which undergoes oxidative decarboethoxylation followed by aromatic hydroxylation and finally glucuronidation (Fig. 1.2).

Occasionally, a metabolic pathway apparently not conforming to this fourfold categorization is encountered, as shown in Fig. 1.3.

However, almost always, closer examination shows that the process was actually a diversion during operation of one of the four standard categories [8,9]. In the example in Fig. 1.3, pulegone is first hydroxylated on an allylic methyl group, followed by internal hemiketal formation and dehydrative aromatization to form menthofuran [10].

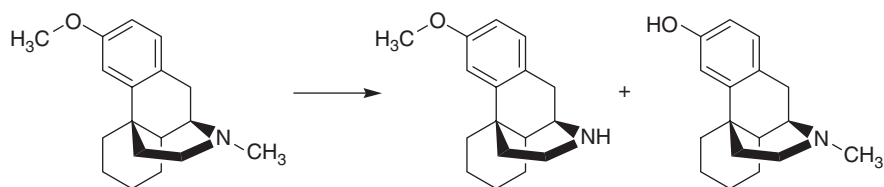


Figure 1.1 N- and O-demethylation of dextromethorphan [6].

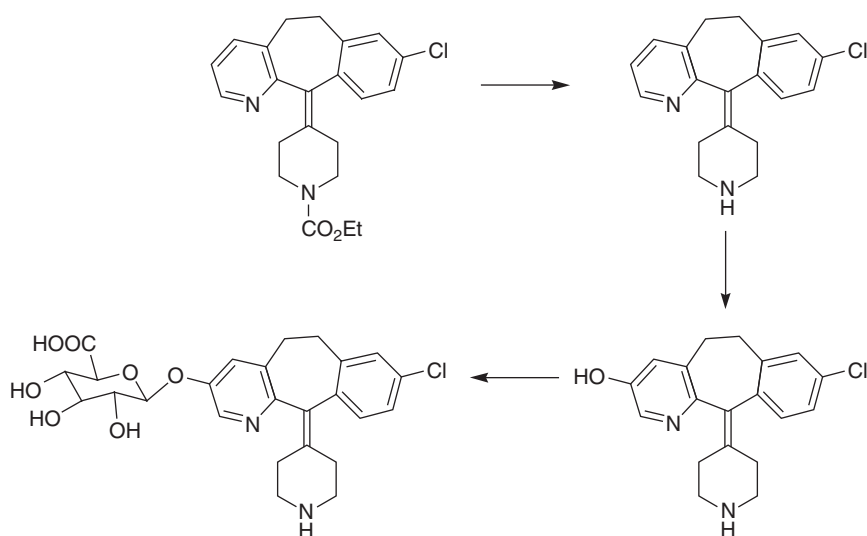


Figure 1.2 Metabolic pathway for loratadine [7].

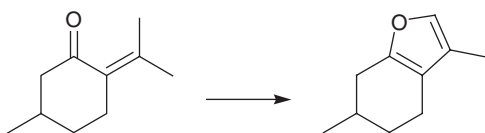


Figure 1.3 Ring closure of pulegone to menthofuran.

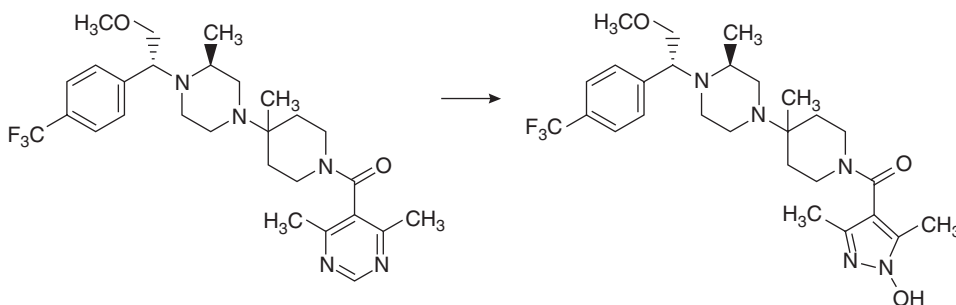


Figure 1.4 Ring contraction of vicriviroc [11].

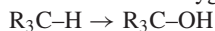
A more vexing example is provided below by the contraction of a six-membered pyrimidine ring to a five-membered pyrazole ring in the anti-HIV drug vicriviroc (Fig. 1.4). Although no explanation for this reaction had been published, one can write a plausible, albeit complex, metabolic pathway linking the parent drug and the metabolite utilizing only known reactions from the four standard categories (details left as an exercise for the reader).

Although many interesting chemical transformations are known in biochemistry, we limit our discussion here only to ones that have been demonstrated to occur with xenobiotics. Table 1.1 summarizes the main chemical reactions of human drug metabolism. Each metabolic reaction has been given a name descriptive of the overall chemical transformation that occurs, regardless of the internal mechanism by which the transformation was accomplished. However, in many cases, the metabolic reaction has a name commonly used in the published literature of drug metabolism. For example, “introduction of an oxygen atom at an aliphatic position” would typically be referred to as *Hydroxylation*, even though an oxygen atom, not a hydroxyl group was added to the molecule. The common name is given in parenthesis. Note that the drugs and metabolites are drawn in their unionized forms to better see the chemical transformation that has occurred. Although Table 1.1 is intended to be reasonably comprehensive for introductory purposes, it is not exhaustive. A number of unusual examples have been compiled elsewhere [8,9].

Finally, it is worth noting that some metabolic reactions can be reversed, leading to the phenomenon of *futile cycling*. A metabolite that is produced through one of the metabolic reactions in Table 1.1 may be reconverted to the parent drug by another metabolic reaction, with no net chemical transformation. An example is the conversion of a tertiary amine to an *N*-oxide (Reaction I.F). Since some *N*-oxides can be reduced to tertiary amines (Reaction II.B), the result is that the amine appears not to have been metabolized, when in fact two metabolic steps occurred [32]. The existence

TABLE 1.1 Chemical Transformations Comprising Drug Metabolism**I. Oxidations**

A. Insertion of an oxygen atom into an aliphatic C–H bond (aliphatic hydroxylation) (Fig. 1.5)



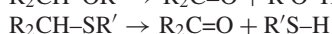
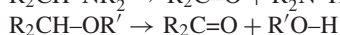
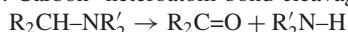
B. Insertion of an oxygen atom into an aromatic C–H bond (aromatic hydroxylation) (Fig. 1.6)

$Ar-H \rightarrow Ar-OH$ Although this reaction is superficially equivalent to Reaction I.A, the underlying mechanism is quite different, justifying a separate designation.

C. Carbon–carbon bond cleavage (C-dealkylation) (Figs. 1.7 and 1.8)



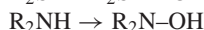
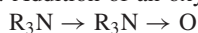
D. Carbon–heteroatom bond cleavage (N, O, or S-dealkylation) (Fig. 1.9)



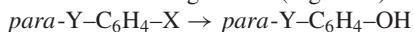
Although dealkylation is sometimes presented as a variant of Reaction I.A in which the alkyl group is merely hydroxylated followed by spontaneous dissociation to the observed amine, alcohol, or thiol and carbonyl products, the actual mechanism is distinct, justifying a separate designation.

E. Addition of an oxygen atom to an alkene or alkyne (epoxidation) (Fig. 1.10)

F. Addition of an oxygen atom to a heteroatom (N- or S-oxidation) (Fig. 1.11)

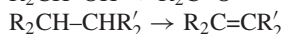
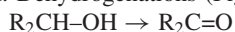


G. Oxidative dehalogenation (Fig. 1.12)



(X = F, Cl; Y = RNH, OH)

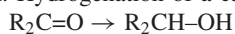
H. Dehydrogenations (Figs. 1.13 and 1.14)



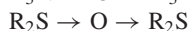
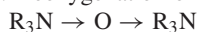
I. Aromatizations (Fig. 1.15)

II. Reductions

A. Hydrogenation of a carbonyl group (Fig. 1.16)



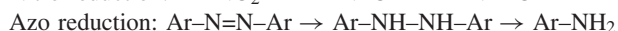
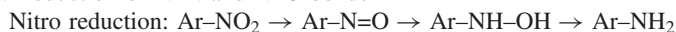
B. Deoxygenation of N- or S-oxides (N- or S-oxide reduction) (Fig. 1.17)



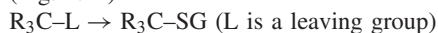
C. Reductive dehalogenation (Fig. 1.18)



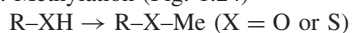
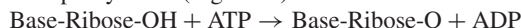
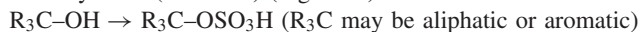
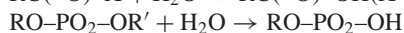
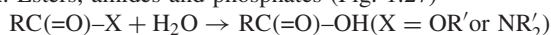
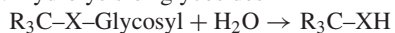
D. Reduction of N–N and N–O bonds



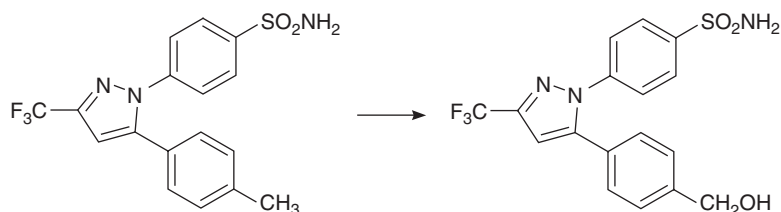
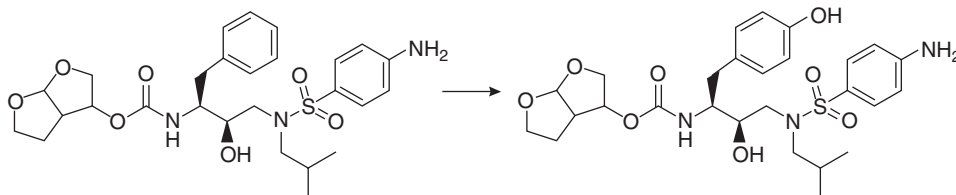
Isoxazole reduction (Fig. 1.19)

TABLE 1.1 (continued)**III. Adductions and Condensations (Conjugations)****A. Acetylation (Fig. 1.20)****B. Amide formation (Fig. 1.21)****C. Glutathionation (Nucleophilic addition of the tripeptide glutathione (GSH (reduced glutathione), Glu-Gly-Cys-SH) to an electrophilic carbon atom in a xenobiotic molecule) (Fig. 1.22)****D. Glycosylation (Fig. 1.23)**

(X = O or N; Glycosyl = glucose, glucuronic acid; R₃C- may be aliphatic or aromatic)

E. Methylation (Fig. 1.24)**F. Phosphorylation (Fig. 1.25)****G. Sulfurylation (Sulfation) (Fig. 1.26)****IV. Hydrolyses****A. Esters, amides and phosphates (Fig. 1.27)****B. Hydrolysis of glycosides**

(X = O or N; Glycosyl = glucose, glucuronic acid; R₃C- may be aliphatic or aromatic)

C. Hydrolysis of epoxides (Fig. 1.28)**Figure 1.5** Benzylic hydroxylation of COX-2 inhibitor celecoxib [12].**Figure 1.6** Phenyl hydroxylation of HIV protease inhibitor darunavir [13].

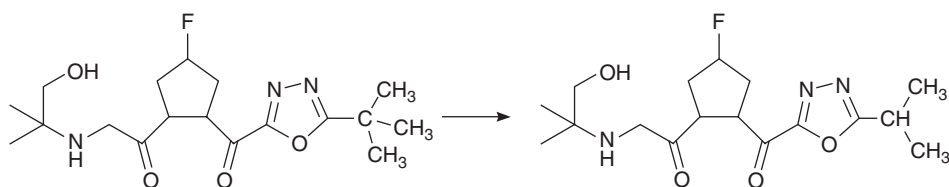


Figure 1.7 C-Demethylation of DPP4 inhibitor LC15-0133 [14].

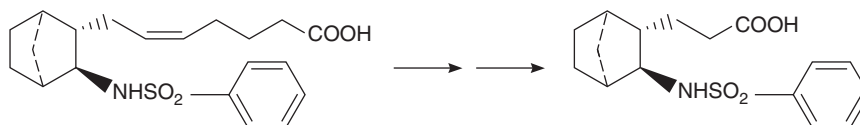


Figure 1.8 β -Oxidation of thromboxane A₂ receptor antagonist (+)-S-145 [15].

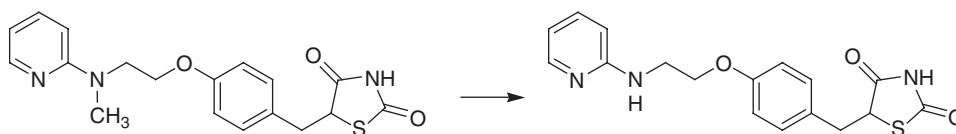


Figure 1.9 N-Demethylation of PPAR γ agonist rosiglitazone [16].

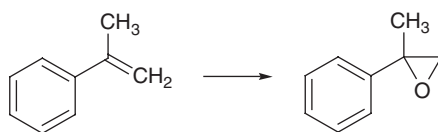


Figure 1.10 Epoxidation of plastics monomer α -methylstyrene [17].

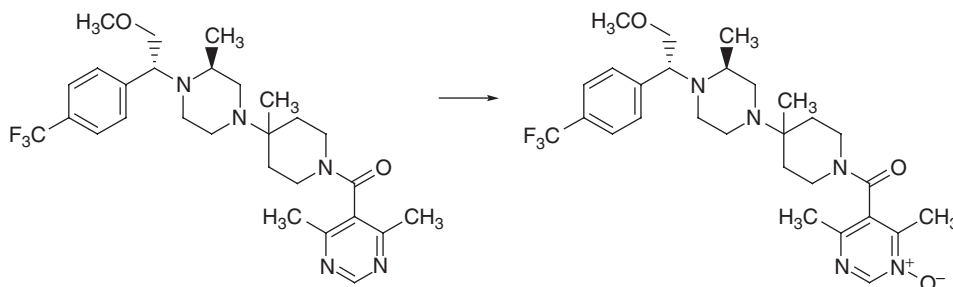


Figure 1.11 N-Oxidation of HIV entry inhibitor vicriviroc [11].

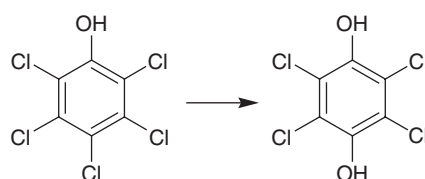


Figure 1.12 Dechlorination of pentachlorophenol [18].

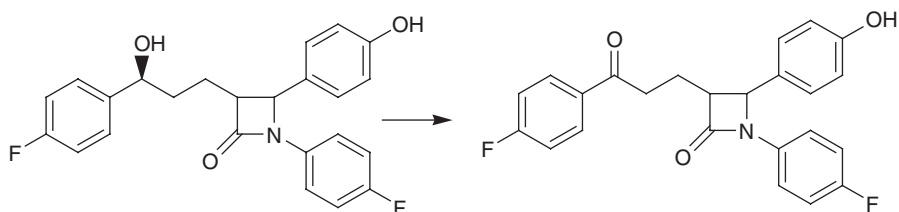


Figure 1.13 Dehydrogenation of anticholesterolemic drug ezetimibe to the corresponding ketone [19].



Figure 1.14 Dehydrogenation of anticonvulsant valproic acid [20].

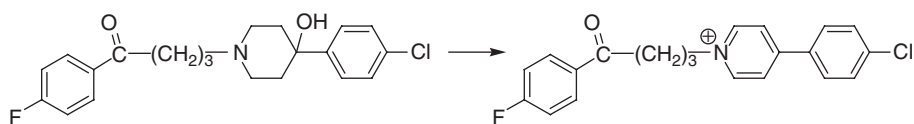


Figure 1.15 Conversion of neuroleptic agent haloperidol to pyridinium ion [21].

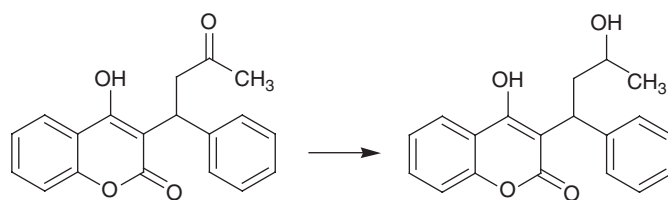


Figure 1.16 Reduction of the keto group of anticoagulant warfarin to a secondary alcohol [22].

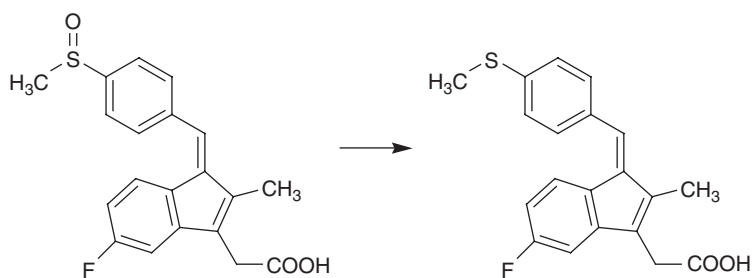


Figure 1.17 Reduction of sulfoxide group of NSAID sulindac to the sulfide [23].



Figure 1.18 Reduction of general anesthetic halothane [24].

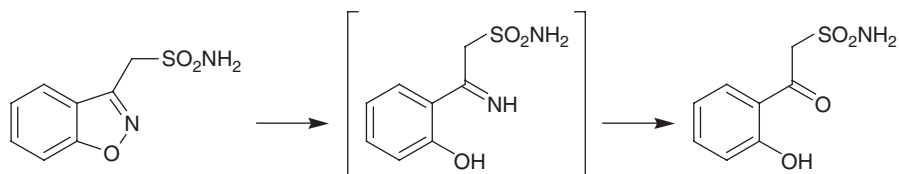


Figure 1.19 Reduction of the anticonvulsant zonisamide [25].

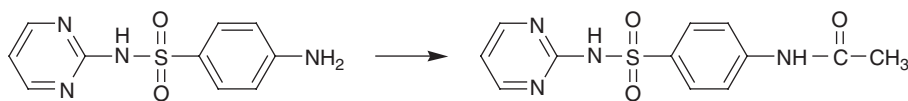


Figure 1.20 N-Acetylation of antiinfective sulfadiazine [26].

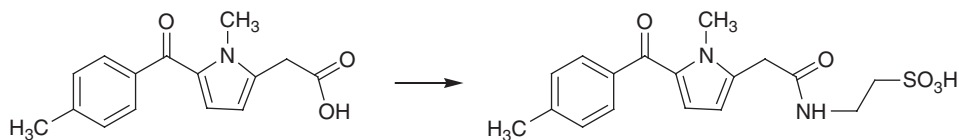


Figure 1.21 Taurine conjugation of NSAID tolmetin [27].

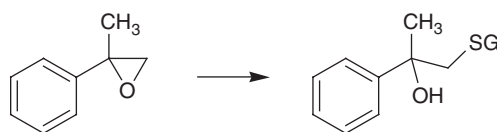


Figure 1.22 Addition of glutathione to α -methyl styrene oxide [17].

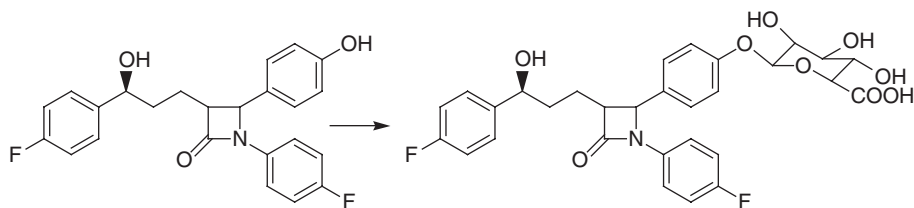


Figure 1.23 Phenolic glucuronidation of anticholesterolemic drug ezetimibe [19].

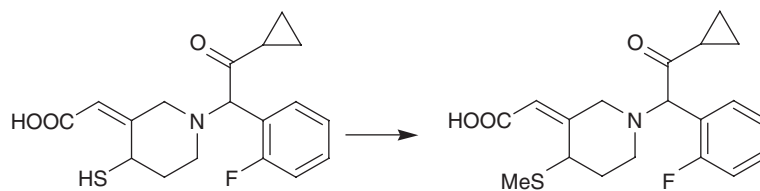


Figure 1.24 S-Methylation of active thiol metabolite of antithrombotic agent prasugrel [28].

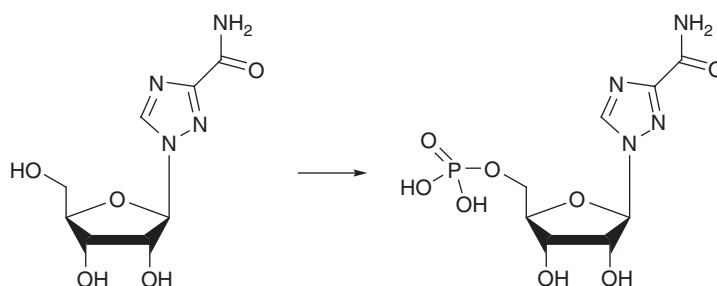


Figure 1.25 Activation of antiviral agent ribavirin by 5'-phosphorylation [29].

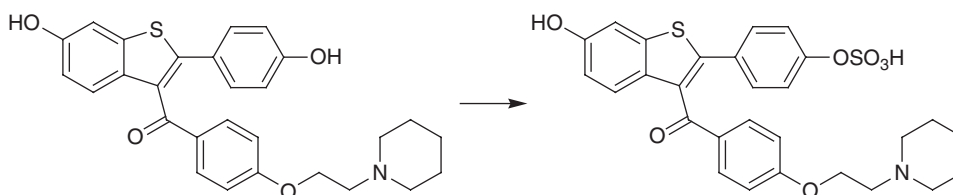


Figure 1.26 Phenolic sulfation of synthetic estrogen raloxifene [30].

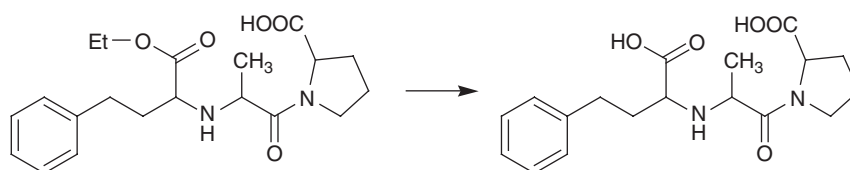


Figure 1.27 Conversion of the prodrug enalapril to active ACE inhibitor enalaprilat [31].

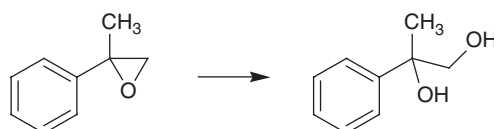


Figure 1.28 Hydrolysis of α -methylstyrene oxide [17].

of the separate oxidation and reduction steps can usually be demonstrated by *in vitro* experiments under controlled conditions. Futile cycling may also occur with the hydroxy-to-keto conversion, sulfation/desulfation, phosphorylation/dephosphorylation, and acetylation/deacetylation [33]. An important variation is seen with glucuronidation of a phenolic drug (Reaction III.D), followed by excretion of the *O*-glucuronide into the intestine through the bile. Intestinal bacteria often efficiently hydrolyze glucuronides back to the parent drug (Reaction IV.B), which may be reabsorbed through the intestinal wall [19]. This *entero-hepatic circulation* may be detected by an anomalous pharmacokinetic (PK) profile, and confirmed by collection of bile to confirm the presence of the glucuronide as well as by *in vitro* experiments showing that the parent drug is readily glucuronidated. Another special case of futile cycling is called *redox cycling*, in which hydroxylation of an aromatic ring on a drug produces a hydroquinone, which is then one-electron oxidized by molecular oxygen to a semiquinone radical and then a quinone [34,35]. Quinones are often easily metabolically reduced back to hydroquinones to start a new cycle, which may be repeated many times. This is an especially undesirable type of metabolism, because it amounts to the drug acting as a catalyst to convert oxygen to dangerous *reactive oxygen species* such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. These several examples of futile cycling show that there can be “hidden” metabolism, and some genetically variant enzymes or drug–drug interactions may interfere with one of the counterbalancing reactions, causing unexpected metabolic effects. Thus, drug-metabolism scientists who are developing drug candidates with potentially reversible metabolic pathways normally investigate whether futile cycling is occurring *in vivo*.

1.4 THE DRUG DISCOVERY AND DEVELOPMENT PROCESS

The study of drug metabolism is a distinct academic science including basic research, specialized scientific societies, and dedicated journals. However, as the name suggests, the biggest application of the science of drug metabolism is in the understanding of the clinical actions of new therapeutic agents and in satisfying regulatory requirements for their registration. For this reason, the majority of metabolism data appearing in the literature for specific drugs results from industrial drug development. Accordingly, we briefly outline the new drug discovery and development process to provide the framework for discussion of this important application of drug metabolism.

1.4.1 Discovery

Basic research elucidates the complex biochemical events that comprise a physiological process such as regulation of blood glucose, and identifies key biochemical control points mediated by enzymes or receptors. Drug discovery begins with a new concept for therapeutic intervention of a disease based on the knowledge gleaned from such research. Most drugs exert their pharmacological action by modulating the activity of one of these enzymes or receptors within cells of the abnormally functioning organ. So the next step is *lead generation*, the design or discovery of a small molecule that will bind to the molecular target in such a way as to modulate its activity. Once a lead has been generated, medicinal chemists typically synthesize hundreds to thousands of analogs by systematically varying the structure to create a compound that has

been optimized with respect to potency and selectivity toward the molecular target, PK characteristics, and safety. Numerous *in vitro* and *in vivo* screening assays are established to determine which compounds have these properties in the acceptable range for the intended medical indication [36]. Since drug discovery normally takes place before the clinical phase, the human PK characteristics of a drug candidate must be inferred from *in vivo* animal models or from various human-derived *in vitro* systems [37]. The data output from these screens is used iteratively to design better molecules. Once a discovery compound has been identified with overall favorable “druglike” characteristics (good potency, selectivity, safety, and PK), it is designated as a *clinical drug candidate* and enters the development process [38].

1.4.2 Preclinical Development

Before a new chemical entity can be tested in humans, its safety in various *in vitro* and *in vivo* pharmacological and toxicity tests must be assessed [39]. This includes administration of high doses of the drug candidate to a rodent species (mice or rats) and a large animal species (dogs or monkeys), as well as *in vitro* tests of genotoxic potential (such as the Ames test). In parallel with the safety testing, a large-scale chemical synthetic process must be developed to produce the tens of kilograms of the drug candidate required to conduct clinical trials. Finally, a clinical formulation must be devised that provides adequate and reproducible exposure of subjects in the clinical trials, and animal PK studies are helpful in this design process. Data from the discovery and preclinical activities form the basis of a petition to government health authorities for permission to begin testing in humans. Once this dossier, called the *Investigational New Drug Application* (IND) in the United States, is approved, clinical trials may proceed [40].

1.4.3 Clinical Development

Clinical trials are formally divided into four distinct, but temporally overlapping phases (I–IV), defined by the objectives of each phase [41].

Phase I tests the safety and tolerability of the drug candidate in normal healthy volunteer subjects. The primary goal is to determine the safety profile and the maximum dose that may be safely given to people. An additional goal is the determination of PK parameters, which are needed to plan the next phases. Other phase I PK studies include metabolism and elimination of the drug [42], detection of circulating metabolites of the drug [43], determination of the effect of food on orally administered drugs [44], formulation testing [45], and checking for PK interactions between coadministered drugs [46]. The most common form of PK drug–drug interaction is the competition of two drugs for the same CL mechanism, especially the CYP superfamily of drug-metabolizing enzymes [47]. These interactions, if present, place limitations on which drugs can ultimately be administered together clinically with the new agent [48]. All clinical PK studies rely on some method of quantitation of drug levels in blood or plasma, and the major bioanalytical technique in use today for this purpose is *liquid chromatography coupled to tandem mass spectrometry* (LC-MS/MS) [49,50]. Present technology permits determination of picogram-per-milliliter levels of drug in the presence of many interfering substances, with sample-to-sample cycle times <2 min.

Phase II investigates in limited numbers of patients whether the drug candidate modulates the pharmacological target (*proof of activity*) and whether this activity actually results in the desired therapeutic benefit (*proof of concept*). If proof of concept is established, several dose levels are next assessed for efficacy in patients with the disease to be treated. The optimum dose level is then chosen, based on a balance of benefit and safety. Although the main goal of phase II clinical trials is the determination of efficacy toward the disease indication, PK is a fundamental part of this goal, because to understand the clinical utility of the new drug candidate, it is necessary to define the temporal relationship between plasma levels and the pharmacodynamics of the beneficial effect (PK/PD). A secondary PK goal in phase II is to determine if the disease state affects the PK of the candidate drug compared to the PK seen in healthy subjects.

With an optimized dose identified, *phase III* tests the drug in much larger groups of patients to establish a comprehensive clinical profile of the new drug candidate. Phase III trials are intended to confirm a statistically significant benefit versus either placebo or, more usually, standard-of-care therapy. Additional phase III studies may investigate the variation of therapeutic response and safety in various severities of the disease and in special populations such as ethnic groups or comorbidities. Once phase III is complete, the sponsor of the drug candidate may submit an application (called the *New Drug Application* in the United States) for approval to government health authorities of all countries in which marketing is desired [51].

Phase IV usually occurs once the drug has been approved and is on the market. It involves testing in even larger populations and for longer periods of time, as well as testing against additional disease indications. However, monitoring patients for long-term safety issues (*pharmacovigilance*) may be the most important activity in phase IV, because the number of patients and the length of treatment allow detection of low frequency and/or long-term effects of the new drug that may not have been evident in phase III.

1.5 THE SIGNIFICANCE AND IMPORTANCE OF DRUG METABOLISM

Drug metabolism is centrally important to the clinical utility and effectiveness of most drugs. Metabolism is the main route of CL for nearly three-quarters of marketed drugs [53]. In fact, even the *absence* of metabolism is an important fact to be known about a drug's clinical profile. The importance of drug metabolism in drug action has four general aspects: CL of the drug from the body, pharmacological activity, toxicity, and drug interactions.

1.5.1 Clearance of Drugs from the Body

Persistence of the drug in the body is usually quantitated as the PK half-life ($t_{1/2}$) or the mean residence time (MRT), both of which are typically given in units of hours or days. Persistence is a function of the availability and capacity of the body's CL mechanisms, of which drug metabolism is often the most important.

In the frequent case that CL occurs by enzymatic metabolism of the drug, the rate of metabolism of the drug by the particular enzyme catalyzing the metabolic reaction

is determined by the Michaelis–Menten equation of enzyme kinetics (chapter titled *Enzyme Kinetics of Drug-Metabolizing Reactions and Drug–Drug Interactions*).

$$\text{Rate} = \frac{V_{\max}[\text{Drug}]}{K_M + [\text{Drug}]} \quad (1.1)$$

In Equation 1.1, $V_{\max}$ is the rate that the enzyme can convert drug to metabolite when drug availability is not limiting (i.e., high drug concentrations), and K_M is a constant that determines the drug-concentration dependence of the enzyme rate. We can simplify Equation 1.1 by assuming that drug concentrations are always low compared to K_M (i.e., $[\text{Drug}] \ll K_M$), leading to Equation 1.2.

$$\text{Rate} = \frac{V_{\max}}{K_M} [\text{Drug}] \quad (1.2)$$

Thus, when the assumption of $[\text{Drug}] \ll K_M$ is obtained (a condition that is often true), the rate of metabolism is proportional to drug concentration (i.e., first-order kinetics), and in that case blood levels of the drug are proportional to dose. Since dose-linear PKs is usually a desirable property of a clinical drug, one of the selection criteria for new drug candidates during the discovery phase may be that K_M -values are high compared to the expected maximal clinical blood levels.

Because metabolizing enzymes are usually concentrated in a CL organ (e.g., liver), CL of a drug from the blood is governed by mass transport to the CL organ in addition to the enzymatic capacity of that organ to remove the drug from blood, as expressed in the following relationship.

$$\text{CL} = \frac{f_u \text{CL}_{\text{int}} Q}{f_u \text{CL}_{\text{int}} + Q} \quad (1.3)$$

In Equation 1.3, CL is the rate at which blood is cleared of the drug by the CL organ, with units of L/h. Intrinsic CL (CL_{int}) is the capacity of the organ to clear the drug in the absence of mass transport restrictions on presentation of the drug to the organ. Q is the rate of blood flow to the CL organ and f_u is the fraction of drug not bound to plasma proteins such as albumin [54].

CL can also be expressed as the concentration-normalized rate, so we may define the intrinsic ability of the enzyme to metabolize the drug as the intrinsic CL (CL_{int}) by dividing Equation (1.2) by $[\text{Drug}]$.

$$\text{CL}_{\text{int}} = \frac{V_{\max}}{K_M} \quad (1.4)$$

Substituting Equation 1.4 into Equation 1.3 yields Equation 1.5, which shows that CL is a function of enzyme kinetics and organ blood flows.

$$\text{CL} = \frac{f_u \frac{V_{\max}}{K_M} Q}{f_u \frac{V_{\max}}{K_M} + Q} \quad (1.5)$$

Equation 1.5 can be further simplified by recognizing that many drugs are so-called “low CL” drugs, meaning that the intrinsic CL is low compared to the blood flow.

$$CL = f_u \frac{V_{\max}}{K_M} \quad (1.6)$$

Since half-life is a more intuitive concept for most scientists than CL, we note that for an ideal drug (i.e., one-compartment PKs), the half-life ($t_{1/2}$) of the drug in the body is related to its CL in a reciprocal fashion,

$$t_{1/2} = \frac{0.693 V_d}{CL} \quad (1.7)$$

where V_d is the volume of distribution. Substituting Equation 1.7 into Equation 1.6 yields the final expression.

$$t_{1/2} = \frac{0.693 V_d}{f_u \frac{V_{\max}}{K_M}} \quad (1.8)$$

Equation 1.8 shows that the values of the enzymatic constants $V_{\max}$ and K_M for a particular enzyme–drug pair will determine whether the drug is low or high CL and, therefore, whether the half-life is long or short. Note that Equation 1.8 was derived for the simple case in which $[Drug] \ll K_M$ and $CL_{\text{int}} \ll Q$ in order to arrive at an equation simple enough to be intuitively understood. Omitting those assumptions would make Equation 1.8 more mathematically complex, but would not alter the qualitative conclusion that the half-life of a drug is inversely related to the enzymatic rate of its metabolism. Equation 1.8 is also used in later sections to rationalize the phenomena of drug interactions, induction, and genetic variation in drug metabolism.

During the drug discovery process, Equation 1.8 provides a means for medicinal chemists to adjust the half-life of their drug candidates by changing the molecular structure to reduce $V_{\max}/K_M$ (to increase half-life) or increase $V_{\max}/K_M$ (to decrease half-life). For instance, if the half-life in animals is very short, it is likely to be short in humans as well. Thus, the chemists would try to design a molecule that is less metabolically labile by identifying the point on the molecule that is susceptible to rapid metabolism (the *metabolic hot spot*; e.g., a methoxy group subject to O-demethylation) and replacing it with a molecular configuration that is more metabolically stable (e.g., substituting OCF_3 for OCH_3). The $V_{\max}/K_M$ ratio (i.e., CL_{int}) of a series of modified molecules can be quickly checked with an appropriate *in vitro* system containing the metabolic enzyme responsible for the metabolism (e.g., cytochrome P450 in liver microsomes). Unfortunately, merely replacing the metabolized group with something that is less easily metabolized does not always lengthen the half-life, since metabolism sometimes shifts to another site on the molecule (*metabolic switching*). Thus, a series of molecules must usually be synthesized and tested to find one that is actually metabolized more slowly, and the ability to test many molecules quickly with metabolic enzymes rather than whole-animal studies greatly facilitates this aspect of drug design. Conversely, a candidate molecule may be metabolized so slowly that the expected human half-life would be unacceptably long. In this case, a common approach is for the chemists to introduce a *metabolic soft spot* to provide an easier point for metabolic enzymes to attack the molecule. Again, a series of modifications would usually be tested with enzymes to find one with the right $V_{\max}/K_M$ ratio, as reflected in the observed half-life.

1.5.2 Pharmacological Activity of Metabolites

It is important to note that CL refers to the elimination from the body of a particular chemical compound, in this discussion the parent drug. Thus, chemical transformation to a metabolite is a form of CL, even though the same mass of drug-related material may remain in the body after the metabolism. Consequently, if the metabolite(s) are pharmacologically active, biological effects may continue even after the parent drug has been cleared. Since metabolites are often only subtly different in chemical structure from the parent drug (e.g., conversion of a tertiary amine to a secondary amine by N-demethylation), it is not surprising that some metabolites retain binding affinity for the biological target. These are called *active metabolites*, and they may contribute substantially to the overall pharmacological effect of the administered drug. For example, in the nonsedating antihistamine loratadine shown in Fig. 1.2, the descarboethoxy metabolite also displays prominent antihistaminic activity [7]. Accordingly, characterization and quantitation of metabolites and their residence times in the body are just as important as they are for the parent drug in developing a full understanding of the clinical profile of a new drug candidate.

Sometimes the parent compound itself has essentially no pharmacological activity and must be converted to an active form by metabolism in the body. Such compounds are called *prodrugs*, and they may be used to circumvent barriers of solubility or intestinal absorption. The inactive prodrug compound may have solubility favorable for preparation of a soluble intravenous formulation or membrane permeability that allows for good oral absorption. Once in the body, enzymes chemically change the prodrug to an active compound which is then available to the biological target. For example, as shown in Fig. 1.27 in Table 1.1, the pharmacologically active free acid enalaprilat is released from the inactive prodrug ester enalapril by intestinal, hepatic and plasma esterases. The difference between a compound producing an active metabolite and a prodrug, then, rests in whether the administered agent itself exhibits intrinsic pharmacological activity, but in both cases metabolism is involved in eliciting the full pharmacological action of the drug. An in-between case is that of the anticancer drug tamoxifen, which has an active four-hydroxy metabolite that is 30–100X more potent than tamoxifen itself. Thus, the extent to which an individual patient metabolizes tamoxifen affects the outcome of treatment [55].

1.5.3 Toxicity of Metabolites

A variation on the concept of active metabolites is the case in which the metabolite has different pharmacological activities from those of the parent drug. These different activities arise from binding of the metabolite to receptors other than those that the parent binds, including being less selective for binding to the particular receptor subtype for which the parent drug was optimized. These additional undesired activities would manifest as *adverse effects* of the drug and could range from mild to severe. Some metabolites can even display frank toxicity, such as cyanide metabolically released from the industrial chemicals acetonitrile and acrylonitrile [56,57] or the nephrotoxin thioformamide formed from the antihelminthic thiabendazole [58].

An important but currently poorly understood type of toxicity due to metabolism results from the production of *reactive metabolites* [59]. These are metabolites that have chemical reactivity that causes them to react with normal physiological compounds present in cells. The normal cellular components with which these metabolites

react are typically nucleophiles (amines, thiols, alcohols, phenols, or nucleobases) and range from small molecules such as glutathione to macromolecules such as proteins or nucleic acids. In each case, the normal component is chemically altered in such a way that its usual function in cellular biology is altered or disrupted. We can, somewhat arbitrarily, further distinguish between reactive metabolites and reactive intermediates. A reactive *intermediate* is so unstable that it immediately reacts with chemical moieties inside the active site of the enzyme that formed it. Conversely, a reactive *metabolite* is stable enough to be released from the active site of the enzyme that formed it and, is thus, potentially able to react with any target molecule in the cell. Numerous examples of reactive intermediates are known, especially with the cytochrome P450 class of enzymes, which are often victims of irreversible metabolism-based inhibition because of the formation of extremely reactive electrophilic intermediates resulting from oxidation of certain drugs. An example is the reactive oxirene resulting from oxidation of the acetylene functional group of ethynylestradiol by CYP3A5 [52]. Likewise, many examples of reactive metabolites have been reported, of which the classic example is *N*-acetyl phenyliminoquinone (NAPIQ), formed by sequential oxidations and conjugations of the commonly used painkiller acetaminophen (paracetamol) [59]. NAPIQ has been shown to covalently bind to an array of intracellular nucleophiles.

With each type of metabolite-derived toxicity, characterization of metabolites would be necessary to fully understand the clinical profile of a drug. International regulatory authorities have recognized this fact by issuing guidances to developers of new drugs, requiring characterization of the toxicological properties of major metabolites circulating in blood in humans. The central dictum is that these major human metabolites must also be present in the circulation of preclinical species used for assessment of safety of new drugs, at exposure levels at least equal to those in humans. Metabolites that are unique to humans may require synthesis and direct dosing for assessment of safety in animals [60,61].

1.5.4 Drug Interactions Related to Metabolic Processes

The final area of importance of human drug metabolism is the occurrence of *PK drug–drug interactions* arising from modulation by one drug (the *perpetrator* drug) of the catalytic activity of a drug-metabolizing enzyme that is involved in the CL of a second drug (the *victim* drug). These modulations take two forms: *inhibition* and *induction* (chapters titled *Enzyme Kinetics of Drug-Metabolizing Reactions and Drug–Drug Interactions*).

Inhibition arises from the fact that drug-metabolizing enzymes are generally nonspecific and typically recognize multiple drugs as substrates. Thus, when two or more drugs are in the body simultaneously, they may act as competitive substrates for one of these enzymes (chapter titled *Enzyme Kinetics of Drug-Metabolizing Reactions and Drug–Drug Interactions*). Modifying Equation 1.8 by the form of the Michaelis–Menten equation that describes competitive inhibition, we can derive Equation 1.9, which shows the effect on the half-life of a drug by a competitive drug, I.

$$t_{1/2} = \frac{0.693V_d}{f_u \frac{V_{\max}}{K_M \left(1 + \frac{[I]}{K_i}\right)}} \quad (1.9)$$

Equation 1.9 shows that the clinical effect of the presence of a second drug I with an inhibition constant K_i is to increase the elimination half-life of a drug, depending on the magnitude of the ratio $[I]/K_i$. Often there is also a concomitant increase in $C_{\max}$. The increased exposure of the patient to the victim drug may result in adverse events or toxicity, so the drug–drug interactions due to inhibition of metabolism are an important safety concern for regulatory authorities. Accordingly, another metabolism-related selection criterion for new drugs is that $[I]/K_i$ should be $\ll 1$, and K_i -values are routinely measured for the most important drug-metabolizing enzymes when a sponsor proposes to bring a new drug candidate to clinical trials. Equation 1.9 assumes *reversibility* of the mutual inhibition between two drugs, so an important adjunct to measurement of K_i -values is the determination that the new drug candidate does not exhibit *metabolism-based inhibition*, which is *irreversible* in nature (also called *time-dependent inhibition*). Assessment of drug–drug interactions due to inhibition of CL by a metabolism-based inhibitor involves a different, more complex mathematical approach [62].

Drugs may also pharmacokinetically interact because of upregulation of the steady-state level of a metabolic enzyme, a phenomenon known as *enzyme induction* (chapter titled ***Transcriptional Regulation of Cytochrome P450 Genes***). The upregulation can occur through a variety of mechanisms, including (i) activation of xenobiotic-sensing receptors resulting in increased expression of the enzyme [63] and (ii) decrease of the cellular degradation rate of the enzyme. Induction usually requires several days of exposure to the inducing drug before the cell fully responds to the stimulus and a new steady-state level of the enzyme is attained. In Equation 1.8, $V_{\max}$ may be factored into $k_{\text{cat}} \cdot E_{\text{tot}}$, where k_{cat} is the catalytic rate constant of the enzyme and E_{tot} is the total amount of enzyme present in the system. If E_{tot} in the CL organ is increased by induction, then the total activity, as measured by $V_{\max}$ will increase. An increase in $V_{\max}$ translates to an increase in CL of the victim drug, a decrease in half-life and a possible decrease in $C_{\max}$ is obtained. This reduced exposure may result in loss of efficacy of the victim drug, so induction of drug-metabolizing enzymes is also an important concern for regulatory authorities. Since the induced enzyme may also be responsible for the metabolism of the inducing drug itself, this phenomenon is sometimes manifest as an adaptive response called *autoinduction*. Sequential doses of an autoinducing drug lead to lower $C_{\max}$ - and AUC-values of that drug. It is possible for autoinduction to be so severe that the drug becomes ineffective after a few doses. Thus, it is important in drug discovery to detect potent enzyme inducers and avoid advancement of such compounds into clinical development. Potent inducers of cytochrome P450 enzymes can be eliminated by higher-throughput *in vitro* assays with gene-reporter assays linked to receptors such as PXR (pregnane X receptor) (Section 1.6.5).

1.6 THE BIOCHEMICAL PROCESS OF DRUG METABOLISM

1.6.1 Anatomical Sites of Drug Metabolism

Where does drug metabolism occur in the body? Some level of drug-metabolism activity can be found in essentially every tissue, but the highest specific activities are associated with organs that interface with the environment: gut, liver, kidneys, skin, lungs, nasal mucosa, and so on. This makes sense if we accept that the object of drug

metabolism is to minimize systemic exposure of the body to xenobiotics. Thus, the first line of defense is to prevent or at least limit entry into the body. This is the function of the various efflux transporters that pump chemicals that have permeated the first layer of cells in these organs. We may consider drug metabolism to be the second line of defense, since generally the enzymes of drug metabolism are intracellular, so that only compounds not efficiently excluded by efflux transporters are subject to drug metabolism. This distinction of first and second lines of defense is somewhat arbitrary, since xenobiotics are not all subject to efflux transport or metabolism during initial organ penetration, but it provides a useful conceptual framework for thinking about the process and is reasonably accurate for many drugs.

Since the greatest absolute amounts of xenobiotics usually arrive by oral ingestion, the intestine and liver are of special importance for the barrier function, and the majority of drug-metabolizing capacity may be found there. An orally ingested xenobiotic must survive stomach acid and intestinal digestive enzymes such as proteases, lipases, and other hydrolases. Then it faces the intestinal wall that is designed to be impermeable to foreign substances, especially highly charged, hydrophilic, or high molecular weight compounds. And those molecules that pass through these two barriers still must elude the efflux transporters. Nonetheless, the majority of xenobiotics actually do survive this gauntlet of exclusion processes and are absorbed into the body, explaining why another type of high capacity barrier (drug metabolism) is necessary in intestine and liver. The process of interception and elimination of molecules that manage to penetrate the intestinal epithelium in order to prevent their ultimate entry into the systemic circulation is called the *first-pass effect*. The physiological purpose of intestine and liver drug metabolism is to *maximize* the first-pass effect. Conversely, the problem faced by the medicinal chemists and pharmaceutical scientists is to *minimize* the first-pass effect, so that a high percentage of the dosed medicine can actually reach the systemic blood stream. Thus, in order to achieve good oral bioavailability, drug discovery teams usually seek to design molecules that are metabolized at only moderate rates.

Once a drug enters the bloodstream, the body begins clearing the compound either by direct excretion or by metabolism followed by excretion. Both of these processes occur in the liver and kidney, but with organ differences. The liver can utilize uptake transporters to bring compounds from the blood into liver cells, where they can be directly excreted through the action of efflux transporters or metabolized by the versatile hepatic drug-metabolizing enzymes. The kidney, on the other hand, can directly excrete a drug into urine by the process of glomerular filtration and by secretion into urine from renal cells by efflux transporters. Kidney also contains a number of drug-metabolizing enzymes, but not the same set or the same densities as liver. Again, for many drugs the direct excretion processes in liver and kidney are ineffective for CL, and molecules must first be metabolized. Metabolism frequently makes a drug more polar and water soluble, facilitating transporter-mediated excretion. In addition, polar metabolites are usually less bound to plasma proteins compared to the parent drug, facilitating their filtration in the kidney. In fact, in this context, metabolism is historically described as occurring in two steps, *phase I* and *phase II*, although more recently this distinction has come into question [64]. Phase I enzymes introduce a polar site into the molecule, for instance, by hydroxylation at a hydrophobic region of the molecule or by dealkylation of an amine or ether functionality to uncover a secondary amine or hydroxyl group. Sometimes phase I metabolism sufficiently increases water solubility that the polar metabolite can either diffuse out of the cell membrane directly into bile or blood

or move to a transporter and be secreted. At other times, however, the metabolite is still too insoluble to be mobilized and continues to mainly associate with cellular membranes. These hydrophobic metabolites may then be recognized as substrates by the phase II enzymes, which add a very polar or even ionic group to make a secondary, conjugated metabolite that is usually excretable in bile or urine. Examples of these more soluble conjugates are glucuronides and sulfates, both of which are negatively charged at pH 7. Thus, we see that metabolism by phase I and/or phase II enzymes followed by excretion of the solubilized metabolites is the most important means of CL of the majority of xenobiotics. A more complete statement of the problem faced by a drug discovery team, then, is to find the right balance between metabolism slow enough that the first-pass effect is low but fast enough that compounds not directly excretable can be metabolized and excreted in a reasonable amount of time.

1.6.2 Subcellular Localization of Drug Metabolism

Taking hepatocytes as the archetypical drug-metabolizing cells, the greatest variety of reactions and capacity for CL are in the smooth endoplasmic reticulum (SER) and the cytosol. Certain special reactions, such as β -oxidation, occur mainly in the mitochondria and peroxisomes. Accordingly, when we wish to study metabolism in an *in vitro* system so that we can control most variables, we use hepatocytes to simultaneously evaluate all possible metabolic routes. Often, however, we want to limit the experimental question to only certain metabolic pathways. In that case, we need to use a subcellular fraction. Hepatocytes can be lysed by homogenization of liver tissue, followed by differential centrifugation to segregate the desired subcellular fraction [65]. By this means, we can prepare the so-called *S-9 fraction* (supernatant from $9,000 \times g$ sedimentation), which contains *cytosol* and liposomal fragments of the smooth endoplasmic reticulum called *microsomes*. *S-9* provides convenient access to almost all important drug-metabolism pathways. Microsomes can be separated from cytosol by more forceful sedimentation of the *S-9* fraction at $105,000 \times g$, allowing us to separately interrogate both the soluble and membrane-bound drug-metabolism enzymes.

1.6.3 The Enzymes of Drug Metabolism

As mentioned above, almost all drug metabolism is mediated by enzymes that fall into a few general classes. Table 1.2 summarizes these classes and a few important features of each enzyme type in the class. In most cases, it is easy to match up an enzyme type with each of the reactions shown in Table 1.1, and the two tables have been arranged in parallel to facilitate this matching. In drug development, it is necessary to identify the enzymes mediating the metabolism of drug candidates, because this information promotes an understanding of several aspects of the clinical behavior of drugs, including genetic variation, drug–drug interactions, time-dependent inhibition, autoinduction, and the effect of disease on a patient’s ability to clear the drug.

Most of the enzymes in Table 1.2 exist in *superfamilies* of closely related proteins, often called *isozymes* or *isoforms*, with different but sometimes overlapping substrate preferences. The enzymes in a superfamily are typically given a designation of the form “*nLm*”, where “*n*” and “*m*” are numbers (1, 2, 3, etc.) and “*L*” is a letter (A, B, C, etc.). The “*n*” designates the family, “*L*” the subfamily, and “*m*” the specific

TABLE 1.2 Summary of Major Enzymes Mediating Human Drug Metabolism

Oxygenases, Oxidases, and Dehydrogenases*Cytochrome P450 (CYP)^a*Typical reaction: $\text{R-H} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{R-OH} + \text{H}_2\text{O} + \text{NADP}^+$

Prosthetic group: Heme

Required cofactors: O_2 , NADPH, CPR, and cytochrome b_5 (with some substrates)

Tissue expression: Liver and most other tissues

Subcellular localization: Endoplasmic reticulum

*Flavin-Containing Monooxygenase (FMO)^b*Typical reaction: $\text{R}_3\text{N} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{R}_3\text{N}^+-\text{O}^- + \text{H}_2\text{O} + \text{NADP}^+$

Prosthetic group: FAD

Required cofactors: O_2 and NADPH

Tissue expression: Liver

Subcellular localization: Endoplasmic reticulum

*Aldehyde Oxidase (AOX)^c*Typical reaction: $\text{RCH=O} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{RCOOH} + \text{H}_2\text{O}_2$

Prosthetic group: Molybdenum, [2Fe-2 S] centers and flavin

Required cofactors: O_2

Tissue expression: Liver and other tissues

Subcellular localization: Cytosol

*Monoamine Oxidase (MAO)^d*Typical reaction: $\text{RCH}_2\text{NH}_2 + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{RCH=O} + \text{NH}_3 + \text{H}_2\text{O}_2$

Prosthetic group: Flavin

Required cofactors: O_2

Tissue expression: Many tissues

Subcellular localization: Mitochondria

*Xanthine Oxidase (XOR)^e*Typical reaction: $\text{xanthine} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{uric acid} + \text{H}_2\text{O}_2$

Prosthetic group: Molybdenum, [2Fe-2 S] centers and flavin

Required cofactors: O_2

Tissue expression: Liver

Subcellular localization: Cytosol

*Alcohol Dehydrogenase (ADH)^f*Typical reaction: $\text{R-CH}_2\text{-OH} + \text{NAD}^+ \rightarrow \text{R-CH=O} + \text{NADH} + \text{H}^+$

Prosthetic group: None

Required cofactors: NAD^+

Tissue expression: Liver

Subcellular localization: Cytosol

*Aldehyde Dehydrogenase (ALDH)^g*Typical reaction: $\text{R-CH=O} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{R-COOH} + \text{NADH} + \text{H}^+$

Prosthetic group: None

Required cofactors: NAD^+

Tissue expression: Liver

Subcellular localization: Cytosol

TABLE 1.2 (continued)**Reductases***Aldo-Keto Reductase (AKR)^h*Typical reaction: RCH=O or $\text{R}_2\text{C=O} + \text{NADPH} + \text{H}^+ \rightarrow \text{RCH}_2\text{OH}$ or $\text{R}_2\text{CHOH} + \text{NADP}^+$

Prosthetic group: None

Required cofactors: NADPH

Tissue expression: Liver and other tissues

Subcellular localization: Cytosol

*Cytochrome P450 (CYP)ⁱ*Typical reaction: $\text{ArN} \rightarrow \text{O} + \text{NADPH} + \text{H}^+ \rightarrow \text{ArN} + \text{H}_2\text{O} + \text{NADP}^+$

Prosthetic group: Heme

Required cofactors: NADPH and CPR (hypoxic conditions)

Tissue expression: Liver and most other tissues

Subcellular localization: Endoplasmic reticulum

*Cytochrome P450 Reductase (CPR)^j*Typical reaction: $\text{ArN} \rightarrow \text{O} + \text{NADPH} + \text{H}^+ \rightarrow \text{ArN} + \text{H}_2\text{O} + \text{NADP}^+$

Prosthetic group: FMN and FAD

Required cofactors: NADPH

Tissue expression: Liver and most other tissues

Subcellular localization: Endoplasmic reticulum

*Quinone Reductase (NQO1)^k*Typical reaction: quinone + NADPH $\rightarrow$ hydroquinone + NADP⁺

Prosthetic group: Flavin Required Cofactor: NADPH

Tissue expression: Liver and other tissues

Subcellular localization: Cytosol

Transferases*N-Acetyl Transferase (NAT)^l*Typical reaction: $\text{Ar-NH}_2 + \text{AcSCoA} \rightarrow \text{Ar-NHCOCH}_3 + \text{CoASH}$

Prosthetic group: None

Required cofactors: AcSCoA

Tissue expression: Liver and other tissues

Subcellular localization: Cytosol

*Adenosine Kinase (AK)^m*Typical reaction: Base-Ribose-OH + ATP $\rightarrow$ Base-Ribose-OPO₂H₂ + ADP

Prosthetic group: None

Required cofactors: ATP

Tissue expression: Liver and other tissues

Subcellular localization: Cytosol

*Catecholamine O-Methyl Transferase (COMT)ⁿ*Typical reaction: A catechol + SAM $\rightarrow$ a guaiacol + S-adenosyl-L-homocysteine

Prosthetic group: None

Required cofactors: SAM

Tissue expression: Liver and other tissues

Subcellular localization: Both cytosol and endoplasmic reticulum

*Glutathione S-Transferase (GST)^o*Typical reaction: $\text{R-X} + \text{GSH} \rightarrow \text{R-SG} + \text{HX}$ (X is a leaving group such as halogen)

(continued overleaf)

TABLE 1.2 (continued)

Prosthetic group: None
Required cofactors: GSH
Tissue expression: Liver, kidney, and other tissues
Subcellular localization: Cytosol
<i>Sulfotransferase (SULT)^p</i>
Typical reaction: $\text{Ar-OH} + \text{PAPS} \rightarrow \text{Ar-OSO}_3\text{H} + \text{adenosine-3', 5'-diphosphate}$
Prosthetic group: None
Required cofactors: PAPS
Tissue expression: Liver and intestine
Subcellular localization: Cytosol
<i>Thiopurine S-Methyl Transferase (TPMT)^q</i>
Typical reaction: A thiopurine-SH (or other R-SH) + SAM $\rightarrow$ a thiopurine-SMe + S-adenosyl-L-homocysteine
Prosthetic group: None
Required cofactors: SAM
Tissue expression: Liver, kidney, and erythrocytes
Subcellular localization: Cytosol
<i>UDP-Glucuronosyl Transferase (UGT)^r</i>
Typical reaction: $\text{R-OH or Ar-OH} + \text{UDPGA} \rightarrow \text{alkyl or aryl } \beta\text{-D-glucuronide} + \text{UDP}$
Prosthetic group: None
Required cofactors: UDPGA
Tissue expression: Liver and intestine
Subcellular localization: Endoplasmic reticulum
Condensation
<i>Amino Acid Conjugation^s</i>
Typical reaction:
$\text{Ar-COOH} + \text{H}_2\text{N-CHR-COOH} + \text{ATP} \rightarrow \text{Ar-CONH-CHR-COOH} + \text{AMP} + \text{H}_4\text{P}_2\text{O}_7$
Prosthetic group: None
Required cofactors: glycine or taurine, ATP, and CoASH
Tissue expression: Liver
Subcellular localization: Mitochondria
Hydrolases
<i>Alkaline phosphatase (AP)^t</i>
Typical reaction: $\text{RO-PO}_3\text{H}_2 + \text{H}_2\text{O} \rightarrow \text{ROH} + \text{HOPO}_3\text{H}_2$
Prosthetic group: None
Required cofactors: None
Tissue expression: Intestinal lumen
Subcellular localization: Epithelial brush border
<i>Amidase (AADAC)^u</i>
Typical reaction: $\text{Ar-NHCOCH}_2\text{R} + \text{H}_2\text{O} \rightarrow \text{Ar-NH}_2 + \text{RCH}_2\text{COOH}$
Prosthetic group: None
Required cofactors: None
Tissue expression: Liver
Subcellular localization: Endoplasmic reticulum
<i>Arylsulfatase (ARS)^v</i>
Typical reaction: $\text{Ar-OSO}_3\text{H} + \text{H}_2\text{O} \rightarrow \text{Ar-OH} + \text{HOSO}_3\text{H}$

TABLE 1.2 (continued)

Prosthetic group: None
 Required cofactors: None
 Tissue expression: Liver
 Subcellular localization: Endoplasmic reticulum and lysosomes

Carboxylesterase (CES)^w

Typical reaction: $\text{RCOOR}' + \text{H}_2\text{O} \rightarrow \text{RCOOH} + \text{R}'\text{OH}$

Prosthetic group: None
 Required cofactors: None

Tissue expression: Liver and many other tissues
 Subcellular localization: Cytosol and endoplasmic reticulum

Epoxide hydrolase (EH)^x

Typical reaction: Epoxide + $\text{H}_2\text{O} \rightarrow \text{trans-1,2-diol}$

Prosthetic group: None
 Required cofactors: None

Tissue expression: Liver and other tissues
 Subcellular localization: Endoplasmic reticulum

 β -Glucuronidase (β -Gluc)^y

Typical reaction: Alkyl or aryl β -D-glucuronide + $\text{H}_2\text{O} \rightarrow$ D-glucuronic acid + alcohol or phenol

Prosthetic group: None
 Required cofactors: None

Tissue expression: Liver, kidney, and intestinal lumen
 Subcellular localization: Endoplasmic reticulum and lysosomes in liver and kidney; secreted by *Escherichia coli* in intestine

^aReference: Volume I, chapter titled *Structure and Function of Cytochrome P450 Enzymes*; also Ref. 66.
 Web Links: http://www.bing.com/reference/semhtml?title=Cytochrome_P450&qpv=cytochrome+P450&src=abop&q=cytochrome+P450&fwd=1.

Accessed 2010 June 1.

http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.14.14.1.

Accessed 2010 June 1.

^bReference: Volume efl, chapter titled *Flavin-Containing Monooxygenase: The Role of Flavin-Containing Monooxygenase in Lead Design and Selection*; also Ref. 67.

Web Links: http://en.wikipedia.org/wiki/Flavin-containing_monooxygenase.

Accessed 2010 June 1.

http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.14.13.8.

Accessed 2010 June 1.

^cReference: Volume I, chapter titled *Molybdenum-Containing Hydroxylases*; also Ref. 68.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.2.3.1.

Accessed 2010 June 1.

^dReference: Volume I, chapter titled *Amine Oxidases and Reductases*; also Ref. 69.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.4.3.4.

Accessed 2010 June 1.

^eReference: Volume I, chapter titled *Molybdenum-Containing Hydroxylases*; also Ref. 70.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.17.3.2.

Accessed 2010 June 1.

^fRef. 71.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.1.1.1.

Accessed 2010 June 1.

^gRef. 72.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.2.1.3.

Accessed 2010 June 1.

^hRefs 73 and 74.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.1.1.2.

Accessed 2010 June 1.

ⁱRef. 75.

Web Links: http://www.bing.com/reference/semhtml?title=Cytochrome_P450&qpv=cytochrome+P450&src=abop&q=cytochrome+P450&fwd=1.

Accessed 2010 June 1.

http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.14.14.1.

Accessed 2010 June 1.

^jRef. 76.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.6.2.4.

Accessed 2010 June 1.

^kRef 35.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.3.1.74.

Accessed 2010 June 1.

^lRef. 77.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=2.3.1.5.

Accessed 2010 June 1.

^mRef. [78]; other kinases such as deoxycytidine kinase (DCK) can also carry out phosphorylations of nucleosides 79

ⁿRef. 80.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=2.1.1.6.

Accessed 2010 June 1.

^oReference: Volume I, chapter titled *Functional Genomics of the Human Glutathione Transferases*; also Ref. 81.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=2.5.1.18.

Accessed 2010 June 1.

^pReference: Volume I, chapter titled *Sulfotransferases*; also Ref. 82.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=2.8.2.1.

Accessed 2010 June 1.

^qRef. 83.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=2.1.1.67.

Accessed 2010 June 1.

^rReference: Volume I, chapter titled *UDP-Glucuronosyltransferases: Pharmacogenetics, Functional Characterization, and Clinical Relevance*; also Ref. 84.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=2.4.1.17.

Accessed 2010 June 1.

^sReference: Volume I, chapter titled *Amino Acid Conjugation: A Novel Route of Xenobiotic Carboxylic Acid Metabolism in Man*; also Ref. 85.

^tRef. 86.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=3.1.3.1.

Accessed 2010 June 1.

^uReference: Volume I, chapter titled *Carboxylesterases*; also Ref. 87.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=3.5.1.4.

Accessed 2010 June 1.

^vRef. 88.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=3.1.6.1.

Accessed 2010 June 1.

^wReference: Volume I, chapter *Carboxylesterases*; also Ref. 89.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=3.1.1.1.

Accessed 2010 June 1.

^xReference: Volume I, chapter titled *Epoxide Hydrolases*; also Ref. 90.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=3.3.2.9.

Accessed 2010 June 1.

^yRefs 91–93.

Web Link: http://www.brenda-enzymes.info/php/result_flat.php4?ecno=3.2.1.31.

Accessed 2010 June 1.

enzyme. The most widely recognized example is the cytochrome P450 (CYP) superfamily (chapter titled *Structure and Function of Cytochrome P450 Enzymes*). The human genome is known to contain 57 functional CYP genes [66], of which approximately a dozen encode enzymes mainly involved in drug metabolism [94]. Arguably, the most important of the cytochrome P450 enzymes is CYP3A4, often truncated to just 3A4 when the context is understood to be a CYP enzyme. Among the “drug-metabolizing” CYPs, it is common for more than one to be capable of metabolizing a particular drug, although one particular CYP enzyme may make the biggest contribution to the metabolism [11]. Thus, for drug-development purposes, a complete description of the enzymes mediating metabolism must include not only which enzyme type is involved (in this case, CYP) but also which particular CYP enzymes are most responsible for the metabolism of the candidate drug (e.g., enzymes 2C9, 3A4, and 3A5). This *reaction phenotyping* is a rigorous process of testing the ability of various CYP enzymes to metabolize the drug candidate, with multiple reagents, techniques and sources of enzymes [94] (see Volume III). Reaction phenotyping is required for most of the important drug-metabolizing enzymes, to the extent that the state of knowledge of each particular enzyme type allows. For instance, as with the CYPs, drugs metabolized by UDP-glucuronosyl transferase (UGT) also require reaction phenotyping [95]. Many drug-metabolizing enzymes have been cloned and heterologously expressed and are commercially available as single enzymes, greatly facilitating reaction phenotyping and other preclinical drug-metabolism purposes [96]. It is also possible to directly isolate the requisite enzymes from human liver [97], but this is seldom done since it is slow, labor intensive, and requires a source of fresh human tissue.

In addition to the existence of multiple enzymes in a given family, some of the enzymes exhibit allelic variants in their genes across populations. Some of these variants translate to proteins that have reduced or no catalytic activity, but in other cases the variant allele is the result of gene duplication leading to overexpression of the active enzyme. This phenomenon is called *genetic polymorphism*, and it can result in individuals who are *poor metabolizers* (homozygous for the defective gene), *intermediate metabolizers* (heterozygous), *extensive metabolizers* (homozygous for the wild-type gene), and *ultrarapid metabolizers* (carriers of the gene-duplication allele). Since the CYP enzymes are so important in human drug metabolism, polymorphism in the CYPs is especially significant [98] (chapter titled *Cytochrome P450 Polymorphisms*). As mentioned in Section 1.5.4, $V_{\max}$ in Equation 1.8 may be factored into $k_{\text{cat}} \cdot E_{\text{tot}}$, allowing us to rationalize the effect of genetic polymorphisms on the half-lives of drugs that mainly depend on the polymorphic enzyme for their metabolism. Some genetic polymorphisms result in expression of inactive enzyme (i.e., $k_{\text{cat}} \sim 0$), while in other cases there is reduced expression (i.e., $E_{\text{tot}} \sim 0$). In either case, $V_{\max}$ is reduced, and individuals carrying such an allele exhibit increased half-life compared to the general population. The opposite is true for the ultrarapid metabolizers, where the active enzyme is overexpressed (i.e., $E_{\text{tot}} \gg$ normal population). These very real effects on the clinical PKs of some drugs in certain individual patients are important reasons to characterize the enzymology of metabolism of new drug candidates. Consequently, during the discovery process, we try to design compounds that do *not* mainly depend on a single, polymorphic enzyme for their CL.

1.6.4 The Role of Transporters in Drug Metabolism

Transporters are ubiquitous proteins in the body, which move molecules from one side of a barrier membrane to the other (see Volume II). Again taking hepatocytes as the typical drug-metabolizing cell, *uptake* transporters move drug molecules from the blood to the interior of the hepatocytes, while *efflux* transporters export drug or metabolite molecules from the cell interior to the blood or bile [99]. Some drug molecules readily penetrate cell membranes by passive diffusion, while others, with poor membrane permeability characteristics, may require the assistance of a transporter. Uptake transporters, then, could be called *phase 0* of drug metabolism, since they move drugs into cells, where the main drug-metabolizing activities are located.

Analogously, efflux transporters could be called *phase III* of drug metabolism. Once metabolism has occurred, removal of drug-derived material is not complete until the metabolites are moved from the interior of the cells to the excreta (bile, feces, and urine). The chemical modifications that make metabolites more water soluble also make them less permeable through cell membranes. Thus, the efflux transporters complete the process for metabolites that cannot independently diffuse through membranes and out of cells. A particularly important efflux transporter is *P-glycoprotein* (P-gp or MDR1), located on the intestinal epithelium, liver canaliculae, kidney proximal tubules, and brain endothelium. This transporter can pump many types of xenobiotic molecules and metabolites out of cells as a means of exclusion or elimination [100].

As with drug-metabolizing enzymes, transporters are subject to the phenomena of competitive inhibition and induction, creating a second type of metabolism-based drug–drug interaction [101]. Also, most, if not all, transporters have allelic variants resulting in polymorphic functionality [102]. This complicated *metabolism-transporter interplay* [103] has drawn the attention of the scientific community, regulatory authorities, and drug developers [100]. Thus, *transporter phenotyping* is now also recognized as an important component of a complete understanding of the behavior of drugs and metabolites in the body.

1.6.5 Biological Aspects of Drug Metabolism

The activities of human drug-metabolizing enzymes are modulated by a number of factors and conditions (see Volume IV). As mentioned in Section 1.5.4, many drug-metabolizing enzymes are upregulated by chronic exposure of liver and other organs to xenobiotics, thereby promoting the metabolic CL of the foreign chemical. This upregulation occurs at the transcriptional level under the control of cytoplasmic or nuclear receptors which sense the presence of the xenobiotics inside cells and activate gene expression for a complement of enzymes and proteins related to drug metabolism [63] (chapter titled ***Transcriptional Regulation of Cytochrome P450 Genes***). Three important receptors for upregulation of drug-metabolizing enzymes are *AhR* (aryl hydrocarbon receptor), *CAR* (constitutive androstane receptor), and *PXR* (pregnane X receptor). The names of these receptors are not informative as to their activators or functions. AhR is a cytoplasmic receptor that is activated by some polycyclic aromatic compounds, such as benzo[*a*]pyrene, resulting in upregulation of CYPs 1A and 1B, GST (glutathione *S*-transferase), NQO1, and UGT1A enzymes (see Table 1.2 for enzyme abbreviations). This set of induced enzymes would be important for metabolic CL of benzo[*a*]pyrene and other aromatic compounds, so that continuous exposure of

a person to inducers such as benzo[*a*]pyrene would result in increased capacity of the person to clear such compounds. CAR is a nuclear receptor activated by phenobarbital and other drugs, resulting in upregulation of CYPs 2B and 3A, GST, and UGT1A enzymes. PXR is particularly important in clinical drug metabolism, since it is activated by many compounds (e.g., dexamethasone and rifampicin) and is the principal mechanism of upregulation of the important enzyme CYP3A4. Other enzymes/transporters upregulated by PXR include SULT1A, UGT1A, and MDR1. Since activation of these receptors can have profound effects on the clinical utility of a drug, *in vitro* assessment of the human induction profile is usually a screening requirement during the discovery process and is normally expected by regulatory authorities during registration of a new drug. If *in vitro* or animal data indicated the likelihood of enzyme induction, phase I clinical studies would be mounted to rigorously define the induction potential.

Physiological conditions such as menstruation and pregnancy affect drug metabolism, possibly necessitating dosage adjustments of medications taken during these times [104,105]. Similarly, drug-metabolizing enzymes and transporters are variably expressed during the stages of fetal development [106] (chapter titled ***Sex Differences in Drug Metabolism***). Enzyme levels continue to change through childhood, adulthood, and old age [107]. The enzymes of drug metabolism are even different in men and women, leading to gender differences in PKs [108]. Section 1.6.3 introduced the idea of genetic polymorphisms in many of the enzymes of drug metabolism, and it follows as a natural consequence that the corresponding alleles occur with higher frequency in some human populations (i.e., *pharmacogenetics*), making the nature and extent of drug metabolism variable across ethnic and genetic groups [109].

Disease states can effect an individual's ability to metabolize drugs, especially in inflammation and infection, where released cytokines can modulate levels of CYP enzymes [110]. Diet can increase or decrease the activities of drug-metabolizing enzymes and transporters, probably due to the effects of phytochemicals operating through several molecular mechanisms [111]. Even the time of day can make a difference in the ability of an individual to clear drugs, since enzyme expression and physiological processes such as blood flows are subject to *circadian rhythms* [112].

Finally, in light of the discussion above, it should be no surprise that the enzymes of animals are different from those of humans, both in amino acid sequences of the proteins and expression levels of the active enzymes (chapter titled ***Species Differences of Drug-Metabolizing Enzymes***). This has implications for human drug development because preclinical drug metabolism information is often used to help design and select drug candidates, but preclinical studies are performed by definition in animals, not humans. Thus, because the animal drug-metabolizing enzymes may have different activities and specificities from those of human, conclusions from animal studies are compromised. *In vitro* drug-metabolism studies with human hepatocytes avoid the problem of interspecies differences, but introduce the problem of *in vitro* to *in vivo* extrapolation. In response to this need for reliable *in vivo* human-relevant drug metabolism information to be available preclinically, various humanized transgenic mouse strains have been developed [113,114], but the reliable use of such models is still being explored.

1.7 THE CHEMICAL CHARACTERIZATION OF DRUG METABOLISM

1.7.1 ADME Studies

The acronym ADME stands for absorption, distribution, metabolism, and excretion. A human ADME study gives a complete description of how much of a drug candidate enters the body by a particular route of administration such as oral dosing, where the compound distributes within the body, what metabolites are formed and in what quantities, and what length of time is required for complete excretion of drug-related materials in feces and urine (see Volume II). A typical human ADME study is based on administration of a low dose of radiolabeled drug candidate (almost always carbon-14), followed by monitoring and quantitating radioactivity in various biofluids, tissues, and excreta to track drug and metabolites as they are absorbed, distributed, metabolized, and excreted.

In reality, of course, for the usual oral route of administration, we do not get complete information in any of these four areas. First, let us consider the “A” part of ADME. Radioactivity, consisting of parent drug and metabolites, recovered in the urine could only have come from intestinally absorbed parent drug and represents a minimum value for absorption. However, radioactivity recovered in the feces cannot be interpreted, since it contains biliary-secreted parent drug, metabolites, and unabsorbed parent drug. The summed radioactivity in urine and feces, then, does not provide an unambiguous estimate of the extent of absorption. Thus, an oral ADME study does not, by itself, necessarily give a reliable estimate of how much of an orally administered drug was intestinally absorbed. A parallel intravascular dose can provide a way to estimate the oral absorption, since the entire dose is known to have reached the systemic circulation in that case. By making the usually reasonable assumption of non-route-dependent elimination processes, we can proportionally scale the observed amounts of drug-related materials appearing in the urine to estimate the fraction of the oral dose that was actually absorbed (provided that the total urinary excretion of parent drug and metabolites was not trivial). Usually, however, an intravascular dose is not included in a human ADME study.

The situation is equally problematic for the “D” in ADME: distribution. In animal studies, it is possible to dissect a dosed animal and measure the radioactivity content of each organ as an indication of the extent and location of distribution of the parent drug in the body. However, this is impossible for a clinical study, so technically a human ADME study is really just an AME study. In most cases, we assume that the distribution of drug to human organs is similar to distribution to animal organs.

The “M” part of the ADME study is probably the most complete and reliable, but even here there may be serious deficiencies with some drugs. Radioactivity allows us to find and quantitate metabolites in blood, urine, feces, and other samples, and modern spectroscopic techniques make exact structure determination virtually assured. However, with some drugs, a fraction of the radioactivity may be retained in the body for a prolonged period, perhaps as covalently attached drug residues from reactive metabolites. In these cases, radioactivity only appears in the excreta after the biological target of the covalent attachment has been broken down by degradation processes. Thus, information about the chemical identity of the reactive metabolite is lost. Furthermore, metabolites recovered in feces may have been produced from unabsorbed parent drug

or further transformed by intestinal microflora, obscuring the origin of these metabolites. Nonetheless, despite these deficiencies, human ADME studies, especially when supplemented by other studies such as animal ADME, human PKs, and enzyme phenotyping, provide the best overall picture of the handling of the drug by the human body. This is the reason that radiolabeled ADME studies are routinely required by regulatory authorities for registrational and product labeling purposes. For example, a recently published human ADME study of [^{14}C]-prasugrel identified over three dozen metabolites, which were rationalized by a scheme of metabolic pathways that showed the process for formation of the metabolite mainly responsible for the pharmacological activity. Exhaustive collection of urine and feces showed that $\sim 95\%$ of the drug and metabolites was excreted within 10 days. Since about 70% was recovered in the urine, intestinal absorption of the oral dose of prasugrel must have been at least 70%. [28].

1.7.2 Metabolite Structure Elucidation

Characterization of the exact chemical structure of the major metabolites of a drug candidate is the foundation for understanding human drug disposition. The chemical structure may provide clues to the possible pharmacological or toxicological properties of the metabolite, and these can be investigated by independent synthesis because the structure is known. In most cases, the structure of each metabolite tells which type of enzyme accomplished the metabolic transformation, as can be demonstrated by comparison of the reactions in Table 1.1 to the enzymes in Table 1.2. It is relatively straightforward from that point to determine which particular enzyme of the family for that type of enzyme was the actual catalyst for the chemical transformation. Once the particular enzyme in the family is identified, then a set of characteristics for that drug immediately become apparent, such as which drugs will likely show interactions, whether polymorphic kinetics are likely to be observed, and whether induction is likely to be a clinical issue. Therefore, rigorous determination of metabolite chemical structure is absolutely essential when characterizing a new clinical candidate.

Metabolite structure elucidation requires the most advanced spectroscopic techniques (Volume V). This is because most such structure determination is accomplished with nano- or microgram quantities of metabolite in a chromatographic mobile phase matrix or with a somewhat impure sample when actual isolation must be accomplished. Chemical reactions are seldom used as a means of determining metabolite structures except in special cases of derivatization to facilitate chromatography or spectroscopy. The workhorse analytical method for metabolite separation and detection is LC combined with *radiometry* [115]. Structural identification of metabolites is mainly accomplished with *mass spectrometry* (MS) coupled with LC, so that metabolites are separated and introduced one-by-one into the inlet of the mass spectrometer (LC-MS). Several types are available depending on the exact information sought [116]. When necessary, *nuclear magnetic resonance spectroscopy* (NMR) is a powerful adjunct to MS that can typically provide structural information which is difficult or impossible to obtain from MS alone, such as the exact position of a hydroxyl group introduced by a CYP enzyme [117].

Since enzymes are involved in almost all metabolic transformations, we typically expect to see stereoselectivity of the metabolism when the parent drug or the metabolite contains one or more stereogenic centers [118]. The significance of *stereochemistry* in metabolism comprises four distinct phenomena. First, a drug which has a stereogenic

center (e.g., *R,S*-warfarin) may show differential rates, metabolic pathways and enzyme preference between the *R*- and *S*-stereoisomers [119]. Second, a drug lacking a stereogenic center may be metabolized in such a manner that a chiral center is introduced, and the enantiomeric metabolites are unlikely to be produced in equal proportions. For example, the achiral drug risperidone is hydroxylated by CYP enzymes to produce both *R*- and *S*-9-hydroxyrisperidone metabolites [119]. Third, a drug containing a stereogenic center may undergo metabolism that eliminates chirality at that carbon atom. For example, the stereogenic secondary alcohol functional group in ezetimibe is oxidized to an achiral ketone (see Fig. 1.13 in Table 1.1). Finally, a drug containing a stereogenic center may undergo *stereochemical inversion*. For example, chiral “profen” drugs such as ibuprofen are metabolically inverted such that the pharmacologically inactive *R*-isomers are converted to the active *S*-isomers [120,121]. In this last example, the subtle change of structure, although important for fully understanding the activity of the drug, would go undetected by ordinary achiral means of chromatography and structural analysis. Therefore, inversion of configuration represents a type of metabolism that must be specifically investigated by chiral methods on the “parent”-drug chromatographic peak.

Since the exact nature of the metabolic reactions observed to occur with a particular drug determines which enzymes are involved and whether active, toxic, or reactive metabolites are generated, a natural exercise is to try to predict preclinically which reactions will be most important for a given drug candidate in humans. Much effort has been devoted to this endeavor and considerable progress has been made, but these computational predictions have limitations and are not yet completely reliable [122]. Nonetheless, predictive software has been documented to be of value in avoidance of certain metabolic liabilities during the design of new drug candidates [123].

1.7.3 Quantitation of Extent of Metabolism

The fraction of the absorbed dose that is metabolized before excretion is called the *extent of metabolism*. Although the fraction of the total drug-related material represented by metabolites in blood is frequently equated with the extent of metabolism, this is clearly not correct, since the concentrations of metabolites in blood compared to the concentration of parent drug are determined not only by the extent of metabolism but also by the PK CLs and volumes of distribution of the metabolites relative to the parent drug. Correct assessment of the extent of metabolism involves summing up the amounts of all metabolites excreted in urine and feces and expressing as a percentage of the administered dose. Since most marketed drugs are orally administered, then when absorption from the intestinal tract is incomplete, unabsorbed parent drug is subject to bacterial metabolism because of the microorganisms in the large intestine. It is often difficult to distinguish whether certain metabolic reactions such as hydrolysis and reduction occurred systemically or in the intestinal lumen. A related ambiguity occurs when a reversible type of metabolism such as glucuronidation or *N*-oxidation occurs in the liver followed by secretion into the intestine through the bile. Bacterial enzymes often can hydrolyze glucuronides and reduce *N*-oxides back to parent drug, which would be recovered in the feces. In that case, we would underestimate the extent of metabolism by assuming that the parent drug recovered in feces had not been metabolized. Sampling of bile in animal studies can alert us to the probability

of reversible metabolites in bile. However, while it is possible to obtain human bile samples as well, this is not routinely done in the absence of a reason more compelling than just detection of reversible metabolism. For these reasons, it may be difficult to reliably assess the true extent of human metabolism in certain cases.

1.7.4 Elimination of Drug-Related Materials from the Body

The “E” of the ADME study is measured as the sum of radioactivity recovered in both urine and feces after exhaustive collection, typically two weeks for an average drug. Obviously, drugs exhibiting a long PK half-life might require more than two weeks to be completely excreted. In other cases, incomplete recovery of radioactivity may indicate that metabolites are retained for a prolonged period, though it might also have just been the result of losses during collection of urine and feces. A reasonable expectation for total recovery of excreta over two weeks from human subjects is about 80–90%, so that recoveries which are significantly lower than that may indicate prolonged retention of metabolites, possibly as drug residues covalently bound to tissues [124]. In animal ADME studies, the residual radioactivity can be measured in carcass to resolve the ambiguity of long-retained metabolites versus simple losses, and this information can be helpful in understanding a human mass balance study with low recovery. A drug that clearly accumulates in the body, as either parent drug or metabolites, is a cause for concern and a possible reason for discontinuation of further clinical development.

1.8 CONCLUSION

Drug metabolism is nominally a chemical event, and the purely chemical aspects of the study of the process, such as separation and rigorous structural identification of the metabolites, require the most advanced chromatographic and spectroscopic chemical techniques. The chemical outcome of drug metabolism is important because the metabolites produced may have pharmacological or toxicological properties of their own, independent of those of the parent drug. However, drug metabolism arises from a complex set of biological phenomena that is only manifest at the end as a chemical transformation of a drug molecule. The biological aspects are equally important as the chemical aspects in contributing to the overall clinical profile of a drug. These biological aspects include the rate of enzymatic conversion of drug to metabolite, which largely determines the biological half-life, the particular enzymes that are responsible for the conversion, because some enzymes are subject to genetic polymorphism, and the regulation of those enzymes and their associated cofactors. Metabolism-based drug–drug interactions, which can seriously compromise the clinical usefulness of a drug, represent a higher-order feature of the biological side of drug metabolism. These clinical consequences of drug metabolism are the reasons that regulatory authorities require a complete description of the metabolic fate of a new drug candidate. Furthermore, the desire to design “druglike” molecules to minimize metabolism-related difficulties in clinical development explains why drug discovery organizations expend considerable effort to characterize the metabolism of research molecules before selecting the best one to enter clinical development.

ABBREVIATIONS

AcSCoA	Acetyl Coenzyme A
AMP	Adenosine Monophosphate
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CoASH	Coenzyme A
CPR	Cytochrome P450 Reductase
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GSH	Reduced Glutathione
NAD ⁺	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADP ⁺	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
PAPS	Phosphoadenosine Phosphosulfate
SAM	S-Adenosine-L-Methionine
UDP	Uridine Diphosphate
UDPGA	Uridine Diphosphate Glucuronic Acid

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2 *In Vitro* and *In Vivo* Models of Drug Metabolism

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2.1	Summary	1
2.2	<i>In vitro</i> models of drug metabolism	2
2.3	<i>In vivo</i> models of drug metabolism	8
2.4	Metabolism studies in drug discovery and development	16
2.5	Future directions	23
	References	24

2.1 SUMMARY

Drug metabolism is the body's inherent ability to remove foreign compounds (xenobiotics) that have either intentionally or unintentionally made their way to the systemic circulation or organs within the body. Understanding the metabolism of newly developed drugs is paramount because of the central role metabolism plays in unraveling the possible toxicity or developability of new drug candidates. For example, the rate of drug metabolism in part determines the bioavailability, clearance, and half-life of a drug, and together, these parameters define how much drug (dose) and how often the drug needs to be dosed. Both these factors (dose size and frequency) can be significant hurdles to new drugs especially if the dose or dosing frequency is too high, making the drug unmanageable to develop or market. Also, metabolism is often considered a detoxification pathway by which the molecules are chemically modified to make them more polar and easily excreted from the body, eventually rendering them pharmacologically inactive. However, there are numerous examples where metabolism renders a compound less soluble (N-methylation reactions), pharmacologically active, or toxic. Therefore, it is always important to understand the full set of metabolic reactions and enzyme involved in the metabolism of a compound in both preclinical models (pharmacokinetic, pharmacological, and toxicological) as well as in human to avoid any unnecessary complications or failures in drug development.

Drug metabolism occurs everywhere within the body to differing extents; however, metabolism occurs predominately in those organs involved in elimination (e.g., kidneys and liver) and those organs to which the drug is first exposed [e.g., gastrointestinal

tract (GI) and liver]. The most common drug-metabolizing organs or compartments are liver, GI tract, kidney, plasma, brain, skin, lung, and nasal mucosa with the liver and GI tract being the most abundant in enzyme level and capacity [1]. Often overlooked are another set of drug-metabolizing enzymes found in the GI tract, which being the microflora that can metabolize foreign compounds as they travel along the lower GI [2–4]. Typically, these microorganisms act through hydrolysis and reductive metabolic pathways, given the environment in the lower GI tract. Therefore, metabolites found in feces often need to be considered as being derived from metabolic reactions of the host organism as well as the microflora.

Clearly, metabolism can occur in many sites and organs within the body, but often specific cell types within the organ are responsible for the majority of metabolic reactions, such as the hepatocyte in the liver and the enterocytes within the intestine.

Focusing even further down, there are numerous subcellular organelles within each organ that contain significant quantities of drug-metabolizing enzymes that participate in the metabolism of xenobiotics, such as the microsomal fraction (derived from the endoplasmic reticulum), cytosol, and mitochondria. The following section describes various *in vitro* and *in vivo* models that are often employed throughout drug discovery and development, as well as the purpose each model system serves to provide valuable information to elucidate the metabolic disposition of new and existing drug molecules. The information contained within will be based on the authors own experiences and numerous seminal publications in the area of *in vitro* and *in vivo* drug metabolism [1,5,6].

2.2 IN VITRO MODELS OF DRUG METABOLISM

In vitro models of drug-metabolism range from single enzymes to whole organs. The models can be categorized into two distinct groups: (i) models that contain intact cells, such as whole organ, slices, or cells and (ii) subcellular fractions. Each model system has its own utility in the study of drug metabolism in either drug discovery and/or development along with a set of advantages and disadvantages, all of which are highlighted in the following sections. General procedures are provided for the isolation and characterization of the most commonly employed *in vitro* model systems, such as microsomes, cytosol, and hepatocytes, which are described in detail by Guengerich and LeCluyse, respectively [7,8].

2.2.1 Individual Enzymes

The single or individual enzyme system is a useful tool to study enzymatic activity in a controlled environment of enzyme content, buffer, and cofactors all of which can be easily manipulated. Generally, the total protein content of such incubations is extremely low and reduces the effects of nonspecific binding, which can be a benefit as opposed to using large quantities of enzyme mixtures to obtain the same amount of enzyme activity. Often, the specific content or quantity of enzyme is known and as such the enzyme activity or turnover can be directly measured as a function of “nanomoles of enzyme” as opposed to the generic measure of “milligrams of protein” or “number of cells.”

Enzymes were initially isolated from a tissue, which is a labor-intensive procedure, leading to significant loss of enzyme content (activity) with each successive isolation

TABLE 2.1 Applications of *In Vitro* Drug Metabolism Models

	Single Enzymes	Subcellular Fractions		Hepatocytes	
		Microsomes	Cytosol	Suspension	Culture
Metabolic stability	—	XXX	X	XX	—
Clearance prediction	X	XXX	—	XXX	X
Metabolic profiling/species comparisons	X	XXX	XX	XXX	X
Metabolite identifica- tion/bioactivation	XX	XXX	X	XX	—
Inhibition studies	XXX	XXX	—	X	—
Induction studies	—	—	—	—	XXX
Reaction phenotyping	XXX	XXX	XX	XX	—
Bioreactor	XXX	XXX	XX	X	X

XXX, commonly used; XX, sometimes used; X, rarely used.

step. Few enzymes today are isolated by this method, however, some are still easy and cost effective to isolate in this manner, such as β -glucuronidase from snails and alkaline phosphatase from bovine liver or intestine. More commonly, enzymes are individually expressed as recombinant enzymes through cDNA-directed expression systems in a variety of host cell lines (mammalian, bacterial, insect, and yeast) and are commercially available from multiple vendors [9–12].

The most commonly employed single-enzyme family is the cytochrome P450 (CYP) family of isozymes, which have been used to evaluate metabolic stability, human hepatic clearance, drug–drug interaction potential (inhibition), reaction phenotyping (i.e., identification of enzymes involved in metabolism of a drug candidate), the effect of allelic variants of drug-metabolizing enzymes on metabolism and elimination, and the potential to undergo bioactivation or covalent binding; serve as bioreactors to generate significant quantities of metabolites for identification purposes; and as a tool to directly study enzyme structure and function [10,12–16]. Table 2.1 illustrates the utility of single-enzyme systems across a range of drug-metabolism studies conducted throughout drug discovery and development. Many of the other individually expressed drug-metabolizing enzymes (e.g., glucuronyltransferases, sulfotransferases (STs), epoxide hydrolases, flavin monooxygenases (FMOs), glutathione *S*-transferases, and *N*-acetyltransferases [11,17]) can also be used for the aforementioned studies; however, they are used less often than the CYP enzymes because of the propensity of most drugs to be substrates of one or more of the CYP isoforms.

In some situations, the results from single-enzyme systems may not always mimic other *in vitro* models or *in vivo*. For example, if the overall metabolism is derived from multiple individual enzymes then the activity from a single enzyme will not properly reflect the overall metabolic clearance. Also, there are numerous examples where mechanism-based inactivation has been observed in recombinant enzyme preparations but not in other *in vitro* models such as liver microsomes likely because of more efficient formation of reactive metabolites in the focused individual enzyme system [18]. In general, the single-enzyme system will tend to overemphasize the importance or potency of metabolic reactions, nonetheless, they are extremely useful in breaking down complex metabolic pathways into individual pathways or enzymes.

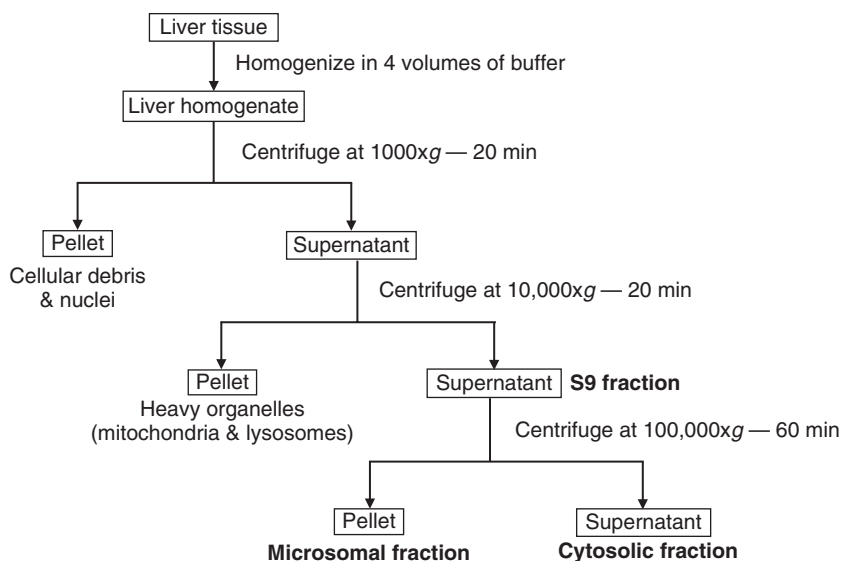


Figure 2.1 Isolation procedure for liver subcellular fractions: S9 fraction, cytosol, and microsomes.

2.2.2 Subcellular Fractions

By use of tissue homogenization and differential centrifugation, it has become possible to obtain various subcellular fractions such as S9, cytosol, and microsomes. Depending on the tissue, either the entire organ or portions of the organ are more appropriate for subcellular isolations. For example, when preparing liver subcellular fractions, the entire liver is used; however, for intestinal or kidney microsomes, generally only certain cell types or tissue layers containing the enzyme(s) of interest are used to prepare subcellular fractions (e.g., enterocytes from the intestine).

Figure 2.1 outlines the general procedure for the isolation of liver subcellular fractions and the procedures for the most common drug-metabolizing organs, liver, and intestine are described in detail in the Refs 8 and 19. Briefly, the procedure begins with cooling the excised liver as quickly as possible to 4°C with buffer and all remaining buffers and steps are conducted at 4°C to ensure the best possible enzyme stability. The liver is then homogenized in four volumes of ice cold buffer and centrifuged at low speed (1000×g) to remove unbroken cells, cellular debris, and nuclei. The supernatant from this step is then centrifuged at 10,000×g to further pellet unwanted organelles such as mitochondria and lysosomes (note this pellet may be used for other drug-metabolism-related experiments). The supernatant from this step is the S9 fraction and is centrifuged at 100,000×g to yield the supernatant (cytosolic fraction) and the microsomes (pellet of endoplasmic reticulum). These subcellular fractions can be stored frozen at −80°C for long periods of time (years) and as such they serve as a convenient source of enzyme for drug-metabolism studies and have become widely available commercially. Owing to their ready availability, subcellular fractions are used in a variety of drug-metabolism- and toxicity-related studies [11–13,20–22].

Most drug-metabolizing enzymes are located in the cytosolic and microsomal fractions as outlined in Table 2.2, although others can be found in lysosomes, peroxisomes,

and mitochondria. The predominate subcellular fraction for evaluating drug metabolism is the microsomal fraction. The microsomal fraction containing the smooth endoplasmic reticulum is the most commonly employed *in vitro* model because most drugs are metabolized by the cytochrome P450s or by glucuronidation, both of which are found in the microsomal fraction. Table 2.1 illustrates how the microsomal model is used in nearly every evaluation except enzyme induction. The cytosolic fraction is used less frequently, however, when the major enzyme involved in metabolism or inhibition (e.g., sulfotransferase or aldehyde oxidase (AO)) of a particular drug is located in the cytosol then this model becomes extremely useful. The S9 fraction also has utility when complex metabolic pathway need to be studied, for example, when multiple different metabolic pathways are involved, such as hydroxylation by CYP followed by sulfation or carbonyl reduction followed by CYP-mediated oxidation and then some form of conjugation. Often when the metabolic pathways become too complex, it is better to use a more appropriate and relevant system such as the hepatocyte model, which is discussed in the following section.

2.2.3 Cellular Systems

Cellular systems can be categorized into two general groups: primary cells isolated from tissues and immortalized cell lines. Both have utility in assessing a variety of drug-metabolism functions; however, the primary cells tend to be employed more often in routine characterization, whereas immortalized cell lines are often used in early drug discovery and in higher throughput assays [23,24]. Primary animal or human hepatocytes isolated from liver tissue is the predominate cell-based model to evaluate drug metabolism (Table 2.1) [23,25]. Like the aforementioned subcellular fractions, hepatocytes can also be cryopreserved and stored for long periods of time, which makes them highly useful for routine or even high throughput assays to assess metabolic stability, metabolite identification, induction, or cytotoxicity. The most common method of isolating hepatocytes from all species is the two-step perfusion method that utilizes a calcium-free buffer in the first step followed by a buffer containing the enzyme collagenase [7,26]. The isolation of hepatocytes from liver tissue is not extremely challenging; however, time and practice are necessary to obtain high quality cells especially from animals other than the rat. Depending on the study type, the hepatocytes can be used as a cell suspension (generally only for 2–3 h) or in cell culture (for several days) [25]. Hepatocytes in suspension represent a fully functional unit of drug metabolism from the liver containing normal concentrations of enzymes and cofactors with the ability to perform complex and multistep metabolic reactions. Therefore, hepatocytes in suspension are commonly used to determine metabolic stability, predict human metabolic clearance, and compare metabolic pathways or metabolite identification across multiple species [22,25,27–30]. Less often, but still of value are studies using hepatocyte suspensions for assessment of drug inhibition, reaction phenotyping, bioreactors for metabolite formation, and cytotoxicity measurements. Unfortunately, when in suspension, the hepatocytes are no longer attached to an extracellular matrix or to one another, which is an inappropriate environment for the cells. Hence, the viability of the cells will begin to decline somewhere around 2–3 h in suspension limiting the length of incubation time and, therefore, the degree or extent of metabolism. Hepatocytes in culture will survive for several days or even weeks because of the artificial environment created in culture with an extracellular matrix and cell-to-cell contact. Hepatocytes in culture

are predominately used to assess enzyme induction (transcriptional activation) and the potential for drug–drug interactions or cytotoxicity [22,26,31,32]. Unfortunately, even in culture, the hepatocytes will begin to dedifferentiate and the phenotypic properties of the cells may start to change within 24–48 h in culture. Most important of these changes are those of the CYP enzymes, which in general will decrease with time in culture; however, some CYPs have been shown to increase and others to decrease and then increase with culture time. Owing to these ever changing levels of the major drug-metabolizing enzymes, many of the other drug-metabolism-related studies are not suited for this model. However, modern advances in cell culturing techniques such as microfluidic flow systems, micropatterned hepatocyte coculture systems, and different three-dimensional cultures such as spheroids have made significant advances in improving the stability and longevity of drug-metabolizing enzymes in culture [33–35].

Immortalized cells are cells that can grow and divide indefinitely under optimal conditions, hence they are attractive as a cellular model, which can provide an inexpensive supply of cells that are phenotypically stable. A variety of cell lines are currently available and used in the assessment of drug metabolism, such as HepG2, Fa2N4, BC2, and HepaRG [23,24,36,37]. Each cell line has a particular or general utility in drug-metabolism studies because each cell line is unique in the expression of drug-metabolizing enzymes (oxidative enzymes such as CYPs or conjugation enzymes), transcription factors (e.g., pregnane X receptor or constitutive androstane receptor), or drug transporters. Therefore, when deciding which cell line to employ, one must first consider the type of evaluation or question is being asked, for example, is the experiment designed to assess metabolite formation/identification or enzyme induction. Although immortalized cell lines are readily available and useful for many applications, there is no single cell line that recapitulates the genotype and phenotype of a primary human hepatocyte and therefore cannot be used as a replacement for primary hepatocytes but can be used in higher throughput models as primary or secondary screens.

2.2.4 Organ Slices

Tissue slices have been prepared from multiple organs, predominately the liver and also intestine, kidney, brain, and lung [11,38,39]. Tissues such as the liver and heart are rigid enough to be sliced directly; however, soft tissues such as the intestine and lung are generally imbedded in agarose and then sliced [39]. Tissue slices, in contrast to the aforementioned *in vitro* models, represent a higher level of structural integrity and architecture containing all of the organ cell types, cellular attachments to basement membranes, and cell-to-cell contacts [40,41]. Liver slices, first noted in the literature in the 1920s and 1930s, are one of the earliest *in vitro* models to evaluate metabolism, pharmacology, and toxicology [42,43]. The early slices were extremely crude and slice thickness was not reproducible, however, they did demonstrate metabolic activity. With the advent of specialized slicing, such as the Krumdieck and Brendel tissue slicers, tissue slice thickness became more reproducible and slices were prepared with minimal tissue damage. Typical liver slices range in thickness from about 200 to 300 μm and generally maintain metabolic function for 24–96 h under appropriate culture conditions. Today, the technology of tissue slicers continues to improve [44], as do the culture conditions, which have moved in the direction of fluidic perfusion techniques [45] as

compared to the earlier roller incubators or multiwell plates, which bathed the tissue slice in media in an atmosphere of oxygen and carbon dioxide.

Tissue slices have many of the same applications as hepatocytes or other cell-based models; however, the advantage of intercellular connections between different cell types within the slice can provide a greater level of cellular communication and function especially in cases where multiple cell types are necessary for complex metabolic or toxicological pathways. As with the cellular models, the enzymatic activity (e.g., CYPs) within the slice begins to decline shortly after being placed in culture. Early applications of tissue slices were the assessment of toxicology and pharmacology, however, drug-metabolism studies such as metabolite identification, species comparisons, and enzyme induction have been prominently noted in the literature [39,41,46–48]. A somewhat controversial application of liver slices is that to predicting metabolic clearance. Concerns have been noted that the slice thickness acts as a diffusional barrier for access of drug and oxygen to the interior of the slice. With some drugs, the rate of metabolism may not be entirely based on metabolic reactions but on the rate of drug diffusion throughout the slice, hence the rate of determination may be compromised by diffusion or transport. Owing to this uncertainty, slices have not been used routinely as a model to assess clearance, however, they do recapitulate the rank order of metabolic rates, just not the values calculated from *in vivo* or other *in vitro* methods such as hepatocytes [40,41].

Tissue slices are not amenable to cryopreservation, therefore, experiments must be conducted on freshly isolated and sliced organs that makes tissue slices not as useful for routine studies, especially when slices are required from species other than the rat. Many of the same drug-metabolism studies that are conducted in liver slices can also be performed using hepatocytes, which can be cryopreserved making the hepatocyte model a more routine model for most applications. An advantage of tissue slices, similar to hepatocytes, is the ability to reproduce complex metabolic processes where more than one metabolic reaction occurs on the molecule (oxidation in combination with conjugation) or when more than a single cell type is required for a reaction or biochemical process.

2.2.5 Organ Perfusion

Organ perfusion experiments can be conducted either *ex vivo* or *in situ*, but it is not technically an *in vitro* method; however, it is covered in this section to distinguish the technique from live intact animal studies. Perfusions have been conducted on many organs such as the liver, heart, lung, kidney, brain, intestine, intestine–liver preparations, and pancreas [49–51]. There are several key references that outline the procedures for organ isolation and perfusion for both liver and intestine [50,52,53]. Owing to the size of these organs, most organ perfusions are generally limited to smaller animals such as rats and rabbits. Organ perfusions, such as the liver or intestine, offer several advantages over other techniques in that the delivery and sampling of the drug under investigation can be by physiological routes, such as the portal vein, bile duct, or intestine. In addition, the concentration of drug, buffer, and rate of drug delivery can all be easily manipulated during the experiment and tailored to the needs of the investigator. A significant disadvantage of the isolated perfused organ method is that only a single organ (i.e., single experiment) can be conducted per animal, which is in contrast to all of the previously described methods.

Liver perfusions or the combination of intestine–liver perfusions provide a good model to assess first-pass metabolism as well as the combination of drug-metabolizing enzymes in conjunction with transporter-mediated efflux (both are mechanisms of drug clearance from the liver). For example, Xu *et al.* [49] used the intestine–liver dual perfusion model to evaluate the first-pass metabolism of salicylamide, while other researchers have used the liver perfusion model as a means to study the interplay between metabolism and transport-mediated elimination or inhibition of transport-mediated elimination [54,55]. There is no doubt that the organ perfusion model replicates many of the delivery, sampling, and connectivities between enzymes and transporters that other *in vitro* models cannot duplicate, making it a powerful tool to study these combinatorial drug disposition and metabolism effects.

2.3 IN VIVO MODELS OF DRUG METABOLISM

In vitro models of drug metabolism are extremely useful to study specific reactions, mechanisms, or structure–activity relationships, however, *in vivo* systems are required to understand additional factors that may contribute to the type of metabolism, rate of metabolism, or other elimination pathways in the overall disposition/exposure of a drug or metabolites. For example, extrahepatic metabolism, protein-binding effects, first-pass metabolism, or the effects of a disease state on drug-metabolizing enzymes [56]. In addition, when metabolites are present in high concentrations, they may need to be quantitated as they may contribute to target pharmacology or off-target liabilities [57,58]. *in vivo* drug-metabolism studies are categorized as preclinical (animal) and clinical (human) studies. In broad terms, the purpose of these studies is to assess the metabolic profile of a drug across animal species and eventually humans. By identifying the *in vivo* metabolites, correlations can be made to the *in vitro* metabolic profiles, assess the possibility of active or toxic metabolites, and determine the elimination pathways of parent drug and metabolites.

2.3.1 Preclinical Animal Studies

The most commonly sampled biological matrices from *in vivo* drug-metabolism studies include plasma, urine, and bile, as well as feces in some situations. Plasma and urine are readily sampled matrices, however, bile collection requires surgically altered animals that have been bile duct cannulated [56]. In such studies, plasma, urine, bile, and feces can be examined for parent drug and metabolites [59,60]. Many drugs are eliminated in bile as parent drug and especially as metabolites. Without the use of bile duct cannulated animals (e.g., intact animals), it would be difficult to assess the overall routes of drug or metabolite elimination, metabolic pathways, or amount of metabolism because of the elimination of bile into the GI tract, which eventually appears in feces. As mentioned previously, identification of parent drug or metabolites is challenging from feces because of the microflora in the GI tract, which can further metabolize compounds or alter metabolic profiles.

In early drug discovery, metabolite identification is conducted with “cold” or non-radiolabeled drug, and it can often be difficult to obtain a full metabolic profile along with metabolite concentrations. However, in late drug discovery or drug development,

radiolabeled compounds (carbon-14 or tritium) are employed as substrates and metabolite identification is more straightforward as even minor metabolites can be observed and quantitated with respect to one another and parent drug. In rarer instances, stable labeled drugs can aid in elucidating the pharmacokinetics, disposition, or metabolism of a drug from *in vivo* studies as well as address mechanistic questions from *in vitro* models [61].

An essential *in vivo* animal study is the mass balance study conducted in rodent and nonrodent species. In this study, animals are dosed either orally or intravenously (or both) with a radiolabeled drug and radioactivity is measured from bile, urine, feces, exhaled $^{14}\text{CO}_2$, animal carcasses, and cage washings [62]. The purpose of this study is to (i) determine the major elimination pathways of drug and drug-related material; (ii) determine the total recovery of radiolabeled material (what goes in should come out); and (iii) conduct metabolic profiling from the various matrices collected to verify that the human metabolites generated are the same as those formed in sufficient quantities in the toxicology models. Sometimes, specific tissues may be collected at various time points for determination of total radioactivity or metabolites to determine tissue distribution (e.g., brain, liver, adrenal). In general, good recovery of radioactivity after a sufficiently long collection period would be 90%, 85%, and 80% for rat, dog, and human studies, respectively [63]. Generally, monkey studies are more challenging and lead to somewhat less recovery of total radioactivity due to issues with incomplete dosing and sample collection.

Additional studies related to drug metabolism in preclinical species can include regulation of drug-metabolizing enzymes, understanding the mechanism of low systemic exposure, and determination of hepatic extraction. Enzyme induction in animals can be assessed by dosing animals for multiple days (generally one to two weeks) after which the liver is removed and the RNA expression or enzyme activity of various drug-metabolizing enzymes (typically CYP1A, 2B, and 3A) are measured. In some situations, the administration of specific or global enzyme inhibitors can help elucidate the enzymes involved in metabolism or determine the mechanism of low exposure. For example, the global CYP450 inhibitor 1-aminobenzotriazole (ABT) can be administered to rats to address (i) gut versus liver contributions to first-pass metabolism [64] or (ii) low systemic exposure due to poor absorption or high first-pass metabolism [65]. Determination of hepatic extraction can be accomplished by infusing the drug via the portal vein and measuring systemic parent drug and/or metabolites [56]. This is a reasonable model when conducted in rats; however, in higher animal species such as the dog or monkey, it generally requires implantation of vascular access ports that lead to the portal vein [65].

2.3.2 Species Differences in Drug Metabolism

Species differences in drug metabolism are common and frequently complicate the extrapolation of metabolite data from preclinical animal models (in particular, pharmacology and toxicology species where active or toxic metabolites, respectively, can have dramatic effects) to the human metabolic profile [66]. Species differences in drug metabolism are generally related to (i) lack of a specific enzyme either in humans or animals; (ii) similar enzyme orthologs between species, which form different metabolite products; or (iii) greatly reduced activity of particular enzymes in preclinical species

that may predominate in humans, for example, AO [66]. Many common species differences in drug metabolism is described in the chapter titled *Sex Differences in Drug Metabolism* of this volume. Some of the most common species difference relate to the CYP family of enzymes [66,67], metabolites formed by N-acetylation where dogs lack this particular enzyme [68,69], or the formation of quaternary *N*-glucuronides where this particular conjugate is typically only formed in humans and monkeys but not in standard preclinical species [70]. For example, the anticonvulsant lamotrigine is formed in humans and monkeys as a major metabolite; however, this same metabolite is not found in standard preclinical species (rat and dog). Interestingly, the quaternary *N*-glucuronide of lamotrigine is the major metabolite formed in guinea pigs [71,72]. Finally, a number of drugs have the ability to induce various sets of drug-metabolizing enzymes in the liver and various other tissues such as the intestine. The major mechanism by which drug-metabolizing enzymes are induced are via transcriptional activation through nuclear hormone receptors or transcription factors. These receptors have been well characterized and shown to have significant species differences with regard to ligand binding and ultimately induction of drug-metabolizing enzymes. Therefore, observation of enzyme induction in any single species has little association to induction in other species, in particular, human enzyme induction [31,32,67,73].

2.3.3 Transgenic and Chimeric Mouse Models

The involvement of a single enzyme, transporter, transcription factor, or a small set of these proteins that are involved in metabolism, bioactivation, disposition, gene expression, or toxicity of a drug can be evaluated through the use of genetically modified knockout or humanized mouse models [74–78]. A large number of knockout mouse models (drug-metabolizing enzymes, drug transporters, and transcription factors involved in the regulation of enzymes and transporters) have been generated through genetic manipulation, Tables 2.3–2.5 [78–84]. The knockout mouse model is extremely useful in understanding the function of a single gene (protein) or the function of multiple enzymes all of which are controlled from a single gene. For example, cytochrome P450 reductase is an obligatory enzyme involved in all cytochrome P450 oxidations, and a cytochrome P450 reductase knockout mouse would be devoid of significant P450-mediated oxidation. The P450 reductase mouse (also known as POR KO or Cpr KO) was useful in distinguishing between cytochrome P450-mediated and FMO-mediated N-oxidation of the antitumor agent C-1311 [85]. When C-1311 was dosed to reductase knockout mice, there were only limited changes to the metabolism of the parent drug and formation of the *N*-oxide was unchanged. These data helped support the presumption that drug–drug interactions due to changes in cytochrome P450 enzyme activity would be greatly reduced when employing this antitumor agent because the drug interacted with FMO and not P450.

Humanized mouse models are created by insertion of a human gene(s) into the mouse genome, or more often, by deletion of the mouse orthologous gene and insertion of the human gene (Tables 2.3–2.5). This second method is generally preferred as it eliminates the confounding factors involved with having both human and animal orthologous genes present and functional at the same time. For example, the transgenic mice expressing the human UGT1A (1–9) locus demonstrated expression of the nine

TABLE 2.2 Subcellular Location of Drug-Metabolizing Enzymes

Reaction	Enzyme	Location
Oxidation	Cytochrome P450 (CYP)	Microsomes
	Flavin monooxygenase (FMO)	Microsomes
	Aldehyde oxidase (AO)	Cytosol
	Xanthine oxidase (XO)	Cytosol
	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria and cytosol
	Monoamine oxidase (MAO)	Mitochondria
	Dihydrodiol dehydrogenase	Cytosol
	β -Oxidation	Mitochondria and peroxisomes
Hydrolysis	Carboxylesterases (esterase/amidase)	Microsomes, cytosol, lysosomes, plasma, blood
	Epoxide hydrolase	Microsomes and cytosol
	Peptidase	Blood and lysosomes
Reduction	Sulfoxide/disulfide reductase	Cytosol
	Quinone reductase	Microsomes and cytosol
	Reductive dehalogenation	Microsomes
	Azo and nitro reductase	Microsomes, cytosol, microflora
	Carbonyl reductase	Microsomes, cytosol, blood
Conjugation	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Methyltransferase	Microsomes, cytosol, blood
	<i>N</i> -Acetyltransferase	Cytosol and mitochondria
	Glutathione conjugation	Microsomes and cytosol
	Amino acid conjugation	Microsomes and mitochondria

human UGT1A genes in a similar pattern as observed in human tissue. Senekeo-Effenberger *et al.* [86] used this transgenic model to demonstrate that human UGT1A1, 3, 4, and 6 were targets of the peroxisome proliferator-activated receptor alpha (PPAR) and that with an appropriate PPAR ligand (WY-14643) dosed to transgenic mice, and these human genes were induced in the liver, GI, or kidney.

Along with the advantages of employing an *in vivo* knockout or humanized model, come a few precautionary notes. There are genetic manipulations that can precipitate inappropriate expression of the human transgene or endogenous mouse genes. For example, the expression of a human gene to evaluate metabolism should be expressed at levels similar to those found *in vivo* in humans. Often the expression of human transgenes can be lower than those found *in vivo* or may be expressed in the wrong tissues. This can be due to a lack of necessary regulatory sites in promoter regions or a lack of cellular components such as coactivators/cofactors from the mouse that do not interact properly with human promoter/gene complexes. In addition, the deletion of a mouse gene (knockout) or insertion of a human gene (humanized) can lead to changes (up or down) in the expression of other mouse genes because of changes in endogenous

TABLE 2.3 Knockout and Humanized Mouse Models of Phase I and II Drug-Metabolizing Enzymes

Gene	Knockout and/or Humanized
Cyp1a1	Knockout
Cyp1a2	Knockout
Cyp1a1/2 with CYP1A1/2	Knockout/humanized
Cyp2a5 with CYP2A6	Knockout/humanized
Cyp2c	Knockout
CYP2C18/19	Humanized
Cyp2d	Knockout
CYP2D6	Humanized
Cyp2e1 with CYP2E1	Knockout/humanized
Cyp3a	Knockout
CYP3A4	Humanized
Cyp3a with CYP3A4 (liver and/or gut expression)	Knockout/humanized
Gstp (α , ω , π , σ , θ , ζ)	Knockout
Sult1e1	Knockout
Ugt1 with UGT1A	Knockout/humanized
UGT2B7	Humanized
Nat1	Knockout
Nat2	Knockout
Nat1 with NAT1	Knockout/humanized
NAT2	Humanized
mEh (microsomal)	Knockout
sEh (soluble)	Knockout
P450 Reductase	Knockout

Source: Taken from Refs 76,78,80,82–84.

metabolic pathways caused by the gene deletion/insertion. Nonetheless, these models offer the advantage of studying a human drug-metabolizing gene (function) or transcription factor in an *in vivo* setting (mouse), thereby avoiding many of the limitations imposed by *in vitro* systems that cannot appropriately recapitulate such affects as protein binding, liver uptake, or the time course of drug/metabolite exposures. The use of these mouse models have significantly increased our knowledge of the expression and function of many enzymes and proteins involved in drug metabolism. The generation of additional knockout or humanized mouse models beyond those currently available will provide tools, much like the plethora of *in vitro* tools, to routinely study drug metabolism in greater detail using an *in vivo* approach.

The chimeric mouse model is a tissue-modified (humanized) mouse model of human ADME (absorption, distribution, metabolism, and excretion) toxicology. In brief, the mouse hepatocytes are destroyed *in vivo* and the liver is repopulated with human hepatocytes that function normally [87]. In this model, mice are genetically altered in such a way that leads to destruction of the native mouse hepatocytes. Two methods for destruction of the existing mouse hepatocytes have been reported: (i) expression of an albumin promoted urokinase-type plasminogen activator (uPA) or (ii) knockout of the fumarylacetoacetate hydrolase genes (Fah). In order for the human hepatocytes to repopulate the mouse liver, the mouse must be immunodeficient. This is accomplished

TABLE 2.4 Knockout and Humanized Mouse Models of Drug Transporters

Gene	Knockout and/or Humanized
Mdr1a	Knockout
Mdr1a/1b	Double knockout
Mdr1a/1b/Bcrp1	Triple knockout
Bsep	Knockout
Mrp1	Knockout
Mrp2	Knockout
Mrp2 with MRP2	Knockout/humanized
Mrp3	Knockout
Mrp4	Knockout
Bcrp1	Knockout
Ost (α)	Knockout
Pept2	Knockout
Mate1	Knockout
Oct1	Knockout
Oct1/2	Double knockout
Oct3	Knockout
Oat1	Knockout
Oat3	Knockout
OATP1B1	Humanized
Oatp1b2	Knockout

Source: Taken from Refs 78 and 83.

TABLE 2.5 Knockout and Humanized Mouse Models of Transcription Factors

Gene	Knockout and/or Humanized
Ahr	Knockout
Ahr with AHR	Knockout/humanized
Car	Knockout
Car with CAR	Knockout/humanized
Pxr	Knockout
Pxr with PXR (SXR)	Knockout/humanized
Car and Pxr	Double knockout
Car and Pxr with CAR and PXR	Double knockout/double humanized
Car and Pxr and Cyp3a with CAR and PXR and CYP3A4	Triple knockout/triple humanized
Ppar (α)	Knockout
PPAR (α)	Humanized
Rxr (α)	Knockout

Source: Taken from Refs 78,81 and 83.

by using existing immunodeficient mice, for example, severe combined immunodeficiency (SCID) mice or genetic manipulation, such as knocking out the recombinant activation gene-2 (RAG-2). After injection of human hepatocytes into the mouse spleen, over time, the mouse liver will become repopulated with human hepatocytes replacing >70–90% of the native mouse hepatocytes.

The chimeric mouse model has been shown to be useful in evaluating the metabolism, disposition, pharmacokinetics, pharmacology, and toxicity of many xenobiotics [87,88]. Using chimeric mice, several authors have been able to demonstrate metabolic profiles that were specific to humans and different from wildtype mice, for example, dexamethasone (CYP3A4), diclofenac (CYP2C9), paclitaxel (CYP2C8), and debrisoquine (CYP2D6) [88]. The model has also been shown to be a predictor of circulating human metabolites especially metabolites derived from multiple metabolic reactions [89]. The chimeric mouse model was also able to predict the human disposition of the drug cefmetazole. In mice and rats, cefmetazole is eliminated in feces (bile), whereas in humans, urinary elimination of cefmetazole is found. When dosed to chimeric mice, cefmetazole was found to be eliminated in urine, similar to the human elimination pathway and different than the wildtype mouse [88]. Drug–drug interactions have also been evaluated in the humanized mouse liver model. For example, the human-specific CYP3A4 inducers rifampicin and rifabutin were shown to induce human CYP3A4 in the chimeric mice but not in wildtype mice. Also, inhibition of human CYP2D6 was demonstrated by using quinidine as an inhibitor and debrisoquine as the probe substrate. The AUC and $C_{\max}$ of 4-hydroxy debrisoquine decreased significantly in the $uPA^{-/-}$ /SCID humanized mice when codosed with debrisoquine and quinidine but did not change in the control $uPA^{-/-}$ /SCID mice [88].

2.3.4 Human

A great deal of metabolism-related information can be obtained through preclinical animal studies and *in vitro* human studies, such as metabolite identification and disposition of metabolites. However, none of these systems can act as a substitute for metabolism studies in healthy volunteers or patients. As quickly as the first human pharmacokinetic study in phase 1, information can be obtained on the identity of metabolites in human plasma and their concentrations relative to the parent drug either from single- or multiple-dose studies. Although these early phase 1 studies provide information on plasma metabolites and exposure, they often do not represent the full metabolic profile or the routes of parent drug and metabolite clearances. The human mass balance/AME (absorption, metabolism, elimination) study provides definitive information on metabolic and clearance pathways of metabolites and parent drug. This experiment, required by regulatory agencies, has several key outcomes: (i) an accounting of the mass balance of a drug and drug-related material through excretion into feces and urine; (ii) identification of rates and routes of elimination and clearance pathways (urine, bile, metabolism); (iii) quantitative identification of metabolites and metabolic pathways; and (iv) qualitatively and quantitatively supporting the use of preclinical toxicology species regarding exposure of metabolites between the preclinical species and humans (i.e., no unique human metabolites not found in preclinical toxicology species or human metabolites with much greater exposures than those found in preclinical species) [90].

In order to achieve these goals, a radioactive dose of the drug is given, generally carbon-14 (^{14}C , C-14) or hydrogen-3 (tritium, ^3H , H-3) and urine and feces are collected for 7–10 days. If significant amounts of parent drug or metabolites are expected to be eliminated in bile (based on animal data) then bile can also be collected and quantitated for parent drug and metabolites, as well as metabolite identification [91]. The study is conducted in healthy volunteers or patients ($\sim N = 4\text{--}6$) as a single oral therapeutic dose or as an intravenous dose if the intended route of clinical use will be intravenous [92,93]. The amount of radioactivity is “as low as feasible” without compromising the ability to identify metabolites and measure elimination routes. The typical amounts of radioactivity for C-14 and H-3 studies are $\sim 50\text{--}100\ \mu\text{Ci}$ and $100\text{--}300\ \mu\text{Ci}$, respectively [94]. Also important is the location of the radiolabeled element in the parent drug molecule. The site of radiolabel should be chemically and metabolically stable so that the radiolabel remains with the parent drug and parent drug/major metabolites and is not lost into the endogenous pool of biochemicals (e.g., release as carbon dioxide). In some situations, typically with compounds with a central amide or ester functionality, the drug molecule can be metabolically “split” into two components. In this case, the radiolabel will be retained in only a portion of the drug and the nonradiolabeled fragment will be difficult to follow and quantitate. When this occurs, two radiolabels are placed in the drug molecule so that both portions of the drug can be traced and a full accounting can be made of metabolic pathways and routes of elimination.

In humans, a successful mass balance study will account for 80–85% of the radioactive dose in urine and feces, which is generally achievable for a compound with a reasonable half-life ($< 50\text{ h}$). Roffey *et al.* have shown that when the human half-life of the drug is longer than 75 h, the ability to recover $> 80\%$ of the radioactive dose decreases dramatically. They found that 80% of drugs with a short half-life achieved 85% or greater recovery, whereas only 28% of drugs with a half-life of $> 75\text{ h}$ achieved 85% recovery [63]. In this situation, the collection period can be extended to collect more of the radioactive dose, but in some situations, the amount of radioactivity being eliminated at later times becomes too low to accurately measure, and it is no longer practical to continue collecting urine or feces. Fortunately, complete recovery of radioactivity from this study is not a requirement as long as the other objectives of the study are met, such as an understanding of routes of elimination and metabolic pathways, as well as exposure to metabolites similar to preclinical toxicology species.

The human radiolabeled mass balance/AME study provides a great deal of information about human *in vivo* metabolic pathways and the identification of metabolites and their exposure. This study provides a definitive and quantitative profile of metabolites found in human plasma. These can be inactive metabolites, pharmacologically active metabolites, or toxic metabolites. The FDA in 2008 published a guidance entitled “Safety Testing of Drug Metabolites” that describes how and when to identify and characterize drug metabolites whose nonclinical toxicity needs to be evaluated [95]. Since its publication in 2008, many other publications by the pharmaceutical industry have described how to practically address the situation of human metabolites that may need to be evaluated for toxicity [96]. Throughout the literature, these are known as *MIST* publications (Metabolites in Safety Testing). Although human *in vitro* systems do a reasonably good job of identifying major metabolic pathways, they cannot recapitulate the phenomenon of absorption, drug or metabolite disposition, elimination

(plasma, urine, bile, feces), or the combined effects of hepatic and extrahepatic (e.g., gut or kidney) metabolism.

2.4 METABOLISM STUDIES IN DRUG DISCOVERY AND DEVELOPMENT

2.4.1 Predicting Human Hepatic Clearance

In vitro metabolism studies using human liver microsomes or human hepatocytes to predict human metabolic clearance from the liver is routinely performed as early as drug discovery screening and throughout drug development. The ability to predict the metabolic clearance is important because the clearance is related to the exposure and half-life of the compound, two components that are essential in predicting the dosing regimen [20,97]. One of the major objectives of the pharmaceutical industry is to develop low dose drugs that are taken once a day thereby improving patient compliance and efficacy. In order to achieve this goal, drugs are often tested in human *in vitro* systems that assess metabolic stability. Compounds that are very rapidly metabolized are indicative of drugs that will have a high metabolic clearance and are therefore undesirable because they will require twice daily or three times a day dosing to achieve efficacy. Compounds that demonstrate no turnover or metabolism *in vitro* may indicate that the drug may have a long half-life in patients. Compounds with extremely long half-lives may be a benefit for some indications; however, they are also a concern should undesirable side effects or toxicity be uncovered for which the effects are prolonged because of the longer exposure time. Therefore, understanding the metabolic stability of both unstable and stable drug candidates is important to our understanding of dosing regimen, efficacy, and safety.

Liver microsomes and hepatocytes are two *in vitro* systems commonly employed to assess metabolic stability by measuring the disappearance of substrate over time [29,98,99]. The simplest method of assessing metabolic clearance is by incubating the compound of interest at a low substrate concentration (typically $<1 \mu\text{M}$) and measuring the disappearance of substrate. This “half-life” method uses the assumption that the substrate concentration is much lower than the K_m of the metabolic reaction(s), hence the low substrate concentration. This half-life method is often utilized in drug discovery during lead optimization because it has the potential to be high throughput when paired with human liver microsomes and provides a quick estimation of human metabolic clearance. The *in vitro* intrinsic clearance is determined from the following equation:

$$CL'_{\text{int}} = \frac{0.693}{\text{In vitro half-life} \times \text{microsomal protein concentration}}$$

For more sophisticated assessments of hepatic clearance, measurements of Michaelis–Menten kinetic parameters (K_m and V_{max}) are determined. From the rate of metabolism, the microsomal or hepatocyte intrinsic clearance (measure of enzyme activity) is determined. The *in vitro* intrinsic clearance (CL'_{int}) is then scaled to whole liver intrinsic clearance (CL_{int}) and incorporated into the following equation (well-stirred model of hepatic extraction) to estimate hepatic clearance (CL_H).

$$CL_H = \frac{Q \times f_u \times CL'_{\text{int}}}{Q + f_u \times CL'_{\text{int}}}$$

where Q = liver blood flow, f_u = fraction unbound in plasma, and intrinsic clearance is defined as $CL'_{int} = V_{max}/K_m$. See the chapter titled Enzyme Kinetics of Drug Metabolizing Reactions and Drug–Drug Interactions of this volume for additional information. This method is much more labor intensive and therefore not often used in drug discovery, however it does eliminate the assumption inherent in the half-life method and the need to use a low substrate concentration.

For drugs that undergo oxidative metabolism (e.g., CYP450 mediated) or glucuronidation, liver microsomes are a simple and effective *in vitro* system to predict hepatic clearance [98,100]. For compounds that undergo extensive phase II metabolism or metabolism via cytosolic enzymes, hepatocyte incubations are the more appropriate *in vitro* system. The same equations as illustrated above are also employed, however, the scaling factors will be different. In order to build confidence in the predicted human hepatic clearance determination from either microsomes or hepatocytes, *in vitro*–*in vivo* correlations of predicted and actual clearance values with several animal species should be performed. Assuming that the metabolism and clearance pathways are similar between the animal species and humans, this *in vitro*–*in vivo* correlation can support or refute the predictability of human *in vitro* hepatic clearance determinations.

2.4.2 Reaction Phenotyping

It is now widely accepted that the fraction of the dose metabolized (f_m) by a given drug-metabolizing enzyme is one of the major factors governing the magnitude of a drug interaction and the impact of a polymorphism on drug clearance. Therefore, pharmaceutical companies routinely determine the enzymes involved in the metabolism of potential new drugs *in vitro* and relate this information to human data on ADME [13,101,102]. This so-called reaction phenotyping or isozyme mapping, usually involves the use of multiple reagents, such as recombinant enzymes, liver microsomes, human hepatocytes, and clinical studies with polymorphic populations [103]. Through an understanding of the enzymes involved in the disposition of a new drug, investigators can predict potential drug interactions, such as those due to metabolism by polymorphic drug-metabolizing enzymes [104,105] (e.g., CYP2D6 or CYP2C9/19) or coadministration with potent inhibitors or inducers of CYP450 enzymes (e.g., a CYP3A4 substrate with ketoconazole or rifampin, respectively).

There are three *in vitro* techniques that can address reaction phenotyping and many researchers will utilize at least two of them if not all three [106]. The first technique utilizes recombinantly expressed single enzymes. In this situation, the drug of interest can be incubated with individual enzymes and turnover of parent or formation of metabolite(s) can be measured. Those enzymes that demonstrate turnover are considered to be involved in the metabolism of the compound. By scaling the amount of turnover with the amount of each particular enzyme in the human liver, the quantitative relative order (or ranking) of importance of each enzyme to the total metabolism of the compound can be calculated [6,13,101]. A second common approach utilizes human liver microsomes and selective chemical inhibitors or inhibitory antibodies for individual CYP450 enzymes. These chemical inhibitors or inhibitory antibodies selectively knockout or eliminate the activity of a single CYP450 enzyme and when compared to uninhibited incubations, the relative contribution of each CYP450 can be determined. For example, furafylline is a selective inhibitor of only CYP1A2 at a concentration of 20 μ and is routinely used to assess the contribution of CYP1A2 to the microsomal

TABLE 2.6 Chemical Inhibitors and Recommended Concentrations for CYP Reaction Phenotyping in Human Liver Microsomes

CYP Form(s)	Inhibitor	Inhibitor Concentrations
CYP1A2	Furafylline α -naphthoflavone	10–30 μ M ^a 1 μ M
CYP2A6	Methoxsalen	1 μ M
CYP2B6	ThioTEPA	50 μ M
CYP2C8	Montelukast	0.1 μ M
CYP2C9	Sulfaphenazole	10 μ M
CYP2C19	Benzylrivanol	1 μ M
CYP2D6 CYP2D6 & CYP3A4/5	Quinidine	<2 μ M and 10 μ M
CYP2E1	Diethyldithiocarbamate	50 μ M
CYP3A4/5 & others	Ketoconazole	1 μ M and 10 μ M
CYP3A4/5	Troleandomycin	50 μ M ^a

^aTime-dependent inhibitors (inhibition potency increases with preincubation in the presence of NADPH).

metabolism during reaction phenotyping studies [13,101]. Table 2.6 lists the common inhibitors used for CYP450 reaction phenotyping studies in human liver microsomes, many of which can also be used for inhibition studies in human hepatocytes. The third *in vitro* technique is termed *correlation analysis* whereby the rate of turnover of the substrate is compared to the rate of turnover of an isoform-selective probe substrate. A good correlation indicates that the enzyme is involved in the metabolism. For example, correlating the rate of metabolism of a drug candidate with the CYP3A4-mediated metabolism of midazolam (formation of 1-hydroxymidazolam is specific to CYP3A4) with preparations of human liver microsomes from 8 to 10 different donors. If the rates of metabolism demonstrate a good correlation, then it is presumed that CYP3A4 is involved in the metabolism of the compound.

It is important that the data are consistent between the three techniques to develop an integrated *in vitro* reaction phenotype for the substrate. In addition, these data are integrated with the overall elimination (metabolism, renal, biliary) of the parent drug so that the impact of a drug interaction can be ascertained. For example, consider two compounds both metabolized only by CYP2D6; however, compound A also undergoes significant renal elimination as parent drug but compound B does not undergo significant renal or biliary elimination. In this situation, compound A will not show a significant drug interaction (increase in parent drug exposure) in poor CYP2D6 metabolizers because renal elimination will provide a secondary elimination pathway. Compound B, however, would be expected to show significant increases in plasma exposure in CYP2D6 poor metabolizers because the drug has no elimination pathway besides CYP2D6-mediated metabolism.

For the human cytochrome P450s, reagents for reaction phenotyping are readily available, such as recombinant enzymes, specific chemical inhibitors/antibodies, and probe substrates for correlation analysis. Therefore, CYP450 reaction phenotyping is routinely performed and required for regulatory submissions. Recombinant enzymes are also available for most of the remaining drug-metabolizing enzymes (UGTs (UDP-glucuronosyltransferase), STs, FMOs), however, specific inhibitors, scaling factors, or probe substrates are not as readily available for many of these enzymes, which

severely limits the amount of information that can be extrapolated to human metabolism or potential drug interactions. Phase II glucuronidation is the second most common metabolic reaction after the CYP450 oxidations and reaction phenotyping is gaining momentum with these enzymes as well [107]. In recent years, new selective substrates and inhibitors have been identified for many of the UGT isoforms [100,108].

2.4.3 Drug–Drug Interactions

2.4.3.1 Enzyme Inhibition. Inhibition of drug-metabolizing enzymes will have a profound effect on any other drugs that are predominately eliminated by the same enzyme. In today's environment of poly pharmacy, many patients take more than one medication at a time and the risk of a drug–drug interaction also increases with the number of comedications. While CYP450 enzymes are the most significant drug-metabolizing enzyme family, inhibition of these enzymes has a major effect on drug concentration or exposure of coadministered drugs. Inhibitory drug interactions often cause exaggerated pharmacology or off-target toxicities because of increased plasma exposures and can result in serious side effects or death in extreme cases. Therefore, pharmaceutical companies and regulatory agencies are keen to reduce potential liabilities associated with inhibition of drug-metabolizing enzymes and eliminate such drugs being developed or marketed.

Two types of inhibition are routinely evaluated in drug discovery and development: reversible inhibition and irreversible inhibition (see Chapter 2 for kinetic details on each type of inhibition). Reversible inhibition is the easiest to determine and often included in high throughput screening in early drug discovery. Reversible inhibition can take the form of multiple mechanisms; however, the most common are competitive inhibition and noncompetitive inhibition (or a combination of the two referred to as *mixed inhibition*). Commonly, drugs that are substrates for P450 enzymes will also be inhibitors of the same enzyme (competitive inhibition), however, drugs can also inhibit CYP450 enzymes but not be substrates (noncompetitive inhibition). A common example is quinidine, which is a potent inhibitor of CYP2D6 but is not a substrate of CYP2D6 (quinidine is a substrate for CYP3A4).

Reversible CYP inhibition is routinely evaluated in early drug discovery by means of rCYP enzymes and fluorescent probes, which are both amenable to high throughput screening. Assays such as this are useful in assessing CYP inhibition potential (IC_{50} determination) or rank ordering of compounds or chemotypes and for the purposes of conducting SAR to eliminate a CYP inhibition liability. In order to determine a more accurate drug–drug interaction potential, most labs will perform inhibition studies with human liver microsomes (IC_{50} or K_i determinations) [106,109]. There is a considerable database of *in vitro* inhibition data related to human liver microsomes and clinical outcomes of drug–drug interactions. These correlations have resulted in a general categorization of potential drug interactions by means of the ratio of inhibitor concentration (efficacious plasma concentration, $[I]$) to the K_i .

$[I]/K_i > 1$ implies that a drug interaction is likely,

$1.0 > [I]/K_i > 0.1$ implies that a drug interaction is possible, and

$[I]/K_i < 0.1$ implies that a drug interaction is unlikely.

Going beyond the CYP enzymes, newer approaches evaluating other enzyme systems, such as glucuronosyltransferases or drug transporters and the use of human hepatocytes to predict drug–drug interactions are showing considerable promise to predict interactions with multiple enzyme systems and integrated pathways more accurately [27,109–111].

The second type of drug interaction known as *mechanism based-* or *metabolism-based inhibition* (MBI) is a result of inactivation of a drug-metabolizing enzyme by a metabolite or metabolic intermediate that forms an adduct with the enzyme (irreversible inhibition) or forms a metabolite-intermediate complex (MI complex) with the enzyme, also known as *quasi-irreversible inhibition* [18]. A classic example of irreversible inhibition was published by Trager *et al.* [112] with CYP1A2 and furafylline while a more recent example of MI-complex formation was published by Takakusa *et al.* [113] using lapatinib and CYP3A4 (both manuscripts provide detailed experimental protocols to assess these types of inhibition). Certain functional groups on drug molecules or natural products have a propensity to undergo reactive metabolite formation and cause irreversible inhibition. These functional groups include, but are not limited to, acetylenes, alkenes, organosulfo-compounds, arylamines, tertiary amines, cyclopropyl amines, hydrazines, furans, thiophenes, dihaloalkanes, and methylene dioxyphenyl containing compounds [114,115]. Whenever such structures are present in a molecule, rigorous testing should be conducted to evaluate the potential for reactive metabolite formation that can lead to irreversible inhibition or covalent binding to other macromolecules such as proteins and nucleic acids.

Drugs that cause irreversible inhibition distinguish themselves from ordinary reversible inhibition by several characteristics: (i) they may display a delayed onset of inhibition, (ii) their inhibition properties are typically greater than what would be expected from ordinary reversible inhibition parameters (IC_{50} or K_i), and (iii) the inhibition effects can persist after the inhibitor has been eliminated because of the fact that enzyme has been destroyed during the inactivation process and new enzyme must be synthesized to regain nominal enzyme levels. The mechanistic differences between reversible and irreversible inhibition require additional experimental methods to appropriately characterize the mechanism and potential drug–drug interaction from irreversible inhibition. For example, a simple test for reversible and irreversible inhibition is the IC_{50} shift method. Here, liver microsomes are preincubated (30 min) with the compound of interest at several concentrations with and without NADPH. Each sample is then diluted ($\sim 10\times$) into an incubation containing a probe substrate and NADPH to measure enzyme activity. A left-ward shift in the IC_{50} plot indicates an irreversible inhibitor. Irreversible inhibition is often referred to as *time-dependent inhibition* because the degree of inhibition increases with time, whereas with reversible inhibitors, the potency of inhibition does not change with time. The reference to time-dependent inhibition is for the most part correct, however, time-dependent inhibition can also be caused by formation of a metabolite that is itself a reversible inhibitor (and would show a similar left-ward shift). In this situation, the inhibition is referred to *reversible inhibition* but does characterize itself as time dependent because of the increasing concentration of the inhibitory metabolite with time. A more characteristic experiment, known as the *preincubation-dilution* method is commonly employed to determine the kinetic parameters of the inactivation process (K_I and K_{inact}) [106]. The K_{inact} is the maximum inactivation rate constant and the K_I is the inhibitor concentration that causes half maximal inactivation (or the dissociation

constant of the initial enzyme-inhibitor complex). Using these kinetic parameters of the inactivation process, a reasonable prediction of the magnitude of the drug interaction can be determined. A review of industry practices and methods to evaluate and interpret irreversible inhibition was recently published [116].

2.4.3.2 Enzyme Induction. The induction of drug-metabolizing enzymes or drug transporters can have significant consequences on the exposure and toxicity of drugs, that is, the efficacy of a drug is reduced if its clearance is increased by enzyme induction. For example, rifampin can decrease the AUC of coadministered drugs by as much as 70–90%, as in the case of (*R*)-verapamil coadministration with rifampin [117]. Another example of a clinically relevant induction drug–drug interaction is patients receiving cyclosporine (organ rejection), who are also treated with rifampin (tuberculosis) [118]. In contrast to inhibition where the drug immediately interacts directly with the enzyme and leads to toxic increases in drug exposure, induction is an indirect effect on the enzyme, generally taking days to result in a clinically relevant effect on drug elimination, which may persist for several days beyond cessation of the inducing agent. Induction of CYPs can also lead to toxicity by increasing reactive metabolite production or by increasing the CYP1A2-mediated activation of procarcinogens [119]. Recognizing the safety and health issues associated with exposure to inducing agents, the US Food and Drug Administration (FDA) and other regulatory agencies have placed more emphasis on determining whether a drug candidate has the potential to induce CYP or UGT enzymes, or drug transporters. In 2006, the FDA published a draft guidance on drug-interaction studies that presented details on the principles and conduct of *in vitro* induction studies. The Pharmaceutical Research and Manufacturer's Association (PhRMA) subsequently published a White Paper that addressed many of these issues and acknowledged the importance of appropriate study design [32].

The most common mechanism of CYP induction is transcriptional gene activation. For drug-metabolizing enzymes, transcriptional activation is mediated by transcription factors such as AhR (aromatic hydrocarbon receptor), CAR (constitutive androstane receptor), and PXR (pregnane X receptor or SXR-steroid X receptor) [120–125]. The general concept for nuclear receptor signaling, as exemplified by PXR, is that in the absence of a ligand (drug), the nuclear receptor is associated with corepressor complexes conferring a basal level of transcription. Drug binding to the ligand-binding domain (LBD) of the nuclear receptors induces conformational changes that lead to the release of corepressors and recruitment of coactivators. Subsequent dimerization with partner molecules (retinoid X receptor, RXR, for CAR/PXR and the AhR nuclear translocator, ARNT, for AhR) lead to chromatin remodeling and subsequent transcriptional activation. Regulation of target gene transcription is achieved through binding of the nuclear receptor DNA-binding domain (DBD) to respective DNA response elements present in the promoter region of target genes (drug-metabolizing enzymes) [125,126]. It should be noted that the LBDs of many nuclear receptors are different between various animal species and human [125]. Therefore, *in vitro* or *in vivo* animal models (e.g., rat and dog) of enzyme induction can be misleading and are generally not employed to assess the potential induction effect in humans.

The mechanisms of CAR- and AhR-mediated gene transcription differs somewhat from that of PXR, which is exclusively an agonist activated process. Both CAR and AhR can be activated by direct ligand binding as described above; however, they can also be activated by ligand binding independent mechanisms. Translocation of CAR

to the nucleus can be initiated by direct agonist binding to the receptor or through a partially elucidated ligand-independent mechanism involving kinases that dephosphorylate CAR. Phenobarbital is an example of a drug that does not bind to CAR yet causes nuclear translocation and transcriptional activation of the target gene, CYP2B6 [123]. Similarly, the inactive AhR resides in the cytoplasm and can be activated on ligand binding or via protein tyrosine kinases [127].

PXR-derived nuclear hormone receptor models (ligand binding and cell-based PXR transactivation assays) are the most common high throughput assays to evaluate enzyme induction due to the simplicity of ligand-based activation and the importance of PXR target genes, such as CYP3A4, CYP2B6, and CYP2Cs, in drug interactions. Although cell-based transcription activation assays exist for CAR (CYP2B6) and AhR (CYP1A); these are used less frequently and most often to address mechanistic questions.

Cultured primary human hepatocytes are the most widely accepted model for assessing the potential of drug candidates to induce human CYP expression [7]. The hepatocyte model is able to better predict the outcome of enzyme induction and drug interactions because of the ability to simultaneously capture multiple nuclear hormone receptor-mediated pathways rather than a single pathway as with the transcriptional activation models. Primary human hepatocyte culture systems have been shown to effectively model human *in vivo* induction responses and are recognized by regulatory agencies as an effective tool for assessing induction potential [128,129]. The enzyme induction data from *in vitro* methods are known to correlate well with clinical observations, provided the *in vitro* experiments are performed at pharmacologically relevant concentrations of drug [130]. In addition to CYP enzymes, numerous studies have been reported using primary hepatocyte culture systems to assess induction of a variety of gene targets from phase II enzymes and drug transporters [131–134]. The pharmaceutical industry and regulatory agencies recommend the analysis of RNA expression and catalytic activity of the major drug-metabolizing enzymes CYP1A2, CYP2B6, and CYP3A4 in freshly isolated or attachable cryopreserved hepatocyte cultures. Results should be obtained from at least three individual donors, treated with the test compound, vehicle, and positive controls for two to three days. A minimum of three test compound concentrations, based on the expected human plasma drug levels are suggested, one of which should be an order of magnitude greater than this concentration. In the absence of knowledge of human plasma levels, concentrations ranging over at least two orders of magnitude should be studied.

Immortalized hepatocytes have been shown to possess many of the characteristics of primary hepatocytes. The advantages of using immortalized cell lines primary hepatocytes for enzyme induction studies include (i) easy access and availability; (ii) the ability to propagate (i.e., continual supply); and (iii) more consistent induction response compared to the variability seen with multiple donors of human hepatocytes. Immortalized cell lines cited throughout the literature include HepG2, HepaRG, Fa2N-4, and BC2 [135,136]. HepG2 and HepaRG cells have been shown to respond, to varying degrees, to CYP1A1/2 and 3A4 inducers [137–139], Fa2N-4 cells respond to rifampicin and β -naphthoflavone in a similar manner as observed for primary human hepatocytes [135,136], and BC2 cells have been reported to respond to CYP1A inducers [140]. While these cell lines can be used for preliminary studies, they do not maintain all of the phenotypic characteristics of human hepatocytes, such as enzyme or receptor function or expression, and their use may result in erroneous conclusions.

Interpretation of *in vitro* induction results can be as simple as compared to known CYP3A4 inducers (e.g., rifampin) or rank ordering of potency (EC_{50}) both of which provide useful information. Accurate predictions of drug interactions incorporate therapeutic drug concentrations, such as therapeutic drug concentrations (C_{max} or C_{ss}) along with induction data. Combining values, such as EC_{50} , E_{max} , and C_{max} (E_{max} mathematical model) have been used to assess induction effects for multiple induction models (transactivation, immortalized hepatocytes, and primary hepatocytes) [136,141,142]. More advanced mathematical models include such parameters as fraction unbound in hepatocyte culture, fraction metabolized, and a scaling factor (d), which allows better correlations between *in vitro* and *in vivo* outcomes of induction [32]. Another method of data interpretation is more empirical and involves correlations between the induction response found *in vitro* and the response *in vivo*. The most utilized form of correlation analysis utilizes the relative induction score (RIS), which has shown utility with both immortalized hepatocytes and primary hepatocytes [32,136].

2.5 FUTURE DIRECTIONS

The science of drug metabolism is continuing to move forward at an extremely fast pace, which is both a blessing and a curse. Without a doubt, the discipline is constantly growing with new information, methodologies, and instrumentation appearing as a continual stream in manuscript and presentation formats. At times, it can be challenging to keep abreast of the plethora of information reported in manuscripts across the field. Although this is a challenging time, it is also an exciting time to contribute to the wealth of information being generated today and to contribute to the future of drug metabolism.

One of the greatest challenges in drug metabolism is the predictive nature of our *in vitro* and animal models to human pharmacokinetics, metabolism, and toxicity. Most models appear to be qualitative or semiquantitative in their ability to accurately predict human outcomes. Moving down this “predictivity scale” to quantitative predictions with *in vitro* and animal models is the future direction of drug metabolism. This goal can be achieved through improvements of our existing models (e.g., hepatocytes), development of new models, and a better understanding of data analysis and data interpretation of both current and new models. Several of the new models that show great promise include the development of genetically modified animals [78,83] and stem cell derived hepatocytes [143,144]. Genetically modified animals provide an opportunity to evaluate human enzymes, receptors, transporters, or combinations of these, sometimes integrated events, in an *in vivo* setting. This should provide a significant improvement on evaluating events precipitated from these biological mediators through individual *in vitro* systems. Stem cell derived hepatocytes would significantly improve our ability to employ human hepatocytes across a number of existing *in vitro* methods, such as metabolite generation, predictions of hepatic clearance, and toxicity. Stem cell derived hepatocytes would provide a renewable and consistent supply of cells that are more stable in culture (expression and function of enzymes) and survive longer in culture so as to be useful in better predicting hepatic clearance or metabolite formation of slowly metabolized compounds, as well as toxic events that take longer to manifest in culture. Once these two advances become more established, the field will once again take a

large step forward, much like it did with the advent of molecular biology techniques in the 1980s and 1990s.

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3 Enzyme Kinetics of Drug-Metabolizing Reactions and Drug–Drug Interactions

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3.1	Introduction	1
3.2	Confounding factors of <i>in vitro</i> experiments	2
3.3	Determination of enzyme kinetics	4
3.4	Graphical presentation of kinetic data to identify atypical kinetic phenomena	13
3.5	Drug–drug interactions due to enzyme inhibition	14
3.6	Conclusions/future perspectives	22
	References	22

3.1 INTRODUCTION

In vitro studies play an important role in characterizing biotransformation reactions. Kinetic parameters are determined during the early phases of drug discovery and development and provide invaluable information needed to predict *in vivo* metabolism and assess potential interactions with enzyme effectors. These *in vitro* studies are routinely used for estimating kinetic parameters, such as K_m and V_{max} . Under linear conditions, the ratio of V_{max} and K_m represents the metabolic intrinsic clearance (CL_{int}), which is assumed to also be representative of the *in vivo* CL_{int} of a compound. Thus, it follows that this calculated CL_{int} value can then be used to extrapolate *in vitro* kinetic data to *in vivo* metabolic clearance and subsequently, the initial dose of a drug to be used in humans. To a great extent, this approach has provided reliable and successful *in vivo* clearance predictions. However, this success is predicated on accurate estimations of *in vitro* enzyme kinetic parameters. This chapter describes the proper conditions for carrying out *in vitro* enzyme kinetic experiments and the potential caveats that may lead to erroneous results. Also described are the various types of kinetic profiles that may be observed and equations that can be used to estimate kinetic parameters based

on the type of kinetics. Finally, methods for determining a compound's potential for drug-drug interactions with other substrates of the same enzyme are described, along with kinetic models to assess inhibition potential.

3.2 CONFOUNDING FACTORS OF *IN VITRO* EXPERIMENTS

3.2.1 General *In Vitro* Incubation Conditions

It is vital to obtain reliable and accurate *in vitro* data that reflect the *in vivo* situation. Therefore, fundamentally sound enzyme kinetics practices must be employed and reaction conditions must be well defined. There are multiple factors to consider when determining which *in vitro* incubation conditions are appropriate and best mimic the *in vivo* system. The most important experimental components in drug metabolism reactions are substrate concentration, incubation time, and protein concentration. An appropriate length of time for an incubation reaction should allow for adequate product formation to ensure precise quantitation. It is equally important that the substrate turnover be linear with respect to incubation time and enzyme concentration. Initial experiments are needed, where product formation is measured at multiple time points in incubations containing different concentrations of protein. From these studies, an incubation time and protein amount can be chosen for all subsequent experiments where the same enzyme source is used. In practice, for many drug-metabolizing enzymes, a protein concentration of 0.1–0.25 mg/mL and an incubation time of 5–10 min will produce a linear reaction with readily detectable conversion of substrate (see below for discussion of the role of protein concentration and nonspecific binding to microsomal proteins). If nonlinearity is observed with respect to velocity, autoinactivation of the enzyme or too much substrate depletion may be occurring. The substrate concentration should be greater than 10-fold excess compared to the enzyme concentration, and <10% of the substrate should be consumed [1,2]. Experiments should include multiple time points (8–10 points), generally ranging from one-fifth to fivefold the K_m value. To this end, iterations of the experiment may be needed to establish the proper experimental conditions of enzyme concentration, time, and substrate concentration range to adequately describe the kinetic profile of the metabolism reaction.

Optimization of other experimental variables is equally as important when designing and conducting *in vitro* experiments to be used to determine kinetic parameters. Other important parameters that may affect metabolic rate and should be given careful consideration include selection of buffer system, buffer ionic strength, incubation pH, and incubation temperature. The *in vitro* experimental variables of temperature, pH, and buffer ionic strength are usually set to reflect human liver physiological conditions. Generally, one tries to employ buffer conditions that mimic the *in vivo* situation. For example, commonly used buffers include 50–100 mM potassium phosphate buffer or tris-hydroxyaminomethane (THAM) at pH 7.4 maintained at 37°C [3]. In addition, cosubstrates such as nicotinamide adenine dinucleotide phosphate (NADPH) or uridine diphosphate glucuronic acid (UDPGA) should not be rate limiting and are typically included at saturating concentrations during the incubation. Typical concentrations of NADPH used range from 0.5 to 2 mM and UDPGA, 3–5 mM. Alternatively, an NADPH-regenerating system may be utilized if one is concerned about maintaining NADPH concentrations, especially over longer incubation periods. However, typically

this is not an issue in incubations up to 1 h since the concentrations of NADPH suggested are significantly above the K_m for NADPH and, thus, depletion due to conversion to NADP is not usually an issue. Because the composition of the incubation matrix could potentially affect various enzymes differently, a systematic evaluation should be conducted to determine optimal experimental conditions [4,5].

Multiple studies have evaluated the effects of different buffering systems on kinetic parameter estimates; however, not all the reported findings are in agreement [6,7]. Irrespective of which buffer is chosen for the incubation mixture, it is important to maintain pH within the physiological range. Yao and Levy [3] have reported differences in *in vitro* K_i values due to small changes in pH from 7.2 to 7.6. These findings suggest that this should be an area of further research in order to optimally define *in vitro* incubation conditions that most accurately reflect the microenvironment of hepatic smooth endoplasmic reticulum.

3.2.2 Effect of Organic Solvents on Enzyme Activity

In an attempt to mimic the microenvironment of the human liver, *in vitro* studies are carried out in aqueous physiological buffers. However, owing to the lipophilic nature of many drug substrates and inhibitors, potential solubility problems arise. To overcome these issues, organic solvents such as methanol, ethanol, acetonitrile, and dimethyl sulfoxide (DMSO) are commonly used to facilitate compound solubilization. The presence of organic solvents in the incubation reaction may, however, alter enzyme–substrate interactions by modifying the native enzyme environment or altering enzyme activity. It is important that one considers these potential effects because any changes resulting from the presence of organic solvent could potentially compromise the reliability and interpretation of the *in vitro* data.

The effect of organic solvents in the incubation reaction on individual cytochrome P450 (CYP) enzyme activity of microsomal, hepatocyte, and recombinant enzyme systems has extensively been investigated [8–11]. The observed effect on individual CYP activity is solvent specific. For example, Vuppugalla *et al.* [10] reported no effect on CYP2B6 or CYP2C8 activity when 0.5% (v/v) methanol was present, whereas the presence of ethanol, acetonitrile, and DMSO showed concentration-dependent inhibition from 0.2% to 5%. Although results typically are in agreement, some discrepancies have been reported. It has been reported that 1% acetonitrile has no effect on CYP1A2 activity in human hepatocytes [11] or on cDNA-expressed recombinant enzymes [8], but it results in increased CYP1A2 activity in human liver microsomes [12,13]. These results suggest that the degree of inhibition may be enzyme-source dependent.

Although numerous studies have reported inhibitory effects on CYP enzyme activity because of the presence of organic solvent, stimulatory effects have been observed [8,14,15]. The presence of acetone (2–4% v/v) or acetonitrile (2–4% v/v) in kinetic studies using human liver microsomes or reconstituted CYP2C9 resulted in increased tolbutamide hydroxylation. The reported stimulatory effect was observed over a wide range of substrate concentrations. Similar findings were described by Tang and others [9] who observed an increase in CYP2C9 activity toward diclofenac 4'-hydroxylation and tolbutamide methyl hydroxylation. However, acetonitrile inhibited celecoxib methyl hydroxylation, suggesting that the stimulatory effect of acetonitrile on CYP2C9 activity is substrate dependent.

A given organic solvent may exhibit differential inhibitory or stimulatory effects on specific CYPs. Although purely aqueous incubation conditions are preferred for *in vitro* CYP metabolism studies, in practice, it is likely that the use of organic solvent will be required to facilitate enhanced solubility of the compound of interest. Thus, the organic solvent concentration must be kept to an absolute minimum and, generally, <1% of the total reaction volume. In addition, it is important to identify suitable solvents that enable *in vitro* studies to be completed with minimal effect on CYP activity.

3.2.3 Nonspecific Microsomal Binding

Human liver microsomes are the most popular and widely used *in vitro* system for evaluating drug metabolism due in part to their reasonable availability, low cost, and ease of use. However, numerous examples exist in the literature of nonspecific binding of drug substrates (and inhibitors) to microsomal proteins. Margolis and Obach [16] reported that the use of high microsomal protein concentrations (especially 0.5 mg/mL and greater) caused alterations of inhibition constants for drugs that were highly lipophilic because of nonspecific binding effects. Therefore, it is desirable to use the lowest amount of protein that yields quantifiable amounts of metabolite in order to minimize these effects [1,2]. Lipophilicity is a characteristic shared by most drugs, and therefore, it is not surprising that many substrates have been shown to readily bind nonspecifically to the protein milieu of the microsomal membrane. The extent of nonspecific binding varies among drug classes and is dictated by the physiochemical characteristics of the drug [17–19]. Drugs that are weak bases, such as amiodarone and amitriptyline, bind extensively to the microsomal membrane, whereas others that are weak acids, such as naproxen and tolbutamide, exhibit relatively little or no microsomal binding [18–20]. When drugs bind nonspecifically to microsomal proteins, the effective free substrate concentrations will be less than the amount added, leading to inaccuracies in the determination of K_m and K_i parameters. The apparent K_m (or K_i value in the case of inhibition experiments) observed will be higher than the true K_m , although there should be no effect on V_{max} [20]. Thus, an overestimation of K_m occurs and *in vivo* drug clearance will be underestimated, unless the actual free substrate concentration in the reaction mixture is determined (i.e., nonspecific binding is measured). Several groups have proposed various correction factors to be applied for nonspecific binding during extrapolation to *in vivo* clearance [19–21]. For drugs that exhibit extensive nonspecific binding, correction for microsomal binding using free fraction in incubation matrices improves the prediction of *in vivo* clearance values calculated from *in vitro* estimates of intrinsic clearance. However, one cannot simply correct the apparent K_m value by multiplying the unbound drug fraction because this rule does not apply to every enzymatic reaction [9].

3.3 DETERMINATION OF ENZYME KINETICS

3.3.1 Michaelis–Menten Kinetics

In typical enzyme-catalyzed reactions, the substrate and product concentrations are much greater than the enzyme concentration such that each enzyme molecule catalyzes the conversion of multiple substrate molecules to product. The conversion of substrate

to product involves the formation of a transition state that occurs at the catalytic, or active, site of the enzyme.

Generally, kinetic characterizations of most enzymatic reactions are governed by hyperbolic kinetics that can be described by the Michaelis–Menten equation (Eq. 3.1).

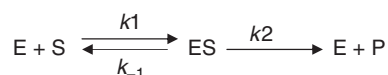
$$v = \frac{V_{\max} [S]}{K_m + [S]} \quad (3.1)$$

The Michaelis–Menten equation is a quantitative description of the relationship that exists among the rate of an enzyme-catalyzed reaction (v), the concentration of substrate $[S]$, and the two parameters, $V_{\max}$ and K_m . In this case, $V_{\max}$ is the maximal velocity of the reaction and K_m is the Michaelis constant. General characterization of the enzymatic reaction can be determined in terms of rate and concentration by means of this equation. However, it is important to keep in mind three general considerations that are made when describing kinetic data modeled after the Michaelis–Menten equation: (i) the enzymatic reaction is operating under steady-state conditions, (ii) rapid equilibrium between the concentration of all the enzyme–substrate intermediates becomes constant soon after initiation of the reaction, and (iii) the catalytic active site of the enzyme contains only one binding site at which one substrate molecule can interact at a given time.

The fundamental concepts underlying these kinetic analyses continue to provide the cornerstone for understanding drug metabolism today, and are routinely used during drug development to determine kinetic constants ($V_{\max}$ and K_m) in the analysis of *in vitro* drug metabolism data. The Michaelis constant, K_m , is strictly defined as the concentration of substrate at which half-maximal velocity of the reaction is achieved. It is important to recognize that the determination of this rate constant results from the combination of several rate constants such that K_m is equal to (Scheme 3.1), where E, S, ES, and P are the concentrations of the enzyme, substrate, substrate-bound enzyme, and enzyme products (metabolites), respectively. The association rate constant of $E + S \rightarrow ES$ is depicted as k_1 , whereas k_{-1} is the dissociation rate constant for $ES \rightarrow E + S$ and k_2 is the dissociation rate constant of $ES \rightarrow E + P$. Often, K_m is incorrectly associated with the affinity of the substrate for the enzyme, but as can be seen from the above discussion, K_m is a composite of three rate constants. More exactly, k_{-1}/k_1 (referred to as K_s or K_d), which is the substrate dissociation constant, reflects substrate affinity.

The definition of $V_{\max}$ is derived from the product of k_{cat} and e_0 ($k_{\text{cat}} \times e_0$), where k_{cat} (or k_2 , as referred to in Scheme 3.1) is the capacity of the enzyme–substrate complex to form product and e_0 is the enzyme concentration. The k_{cat} parameter is a reflection of the number of molecules of substrate that one molecule of enzyme can convert to product per unit time. For this reason, k_{cat} is also referred to as the *catalytic constant* or the *turnover number*.

The velocity of the reaction can be characterized as a hyperbolic, saturating profile. The key features of the substrate concentration versus reaction velocity plot are depicted



Scheme 3.1

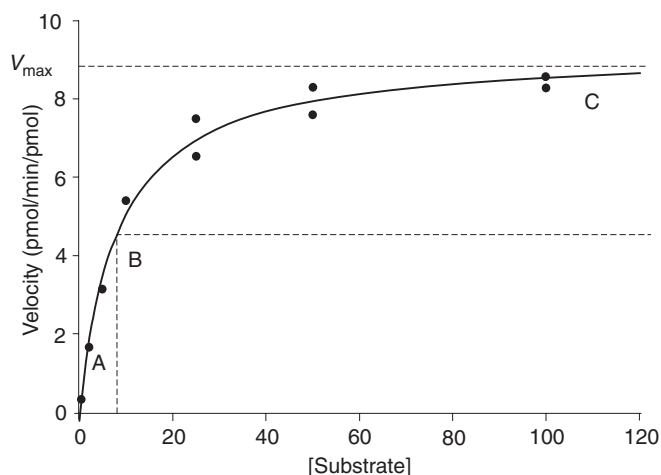


Figure 3.1 Hyperbolic saturation curve depicting typical Michaelis–Menten kinetics.

in Fig. 3.1. At high substrate concentrations, the rate of the reaction, represented by the point C, is almost equal to $V_{\max}$, and rate differences at reasonably similar substrate concentrations are negligible. It should be noted that $V_{\max}$ is only an estimate, as the maximal velocity of the enzymatic reaction is never reached at a finite substrate concentration. If, however, the Michaelis–Menten plot is extrapolated to infinitely high substrate concentrations, the extrapolated rate is equal to $V_{\max}$. At this point, the reaction becomes nearly independent of substrate concentration, and the rate is said to be zero order. At lower substrate concentrations, such as the areas of the curve before K_m (point B), the lower reaction velocities are indicative of only a portion of the enzyme molecules being occupied by substrate. Therefore, at low substrate concentrations (i.e., point A), the rate of the reaction is directly proportional to substrate concentration and the reaction is said to be first order.

Estimation of K_m and $V_{\max}$ is useful during the drug development process since these parameters can be used to estimate the *in vitro* intrinsic clearance (CL_{int}) for a drug, which in turn can be extrapolated to estimate the *in vivo* CL_{int} . The extrapolated parameter (CL_{int}) can then be used to predict *in vivo* hepatic clearance and subsequently total (systemic) clearance, and drug dosing. To do this, one makes the assumption that *in vivo*, the substrate concentration will be much lower than the K_m . Under these conditions, the $[S]$ term drops out of the denominator and Equation 3.1 becomes Equation 3.2.

$$v = \frac{V_{\max}[S]}{K_m} \quad (3.2)$$

In Equation 3.2, the velocity is proportional to $[S]$ and is equal to the product of CL_{int} and $[S]$ (Eq. 3.3).

$$v = CL_{\text{int}}[S] \quad (3.3)$$

Since *in vivo* CL_{int} is defined as the ability to clear drug in the absence of blood flow or protein-binding restrictions, the *in vivo* CL_{int} should be equal to the *in vitro*

CL_{int} . Because Equations 3.2 and 3.3 are analogous, the equations can be rearranged as (Eq. 3.4)

$$CL_{int} = \frac{V_{max}}{K_m} = \frac{v}{[S]} \quad (3.4)$$

This equation then allows derivation of CL_{int} following estimation of the *in vitro* enzyme parameters, K_m and V_{max} .

3.3.2 Non-Michaelis-Menten Kinetics

Unfortunately, not all enzyme reactions carried out by drug-metabolizing enzymes can be adequately described by Michaelis–Menten kinetics (i.e., they do not exhibit a hyperbolic kinetic profile). These types of kinetic profiles have been observed with several of the CYP drug-metabolizing enzymes, most commonly, CYP3A4, and also with most other drug-metabolizing CYPs. However, atypical kinetic profiles have also been observed with other important drug-metabolizing enzymes such as the UGTs (UDP-glucuronosyltransferases) [22–24].

Most commonly, atypical kinetic profiles observed with drug-metabolizing enzymes have been attributed to the binding of multiple molecules of a substrate (homotropic) or two chemically different substrate molecules (heterotropic) within the enzyme active site. The binding of one molecule can change the kinetics of a second substrate molecule binding within the active site and is frequently referred to as *cooperativity*. This cooperativity is characterized as being homotropic or heterotropic depending on the chemical species present in the enzyme active site. This cooperativity can be either positive or negative, depending on the effects elicited by the second substrate molecule. Although the term *allosterism* is generally used to describe cases where a second molecule binding distant to the substrate molecule causes an alteration in the functioning of the enzyme, it is also sometimes used to describe the kinetic changes due to a second substrate molecule binding since it is separate from the original substrate but may be still within the active site. However, it should also be realized that these types of kinetics may also be due to the presence of multiple species of the enzyme (e.g., conformers) that exhibit different kinetic characteristics with a substrate.

3.3.2.1 Sigmoidal Kinetics. Numerous examples of activation, or positive cooperativity, in drug-metabolizing enzymes are found throughout the literature [25]. Activation occurs when enzyme activity for a substrate is increased either by the presence of another compound or a single compound activating its own metabolism. Autoactivation is an example of homotropic cooperativity and is characterized by a sigmoidal kinetic profile. This profile is thought to occur because of the simultaneous binding of two molecules of the substrate within the enzyme active site, with the metabolism of one of the substrate molecules exhibiting a low K_m and low V_{max} and the other exhibiting a higher K_m and higher V_{max} (Fig. 3.2). A useful tool in establishing the presence of sigmoidicity in a kinetic plot is to identify deviations from linearity when the data are presented in an Eadie–Hofstee plot. A marked increase in the curvature, or hooked nature, of the line is indicative of a sigmoidal profile (Fig. 3.3). These data are indicative of positive cooperativity and suggest the presence of multiple interactions between the substrate and enzyme. Altered affinity and rate of product formation for other substrate binding sites may result from these interactions.

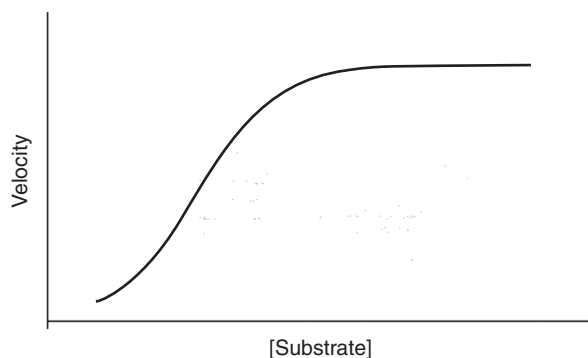


Figure 3.2 Sigmoidal saturation curve of velocity versus substrate concentration.

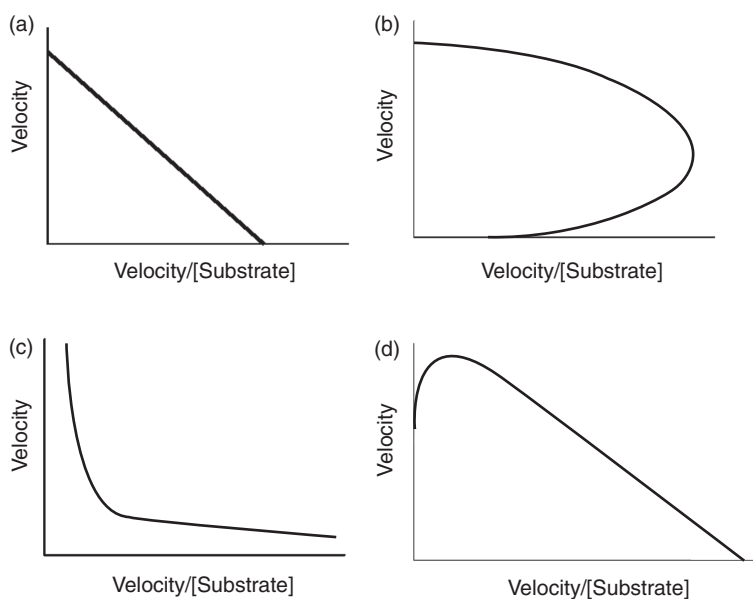


Figure 3.3 Eadie–Hofstee plots of typical and atypical kinetics. (a) Hyperbolic (Michaelis–Menten kinetics), (b) sigmoidal kinetics, (c) biphasic kinetics, and (d) substrate inhibition kinetics.

One of the most commonly used equations to describe sigmoidal kinetic profiles is the Hill equation [26] and is commonly used to mathematically describe sigmoidal kinetic profiles (Eq. 3.5).

$$v = \frac{V_{\max} [S]^n}{K' + [S]^n} \quad (3.5)$$

Although the K' parameter is analogous to K_m , it is not equivalent (unless $n = 1$) as it is composed of both K_m and the interaction factors [27]. The coefficient n , referred to as the *Hill coefficient*, reflects the degree of cooperativity observed. It should be

noted that n does not relate to the number of binding sites within the enzyme. Values of $n > 1$ are indicative of positive cooperativity, and the greater the n value, the greater the sigmoidicity of the kinetic profile.

Although applicable to most sigmoidal kinetic data, the Hill equation provides limited information about the actual kinetic processes and the associated rate constants. In an attempt to overcome these limitations and gain additional insight into the substrate–enzyme interaction, more complex kinetic models have been proposed [28,29]. These models are based on the assumption of two equivalent substrate binding sites within the enzyme active site. Korzekwa *et al.* [29] derived velocity equations to fit sigmoidal kinetic observations, thereby allowing one to estimate two K_m values (K_{m1} and K_{m2}) and two V_{max} values (V_{max1} and V_{max2}) (Eq. 3.6), ostensibly representing the kinetic processes at each of the binding sites.

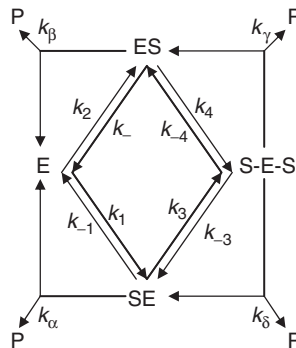
$$v = \frac{\frac{V_{max1}}{K_{m1}} + \frac{V_{max2}}{K_{m1}K_{m2}}}{1 + \frac{[S]}{K_{m1}} + \frac{[S]^2}{K_{m1}K_{m2}}} \quad (3.6)$$

A more detailed model that provided additional insights into the micro rate constants of the various processes was proposed by Shou *et al.* [27], again modeling two binding sites within the enzyme active site. A kinetic scheme was proposed that discriminates all possible equilibrium and rate steps of each of the enzyme–substrate complexes possible within the two binding sites.

The following equation (Eq. 3.7) was derived based on Scheme 3.2 for estimation of the associated kinetic parameters:

$$\frac{v}{[E]_{total}} = \frac{\frac{k_\alpha}{K_{S1}} + \frac{k_\beta}{K_{S2}} + \frac{[S]}{K_{S1} \times K_{S3}} \times (k_\delta + k_\gamma)}{\frac{1}{[S]} + \frac{1}{K_{S1}} + \frac{1}{K_{S2}} + \frac{[S]}{K_{S2} \times K_{S4}}} \quad (3.7)$$

3.3.2.2 Biphasic Kinetics. Biphasic kinetic profiles are another example of homotropic cooperativity, but unlike sigmoidal kinetics, biphasic kinetics profiles do not reach saturation, but rather exhibit two distinct phases (Fig. 3.4). The first phase is observed at low substrate concentrations and is more characteristic of hyperbolic



Scheme 3.2

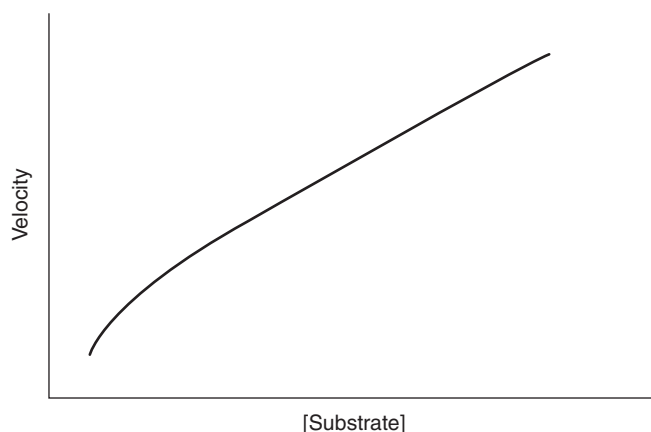


Figure 3.4 Biphasic saturation curve of velocity versus substrate concentration.

kinetics. At increasing substrate concentrations, the second phase becomes evident as the velocity of the reaction continues to increase linearly, with no evidence of saturation. However, one must be cognizant that “apparent” biphasic kinetic profiles may appear in multienzyme systems, where two enzymes are producing the same metabolite but with dramatically different kinetics, such that one enzyme is a high affinity–low capacity enzyme and the second enzyme is a low affinity–high capacity enzyme. In true biphasic kinetics, the same enzyme is operating with both a low- and high-affinity binding component [29]. If a biphasic kinetic profile is observed within a single enzyme system, it most likely results from multiple binding regions within the active site, or the substrate binds within the active site in multiple orientations. Regardless, the binding orientation responsible for the semihyperbolic portion of the profile will result in a low K_m , low V_{max} component, whereas the other binding orientation will produce a high K_m and high V_{max} . A kinetic equation has been developed, essentially a combination of the Michaelis–Menten equation and a linear equation, to describe biphasic kinetics (Eq. 3.8):

$$v = \frac{(V_{max1}[S]) + (CL_{int}) \times [S]^2}{(K_{m1} + [S])} \quad (3.8)$$

From this equation, the parameters derived from fitting the equation to the initial curved portion of the plot provide estimates of V_{max1} and K_{m1} and are reflective of low substrate concentrations. The CL_{int} parameter describes the linear portion of the profile that occurs at high concentrations and can be determined from the ratio of V_{max2}/K_{m2} (i.e., the slope of the linear portion of the kinetic profile).

3.3.2.3 Substrate Inhibition. Substrate inhibition occurs when the rate of product formation plateaus at a point and then decreases with increasing substrate concentrations (Fig. 3.5). To model substrate inhibition and derive kinetic parameter estimates, an equation has been derived based on the uncompetitive inhibition model (Eq. 3.9). From this equation, one can estimate the kinetic parameters K_m , V_{max} , and K_{si} (i.e., inhibition constant for the second substrate molecule).

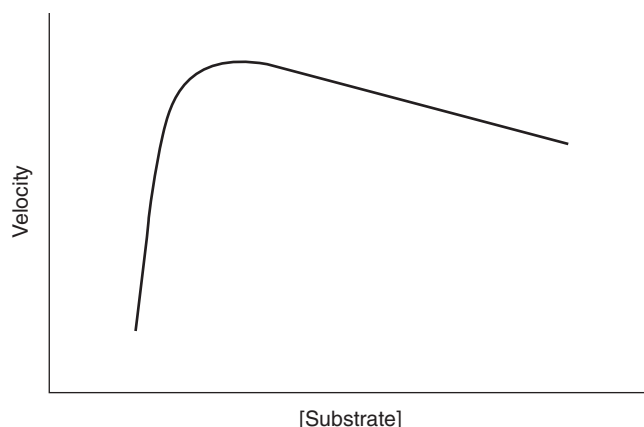
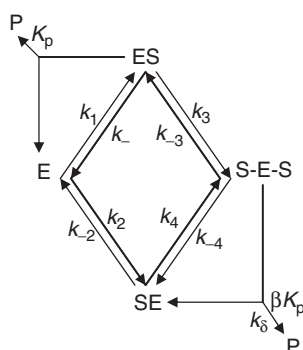


Figure 3.5 Substrate inhibition saturation curve of velocity versus substrate concentration.

$$v = \frac{V_{\max}[S]}{K_m + [S] + \frac{[S]^2}{K_{si}}} \quad (3.9)$$

However, this kinetic model, apart from the K_m and $V_{\max}$ estimations, only provides insight into the inhibition constant of the substrate and not into more of the micro rate constants of formation and dissociation of the various possible species present. To better describe the various rate constants associated with all possible species and processes involved in substrate inhibition, a more complex kinetic model was proposed (Scheme 3.3) [30].

This model is based on the premise that one binding site is catalytic and the other inhibitory and that substrate molecules have access to both these sites. When the substrate (S) binds to the catalytic site of the enzyme (i.e., ES), the rate and $V_{\max}$ of product formation ($ES \rightarrow P$) are determined by $k_p[ES]$ and $k_p[E]_{\text{total}}$, respectively. In contrast, once excess S binds to the nonproductive, inhibitory site (SE) the rate of S-E-S is no longer the same as ES and is reduced by a factor β . This model incorporates sequential binding of substrate molecules such that the substrate inhibition site cannot



Scheme 3.3

be occupied until the active site is filled and the second site may be independent from the active site.

On the basis of this scheme, an equation was derived to describe substrate inhibition, based on the assumption that the rates of formation and of decomposition of the various enzyme-substrate complexes are equivalent (Eq. 3.10) [30].

$$v = \frac{V_{\max} \left[\frac{1}{K_S} + \frac{\beta \times [S]}{(\alpha \times K_i \times K_S)} \right]}{\frac{1}{S} + \frac{1}{K_S} + \frac{1}{K_i} + \frac{[S]}{(\alpha \times K_S \times K_i)}} \quad (3.10)$$

In this equation, K_S is approximately equal to K_m , and K_i is the dissociation constant of the substrate binding to the inhibitory site. Alpha (α) represents the factor by which the dissociation of substrate (K_S and K_i) at both sites is altered when a second molecule is bound, whereas β is the factor by which $V_{\max}$ changes on binding of a second substrate molecule. It is evident that the use of this equation allows for additional parameter estimations and provides useful information as to the degree to which binding of the second substrate molecule alters K_m and $V_{\max}$. However, it should be noted that because of the number of parameters to be estimated, properly fitting data to this equation requires acquisition of a substantial number of data points.

3.3.2.4 Heteroactivation Kinetics. During *in vitro* studies to evaluate potential drug-drug interactions, one typically expects to observe inhibition using microsomal or expressed enzyme systems. However, it is now well established that *in vitro* coincubation of two different compounds can also result in increased metabolism of one substrate rather than inhibition [25,29] (Fig. 3.6). Again, this phenomenon

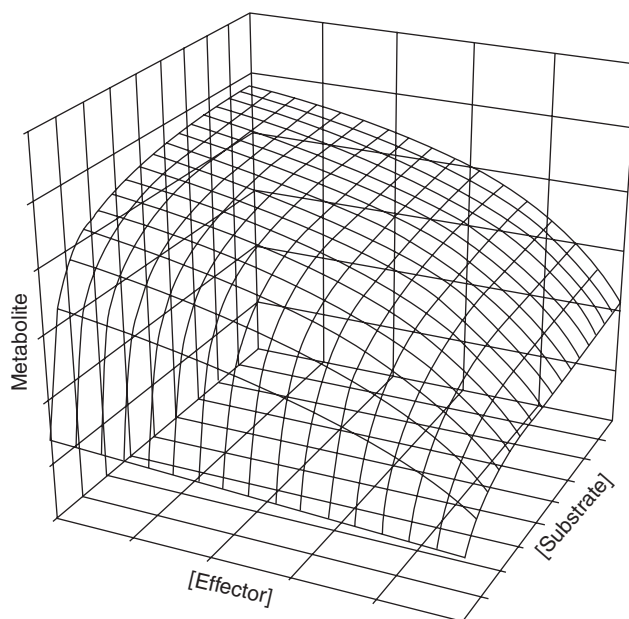
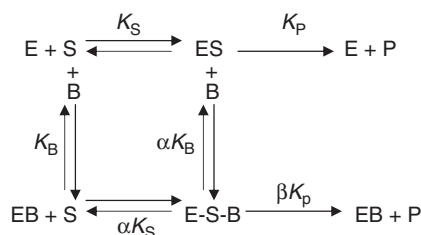


Figure 3.6 Heteroactivation plot depicting the relationship between the metabolite, effector molecule, and substrate.



Scheme 3.4

has been attributed to the simultaneous binding of two different molecules, substrate and effector, within the active site. However, it should be realized that the “effector” may also be metabolized itself. This positive cooperativity typically results in both a decrease in K_m and an increase in V_{max} . This heteroactivation is depicted in Scheme 3.4.

This kinetic scheme has been described by a complex equation (Eq. 3.11) that allows for determination of the kinetic parameters above:

$$v = \frac{V_{max} \times [S]}{K_m \left(\frac{1 + \frac{[B]}{K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right) + [S] \left(\frac{1 + \frac{[B]}{\alpha K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right)} \quad (3.11)$$

where B is the concentration of activator, α is the factor by which the K_m for substrate changes, and β can be thought of as the factor by which V_{max} changes. This equation allows for fitting multiple curves representing different activator concentrations and has a manageable number of parameters including the usual K_m and V_{max} for substrate in the absence of activator.

3.4 GRAPHICAL PRESENTATION OF KINETIC DATA TO IDENTIFY ATYPICAL KINETIC PHENOMENA

The most appropriate method of generating enzyme kinetic parameter estimates is the use of computer-generated, nonlinear regression analysis of the untransformed data using a suitable model. However, in the analysis of enzyme kinetics data, much value can be gained by examining a graphical representation of the kinetic data. Graphical plotting of the data can provide additional insights into interpretation of whether the data are truly hyperbolic and follow Michaelis–Menten kinetics or whether atypical kinetic phenomena may be operational. Thus, graphical presentations of the data can aid in determining the correct kinetic model to apply in estimating kinetic parameters.

3.4.1 Eadie–Hofstee Plot

The Eadie–Hofstee plot is based on the transformation of the Michaelis–Menten equation to give the equation of a straight line. In this case, the v is plotted along the y-axis and $v/[S]$ along the x-axis (Fig. 3.3). The slope of the best-fit line is equal to $-K_m$, the y-intercept = V_{max} , and the x-intercept = V_{max}/K_m . As with other graphical

representations, the Eadie–Hofstee plot is not without caveats. The Eadie–Hofstee plot has the disadvantage that the velocity appears in both coordinates. Because the x -axis contains an element of the velocity, it no longer reflects a truly independent variable. As a result, any experimental errors will be reflected on both axes. Therefore, otherwise seemingly good data can appear worse. On the other hand, this plot best reveals the presence of atypical kinetic profiles based on the shape of the data-fit obtained. For example, a linear Eadie–Hofstee plot of the data would be representative of hyperbolic (Michaelis–Menten) kinetics, a “hooked” Eadie–Hofstee plot suggests sigmoidal autoactivation kinetics, a biphasic Eadie–Hofstee plot suggests biphasic kinetics, and an Eadie–Hofstee plot where the plot rises and then falls similar to the substrate versus velocity plot of substrate inhibition would, of course, suggest that substrate inhibition is occurring.

3.5 DRUG-DRUG INTERACTIONS DUE TO ENZYME INHIBITION

Drug–drug interactions continue to be an obstacle for the pharmaceutical industry, and thus, accurate predictions of potentially adverse clinical outcomes become increasingly important in drug discovery and development. The determination of *in vitro* enzyme inhibition kinetics is an important predictor of potential drug–drug interactions. Enzyme inhibition is the decrease in the rate of substrate turnover in the presence of an inhibitory effector molecule. Enzyme inhibitors fall into two broad classes—reversible and irreversible [30]. Reversible inhibitors bind to enzymes through weak, noncovalent interactions such as hydrogen bonds, hydrophobic interactions, and ionic bonds. The sum of the multiple weak interactions between the inhibitor and the enzyme active site results in strong, specific but still reversible binding [31]. In contrast, irreversible, or mechanism-based, inhibitors cause enzyme inactivation through covalent or quasi-irreversible modification of the enzyme structure. The following sections discuss both classes of inhibition, as well as the meaning of the inhibition kinetic parameters, IC_{50} and K_i .

3.5.1 Reversible Inhibition

3.5.1.1 Competitive Inhibition. The simplest and most commonly observed type of reversible enzyme inhibition is that of competitive inhibition. As the name implies, a substrate and inhibitor are competing for the enzyme active site, and their binding is mutually exclusive. The inhibitor may bind to the same binding region of the substrate or partly mask it. In either case, when the inhibitor is bound to the enzyme, the substrate is unable to bind in a productive orientation and the complex is incapable of catalysis of substrate. Competitive inhibition can be overcome by sufficiently high concentrations of substrate. The kinetic scheme for this type of inhibition is shown in Scheme 3.5.

In case of competitive inhibition, there is no change in V_{max} ; however, an increase in K_m is observed. When comparing inhibition potential of several compounds, it often helps to characterize the inhibition in terms of CL_{int} . Therefore, one can characterize competitive inhibition as resulting in a decrease in CL_{int} (V_{max}/K_m), with no change in the apparent V_{max} . The equation for competitive inhibition is shown in Equation 3.12.



Scheme 3.5

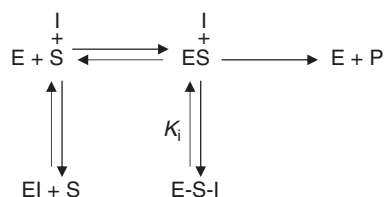
$$v = \frac{V_{\max} \times [\text{S}]}{K_m \left(1 + \frac{[\text{I}]}{K_i}\right) + [\text{S}]} \quad (3.12)$$

In this equation, $V_{\max}$ and K_m have their usual meanings and $[\text{I}]$ is the free inhibitor concentration. K_i is the inhibition constant, and $K_i = [\text{E}][\text{I}]/[\text{EI}]$, which when substituted into Equation 3.12, is in the same form as the Michaelis-Menten equation. From Equation 3.12, it becomes apparent that in the case of competitive inhibition, K_m is increased by a factor $(1 + [\text{I}]/K_i)$ and $V_{\max}$ remains unchanged.

3.5.1.2 Noncompetitive Inhibition. Purely noncompetitive inhibition is rarely observed in enzyme kinetics studies, except in the case of very small inhibitors (e.g., protons, metal ions, and small amides), but is included here for completeness. Noncompetitive inhibition is a form of mixed inhibition where the substrate and inhibitor bind reversibly and independently. In this case, the binding of the inhibitor to the enzyme reduces its activity but has no effect on the binding of the substrate. The kinetic scheme for noncompetitive inhibition is shown in Scheme 3.6.

An example of noncompetitive inhibition would be when the inhibitor binds to the enzyme somewhere other than the active site, thereby changing the enzyme's three-dimensional structure such that the active site is still able to bind substrate but is no longer in the optimal configuration to stabilize the transition state and catalyze the reaction. This type of inhibition results in a decrease in $V_{\max}$ and no apparent change in K_m . On closer examination, most situations originally thought to involve noncompetitive inhibition were actually cases of mixed inhibition. The equation for noncompetitive inhibition is shown below (Eq. 3.13).

$$v = \frac{V_{\max}[\text{S}]}{K_m \left(1 + \frac{[\text{I}]}{K_i}\right) + [\text{S}] \left(1 + \frac{[\text{I}]}{K_i}\right)} \quad (3.13)$$



Scheme 3.6

3.5.1.3 Uncompetitive Inhibition. Uncompetitive inhibition occurs when an enzyme-inhibitor reversibly binds only to the enzyme-substrate (E-S) complex yielding an inactive enzyme-substrate-inhibitor (E-S-I) complex; the inhibitor does not bind to the free enzyme. However, uncompetitive inhibition is also not common. The kinetic model for this type of inhibition is shown in Scheme 3.7.

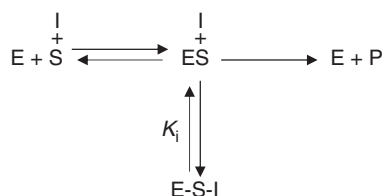
In this case, the reduction in the effective concentration of the ES complex results in a decreased K_m value and proportional decrease in V_{max} . As a result, the inhibitor has no net effect on V_{max}/K_m (i.e., enzyme efficiency or CL_{int}). The equation to describe uncompetitive inhibition constants, as well as values for K_m and V_{max} , is presented below (Eq. 3.14).

$$v = \frac{\left[\frac{V_{max}}{1 + \frac{[I]}{K_i}} \right] + [S]}{\left[\frac{K_m}{1 + \frac{[I]}{K_i}} \right] + [S]} \quad (3.14)$$

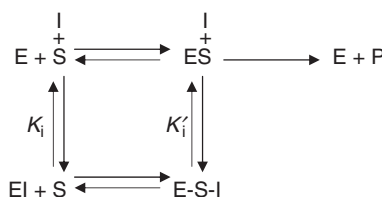
3.5.1.4 Mixed Inhibition. The effects of mixed inhibition on enzyme reactions are such that both specific and catalytic effects are present. As a result, this type of inhibition is characterized by a decrease in the maximal velocity (V_{max}) and an increase in the concentration at which half-maximal velocity (K_m) is achieved. This type of inhibition can be reduced, but not completely overcome, by increasing concentrations of substrate. The kinetic scheme for mixed inhibition is shown in Scheme 3.8, where K_i and K'_i represent the competitive and uncompetitive inhibition components, respectively.

From the kinetic scheme, it is clear that the mechanism that results in mixed inhibition is much more complex than that of others. In mixed inhibition, the substrate can bind to the enzyme-inhibitor complex, which can subsequently dissociate to form an enzyme-substrate complex capable of undergoing catalysis to produce product.

There are multiple binding mechanisms that may account for the observed effects on V_{max} and K_m in the presence of the inhibitor. First, the inhibitor can compete with



Scheme 3.7



Scheme 3.8

the substrate for binding to the enzyme. Second, the inhibitor may bind to an enzyme molecule that subsequently binds to the substrate. Third, the inhibitor can bind to an enzyme-substrate complex and affect catalytic turnover. The effects of the mixed inhibition on the velocity of the reaction can be described by the following equation (Eq. 3.15).

$$v = \frac{V_{\max} \times [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad (3.15)$$

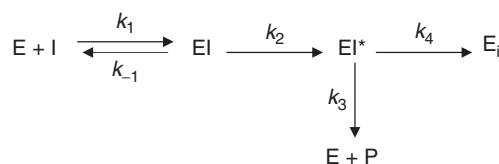
3.5.2 Irreversible Inhibition

3.5.2.1 Mechanism-Based Inhibition. Unlike reversible inhibition that involves rapid association and dissociation of drugs and enzymes, time-dependent inhibition involves irreversible covalent binding or quasi-irreversible tight binding of a chemically reactive intermediate to the heme of the CYP or to the protein itself [32]. In general, a time-dependent inhibitor is defined as a compound that exhibits increased inhibition when incubated with the enzyme before the addition of the substrate. Therefore, mechanism-based inhibitors qualify as a subset of time-dependent inhibitors, in which experimental evidence supports the formation of a chemically reactive metabolite capable of inactivating the enzyme [33].

The *in vitro* characterization of a mechanism-based inhibitor must satisfy seven criteria (see below) and includes the determination of three key parameters: the maximum inactivation rate constant (k_{inact}), the inactivator concentration that produces half-maximal rate of inactivation (K_I), and the efficiency of inactivation relative to metabolism, or partition ratio (r). The necessary criteria defining mechanism-based inhibition include (i) time dependence, (ii) saturation, (iii) substrate protection, (iv) irreversibility, (v) stoichiometry of inactivation, (vi) involvement of a catalytic step, and (vii) inactivation occurring before release of the activated species [34]. The kinetic scheme for mechanism-based inhibition is depicted below (Scheme 3.9), where EI^* is the reactive form of EI , P is the metabolite product, and E_i is the inactivated enzyme. From the model, it is apparent that the enzyme kinetics involving mechanism-based inhibition are complex and are governed by the first-order rate constants k_1 , k_{-1} , k_2 , k_3 , and k_4 [35].

A rate equation representing the loss of functional enzyme with time was described by Kitz and Wilson [36] (Eq. 3.16).

$$\frac{d[E]_t}{dt} = \left(\frac{k_{\text{inact}}[I]}{K_I + [I]} \right) [E]_t \quad (3.16)$$



Scheme 3.9

$[E]_t$ represents the active enzyme concentration at time t ; $[I]$, the inactivator concentration; k_{inact} , the maximum inactivation rate constant; and K_I , the inactivator concentration that produces half-maximal rate of inactivation. From Equation 3.16, it is evident that the rate of P450 enzyme inactivation is proportional to the inactivator concentration and can be saturated at high concentrations [37]. Both k_{inact} and K_I are derived from complex relationships between the first-order rate constants (Eqs. 3.17 and 3.18, respectively).

$$k_{\text{inact}} = \frac{k_2 k_4}{k_2 + k_3 + k_4} \quad (3.17)$$

$$K_I = \left[\frac{k_{-1} + k_2}{k_1} \right] \left[\frac{k_3 + k_4}{k_2 + k_3 + k_4} \right] \quad (3.18)$$

The effects of a mechanism-based inhibitor on the velocity of the reaction can be described by Equation 3.19, where k_{cat} is defined by Equation 3.20.

$$v = \frac{k_{\text{cat}}[E]_t[I]}{K_m + [I]} \quad (3.19)$$

$$k_{\text{cat}} = \frac{k_2 k_3}{k_2 + k_3 + k_4} \quad (3.20)$$

The ratio of the metabolite formation rate to the inactive enzyme formation rate is referred to as the *partition ratio* (r) and is described in Equation 3.21.

$$r = \frac{k_{\text{cat}}}{k_{\text{inact}}} = \frac{k_3}{k_4} \quad (3.21)$$

The partition ratio can be used as a measure of efficiency of the mechanism-based inactivation process, thereby providing useful information regarding the inactivation potential of compounds in early drug discovery.

Experimental conditions employed to determine the kinetics of mechanism-based enzyme inactivation involve evaluation of the time dependence of the reaction. In these experiments, a preincubation (primary incubation) for specific time periods is conducted in the presence/absence of increasing concentrations of the putative inactivator and NADPH, followed by CYP activity measurement by dilution into a secondary incubation [38]. Typical results are depicted in Figs. 3.7 and 3.8.

The half-lives for inactivation of probe substrate at each concentration of inactivator can be calculated from the slopes of the individual lines to give k_{obs} , the pseudo first-order rate constant of inactivation at inactivator concentration $[I]$ (Fig. 3.9). A double reciprocal plot of $1/k_{\text{obs}}$ versus $1/[I]$, known as *Kitz-Wilson plot*, can be constructed to obtain the inactivation parameters k_{inact} and K_I (Fig. 3.9) [36]. In the case of a saturation reaction, the concentration of the inactivator is infinite and there is a finite half-life, and the point where the plotted line intersects the y-axis is equal to $0.693/k_{\text{inact}}$. However, with commercially available graphing software, the inactivation parameters also can be determined by a direct plot of k_{obs} versus inactivator concentration followed by non-least-squares fitting [37].

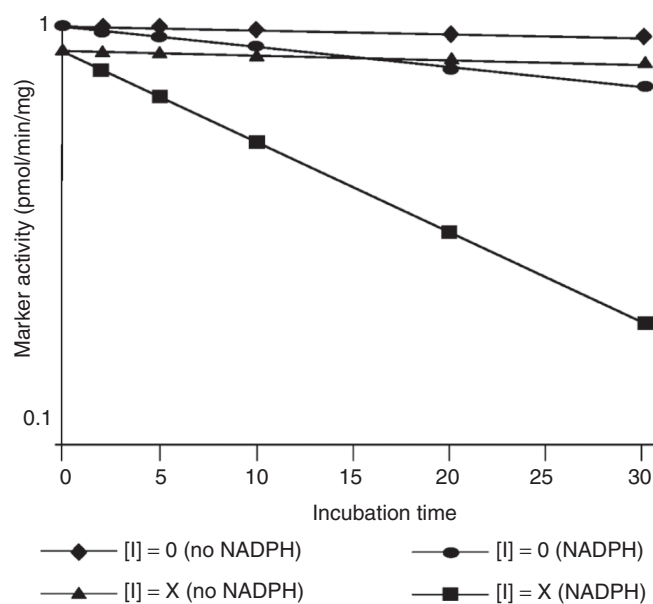


Figure 3.7 Evaluation of the time dependence of a mechanism-based inactivator in the presence/absence of NADPH.

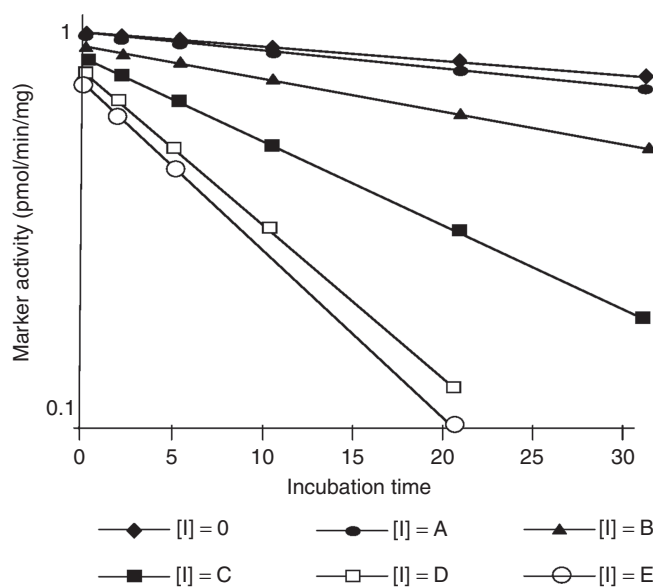


Figure 3.8 Evaluation of the time dependence of a mechanism-based inactivator in increasing concentrations of a putative inactivator.

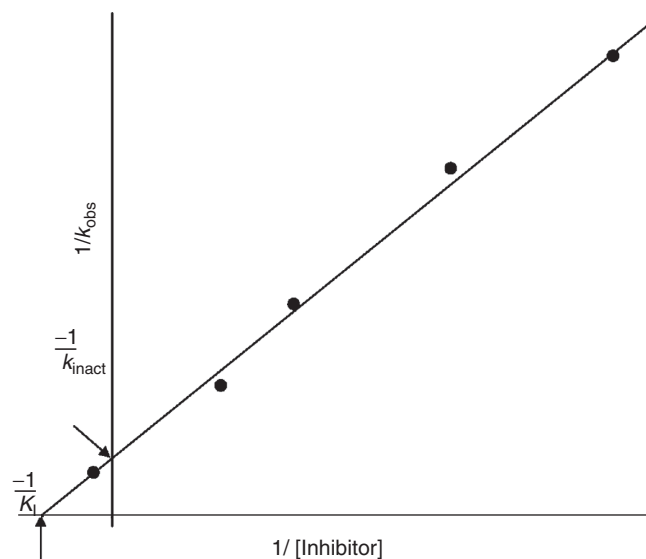


Figure 3.9 Kitz–Wilson double reciprocal plot depicting the relationship between k_{inact} and K_i .

3.5.3 Context of IC_{50} and K_i Parameters

The parameters of IC_{50} and K_i are used to describe the inhibitory potential of a compound and are routinely determined in early drug discovery to assess the potential inhibition liabilities for new molecular entities. Estimation of K_i follows the same guidelines as for estimating K_m ; multiple substrate concentrations (usually three to four) and multiple inhibitor concentrations (usually four to five) should be used. The range of inhibitor concentrations also should be at least threefold above and below the estimated K_i value [39]. An appropriate definition or specification of the type of enzyme inhibition (e.g., competitive, noncompetitive, mixed) must be selected in order to determine an accurate estimation of K_i . Guidelines for the selection of a proper model have been previously described [37]. However, alternative methods for selecting an inhibition model have been reported [40].

Velocity equations for K_i determinations result in a 3D plot because of the presence of two variable parameters, substrate [S] and inhibitor [I] concentration. Linear transformation of the data, in the form of an Eadie–Hofstee plot (v vs [I]) or as a Dixon plot ($1/v$ vs [I]), allows for easier visualization of the data. The K_i value cannot be determined solely from these graphical representations; however, they are helpful in providing insight into possible inhibition mechanisms involved. In the Dixon plot, for example, if the lines intersect above the x -axis, it suggests competitive inhibition, whereas if the intersection is on the x -axis, it suggests mixed inhibition (Fig. 3.10). To avoid inaccuracies, K_i should always be determined by simultaneous nonlinear regression of the entire untransformed data set using a suitable software program.

In comparison to the K_i value, which represents the dissociation constant of the enzyme–inhibitor complex, an IC_{50} is simply defined as the inhibitor concentration that decreases the biotransformation of a substrate at a single, specified concentration by 50%. The two inhibition parameters cannot be used interchangeably. The IC_{50}

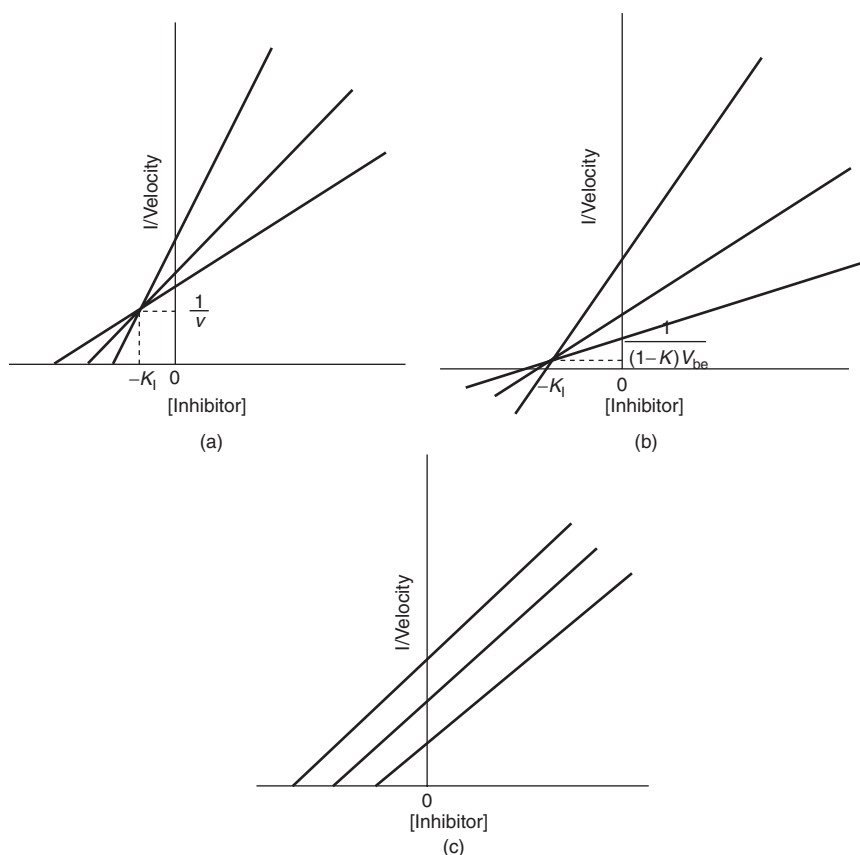


Figure 3.10 Dixon plots showing (a) competitive, (b) mixed, and (c) uncompetitive inhibition.

parameter is dependent on the concentrations of the substrate, inhibitor, and enzyme studied, whereas K_i values are independent of the substrate and thus more readily comparable across laboratories. IC_{50} plots are conventionally plotted as rate versus $\log [I]$, and the resulting curve can be described by Equation 3.22, where v_0 is the control rate, b is the background activity, S is the slope factor, and IC_{50} is the inhibitor concentration that reduces the velocity by 50%.

$$v = \frac{v_0}{1 + \left[\frac{[I]}{IC_{50}} \right]^S} + b \quad (3.22)$$

As with K_i determinations, the IC_{50} value should always be calculated using non-linear regression of the untransformed data.

Owing to the complexity and time-consuming nature of K_i determinations, it is often favorable in drug discovery and development to determine IC_{50} values. The relationship between the K_i and the IC_{50} of an enzymatic reaction is influenced by the type of inhibition (Table 3.1). For example, with competitive and uncompetitive inhibitions, if the IC_{50} determination is made at $[S] \approx K_m$, then $K_i \approx \frac{1}{2}IC_{50}$, whereas for noncompetitive and mixed inhibitions, the IC_{50} and K_i values will be equal [41].

TABLE 3.1 Effects of Various Types of Reversible Inhibition on Kinetic Parameters and the Relationship Between K_i and IC_{50}

Type of Inhibition	Change in Kinetic Parameter by Inhibition			Relationship Between K_i and IC_{50} Values	
	$V_{\max}^{\text{app}}$	K_m^{app}	$V_{\max}^{\text{app}}/K_m^{\text{app}}$	General Equation	If $[S] \approx K_m$
Competitive	$\leftrightarrow$	$\uparrow$	$\downarrow$	$IC_{50} = K_i \left(1 + \frac{[S]}{K_m} \right)$	$\frac{1}{2} K_i = \frac{1}{2} IC_{50}$
Noncompetitive	$\downarrow$	$\leftrightarrow$	$\downarrow$	$IC_{50} = K_i$	$K_i = IC_{50}$
Uncompetitive	$\downarrow$	$\downarrow$	$\leftrightarrow$	$IC_{50} = K_i' \left(1 + \frac{K_m}{[S]} \right)$	$\frac{1}{2} K_i = \frac{1}{2} IC_{50}$
Mixed	$\downarrow$	$\uparrow$	$\leftrightarrow$	$IC_{50} = K_i' \left(\frac{K_m + [S]}{K_m + [S]} \right)$	$K_i = IC_{50}$

3.6 CONCLUSIONS/FUTURE PERSPECTIVES

The determination of enzyme kinetics for drug metabolism reactions is an integral part of drug discovery and development. When carried out carefully and under the proper conditions, one can use these *in vitro* kinetic parameters in making predictions of a drug's potential *in vivo* pharmacokinetics and thus the “druggability” of the compound. Although most compounds follow hyperbolic, saturable (i.e., Michaelis–Menten) kinetics, one can also observe atypical kinetic profiles such as sigmoidal (autoactivation), biphasic, and substrate inhibition kinetics. Thus, it is critical to apply the correct kinetic model to enzyme kinetics data analyses to avoid making inaccurate conclusions about a compound's kinetics. *In vitro* determinations of a drug's potential for drug–drug interactions due to enzyme inhibition are also critical in assessing the potential *in vivo* utility of a compound. Again, one must assure use of the proper kinetic model when assessing propensity to inhibit drug-metabolizing enzymes.

As increasing numbers of compounds are being assessed for their metabolism characteristics during the discovery and development phase, one must continually look to more efficient methods for carrying out these analyses. For example, many high throughput technologies have been applied to ADME (absorption, distribution, metabolism, and excretion) studies such as 96-well-plate automation, parallel LC–MS methods, automated data processing, and sample pooling strategies [42]. Lastly, work will continue toward refining the *in vitro*–*in vivo* correlation models in an effort to better predict human *in vivo* metabolism from *in vitro* data. As we learn more about additional *in vivo* factors that control a drug's rate of metabolism *in vivo* and about intersubject variability in metabolism, these factors can be incorporated into models with the hope of improved predictions.

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4 Sex Differences in Drug Metabolism

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4.1	Summary	1
4.2	Introduction	2
4.3	Sex differences in hepatic drug metabolism	2
4.4	Sex-dependent expression of hepatic drug-metabolizing enzymes	4
4.5	Hormonal determinants of sex-dependent expression of hepatic cytochrome P450s	7
4.6	Molecular determinants of sex-dependent expression of hepatic cytochrome P450s	10
4.7	Concluding remarks	12
	Acknowledgment	12
	References	13

4.1 SUMMARY

Sex differences in drug metabolism are recognized as a major determinant of sex differences in pharmacokinetics and an important contributor to interindividual differences in drug metabolism and, in some cases, drug action. Historically, sex differences in drug metabolism were widely studied in rat and mouse models in the 1960s and 1970s. Sex differences in human drug metabolism have also been observed with a variety of drugs; however, the quantitative differences in rates of metabolism between men and women are typically much smaller than those seen in rats. The biochemical basis of sex differences in hepatic drug metabolism was largely unknown until the 1980s when it was discovered that expression of some of the drug-metabolizing enzymes, including those in the cytochrome P450 (CYP) family, is sex-dependent in mouse and rat liver. Thus, the expression of certain rat CYP enzymes is male specific (e.g., CYP2C11), whereas others are female specific (e.g., CYP2C12) or female predominant (e.g., CYP2A1). The major gonadal hormones, testosterone and estradiol, regulate the sex-dependent expression of rat hepatic CYP enzymes indirectly through their effects on the hypothalamus and its regulation of the sexually dimorphic, ultradian

rhythm of pituitary growth hormone (GH) secretion, which ultimately dictates the sex-dependent patterns of expression of select CYPs and certain other drug-metabolizing enzymes. In male rats, the intermittent, pulsatile pattern of GH secretion stimulates the expression of male-specific liver enzymes, whereas the more continuous pattern of GH release in females suppresses the expression of male-specific enzymes while inducing female-specific enzymes. Studies conducted in the past decade have identified essential molecular mediators of the effects of GH on sex-dependent liver CYP enzymes and their genes, in particular, the transcription factors—signal transducer and activator of transcription (STAT) 5b and hepatocyte nuclear factors (i.e., HNF4 α). The current view is that STAT5b and HNF4 α coordinately regulate the action of GH and its resultant effect on the sex-dependent expression of hepatic *CYP* genes and thus provide a molecular basis for sex differences in drug metabolism.

4.2 INTRODUCTION

Individual variability in drug metabolism and drug response is well documented in the biomedical literature [1–3]. This variability is manifested as qualitative and/or quantitative differences in drug response within a group of patients (i.e., interindividual variability) or in the same patient (i.e., intraindividual variability). Within a population, patient-specific characteristics such as sex [4], age [5], and genetics [6,7] have been identified as determinants of interindividual variability in drug action. Intraindividual differences in drug action can be induced by administration of a second drug leading to drug–drug interactions, may be caused by deterioration of organ function, or may be by progression in the severity of a disease state, among others [8]. The pharmacological basis for variability in drug response can be attributed to interindividual differences in pharmacokinetics (i.e., drug absorption, distribution, metabolism, and excretion) and/or pharmacodynamics (i.e., the physiological, biochemical, cellular, and molecular actions of a drug). Indeed, patient-specific characteristics are known to influence the biological processes that govern drug absorption, distribution, metabolism, excretion, and functionality of therapeutic targets (e.g., receptors, enzymes) and their associated signal transduction pathways.

Sex differences in drug action were first observed in the 1930s, where adult female rats were found to require only half the dosage of amobarbital (Amytal), as compared to adult male rats, to elicit an anesthetic effect [9], and the duration of action of certain barbiturates (e.g., amobarbital and pentobarbital) was found to be longer in female than in male rats [10]. Subsequent animal and human studies have shown sex differences in the pharmacokinetics and pharmacodynamics of various other drugs [11–15]. Considerably more is known about sex differences in pharmacokinetics, especially sex differences in drug metabolism in the liver than about sex differences in pharmacodynamics. The overall aim of this chapter is to provide an overview of our current understanding of sex differences in hepatic drug metabolism.

4.3 SEX DIFFERENCES IN HEPATIC DRUG METABOLISM

4.3.1 Rat Hepatic Drug Metabolism

Sex differences in hepatic drug metabolism were widely studied in the 1960s and 1970s in experiments involving the incubation of rat hepatic microsomes (liver membrane

TABLE 4.1 Metabolism of Drugs and Other Substrates Catalyzed by Hepatic Microsomes Isolated from Male and Female Rats

Drug/ Substrate	Reaction	Strain of Rat	Sex Difference (Male/Female Ratio)	References
5 α -androstane- 3 α ,17 β -diol-3, 17-disulfate	15 β -Hydroxylation	Long-Evans	<0.05	21
Aminopyrine	N-demethylation	Sprague-Dawley ^a	3.5 4.0	22 23
Aniline	Hydroxylation	Sprague-Dawley ^a	1.1	22
Diazepam	3-Hydroxylation	Wistar ^a	5.2	16
		Wistar ^b	0.7	16
		BD ^a	10	16
	N-desmethylation	Wistar ^a	0.9	16
		Wistar ^b	0.9	16
		BD ^a	2.6	16
Diphenhydramine	N-demethylation	Sprague-Dawley ^a	2.4	23
Hexobarbital	Hydroxylation	Sprague-Dawley ^a	4.2 3.0	22 23
Imipramine	N-demethylation	Sprague-Dawley ^a	2.5	24
	2-Hydroxylation	Sprague-Dawley ^a	0.7	24
	N-oxidation	Sprague-Dawley ^a	2.9	24
Indinavir	Oxidation ^c	Sprague-Dawley ^a	3.3	25
Lidocaine	N-demethylation	Sprague-Dawley ^a	8.5	24
	3-Hydroxylation	Sprague-Dawley ^a	0.7	24
Meperidine	N-demethylation	Sprague-Dawley ^a	2.4	23
3,4-Methylenedioxy- methamphetamine	N-demethylation	Sprague-Dawley ^a	3.3	18
Morphine	N-demethylation	Sprague-Dawley ^a	2.6	23
Pentobarbital	Oxidation	Sprague-Dawley ^a	2.6	23

^aPostpubertal rats.^bNeonatal rats.^cTotal oxidation products.

preparations enriched in endoplasmic reticulum) with drugs, other foreign chemicals, or endogenous lipophilic substrates (e.g., endogenous steroids and fatty acids). As illustrated in Table 4.1, drugs such as diazepam, lidocaine, and indinavir are metabolized more rapidly by liver microsomes isolated from postpubertal male rats than by those from postpubertal female rats. In contrast, other substrates (e.g., the steroid 5 α -androstane-3 α ,17 β -diol-3,17-disulfate) are more rapidly metabolized by hepatic microsomes from female rats. However, not all substrates (e.g., aniline) exhibit sex difference in hepatic metabolism. Table 4.1 also shows that the sex dependence of drug metabolism can be pathway specific; for example, lidocaine N-demethylation, but not lidocaine 3-hydroxylation, is preferentially catalyzed by hepatic microsomes from male rats. With some drugs, sex differences in hepatic metabolism are strain specific; for example, liver microsomes prepared from BD rats but not those from Wistar rats exhibit sex differences in diazepam N-desmethylation. Sex differences in hepatic drug metabolism may also vary as a function of age (Table 4.1), typically being

manifested during the pubertal and adult periods but not prepubertally [16] and present at a reduced level during senescence [17]. Sex differences in hepatic drug bioactivation can lead to deleterious consequences, as exemplified by the finding that the enhanced N-demethylation of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) by hepatic microsomes from male rats is associated with an increased susceptibility to the acute toxicity of MDMA, when compared to female rats [18]. In addition to phase I oxidative drug metabolism, sex differences in phase II metabolism affecting glucuronidation, sulfation, and glutathione conjugation, have also been reported in animal studies [19,20].

4.3.2 Human Hepatic Drug Metabolism

Sex differences in human hepatic drug metabolism have been investigated in clinical pharmacokinetic studies that compare the clearance of a drug in men and women. Sex differences characterize the clearance of certain drugs, including cyclosporine [26], erythromycin [27], nifedipine [28], triazolam [29], midazolam [30,31], alprazolam [32], and verapamil [33]. However, in some cases, conflicting data exists with regard to the effect of sex on drug clearance [34–36]. Possible reasons for the conflicting data include (i) the small sample size employed in some of the studies resulting in a lack of statistical power to detect a sex-based difference and (ii) the role of confounding variables, such as age [12,35], ethnicity [37], and other biological determinants of drug clearance, which can include the level of expression of uptake and efflux transporters, although it is debatable whether human hepatic P-glycoprotein expression differs between males and females [38], given the conflicting data in the literature [39,40]. With the increased availability of human hepatic microsomes for research, the *in vitro* metabolism of drugs and other substrates can readily be assessed using microsomes isolated from liver tissue samples obtained from male and female donors. As shown in Table 4.2, in general, sex differences are not apparent or are relatively modest in human hepatic microsomal drug metabolism. An exception is *S*-mephenytoin N-demethylation, whose metabolism is substantively greater in hepatic microsomes from females than in those from males, but this occurs only in samples from Hispanic donors [37]. Overall, sex differences occur in hepatic phase I and phase II drug metabolism, and the differences are generally larger and hence more apparent in rats than in humans.

4.4 SEX-DEPENDENT EXPRESSION OF HEPATIC DRUG-METABOLIZING ENZYMES

The underlying biochemical basis for sex differences in drug metabolism came to light when it was discovered in the 1980s that many of the rat hepatic CYP enzymes (Table 4.3) are expressed in a sex-dependent manner [47]. Since then, sex-dependent expression of other rodent drug-metabolizing enzymes, including UDP-glucuronosyltransferases [48–50], sulfotransferases [49,51,52], and glutathione *S*-transferases [49,53,54], has also been reported. The available experimental evidence suggests that certain human hepatic CYP enzymes may also be expressed differentially in men and women. Overall, among the drug-metabolizing enzymes, the hepatic CYPs are the best characterized with respect to their sex-dependent expression. Thus, the

TABLE 4.2 Effect of Sex on Metabolism of Drugs and Other Substrates Catalyzed by Human Hepatic Microsomes

Drug	Reaction	Effect	References
Aminopyrine	N-demethylation	M = F	41
Chlorzoxazone	6-Hydroxylation	M = F	42
Coumarin	7-Hydroxylation	M = F	42
Dextromethorphan	O-demethylation	M = F	42
Diclofenac	4'-Hydroxylation	M = F	42
Erythromycin	N-demethylation	F > M (1.2-fold)	43
7-Ethoxyresorufin	O-demethylation	M = F	42
Ifosfamide	N-dechloroethylation	F > M (2.2-fold)	44
Lauric acid	12-Hydroxylation	M = F	42
S-mephenytoin	N-demethylation	F > M (7.8-fold) ^a	37
		M = F ^{b,c}	37
		M = F ^d	42
	4'-Hydroxylation	M = F	42
Nicotine	C-oxidation	M = F	45
S-oxazepam	Glucuronidation	M > F (1.7-fold)	46
Paclitaxel	6 α -Hydroxylation	M = F	42
Testosterone	6 β -Hydroxylation	M = F	42

^aHispanic.^bAfrican-American.^cCaucasian.^dSamples from Hispanics, African-Americans, and Caucasians.

remainder of this chapter focuses on hepatic CYP enzymes and the various factors that regulate their sex-dependent expression.

4.4.1 Rat Cytochrome P450s

As summarized in Table 4.3, multiple CYP enzymes are expressed constitutively in a sex-dependent manner in rat liver, and their expression is developmentally regulated. Interestingly, a catalytic function of many of these enzymes is the oxidative metabolism of sex steroid hormones such as testosterone. The prototype male-specific P450 enzyme of rat liver is CYP2C11 [55–57, 75]. CYP2C11 is essentially undetectable in both male and female rat liver before puberty, but its expression increases rapidly at puberty (at approximately day 35 of age) in males only and reaches a maximum during early adulthood [58,59]. A similar pattern of postnatal developmental expression has been described for several other male-specific rat hepatic CYP enzymes, that is, CYP2A2 [60,61], CYP2C13 [62], and CYP3A18 [63,64]. In contrast, hepatic expression of the male-specific CYP3A2 is detectable during the prepubertal period in both sexes, whereas at the onset of puberty, the level of CYP3A2 is increased in male liver, but at about the same time it decreases to barely quantifiable levels in females [58]. Another sex-dependent rat hepatic CYP is the female-specific CYP2C12 (Table 4.3). Although CYP2C12 is expressed at low levels in the livers of both sexes prepubertally, its expression is increased at puberty in female rat liver but is suppressed in male rats [58,65]. The female-predominant enzymes CYP2A1, CYP2A7, and CYP3A9 (Table 4.3) comprise a third group of sex-dependent liver P450s. Expression is either similar (CYP2A1

TABLE 4.3 Sex-Dependent Hepatic Expression of Rat Cytochrome P450 Enzymes^a

Enzyme	Catalytic Reaction	Developmental Expression	
		Prepuberty	Puberty
<i>Male Specific</i>			
CYP2A2	Testosterone 15 α -hydroxylation	None	↑ in male
CYP2C11	Testosterone 2 α -hydroxylation	None	↑ in male
CYP2C13	Testosterone 15 α -hydroxylation	Minimal	↑ in male
CYP3A2	Testosterone 6 β -hydroxylation	Male ~ female	↑ in male, ↓ in female
CYP3A18	Testosterone 6 β -hydroxylation	Minimal	↑ in male
CYP4A2	Lauric acid ω -hydroxylation	None ^b	↑ in male ^b
<i>Female Specific</i>			
CYP2C12	5 α -Androstane-3 α ,17 β -diol-3,17-disulfate 15 β -hydroxylation	Minimal/moderate	↑ in female
			None in male
<i>Female Predominant</i>			
CYP2A1	Testosterone 7 α -hydroxylation	Male ~ female	Female > male
CYP2C7	Retinoic acid 4-hydroxylation	Male ~ female	Female > male
CYP3A9	Testosterone 6 β -hydroxylation	↓	Female > male

^aBased on Refs 21,55–73.^bBased on data for renal CYP4A2 [74].

and CYP2C7) or absent (CYP3A9) in both sexes during prepuberty, and the levels of these enzymes are greater in postpubertal female rats than in postpubertal male rats [58,62,64].

4.4.2 Human Cytochrome P450s

In contrast to the rat CYP enzymes, relatively little is known about the sex-dependent expression of human CYPs. With the available information in the literature, many of the conclusions have been drawn from *in vivo* pharmacokinetic studies that measured the plasma clearance of an enzyme-selective substrate (e.g., midazolam for CYP3A enzymes [31]) or urinary formation of a biomarker (e.g., 6 β -hydroxycortisol for CYP3A enzymes [76]). In other cases, conclusions regarding the sex dependence of human enzyme levels have been drawn from *in vitro* metabolism studies (Table 4.2) based on the conversion of an enzyme-selective substrate to its metabolite (e.g., microsomal formation of 6 β -hydroxytestosterone as a catalytic marker for CYP3A [42]). More limited information is available on the immunoreactive protein levels of human CYP enzymes in livers from both sexes. In a panel of 94 liver samples (48 from males and 46 from females), the median level of CYP3A4 was approximately twofold greater in liver samples from females than in samples from males [40]. By comparison, CYP2B6 protein levels were found to be approximately twofold higher in livers from Caucasian women ($N = 15$) than from Caucasian men ($N = 28$), whereas it was approximately fourfold higher in liver samples from Hispanic women ($N = 3$) than in those from Hispanic men ($N = 4$) [37]. In another study of human livers from donors of unknown ethnic origin, multiple linear regression analysis indicated that sex was a

predictor of hepatic CYP2B6 protein levels, but only accounted for 14% of the variance [77]. Overall, it appears that sex differences occur in hepatic expression of human CYP, namely, CYP3A4 and CYP2B6, although the effects are rather modest and considerably less pronounced when compared to the sex differences in the expression of rat hepatic CYP enzymes (Table 4.3).

4.5 HORMONAL DETERMINANTS OF SEX-DEPENDENT EXPRESSION OF HEPATIC CYTOCHROME P450s

A hormonal basis for sex differences in drug metabolism was apparent from early studies showing that (i) castration of postpubertal male rats decreased hepatic microsomal CYP-mediated metabolism of aminopyrine and hexobarbital and (ii) exogenous administration of an androgen (methyltestosterone) fully reversed the suppressive effects of castration [22,78]. These observations paved the way for subsequent studies by various research groups elucidating the hormonal factors that determine the sex-dependent expression of CYP enzymes in rat liver. Collectively, the results of those studies indicate that in addition to gonadal hormones, other hormones also regulate the sex-dependent expression of rat hepatic CYPs. Among these, GH is the most important and the best characterized [79].

4.5.1 Gonadal Hormones

4.5.1.1 Androgen. Androgen has distinct effects on the hepatic expression of the male-specific and female-specific rat CYP enzymes. In male rats, androgen is required for the pubertal expression of the male-specific forms, such as CYP2A2 [60], CYP2C11 [55,58,59,80], CYP2C13 [66], CYP3A2 [58,81], CYP3A18 [82], and CYP4A2 [67], as shown in experiments involving castration of neonatal male rats and exogenous administration of testosterone. Thus, exposure to androgen early in life (e.g., neonatal period) is sufficient to ensure enzyme expression later in adult life in a process known as *neonatal programming* [83]. Full adult expression levels of the male-specific enzymes are achieved in rats exposed to androgen during both the neonatal and peripubertal periods [47]. In contrast to the positive regulation of the male-specific rat hepatic CYP enzymes by androgen, this hormone negatively regulates the hepatic expression of the female-specific CYP2C12, as shown by the decreased enzyme levels in intact, adult female rats administered with exogenous androgen [65,84]. An important conclusion from the available experimental evidence [47,85] is that androgen controls hepatic expression of the sex-dependent rat hepatic CYP enzymes in an indirect manner through its action on the hypothalamic pituitary axis, which results in a sex-specific pattern of pituitary GH secretion (Section 4.5.2).

4.5.1.2 Estrogen. Estrogen also has distinct effects on the expression of sex-dependent rat liver CYP enzymes. In female rats, ovariectomy and exogenous estrogen replacement experiments show that estrogen is required for liver expression of the female-specific CYP2C12 [58,65] and the female-predominant CYP2C7 [80] and CYP3A9 [86]. However, estrogen is not absolutely required for hepatic expression of these enzymes, as these enzymes are still expressed, albeit at low levels, in ovariectomized rats. In intact and castrated male rats, estrogen suppresses the hepatic

expression of the male-specific CYP2C11 [59,80,84]. The effects of estrogen on these sex-dependent CYP enzymes also involves its effects on the sex-specific pattern of pituitary GH secretion [47,85].

4.5.2 Growth Hormone

GH is a 191-amino-acid protein hormone that is secreted by the anterior pituitary gland. Its secretion is rhythmic and episodic over a 24-h period in mammals, including rats [87] and humans [88]. The ultradian rhythm of pituitary GH secretion can be influenced by various factors, including age [89], prolonged food deprivation [90], and sex steroids, particularly, estrogen [91]. There is a distinct sexual dimorphic pattern of GH secretion, which becomes apparent at the onset of puberty [92]. In adult male rats, the plasma GH profile is characterized by well-defined peaks (up to 250 ng GH/mL) and troughs where the levels are very low or undetectable (Fig. 4.1). The period between peaks typically lasts 3–4 h. By comparison, in female rats, GH is secreted in a more continuous manner such as the plasma GH peaks are lower (75 ng/mL), but circulating hormone levels are nearly always detectable (Fig. 4.1).

An initial clue that GH plays a role in regulating hepatic CYP expression was provided by the findings that GH administration decreased total CYP content and hepatic microsomal metabolism of CYP substrates [94]. It is now widely recognized that the sexual dimorphic pattern of GH secretion is the major hormonal determinant

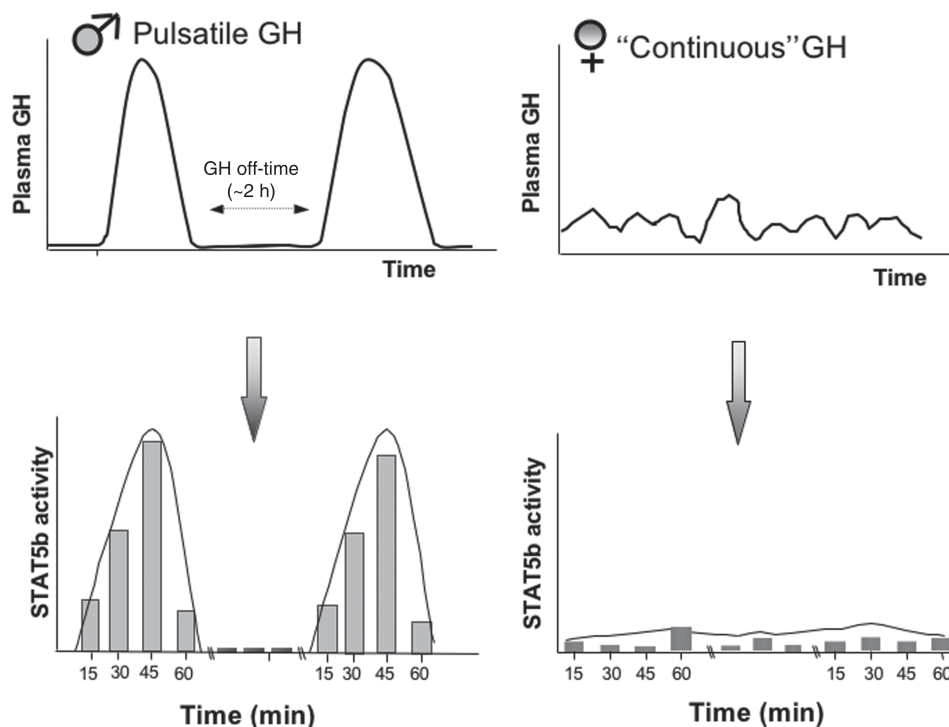


Figure 4.1 Sex dependence of the temporal pattern of plasma GH secretion and hepatic STAT5b activity in male and female rats [93]

TABLE 4.4 Responsiveness of Sex-Dependent Rodent Hepatic Cytochrome P450 Genes to Hypophysectomy and Exogenous Administration of GH^a

Rodent Hepatic Cytochrome P450 Genes	Hypophysectomy		Exogenous Administration of GH	
	Male	Female	Intermittent ^b	Continuous ^c
	Effect on Gene Expression		Effect on Gene Expression	
Rat, male-specific (class I) <i>CYP2C11</i>	↓	↑	↑	↓
Rat, male-specific (class II) <i>CYP2A2</i> , <i>CYP2C13</i> , and <i>CYP3A2</i>	No effect	↑	↓	↓
Rat, female-specific (class I) <i>CYP2C12</i>	↓	↓	↓	↑
Mouse, male-specific (class IA) <i>Cyp2d9</i>	↓	No effect	↑	↓
Mouse, male-specific (class IB) <i>Cyp7b1</i>	↓	↓	↑	↓
Mouse, male-specific (class IC) <i>Cyp4a12</i>	↓	↑	↑	↓
Mouse, female-specific (class II) <i>Cyp2a4</i> and <i>Cyp2b9</i>	↑	No effect	↓	↑

^aAs summarized in Ref. 79.^bIntermittent GH administration to hypophysectomized male rats.^cContinuous GH administration of intact male rats.

of the sex-dependent expression of rat hepatic CYPs [47]. On the basis of the available evidence, it appears that the interpulse period devoid of GH is a key signal in the masculinization of CYP expression in rat liver [95,96]. As shown in Table 4.4, the GH-regulated CYP enzymes can be categorized into different classes, based on their response to hypophysectomy [79,127,128]. The expression of a male class I gene (i.e., *CYP2C11*) is decreased in hypophysectomized male rats, whereas it is increased in hypophysectomized female rats but only to a level comparable to that in hypophysectomized male rats. *CYP2C11* levels are increased by intermittent administration of GH and decreased by continuous GH treatment. A corresponding pattern of response is seen for a female class I gene (e.g., *CYP2C12*), except that decreased expression occurs in hypophysectomized female rats and by intermittent GH administration. By comparison, hypophysectomy of female rats increases the expression of class II male-specific genes (i.e., *CYP2A2*, *CYP2C13*, and *CYP3A2*), whereas GH, in particular, continuous infusion of GH in male rats, represses the expression of these genes.

GH regulates the clearance of certain drugs that undergo CYP-mediated clearance [97–99], and also it appears that the sex-dependent pattern of GH secretion controls the expression of human CYP enzymes such as *CYP3A4*, which appears to be expressed at a somewhat greater level in livers from women than in livers from men (Section 4.4.2). As demonstrated in primary cultures of human hepatocytes, continuous GH treatment (to reflect the female pattern of GH secretion) increases *CYP3A4* expression [100,101], whereas intermittent GH administration (i.e., two daily doses, to mimic the pulsatile, male pattern of GH secretion) decreases *CYP3A4* expression, although the effect was

greater in hepatocytes isolated from males than in those from females [101]. The suppressive effect of intermittent GH has also been reported *in vivo*. In a human study conducted in male and female subjects with GH deficiency, GH infusion was associated with an increase in CYP3A activity, whereas GH administered as three daily doses was associated with a decrease in CYP3A activity, as assessed by the erythromycin breath test [102]. In another study conducted in GH-deficient boys and girls, administration of GH once daily for 30 days decreased CYP3A activity, as assessed by the urinary ratio of 6 β -hydroxycortisol/free cortisol, but this response was only observed in boys [103]. Interestingly, the administration of GH by constant infusion over a seven-day period increases CYP3A4 mRNA and protein levels in transgenic male mice [104]. Overall, it appears that the mode of GH administration (intermittent vs continuous) exerts differential effects on human CYP expression in a manner similar to that documented for rat CYP enzymes.

4.6 MOLECULAR DETERMINANTS OF SEX-DEPENDENT EXPRESSION OF HEPATIC CYTOCHROME P450s

Studies in the past decade have increased our understanding of the underlying molecular mechanisms through which GH controls the sex-dependent expression of hepatic CYP enzymes. GH exerts its action by binding to the growth hormone receptor (GHR), which is a member of the superfamily of cytokine receptors [105]. This leads to a conformational change of GHR and activation of a tyrosine kinase known as Janus kinase 2 (JAK2), which catalyzes the tyrosine phosphorylation of various intracellular proteins, including GHR and those belonging to the STAT family, such as STAT5b [106]. The GHR-bound, tyrosine phosphorylated STAT5b dimerizes and translocates to the nucleus where it binds to DNA response elements of target genes and stimulates gene transcription [105,107].

Whereas STAT5b is a direct transcriptional mediator of GH action, GH may also exert indirect effects on gene expression, through various HNFs, including HNF4 α [108], a member of the nuclear receptor superfamily [109]. The available evidence to date indicates that STAT5b and HNF4 α are molecular determinants of the sex-dependent expression of hepatic CYP enzymes [79]. These factors are likely to regulate hepatic CYPs and other sex-specific genes both directly and indirectly, via a complex transcriptional regulatory network, as indicated by the complex time course that characterizes the feminization of these sex-specific genes in male mice infused with GH continuously over a 14-day period [110]. Several other liver-expressed transcription factors show strong sex differences in their expression, including the female-specific factor Cux2 (Cutl2) [111] and the male-specific factor Bcl6 [112]. These factors are both dependent on GH and STAT5b for their sex-specific expression, suggesting that they may play a key role in this transcriptional regulatory network by regulating downstream targets of GH and STAT5b.

4.6.1 STAT5b

A hormonal basis for the tyrosine phosphorylation of STAT5b is provided by the experimental findings indicating that hepatic STAT5b activity is (i) greater in adult

TABLE 4.5 STAT5b and HNF4 α Regulate Sex-Dependent Mouse Hepatic Cytochrome P450 Gene Expression^a

Hepatic Cytochrome P450 Genes	Male		Female	
	STAT5b	HNF4α	STAT5b	HNF4α
	Deficiency	Deficiency	Deficiency	Deficiency
Effect on Gene Expression				
<i>Male-Specific Genes</i>				
<i>Cyp2d9</i>	↓	↓	↑	↓
<i>Cyp7b1</i>	↓	↓	↓	↓
<i>Cyp4a12</i>	↓	↓	No effect	No effect
<i>Female-Specific Genes</i>				
<i>Cyp2a4</i>	↑	↑	No effect	No effect
<i>Cyp2b9</i>	↑	↑	No effect	No effect

^aBased on Refs 110,118, and 119.

male rats than in adult female rats, (ii) abolished by hypophysectomy, (iii) stimulated by intermittent administration of GH but decreased by continuous administration of GH in hypophysectomized male rats, and (iv) stimulated by continuous GH infusion in hypophysectomized female rats [113,114]. Furthermore, there is a direct temporal relationship between the occurrence of a plasma GH pulse and the activation of hepatic STAT5b activity [115]. As illustrated in Fig. 4.1, in adult male rats, STAT5b is activated by each pulsatile secretion of GH, such that the time for peak STAT5b activity corresponds to the time for peak plasma GH levels and little or no STAT5b activity is detected during the interpulse, GH-free period. In contrast, in adult female rats, there is a very low but persistent level of STAT5b activity over time, similar to the temporal pattern of the low level, continuous release of plasma GH.

STAT5b is essential for the sex-dependent expression of hepatic CYP enzymes and many other sex-dependent genes, as determined in mice genetically deficient in STAT5b. Thus, the sexual dimorphism of hepatic CYP genes is abolished in the absence of STAT5b [116]. As shown in Table 4.5, when compared to the wild-type control male mice, STAT5b-deficient mice have decreased hepatic gene expression of male-specific CYPs (i.e., *Cyp2d9*, *Cyp4a12*, and *Cyp7b1*), but increased levels of female-specific CYPs (i.e., *Cyp2a4*, *Cyp2b9*, and *Cyp2b13*) [110,117]. In female mice, STAT5b deficiency leads to increased hepatic expression of the male-specific *Cyp2d9*, leads to decreases of the male-specific *Cyp7b1*, and has little or no effect on the female-specific *Cyp2a4* or *Cyp2b9* [118]. However, not all sex-dependent CYP genes are regulated by STAT5b because STAT5b deficiency does not influence the hepatic expression of the female-specific *Cyp3a16*, *Cyp3a41*, and *Cyp3a44* [118]. Moreover, hepatic *Cyp2d9* and *Cyp4a12* levels are increased in response to intermittent GH administration in hypophysectomized, STAT5b-expressing male mice but not in hypophysectomized, STAT5b-deficient male mice [110,117]. Collectively, these findings indicate positive regulation of male-specific CYP genes and negative regulation of a subset of female-specific CYP genes by GH pulse-activated STAT5b.

4.6.2 HNF4 α

HNF4 α (gene designation *NR2A1*) is a transcription factor initially known for its role in liver-specific gene expression [120]. It is expressed at high levels in liver and several tissues, and it is constitutively localized in the nucleus [121]. Among its many functions, HNF4 α has been shown to regulate the expression of CYP genes by interacting with various nuclear receptors including constitutive androstane receptor and pregnane X receptor [122,123]. HNF4 α also play a role in hepatic expression of sex-dependent CYP genes [110,124]. As summarized in Table 4.5, HNF4 α deficiency in male mice is associated with a decrease in hepatic levels of the male-specific genes *Cyp2d9*, *Cyp7b1*, and *Cyp4a12*, and an increase in that of the female-specific *Cyp2a4*. By comparison, HNF4 α deficiency in female mice decreases the low level of expression of *Cyp2d9*, whereas it has no effect on the male-specific *Cyp7b1* and *Cyp4a12* and the female-specific *Cyp2a4* and *Cyp2b9*. This pattern of response to HNF4 α deficiency is similar to that of STAT5b deficiency (Table 4.5) and is consistent with the idea that STAT5b is not the sole regulator of the sex-dependent hepatic *Cyp* genes [125,126]. The current view is that HNF4 α functions cooperatively with GH pulse-activated STAT5b to achieve the full expression of male-specific CYP genes [79], a view that is supported by microarray analysis showing global similarities in the dependence of sex-specific genes on STAT5b and HNF4 α in mouse liver [119].

4.7 CONCLUDING REMARKS

Sex differences in hepatic drug metabolism are well documented in rat and mouse models and are increasingly recognized as making a key contribution to sex differences in pharmacokinetics. The underlying mechanisms responsible for the sex differences in the hepatic expression of drug-metabolizing enzymes, in particular, CYPs, are not fully understood but involve complex patterns of hormonal control and intracellular signaling pathways. It is now well established that the sexually dimorphic pattern of plasma GH secretion is a major regulator that controls the sex-dependent expression of CYPs. Studies over the past few years have revealed that sex differences in the liver extend well beyond enzymes of drug metabolism to include more than 1000 genes [127,128], and the transcription factors STAT5b and HNF4 α have been identified as essential determinants of the sex-dependent expression of a large fraction of these genes, including many CYPs. Several other GH-regulated liver transcription factors that show strong sex differences in their expression, as well as a dependence on STAT5b, have also been identified and seem likely to be part of a complex transcriptional regulatory network that regulates the sex-dependent expression of CYPs and many other liver-expressed genes. Much has been learned but much more remains to be elucidated regarding the complex signaling pathways involved in the cellular and molecular actions of GH and how they contribute to sex differences in drug metabolism.

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5 Species Differences of Drug-Metabolizing Enzymes

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5.1 Overview	1
5.2 Introduction	2
5.3 Species differences in enzyme induction	3
5.4 Cytochrome P450s	7
5.5 Glucuronosyltransferases (UGTs)	21
5.6 Sulfotransferases (SULTs)	23
5.7 Flavin-containing monooxygenases (FMOs)	26
5.8 Summary	28
References	28

5.1 OVERVIEW

This chapter introduces the reader to the basic differences in drug-metabolizing enzymes (DMEs) between humans and the most commonly employed preclinical animal models. The phenotypic differences in metabolism that are observed between species are rooted in each species genotype; however, it is generally not possible to determine the observed phenotype from the specific genotype. These genotypic differences are largely driven by the different dietary and environmental pressures that each species has been exposed to and ultimately lead to an expressed specific phenotype. This chapter explores both phenotypic and genotypic differences among species in order to give an overview of the many factors that alter the metabolic response between different organisms.

The ability of an organism to respond to the numerous potentially toxic chemicals that it encounters is controlled by a “xenobiotic response,” which can roughly be broken down into two parts, xenobiotic-sensing receptors and DMEs. The xenobiotic-sensing receptors covered here are the aryl hydrocarbon receptor (AHR), the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR). These receptors control the expression of the DMEs that functionally modify xenobiotics in an attempt to detoxify and excrete them from the body. The enzymes covered in this chapter are the cytochrome P450s (CYPs), the uridine 5'-diphospho-glucuronosyltransferases (UGTs), the sulfotransferases (SULTs), and the flavin-containing monooxygenases (FMOs).

Although the overall goal of the “xenobiotic response” is the same for all mammalian species, the systems involved have diverged considerably over time. This divergence has led to differences in the primary genetic sequence of each gene resulting in differences in basal enzyme expression and induction, altered enzyme kinetics and substrate specificity, and ultimately the observed differences in the absorption, distribution, metabolism, excretion (ADME), and toxicity of xenobiotics.¹

5.2 INTRODUCTION

Animal models are commonly used in preclinical drug development to predict the pharmacokinetic and drug metabolism behaviors of new chemical entities (NCEs) in humans. One of the challenges drug metabolism scientists are facing is the extrapolation of metabolic data from animal studies to humans to incorporate into the overall ADME data set. Interspecies differences in drug metabolism have been recognized for many years, with some of the earlier studies investigating the ratio of ortho- to para-hydroxylation of aniline and 1,1'-biphenyl across species. These studies noted differences not only between species but also between different strains of animals, males and females, and the young and old [1–3].

The challenge in predicting human pharmacokinetics and drug metabolism from preclinical animal models is twofold in that there are significant differences in drug metabolism between species (the focus of this chapter) as well as a large amount of variability among human beings due in part to differences in enzyme and receptor expression levels and single nucleotide polymorphisms (SNPs). In order to make an informed decision as to which preclinical species will best predict human drug metabolism, it is important to gain a better understanding of the underlying mechanisms that explain species differences in drug metabolism. These differences are highly related to enzyme (isoform) composition, gene regulation, protein expression, and catalytic activities of the DMEs. The consequences of which can include altered bioavailability, changes in biological half-life, altered prodrug activation, and differential formation of active and/or toxic metabolites [4–7].

This chapter aims to provide the reader with a basic knowledge of the differences between preclinical species for the enzymes most critical in drug metabolism. The most important enzyme system for drug metabolism is the CYP superfamily, in particular, the CYP1, CYP2, and CYP3 families [8–10], and the bulk of the chapter focuses on these enzymes. The UGTs, SULTs, and FMOs are also covered but in lesser detail. Understanding species differences in drug metabolism is helpful for scientists to select the animal models from which the metabolism data can be most accurately extrapolated to humans. Unlike certain physiologic parameters, such as blood circulation and the ratio of organ weight to body weight, for which one can apply an allometric relationship across species in order to predict human pharmacokinetics from preclinical data, no such relationship can be applied to the biochemical parameters that govern the differences seen in the function of DMEs across species [11]. Therefore, it can be generally stated that the more extensively an NCE is metabolized, the more inaccurate

¹For the purpose of this chapter, all gene names for human, monkey, and dog are represented in all-capitalized letters. Gene names for rodents have only the first letter capitalized. Protein names are represented by all-capitalized letters for all species.

the empirical approach of allometric scaling in predicting human pharmacokinetics. In a retrospective analysis, the accuracy of quantitative approaches for the prediction of human pharmacokinetics was determined using 50 compounds for which extensive preclinical data as well as clinical data were available. One of the major conclusions from this analysis was that when a compound was cleared almost exclusively by P450-mediated pathways, scaling from *in vitro* human liver microsome (HLM) data was as predictive as single-species scaling of *in vivo* clearance data from rats, dogs, or monkeys [12].

In order to give the reader an overview of the biochemical basis for the functional differences between the major DMEs that are observed across species, the authors have attempted to focus on four major factors that differ between species for each enzyme subfamily discussed: species differences in inducible enzyme expression, similarity and dissimilarity of primary protein sequence, quantitative and qualitative differences in enzyme expression, and substrate specificity for the metabolism of clinically used drugs and other xenobiotics. The primary protein sequence analysis comes in the form of an accompanying table comparing the percentage amino acid identity for each subfamily discussed in the text (Tables 5.1–5.12). All the amino acid sequences used for the tables were collected from the UniProt database [13] (<http://www.uniprot.org/>), and the percentage homology scores were generated using the ClustalW2 [14] sequence analysis tool from the European Bioinformatics Institute website (<http://www.ebi.ac.uk/Tools/clustalw2/>). Sequences were collected and aligned for human, monkey, dog, rat, mouse, rabbit, guinea pig, and hamster. The tables are limited to sequences that were deposited in the UniProt database and therefore are not representative of every known DME or of every DME discussed in the text. Instead of being all encompassing, these tables are meant as a reference to give the reader an idea of the homology between the different enzyme families and subfamilies at the amino acid level while maintaining consistency with a single database in order to avoid some of the idiosyncrasies that arise during the automated/manual curation of sequence databases by different sources. It should also be noted that the amino acid identity does not necessarily translate into catalytic specificity as a single amino acid substitution is sufficient to alter substrate specificity [15], and many considerations must be made when attempting to determine the appropriate preclinical species for drug metabolism studies. These considerations include differences in the expression levels and inducibility of individual DMEs, tissue- and gender-specific expression, and differences in substrate specificity, as well as factors that may not be related to drug metabolism at all. Turpeinen *et al.* note that if hepatic metabolism were the only factor in choosing a preclinical species, in many cases, the rat is unlikely to be an appropriate model even though it is a very common nonprimate preclinical species; however, it might also be argued that there is no global need for selecting the animal model that is metabolically closest to man. For example, in studies designed for establishing safety margins, one could select an animal model that is likely to result in high amounts of metabolites and potentially improve the safety factors for risk analyses [6].

5.3 SPECIES DIFFERENCES IN ENZYME INDUCTION

Many of the DMEs display inducible expression levels in what is considered an adaptive metabolic response. This inducible expression is governed principally by three

TABLE 5.1 Percentage Amino Acid Homology for CYP1A Subfamily in Various Species

	Human CYP1A1	Human CYP1A2	Cynomolgus Monkey CYP1A1	Cynomolgus Monkey CYP1A2	Rhesus Monkey CYP1A1	Dog CYP1A1	Dog CYP1A2	Rat Cyp1a1	Rat Cyp1a2	Mouse Cyp1a1	Mouse Cyp1a2	Rabbit Cyp1a1	Rabbit Cyp1a2	Guinea Pig Cyp1a1	Guinea Pig Cyp1a2	Hamster Cyp1a1	Hamster Cyp1a2
Human CYP1A1	100	71	94	71	94	81	67	79	66	80	66	76	67	77	66	79	65
Human CYP1A2	71	100	71	92	71	72	79	66	73	67	71	69	77	67	76	66	72
Cyno CYP1A1	94	71	100	72	99	82	67	78	66	79	66	76	68	77	66	77	65
Cyno CYP1A2	71	92	72	100	72	72	80	68	75	68	73	70	79	68	77	66	74
Rhesus CYP1A1	94	71	99	72	100	82	67	78	66	79	66	76	68	77	66	77	65
Dog CYP1A1	81	72	82	72	82	100	73	76	65	77	65	77	68	75	67	76	66
Dog CYP1A2	67	79	67	80	67	73	100	63	71	63	70	64	75	64	71	62	71
Rat Cyp1a1	79	66	78	68	78	76	63	100	68	93	67	76	65	76	64	86	65
Rat Cyp1a2	66	73	66	75	66	65	71	68	100	69	93	63	73	65	73	66	88
Mouse Cyp1a1	80	67	79	68	79	77	63	93	69	100	70	76	66	76	64	87	68
Mouse Cyp1a2	66	71	66	73	66	65	70	67	93	70	100	63	73	64	74	65	88
Rabbit Cyp1a1	76	69	76	70	76	77	64	76	63	76	63	100	71	72	65	75	62
Rabbit Cyp1a2	67	77	68	79	68	68	75	65	73	66	73	71	100	65	73	64	71
Guinea Pig Cyp1a1	77	67	77	68	77	75	64	76	65	76	64	72	65	100	69	75	63
Guinea Pig Cyp1a2	66	76	66	77	66	67	71	64	73	64	74	65	73	69	100	63	73
Hamster Cyp1a1	79	66	77	66	77	76	62	86	66	87	65	75	64	75	63	100	68
Hamster Cyp1a2	65	72	65	74	65	66	71	65	88	68	88	62	71	63	73	68	100

transcription factors: AHR, a ligand-activated member of the Per-Arnt-Sim superfamily [16], and CAR [17] and PXR [18,19], two members of the nuclear receptor superfamily. These three receptors work in concert to alter the expression of a separate but overlapping set of DME genes in response to various xenobiotics. The DME genes controlled by these receptors appear to be relatively consistent across species. The AHR has been historically known as a regulator of xenobiotic metabolism and a mediator of dioxin toxicity but it has also been implicated in many other physiologic roles [20,21]. The AHR is generally associated with the induction of the CYP1 genes, but it is also believed to regulate the expression of some of the UGT1A enzymes [22,23]. Regulation of these genes by the AHR occurs by heterodimerization of the AHR with the aryl hydrocarbon receptor nuclear translocator (ARNT) [24] and by binding to dioxin response elements (DREs) upstream of target genes [25]. Although CAR and PXR seem to be more intimately linked to the overall xenobiotic response than AHR, they have also been implicated in physiologic processes, most notably, the regulation of hepatic energy metabolism [26]. CAR and PXR regulate the expression of many CYP2B, CYP2C, CYP3A, UGT, and SULT genes by forming a heterodimer with the retinoid X receptor (RXR) and binding to a number of different promoter elements upstream of their target genes [27].

Although the battery of DME genes regulated by these receptors is fairly well conserved across species, there are considerable species differences in the ligands that activate the receptors. The ingestion of polycyclic aromatic hydrocarbons (PAHs) and β -naphthoflavone in rat, mouse, monkey, and dog leads to an increase in the level of CYP1A proteins in numerous tissues, such as liver, lung, and intestine [28–30]. The human CYP1B1 gene is also induced by dioxin [31]. Omeprazole has been reported to induce CYP1A2 in man by activating AHR, but the mechanism is not fully understood. The induction of Cyp1a genes by omeprazole does not occur in rat and mouse and is one of the few examples of species-specific activation of AHR [32]. The AHR has also shown considerable species differences in its affinity for dioxin. Guinea pigs show a 1000-fold lower sensitivity to dioxin-induced toxicity when compared to hamster [33]. There are two Ahr alleles in mouse Ahr^b and Ahr^d. The Ahr^d encodes a receptor with 10-fold lower affinity for dioxin [20,34]. The human AHR shows a further 10-fold reduction in affinity for dioxin when compared to the mouse Ahr^d allele [35]. Therefore, the C57BL/6 strain of mice that express the AHR^b enzyme [36] is likely not to be a good model for investigations into human AHR activation.

The nuclear receptors CAR and PXR show a greater degree of alternative ligand binding across species compared to AHR. Phenobarbital is a potent activator of CAR in most species but it does not physically bind to CAR, instead it activates CAR by inducing the translocation of the receptor from the cytoplasm to the nucleus [37]. Lack of binding to CAR is probably the reason why activation by phenobarbital is so well conserved among species. CAR ligands, on the other hand, show a much wider array of effects on the receptor. For example, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is a potent activator of mouse CAR but not human or rat [38], 2,4,6-triphenyldioxane-1,3 (TPD) is specific to rat [39], and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) is specific to human [40]. The antifungal clotrimazole is an inverse agonist of human CAR but an agonist of rat CAR [41]. The anti-nausea agent meclizine is also an inverse agonist of human CAR but is a potent agonist of mouse CAR [42]. PXR can bind to a large variety of chemicals because of its large and flexible ligand-binding pocket [43].

Rifampicin is a potent PXR ligand in humans, dogs, and rabbits [30,44,45]. In mice and rats, the prototypical PXR agonists are dexamethasone and pregnenolone 16 α -carbonitrile (PCN) [44,45]. Troglitazone is an activator of human PXR and inducer of human CYP3A4 but does not induce Cyp3a in rodent models or activate rodent PXR [46]. Clotrimazole is an agonist of human, mouse, and rat PXR [41,47], and hyperforin, an active component of St. John's Wort, is an agonist of human PXR but not rat [48].

As discussed in greater detail later, the evolution of adaptive responses to xenobiotics is likely to be greatly influenced by diet. Phylogenetic analyses of CAR and PXR support this hypothesis. CAR and PXR diverged from the chicken X receptor (Cxr) and showed cross talk in the genes that they regulate and the ligands that activate them, creating some redundancy in the expression pathways that regulate DMEs in higher organisms [49] presumably as a means of responding to a larger cadre of exogenous chemicals that these organisms were exposed to. Both of these nuclear receptors show high ratios of nonsynonymous (Ka) to synonymous (Ks) substitutions (the ω -ratio) within their ligand-binding domains when compared to other members of the nuclear receptor superfamily [50]. This ratio is highly suggestive of positive selection for binding to unique ligands and is not surprising when one considers that these two receptors are the most intimately related to the expression of the DMEs most important to xenobiotic metabolism. The high ω -ratio generally equates to rapid evolution and can again likely be traced back to the evolutionary pressure that diet places on this system. In light of this, it is somewhat surprising that there is almost no variation seen in the ligand-binding domains of CAR or PXR among humans [51,52], with most of the SNPs being found within the Japanese population [53,54]. This observation suggests that there are ligands applying negative pressure to the evolution of the CAR and PXR ligand-binding domains in the human population [50]. Phylogenetic analysis of the AHR suggests that ligand binding is a function of the receptor, which arose during vertebrate evolution [20] and has likely developed as a secondary role to the many physiologic processes that the receptor has been implicated in, such as fetal vascular development [55] and cardiac hypertrophy [56].

Further functional differences in these receptors across species can be found in the differential splicing of the primary transcripts. CAR has been shown to generate splice variants in mouse [57] and human [58–62]. The alternatively spliced mouse receptor lacked the ability to bind DNA and did not show any transcriptional or inhibitory properties [57]. A larger number of alternatively spliced CAR transcripts have been described in humans, a few of which have shown functional differences when compared to the reference form of the receptor, including a switch from constitutive activity to ligand-activated activity for CAR2 and CAR3 as well as the ability of CAR2 to recognize unique ligands [63–65]. A number of human PXR splice variants have also been described [66]. The most abundant variant, hPXR.2, is missing 37 amino acids from the ligand-binding pocket and displays significantly diminished ligand-activated transcriptional activity [67,68]. The intronic sequences that signal for the alternative exon splicing do not appear to be well conserved across species, for example, the splice acceptor site for CAR2 has been shown to be conserved across human, rhesus monkey, and guinea pig, but not in mouse, rat, or marmoset [64]. These observations suggest that changes in the genetic code that control pre-mRNA processing are another means by which the diversity of xenobiotic response systems and the ability to adapt to environmental pressures can be increased in a species.

5.4 CYTOCHROME P450s

5.4.1 Overview

The CYPs are hemoproteins that are among the most ancient proteins known and are expressed in almost all living organisms today, from archaeobacteria to humans [69]. They are a fundamental enzyme family for the anabolic and catabolic metabolism of many of the endogenous chemicals required for life and also play critical roles in the biotransformation (bioactivation and detoxification) of a wide variety of xenobiotic chemicals [70,71]. The CYPs are believed to have evolved from a single ancestral gene 3.5 to 4.0 billion years ago [72]. Around 2.8 billion years ago, when oxygen levels began to increase dramatically in the atmosphere, the CYPs likely became involved in the detoxification of oxidative stress [1]. When plants and animals diverged ~1.2 billion years ago, it is believed that an “animal–plant warfare” began, whereby plants evolved toxic or unpalatable compounds to deter animals from eating them and in turn animals evolved new CYP genes to detoxify these chemicals [73]. The “animal–plant warfare” hypothesis is further supported by the observation that there was a large increase in the number of CYP genes following the evolutionary divergence of organisms with a gastrointestinal tract, which led to an increased exposure to xenobiotics from diet [70]. The unique selective pressure that diet places on the animal CYP superfamily and the overall xenobiotic response machinery is likely to be a major evolutionary drive of the functional differences observed between species. This is probably of particular importance for the CYP families 1, 2, and 3, which appear to be most important for xenobiotic metabolism and overall ADME [74], as well as the xenobiotic-sensing receptors that govern their expression.

The CYPs are encoded by a superfamily of genes and exhibit a high degree of structural and functional similarity across a vast array of species. They are localized in the smooth endoplasmic reticulum of the cell and distributed throughout the body, particularly, in the liver, intestine, nasal epithelia, and skin [9,75]. The liver and intestine are the predominate sites for P450-mediated drug elimination and are also a major site of drug–drug interactions (DDIs). The CYPs have been characterized in many species of organisms, including bacteria, fungi, plants, fish, and mammalian systems [76]. There are more than 6500 known CYP enzymes organized into 708 families, of these approximately 2279 among 99 families exist in animals. The approximately 50 CYP enzymes described in man have the most clinical relevance, in particular, approximately the 22 genes in families 1, 2, and 3 that are responsible for the majority of the metabolism of drugs and environmental pollutants [74]. The CYP enzymes from other families are largely involved in endogenous processes, particularly, biosynthesis of hormones, fatty acids, and vitamins [77]. Notably, microbial CYPs are also of clinical importance due mainly to the metabolic capacity that exists in the microflora of the gut. Differences in the composition of these microbes can account for some of the intra- and interindividual differences in drug metabolism and can lead to unexpected metabolic outcomes [78,79]. Gut microflora have been implicated in the metabolism of a number of different drugs including sulfasalazine, sulfinpyrazone, metronidazole, digoxin, isosorbide, flucytosine, and levodopa [1,80].

There are a number of animal species that have been used as preclinical models of drug metabolism; monkey, dog, rat, and mouse see the most widespread use, while rabbit, guinea pig, and hamster are used to a significantly lesser degree. There

are a number of observed differences in the CYPs across these species, including nucleotide/protein sequence, regulation, genetic polymorphism, substrate catalytic specificity, and inhibitor selectivity. 7-Ethoxycoumarin *O*-deethylase (ECOD) activity is catalyzed by several CYP isoforms and can be used as a general measure of xenobiotic-metabolizing capacity [81,82]. This method was applied in an *in vitro* study comparing overall CYP activity between human and several preclinical species, using liver microsomes. The result of the study showed that when compared to humans, ECOD activity was approximately 8.6%, 66.7%, 622%, 1086%, and 508% for mouse, rat, rabbit, dog, and monkey, respectively [6]. This result demonstrates the large range in activity levels that can be found between species and serves as a good example as to why it is valuable to have a good mechanistic understanding of the individual enzymes across species and how they differ in substrate specificity and metabolite formation.

5.4.2 CYP1A

The CYP1A subfamily (Table 5.1) consists of two members, CYP1A1 and CYP1A2. These enzymes have been extensively studied because of their importance in the bioactivation of mutagens and carcinogens. CYP1A activity can be monitored using an ethoxyresorufin *O*-deethylase (EROD) assay [83]. EROD activity in mouse, rat, rabbit, dog, and monkey liver microsomes has been shown to be approximately 374%, 102%, 413%, 630%, and 983%, respectively, of the activity found in humans [6]. The CYP1A enzymes are responsible for the species-specific metabolism of caffeine, where in humans the major metabolite is paraxanthine generated through N3-demethylation. Monkeys generate mostly theophylline by N7-demethylation, while rat and mouse favor C8-hydroxylation to 1,3,7-trimethyluric acid, and rabbits show approximately equal formation of paraxanthine, theophylline, 1,3,7-trimethyluric acid, and theobromine [84].

CYP1A1 is responsible for bioactivation of many environmental carcinogens, such as the PAHs benzo[*a*]pyrene and 3-methylchlanthrene, to highly carcinogenic diol epoxides [85]. The catalytic activities of the CYP1A enzymes are well conserved across mammals. In humans, CYP1A1 is not constitutively expressed (or its basal expression is very low) but is instead expressed only after induction, likely through the AHR pathway [86]. Since CYP1A1 is expressed in very low levels in human liver, the specific activity for the metabolism of PAHs in rodents is more efficient than the activity seen in uninduced human livers [87–89].

CYP1A2 is expressed almost exclusively in the liver of humans, rats, and mice [90,91]. In contrast, monkeys and dogs express CYP1A2 at low levels in the liver in the absence of treatment with an inducer [30,92]. The functional characteristics of CYP1A2 are well conserved across species [4]. In contrast to PAHs, carcinogenic heterocyclic amines are metabolized predominately by CYP1A2 [93,94]. For example, carcinogenic 2-amino-3,8-dimethylimidazol[4,5-*f*]quinoxaline (MeIQx) is metabolized in a species-specific pattern. Rodent CYP1A2 catalyzes both C- and N-oxidation of MeIQx, whereas human CYP1A2 only metabolizes N-hydroxylation at the amine group (a bioactivation pathway to carcinogenicity) [95]. In contrast, the cynomolgus monkey is resistant to MeIQx-related carcinogenicity due to low basal expression levels of CYP1A2 [96]. Therefore, the monkey is not a valuable model for comparison to human.

Human CYP1A2 accounts for 13% of the total liver CYP content and is involved in the metabolism of 4% of drugs in the market, including phenacetin, tacrine, ropinirole, acetaminophen (APAP), riluzole, theophylline, and caffeine. Furaflavone is a selective inhibitor of CYP1A2 in humans, mice, rats, and dogs but is not an inhibitor of CYP1A2 in monkeys. [9,97]. Phenacetin and caffeine have been commonly used as the marker substrates for CYP1A2 in several species. Although there is a strong similarity in amino acid sequence for CYP1A2 and similar catalytic activities toward many drugs have been demonstrated between humans and many animal species, the expression of these enzymes varies considerably among species. For example, dogs and monkeys have low expression levels of the enzyme in the liver and may not serve as good models of CYP1A2-mediated drug metabolism and toxicity in humans in the drug development process [98].

5.4.3 CYP1B

CYP1B1 is the only member of the CYP1B family (Table 5.2). Human CYP1B1 is constitutively expressed in normal tissues, such as lung, liver, heart, brain, kidney, placenta, and prostate [31]. Expression of CYP1B1 is species dependent. Rat Cyp1b1 is expressed in liver and lung, while mouse Cyp1b1 is present in testis, kidney, lung, brain, and heart but not in liver [90,99]. CYP1B1 is differentially expressed within the tumor microenvironment of many cancers, showing much higher expression in tumor cells compared to the surrounding tissues [100,101]. It is also an important factor in determining risk associated with hormone-mediated cancers and catalyzes estrogens to form active 4-hydroxylated derivatives that may cause breast cancer [102]. Similar to CYP1A1, human CYP1B1 and rodent CYP1B1 can bioactivate carcinogenic PAHs to DNA-reactive species associated with toxicity, mutagenesis, and carcinogenesis [103]. Currently, little is known about the activity of CYP1B1 in most preclinical species, and further investigations are necessary.

5.4.4 CYP2A

Table 5.3 shows the percentage amino acid homology for the CYP2A subfamily across species. The human CYP2A subfamily includes CYP2A6, CYP2A7, and CYP2A13. Human CYP2A6 is expressed predominately in liver where it accounts for about 4% of total hepatocyte CYP content [9] but has also been found in the nasal mucosa, trachea, lung, and skin [104,105], while CYP2A13 is mainly expressed in the respiratory

TABLE 5.2 Percentage Amino Acid Homology for CYP1B Subfamily in Various Species

	Human CYP1B1	Cynomolgus Monkey CYP1B1	Dog CYP1B1	Rat Cyp1b1	Mouse Cyp1b1
Human CYP1B1	100	94	84	80	81
Cyno CYP1B1	94	100	86	84	83
Dog CYP1B1	84	86	100	80	81
Rat Cyp1b1	80	84	80	100	93
Mouse Cyp1b1	81	83	81	93	100

TABLE 5.3 Percentage Amino Acid Homology for CYP2A Subfamily in Various Species

	Human CYP- 2A6	Human CYP- 2A7	Human CYP- 2A13	Cyno- molgus Monkey CYP2A23	Cyno- molgus Monkey CYP2A24	Rhesus Monkey CYP2A23	Rhesus Monkey CYP2A24	Dog CYP- 2A13	Dog CYP- 2A25	Rat Cyp- 2a1	Rat Cyp- 2a2	Rat Cyp- 2a3	Mouse Cyp2a4	Mouse Cyp2a5	Mouse Cyp2a12	Mouse Cyp2a22	Rabbit Cyp2a10	Rabbit Cyp2a11	Hamster Cyp2a8	Hamster Cyp2a9
Human CYP2A6	100	93	93	92	94	92	94	89	85	69	64	85	83	85	69	70	84	84	73	69
Human CYP2A7	93	100	91	89	93	89	93	87	83	69	65	84	82	83	70	70	82	83	72	69
Human CYP2A13	93	91	100	94	93	93	94	92	87	72	67	88	86	88	72	73	87	87	75	71
Cyno CYP2A23	92	89	94	100	93	98	93	91	87	72	68	88	85	87	72	73	86	86	75	71
Cyno CYP2A24	94	93	93	93	100	93	98	89	86	70	66	86	84	85	71	71	85	84	75	70
Rhesus CYP2A23	92	89	93	98	93	100	93	90	87	71	68	87	85	87	72	73	86	86	75	71
Rhesus CYP2A24	94	93	94	93	98	93	100	90	87	70	66	87	85	86	71	72	85	85	76	70
Dog CYP2A13	89	87	92	91	89	90	90	100	93	71	66	90	87	89	71	71	87	88	73	71
Dog CYP2A25	85	83	87	87	86	87	87	93	100	70	66	86	83	85	70	71	84	84	72	69
Rat Cyp2a1	69	69	72	72	70	71	70	71	70	100	88	71	69	70	88	86	69	69	65	83
Rat Cyp2a2	64	65	67	68	66	68	66	66	66	88	100	67	65	66	82	80	65	64	61	76
Rat Cyp2a3	85	84	88	88	86	87	87	90	86	71	67	100	94	96	72	72	86	86	76	71
Mouse Cyp2a4	83	82	86	85	84	85	85	87	83	69	65	94	100	97	69	70	84	85	74	70
Mouse Cyp2a5	85	83	88	87	85	87	86	89	85	70	66	96	97	100	70	71	86	86	76	70
Mouse Cyp2a12	69	70	72	72	71	72	71	71	70	88	82	72	69	70	100	93	69	70	65	81
Mouse Cyp2a22	70	70	73	73	71	73	72	71	71	86	80	72	70	71	93	100	69	70	65	80
Rabbit Cyp2a10	84	82	87	86	85	86	85	87	84	69	65	86	84	86	69	69	100	98	74	68
Rabbit Cyp2a11	84	83	87	86	84	86	85	88	84	69	64	86	85	86	70	70	98	100	74	68
Hamster Cyp2a8	73	72	75	75	75	75	76	73	72	65	61	76	74	76	65	65	74	74	100	63
Hamster Cyp2a9	69	69	71	71	70	71	70	71	69	83	76	71	70	70	81	80	68	68	63	100

tract [106]. Rat Cyp2a1 (female dominant) and Cyp2a2 (male dominant) are expressed in the liver [107,108], while rat Cyp2a3 is constitutively expressed in the esophagus, lung, and nasal epithelium but is absent in liver [109]. Mouse Cyp2a5 is expressed in the olfactory mucosa, liver, lung, kidney, brain, and small intestine [110]. Cyp2a4 is a predominately female isoform found in mouse liver [111], and mouse Cyp2a12 has been found in the liver but not in the olfactory mucosa [112].

The human CYP2A enzymes show different substrate specificity when compared to the CYP2A of animal species. Human CYP2A6 is involved in the metabolism of a wide range of xenobiotics, including coumarin, nicotine, cyclophosphamide, fadrozole, and aflatoxin B1 [4,113]. Activation of aflatoxin B1 to its carcinogenic/toxic AFB1 (aflatoxin B1)-8,9-epoxide and AFM1 (aflatoxin M1)-8,9-epoxide by CYP2A13 is likely to play a role in the development of lung cancer after aflatoxin inhalation [114]. CYP2A13 is also involved in the metabolism of nicotine and cotinine [115]. Unlike their human orthologs, rodent CYP2A enzymes catalyze the 7 α - and 15 α -hydroxylation of steroids. For example, CYP2A1 is highly specific for the catalysis of 7 α -hydroxylation of testosterone, while CYP2A2 catalyzes both the 15 α - and the 7 α -hydroxylation of testosterone [113,116]. All CYP2A isoforms appear to catalyze coumarin 7-hydroxylation (a marker substrate) but exhibit regioselectivity of the hydroxylation at all six possible positions [117]. Using coumarin 7-hydroxylase activity as a measure of overall CYP2A function [118] in mouse, rabbit, dog, and monkey liver microsomes, it was found that these species had approximately 18%, 8.7%, 2.9%, and 75%, respectively, of the activity found in HLM preparations. In the same study, coumarin 7-hydroxylase activity in rats was found to be below the limit of quantification [6], and in a similar study, it was found to be detectable only when large amounts of substrate were used [119]. This is because in rats, coumarin metabolism occurs mainly through a 3,4-epoxidation reaction catalyzed by CYP2A1 [120]. This regioselective difference in coumarin metabolism between humans and rats also explains the differences in coumarin toxicity between the species; coumarin shows hepatotoxicity and tumor induction in rats because of the epoxide metabolite [117]. In mice, coumarin 7-hydroxylase activity is carried out by CYP2A5 [121] and varies across different strains. The DBA/1J and DBA/2J strains show high 7-hydroxylase activity unlike the C57BL/6J strain that demonstrates low activity at this site [122].

5.4.5 CYP2B

The homology of the CYP2B subfamily across species is presented in Table 5.4. The human CYP2B family includes CYP2B6 and the pseudogene CYP2B7. CYP2B6 is mainly expressed in liver and is involved in the metabolism of a large number of drugs in the market, such as the anticancer drugs cyclophosphamide and tamoxifen, the anesthetics ketamine and propofol, and the procarcinogen aflatoxin B1 [117]. Using pentoxyresorufin *O*-depentylase (PROD) activity as a measurement of overall CYP2B activity [83], it was found that activity in mouse, rat, rabbit, dog, and monkey microsomes was approximately 1000%, 517%, 4827%, 3793%, and 827% of human microsomal activity, respectively [6]. In the same study, using the more specific bupropion hydroxylase assay [123,124], the CYP2B activity was found to be 47%, below quantitation limit, 1084%, 146%, and 605% for the same species compared to humans. The difference in these two assays highlights the large variability that can be seen in the metabolism of different substrates by similar enzymes. The basal expression of

TABLE 5.4 Percentage Amino Acid Homology for CYP2B Subfamily in Various Species

	Human CYP2B6	Cyno- molgus Monkey CYP2B6	Rhesus Monkey CYP2B30	Dog CYP2B11	Rat Cyp2b1	Rat Cyp2b2	Rat Cyp2b3	Rat Cyp2b12	Rat Cyp2b15	Mouse Cyp2b9	Mouse Cyp2b10	Mouse Cyp2b13	Mouse Cyp2b19	Rabbit Cyp2b4	Rabbit Cyp2b5
Human CYP2B6	100	91	91	78	75	74	67	72	72	71	75	71	74	77	75
Cyno CYP2B6	91	100	99	80	75	74	66	72	71	71	73	71	74	78	77
Rhesus CYP2B30	91	99	100	80	75	75	67	72	72	71	74	71	74	78	77
Dog CYP2B11	78	80	80	100	74	74	67	71	70	71	74	72	73	79	78
Rat Cyp2b1	75	75	75	74	100	97	76	80	80	80	90	82	82	77	76
Rat Cyp2b2	74	74	75	74	97	100	76	79	80	80	88	80	82	77	76
Rat Cyp2b3	67	66	67	67	76	76	100	71	71	76	75	76	74	68	68
Rat Cyp2b12	72	72	72	71	80	79	71	100	89	77	78	77	87	72	71
Rat Cyp2b15	72	71	72	70	80	80	71	89	100	76	78	76	86	74	72
Mouse Cyp2b9	71	71	71	71	80	80	76	77	76	100	83	89	79	72	71
Mouse Cyp2b10	75	73	74	74	90	88	75	78	78	83	100	83	81	77	75
Mouse Cyp2b13	71	71	71	72	82	80	76	77	76	89	83	100	79	72	72
Mouse Cyp2b19	74	74	74	73	82	82	74	87	86	79	81	79	100	76	74
Rabbit Cyp2b4	77	78	78	79	77	77	68	72	74	72	77	72	76	100	97
Rabbit Cyp2b5	75	77	77	78	76	76	68	71	72	71	75	72	74	97	100

CYP2B6 has been reported to be extremely variable in humans [125–127], and the gene is also highly inducible through the CAR/PXR pathway, making the extrapolation from preclinical species even more difficult.

Mouse Cyp2b9 (female) and Cyp2b10 (male) are sexually dimorphic and lack catalytic activity. The rat Cyp2b subfamily mainly includes Cyp2b1 and Cyp2b2, which have very similar substrate specificities. Both are expressed constitutively in liver and in the extrahepatic tissues, intestine, and lung. Dog CYP2B11 catalyzes the N-demethylation of dextromethorphan, N1-demethylation of diazepam, and the 4'-hydroxylation of mephenytoin [128,129]. Rat, rabbit, and dog CYP2B isoforms all metabolize steroids (androstenedione, testosterone, and progesterone); however, each of these enzymes show unique steroid hydroxylation profiles [130]. 7-Ethoxy-4-trifluoromethylcoumarin and 7-pentoxoresorufin are also metabolized by CYP2B isoforms. K_m and V_{max} values for the enzymes are conserved in liver microsomes across human, mouse, rat, dog, and rabbit species but not in monkey species [7]. Cyclophosphamide, an anticancer prodrug, is activated in the liver by 4-hydroxylation and deactivated by N-dechloroethylation, both of which were observed in rat (CYP2B1) and man (CYP2B6) [131].

5.4.6 CYP2C

The CYP2C subfamily is the largest subfamily and contains multiple isoforms in all species (Table 5.5). In humans, CYP2C isoforms make up ~20% of the total CYP content in liver [132] and are involved in the metabolism of about 16% of drugs in the market [133]. CYP2C8 and CYP2C9 are the major expressed CYP2C forms in humans (>85%), while CYP2C19 expression is low (1%) [134]. Relative to human microsomes, CYP2C activity in mouse, rat, rabbit, dog, and monkey has been found to be 0.6%, 39%, 34%, below the limit of quantitation, and 47%, respectively, by the tolbutamide hydroxylase assay (OH-TOL) [135,136] and 43%, 28%, 50%, below the limit of quantitation, and 172%, respectively, by the omeprazole 5-hydroxylase (5-OH-OME) assay [6,137].

CYP2C8 is expressed mainly in the liver but has also been detected in the kidney, adrenals, and small intestine [138]. It catalyzes the metabolism of retinol and retinoic acid, arachidonic acid, and the anticancer drug paclitaxel (a selective marker) [9,139]. CYP2C9 shows a pattern of expression that is very similar to that of CYP2C8 [138] and metabolizes many clinically important drugs including the diabetic drugs tolbutamide (a selective marker); the anticonvulsant phenytoin; the anticoagulant warfarin; many anti-inflammatory drugs, such as ibuprofen, diclofenac, piroxicam, and tenoxicam; the antihypertensive losartan; the antidiabetic glipizide; and the diuretic torasemide [140]. CYP2C19 is detected in the liver and small intestine [138], is highly polymorphic [141], and has been shown to metabolize *S*-mephenytoin (a selective marker), omeprazole, imipramine, diazepam, barbiturates, and proguanil [9,142–144].

Mouse has more than 10 Cyp2c members. These Cyp2c isoforms have similar catalytic activity in the oxidation of arachidonic acid and hydroxyeicosatetraenoic acids [145]. Among the mouse Cyp2c isoforms, CYP2C37 is the most abundant form in the liver and CYP2C29 is the most abundant in the lung, while CYP2C40 is the major CYP2C found in both kidney and intestine [145–147]. All these enzymes catalyze tolbutamide. The rat Cyp2c family includes at least seven isoforms. They are all expressed in the liver and oxidize dihydropyridines, aflatoxin B1, and steroids [148].

TABLE 5.5 Percentage Amino Acid Homology for CYP2C Subfamily in Various Species

	Human CYP2C8	Human CYP2C9	Human CYP2C18	Human CYP2C19	Cyno- molgus Monkey CYP- 2C20	Cyno- molgus Monkey CYP- 2C43	Cyno CYP- 2C75	Cyno CYP- 2C76	Dog CYP- 2c21	Dog CYP- 2C41	Rat Cyp- 2c6	Rat Cyp- 2c7	Rat Cyp- 2c11	Rat Cyp- 2c12	Rat Cyp- 2c13	Rat Cyp- 2c23	Mouse Cyp- 2c29	Mouse Cyp- 2c37	Mouse Cyp- 2c38	Mouse Cyp- 2c39	Mouse Cyp- 2c40
Human CYP2C8	100	77	77	77	92	78	76	70	66	70	71	68	73	64	64	60	71	68	71	71	65
Human CYP2C9	77	100	81	91	78	92	93	71	68	75	75	71	76	66	66	61	75	73	72	72	66
Human CYP2C18	77	81	100	81	76	80	81	71	69	75	77	71	77	67	68	61	76	73	74	74	66
Human CYP2C19	77	91	81	100	78	90	91	72	70	74	75	71	74	67	66	61	74	72	72	72	65
Cyno CYP2C20	92	78	76	78	100	77	76	69	67	70	71	68	74	66	65	60	71	69	71	71	65
Cyno CYP2C43	78	92	80	90	77	100	93	71	68	74	72	70	74	65	65	61	73	71	71	71	65
Cyno CYP2C75	76	93	81	91	76	93	100	70	69	74	74	71	74	65	66	61	75	71	72	72	65
Cyno CYP2C76	70	71	71	72	69	71	70	100	77	70	69	63	69	60	60	58	68	65	65	65	61
Dog CYP2c21	66	68	69	70	67	68	69	77	100	69	66	63	65	61	60	58	66	65	66	66	60
Dog CYP2C41	70	75	75	74	70	74	74	70	69	100	71	66	70	65	66	61	70	70	67	68	66
Rat Cyp2c6	71	75	77	75	71	72	74	69	66	71	100	75	72	67	69	59	82	76	77	79	67
Rat Cyp2c7	68	71	71	71	68	70	71	63	63	66	75	100	69	68	69	57	83	70	79	80	68
Rat Cyp2c11	73	76	77	74	74	74	74	69	65	70	72	69	100	65	65	60	73	71	71	71	64
Rat Cyp2c12	64	66	67	67	66	65	65	60	61	65	67	68	65	100	80	54	70	71	69	69	77
Rat Cyp2c13	64	66	68	66	65	65	66	60	60	66	69	69	65	80	100	54	70	72	70	70	79
Rat Cyp2c23	60	61	61	61	60	61	61	58	58	61	59	57	60	54	54	100	60	59	58	59	52
Mouse Cyp2c29	71	75	76	74	71	73	75	68	66	70	82	83	73	70	70	60	100	76	84	84	68
Mouse Cyp2c37	68	73	73	72	69	71	71	65	65	70	76	70	71	71	72	59	76	100	74	74	71
Mouse Cyp2c38	71	72	74	72	71	71	72	65	66	67	77	79	71	69	70	58	84	74	100	91	70
Mouse Cyp2c39	71	72	74	72	71	71	72	65	66	68	79	80	71	69	70	59	84	74	91	100	70
Mouse Cyp2c40	65	66	66	65	65	65	65	61	60	66	67	68	64	77	79	52	68	71	70	70	100

Rhesus monkey CYP2C43, CYP2C74, and CYP2C75 (not shown) display 99% amino acid homology to the cynomolgus monkey CYP2C43, CYP2C20, and CYP2C75 sequences, respectively.

Cyp2c11 (male), Cyp2c12 (female), and Cyp2c13 (male) are gender specific. The dog CYP2C21 and CYP2C41 enzymes are found in the liver, and the latter has been shown to be highly polymorphic. The substrate specificity (tolbutamide, warfarin, and *S*-mephenytoin) of the two dog CYP2C isoforms is impaired when compared to that of humans [149]. Monkey has genes encoding for CYP2C20 and CYP2C43, which are expressed in the liver. CYP2C43, but not CYP2C20, was able to metabolize *S*-mephenytoin, a probe of human CYP2C19, and was not able to metabolize tolbutamide, a probe substrate of human CYP2C9, suggesting that CYP2C43 appears to be functionally related to CYP2C19 [9,150]. Substrate specificities are largely different among the species, particularly, CYP2C forms in dog as a model for human. The stereoselective metabolism of mephenytoin, a marker substrate for CYP2C isoforms shows differences between species. In rabbit, dog, and rat, the *R*-enantiomer is preferentially 4'-hydroxylated two- to sixfold higher than that of the *S*-form. In female mice, both the enantiomers are equally good substrates; and in the rat, CYP2C11 preferentially metabolizes the *S*-enantiomer. However, both human and monkey CYP2C forms preferentially 4'-hydroxylate *S*-mephenytoin with similar K_m values, suggesting that monkeys may be useful as a human model in the metabolism of *S*-mephenytoin [151].

5.4.7 CYP2D

Amino acid homologies for the CYP2D subfamily are presented in Table 5.6. Human CYP2D6, the only active human CYP2D isoform, makes up approximately 1–3% of the total CYP content in the liver and catalyzes the biotransformation of ~30% clinically used drugs. CYP2D6 mainly catalyzes antidepressants (desipramine), β -blockers (propranolol), the antiarrhythmics (sparteine), and dextromethorphan, as well as methadone [152]. Using the dextromethorphan O-demethylation reaction as a selective marker of CYP2D activity [153,154], mouse, rat, rabbit, dog, and monkey microsomes displayed approximately 141%, 96%, 148%, 45%, and 234% of the human microsome activity, respectively [6].

SNPs have been extensively studied in the CYP2D6 enzyme and have been shown to cause a large degree of variability in drug turnover rates for CYP2D6 substrates [155]. CYP2D6 is also known to undergo gene deletion and duplications in humans, a genetic process known as *copy number variation* (CNV), the result of which leads to variable enzyme expression and consequently altered activity [156]. The gene has been reported to be multiplied up to 12-fold in humans, resulting in an ultrarapid metabolizer phenotype [157]. Data on copy number variation of DMEs in preclinical species and what consequences these genetic differences may incur for extrapolations to humans is currently lacking, but it is known that CNVs exist in other species and it appears as though the mechanisms by which they occur are conserved [158]. This issue is further complicated by the fact that the well-documented CYP2D6 SNPs are carried over to the duplicated copies of the gene [159]. The ramifications of CNVs and the combination of CNVs and SNPs on extrapolating drug metabolism data from preclinical species to humans remain unclear and may be very difficult to assess due to conventional laboratory breeding techniques but it is worth noting.

There are as many as nine mouse Cyp2d isoforms but little is known about their catalytic functions. Rats have six Cyp2d isoforms expressed in various tissues, such as liver, kidney, and brain. Cyp2d1 is the likely rat orthologue of human CYP2D6 and shows a similar substrate specificity [160]. Dog CYP2D15 is the major CYP2D form

TABLE 5.6 Percentage Amino Acid Homology for CYP2D Subfamily in Various Species

	Human CYP2D6	Cyno- molgus Monkey CYP2D17	Rhesus Monkey	Dog CYP- 2D15	Rat Cyp- 2d1	Rat Cyp- 2d3	Rat Cyp- 2d4	Rat Cyp- 2d10	Rat Cyp- 2d18	Rat Cyp- 2d26	Mouse Cyp2d9	Mouse Cyp2d10	Mouse Cyp2d11	Mouse Cyp2d26	Guine Pig Cyp2d16	Hamster Cyp2d20	Hamster Cyp2d27	Hamster Cyp2d28
Human CYP2D6	100	93	93	74	71	72	77	71	76	71	69	69	66	70	72	72	71	69
Cyno CYP2D17	93	100	98	76	71	72	76	71	76	72	70	70	67	72	72	73	73	70
Rhesus CYP2D42	93	98	100	75	71	72	76	71	76	72	70	71	67	71	72	74	72	70
Dog CYP2D15	74	76	75	100	65	67	70	64	70	66	63	64	61	68	68	67	66	67
Rat Cyp2d1	71	71	71	65	100	79	72	95	72	73	82	83	80	74	68	75	74	76
Rat Cyp2d3	72	72	72	67	79	100	75	79	75	77	78	79	75	78	70	77	76	76
Rat Cyp2d4	77	76	76	70	72	75	100	72	99	72	70	71	68	72	70	72	72	72
Rat Cyp2d10	71	71	71	64	95	79	72	100	73	72	81	83	79	73	67	75	74	76
Rat Cyp2d18	76	76	76	70	72	75	99	73	100	72	70	72	69	71	69	72	71	72
Rat Cyp2d26	71	72	72	66	73	77	72	72	72	100	72	73	70	88	71	82	81	72
Mouse Cyp2d9	69	70	70	63	82	78	70	81	70	72	100	87	85	75	68	73	72	75
Mouse Cyp2d10	69	70	71	64	83	79	71	83	72	73	87	100	87	74	67	75	73	77
Mouse Cyp2d11	66	67	67	61	80	75	68	79	69	70	85	87	100	71	64	72	71	73
Mouse Cyp2d26	70	72	71	68	74	78	72	73	71	88	75	74	71	100	70	79	79	73
Guinea Pig Cyp2d16	72	72	72	68	68	70	70	67	69	71	68	67	64	70	100	70	69	67
Hamster Cyp2d20	72	73	74	67	75	77	72	75	72	82	73	75	72	79	70	100	95	73
Hamster Cyp2d27	71	73	72	66	74	76	72	74	71	81	72	73	71	79	69	95	100	71
Hamster Cyp2d28	69	70	70	67	76	76	72	76	72	72	75	77	73	73	67	73	71	100

and also demonstrates enzymatic activities similar to human CYP2D6, such as 1'-hydroxylation of bufuralol and quinidine metabolism [7,98]. Cynomolgus monkey has CYP2D17 an isoform that is 93% identical to human CYP2D6 at the amino acid level. It metabolizes bufuralol and dextromethorphan and is strongly inhibited by quinidine. The marmoset monkey has two known isoforms of CYP2D: CYP2D30 and CYP2D19. Both enzymes have shown regioselectivity of debrisoquine hydroxylation at various positions. A comparison of the CYP2D marker activity, bufuralol 1'-hydroxylation, in liver microsomes of different species showed that rat, mouse, rabbit, and monkey CYP2D6 exhibit a K_m value about 10 times lower than that of human CYP2D6, whereas the V_{max} value is about 6–10 times higher than that of the human enzyme. The kinetic behavior of dog CYP2D15 and human CYP2D6 was shown to be very similar for the catalysis of bufuralol, metoprolol, and dextromethorphan [7].

5.4.8 CYP2E

CYP2E1 is the only member of the CYP2E subfamily (Table 5.7) and is expressed in many tissues such as the nose, oropharynx, lung, and liver. CYP2E1 plays a detoxification role in the metabolism of alcohol to reduce alcohol levels after excessive intake and is also involved in the metabolism of ketones and fatty acids associated with diabetes and obesity [161]. CYP2E1 metabolizes small molecule drugs such as APAP, enflurane, caffeine, and chlorzoxazone (a marker substrate) [162]. In addition to these drugs, some environmental carcinogens and toxicants (benzene, styrene, acrylonitrile, and nitrosoamines) are also metabolized by CYP2E1 to superoxide radicals that cause liver injury and bind to DNA or cellular proteins [163,164]. CYP2E1 is inducible but the mechanism differs from the increased mRNA transcription that occurs after activation of the xenobiotic receptors AHR, CAR, and PXR. Instead, this inducibility occurs through increased translational efficiency and decreased protein turnover [165,166]. CYP2E1 induction by alcohol has been demonstrated in both animals [167] and man [168].

CYP2E1 is known to be highly conserved in mammals [169], and the metabolic pattern of CYP2E1 in humans appears to be very similar to that observed in rodents, and therefore, rodents and rat, in particular, may be an appropriate model to study CYP2E1 in man [98]. In further support of the hypothesis that there is a high level of functional conservation of CYP2E1 across species, it has been shown that in a chlorzoxazone 6-hydroxylase (6-OH-CLZ) assay [170], CYP2E1 activity in mouse, rat, rabbit, dog, and monkey was 150%, 47%, 45%, 45%, and 78% of the average human activity, respectively [6]. This result demonstrates that there is much less variability in chlorzoxazone metabolism than what is generally seen for marker substrates of the other CYP subfamilies. Although chlorzoxazone is considered to be a very good marker substrate for CYP2E1 in mouse, rat, rabbit, dog, monkey, and man, other enzymes have been implicated in its metabolism. Dog CYP1A has been shown to be catalytically active in the metabolism of chlorzoxazone, and human and rodent CYP1A2 may also contribute to chlorzoxazone metabolism [7].

5.4.9 CYP3A

The CYP3A subfamily (Table 5.8) plays a very important role in the metabolism of xenobiotics and catalyzes more than 50% of therapeutic drugs [171], such as

TABLE 5.7 Percentage Amino Acid Homology for CYP2E Subfamily in Various Species

	Human CYP2E1	Cynomolgus Monkey CYP2E1	Rhesus Monkey CYP2E1	Dog CYP2E1	Rat Cyp2e1	Mouse Cyp2e1	Rabbit Cyp2e1	Hamster Cyp2e1
Human CYP2E1	100	91	94	77	78	78	79	78
Cyno CYP2E1	91	100	95	73	77	76	76	75
Rhesus CYP2E1	94	95	100	76	80	79	79	79
Dog CYP2E1	77	73	76	100	76	76	75	76
Rat Cyp2e1	78	77	80	76	100	93	81	90
Mouse Cyp2e1	78	76	79	76	93	100	80	88
Rabbit Cyp2e1	79	76	79	75	81	80	100	79
Hamster Cyp2e1	78	75	79	76	90	88	79	100

TABLE 5.8 Percentage Amino Acid Homology for CYP3A Subfamily in Various Species

	Human CYP3A4	Human CYP3A5	Human CYP3A7	Human CYP3A43	Cynomolgus Monkey CYP3A8	Dog CYP- 3A12	Rat Cyp- 3a1	Rat Cyp- 3a2	Rat Cyp- 3a9	Rat Cyp- 3a18	Mouse Cyp- 3a11	Mouse Cyp- 3a13	Mouse Cyp- 3a16	Mouse Cyp- 3a25	Mouse Cyp- 3a41	Rabbit Cyp- 3a6	Guinea Pig Cyp3a14	Guinea Pig Cyp3a15	Guinea Pig Cyp3a17	Hamster Cyp3a10	Hamster Cyp3a31
Human CYP3A4	100	84	88	75	93	79	73	72	76	68	72	75	70	71	71	75	71	71	70	67	69
Human CYP3A5	84	100	81	75	83	78	72	70	75	68	73	74	70	71	72	74	70	69	69	67	68
Human CYP3A7	88	81	100	71	89	74	70	69	72	65	70	72	68	67	68	72	69	68	67	64	66
Human CYP3A43	75	75	71	100	73	72	63	63	67	64	64	67	63	66	63	70	64	63	63	61	62
Cyno CYP3A8	93	83	89	73	100	77	71	71	75	67	71	74	69	69	70	74	71	69	69	66	68
Dog CYP3A12	79	78	74	72	77	100	69	69	75	66	70	73	67	68	69	74	68	67	66	66	66
Rat Cyp3a1	73	72	70	63	71	69	100	88	72	69	87	70	83	70	86	70	70	69	69	68	81
Rat Cyp3a2	72	70	69	63	71	69	88	100	71	67	85	69	81	67	83	68	68	67	67	66	81
Rat Cyp3a9	76	75	72	67	75	75	72	71	100	68	73	91	69	71	71	74	70	69	69	68	70
Rat Cyp3a18	68	68	65	64	67	66	69	67	68	100	69	67	66	90	67	64	67	66	65	79	65
Mouse Cyp3a11	72	73	70	64	71	70	87	85	73	69	100	70	87	70	91	71	70	68	68	69	81
Mouse Cyp3a13	75	74	72	67	74	73	70	69	91	67	70	100	66	69	67	72	68	68	67	67	68
Mouse Cyp3a16	70	70	68	63	69	67	83	81	69	66	87	66	100	67	87	68	67	66	66	66	76
Mouse Cyp3a25	71	71	67	66	69	68	70	67	71	90	70	69	67	100	69	66	69	68	67	80	66
Mouse Cyp3a41	71	72	68	63	70	69	86	83	71	67	91	67	87	69	100	70	68	66	67	67	78
Rabbit Cyp3a6	75	74	72	70	74	74	70	68	74	64	71	72	68	66	70	100	71	69	68	65	68
Guinea Pig Cyp3a14	71	70	69	64	71	68	70	68	70	67	70	68	67	69	68	71	100	96	93	66	69
Guinea Pig Cyp3a15	71	69	68	63	69	67	69	67	69	66	68	68	66	68	66	69	96	100	92	66	67
Guinea Pig Cyp3a17	70	69	67	63	69	66	69	67	69	65	68	67	66	67	67	68	93	92	100	66	68
Hamster Cyp3a10	67	67	64	61	66	66	68	66	68	79	69	67	66	80	67	65	66	66	66	100	66
Hamster Cyp3a31	69	68	66	62	68	66	81	81	70	65	81	68	76	66	78	68	69	67	68	66	100

terfenadine, midazolam, diazepam, quinidine, lidocaine, carbamazepine, nifedipine, tacrolimus, dapsone, erythromycin, and dextromethorphan [172]. In addition, CYP3A is also involved in the metabolism of endogenous substances such as steroids, bile acids, and retinoic acid. Human CYP3A enzymes (CYP3A4 and CYP3A5) are expressed in liver, stomach, lung, intestine, and kidney and are the most abundant CYP isoforms in human liver [173]. Using midazolam 1'-hydroxylase (1'-OH-MDZ) as a measure of CYP3A activity [174], mouse, rat, rabbit, dog, and monkey microsomal levels of activity were found to be approximately 103%, 9.2%, 20%, 137%, and 128%, respectively, of the average human activity [6]. Midazolam is a marker substrate of human CYP3A4 and shows significant interspecies variability in metabolism. Higher rates of midazolam 1'-hydroxylation were observed in dogs and monkeys in comparison with human; however, no significant differences were observed between cynomolgus and rhesus monkeys [175].

Mouse has at least six Cyp3a isoforms of which Cyp3a11 is believed to be the orthologue of human CYP3A4. The mouse Cyp3a41 and Cyp3a42 isoforms are preferentially expressed in females [176]. The catalytic activity of the mouse CYP3A toward clinically used drugs has not been extensively evaluated, but it has been shown that some chemicals that are metabolized by human CYP3A4 can be also catalyzed by mouse CYP3A, such as aflatoxin B1 and ethylmorphine [177]. Rats have six Cyp3a isoforms that are expressed in a gender-specific manner. For example, Cyp3a2 and Cyp3a18 are male specific, whereas Cyp3a9 is a female-dominant isoform [178]. Oral bioavailability does not generally correlate well between human and rat forms, and this may be due to higher Cyp3a expression in rat intestine and consequently increased first-pass metabolism [5]. Rats are also not an appropriate model for human CYP3A induction because of the lack of induction response of Cyp3a1/2 to rifampin, a known inducer of human CYP3A [179]. In addition, the metabolism of some CYP3A substrates in rats is different from that in human. For example, nifedipine and dihydropyridine are not metabolized by rat CYP3A [4,10]. The dog CYP3A family has two isoforms, CYP3A12 and CYP3A26. Dog CYP3A has shown similar catalytic activities toward the marker substrates for human CYP3A4 [129,180]. Cynomolgus monkey CYP3A8 is expressed largely in the liver and shows 93% amino acid sequence homology to human CYP3A4 and is identical to rhesus monkey CYP3A64 [181]. CYP3A64 is capable of metabolizing testosterone, midazolam, nifedipine, and 7-benzoxo-4-trifluoromethylcoumarin, which are also substrates for human CYP3A4 and CYP3A5. However, species differences in the CYP3A catalytic activities are observed among rats, rabbits, dogs, and humans.

Testosterone 6 β -hydroxylation is a commonly used marker activity for CYP3A enzymes in all species. There is no significant difference in the reaction among the species, with exception of dogs (higher K_m value) and female rats (higher K_m and lower V_{max} values) [7]. Rats demonstrate a sex-related difference in CYP3A activity. Rat CYP3A not only shows higher rates of the 6 β -hydroxylation than human CYP3A but also generates 2 β - and 16 β -hydroxyltestosterone. On the basis of kinetic similarity in liver microsomes for testosterone 6 β -hydroxylation, mouse and male rat appear to be the most similar species to human. Mouse microsomal testosterone 6 β -hydroxylation is higher in females than in males probably due to the catalytic activity of CYP3A41, a female-specific form [182]. Lidocaine metabolism provides another strong example of interspecies differences in CYP3A metabolism. In humans, lidocaine undergoes an N-deethylation catalyzed by CYP3A4 [183,184]; however, in rats, the major metabolite

is formed through 3-hydroxylation catalyzed by a member of the CYP2D subfamily [1,185]. In general, CYP3A enzymes in all species are involved in the metabolism of xenobiotics and show species-related differences in substrate specificity, expression, induction, and inhibition in various organs and tissues, which together contribute to altered drug clearance, efficacy, and toxicity across species. This wide degree of inter-species variation makes the extrapolation of CYP3A data from animals to man quite difficult.

5.5 GLUCURONOSYLTRANSFERASES (UGTs)

The UGTs catalyze the addition of a glucuronic acid moiety to nucleophilic substrates using uridine diphosphate/glucuronic acid (UDPGA) as a cofactor. The UGT superfamily is composed of a large multiplicity of forms, each with a broad and overlapping substrate selectivity and wide tissue distribution [186,187]. Three major subfamilies responsible for drug metabolism are present to date: UGT1A (Table 5.9), UGT2B (Table 5.10), and UGT2A (Table 5.11). Similar to CYPs, UGTs are localized in the endoplasmic reticulum membrane; however, the active site is exposed on the luminal side of the endoplasmic reticulum, while CYPs are on the cytosolic side [188]. Many endogenous and xenobiotic compounds are able to be glucuronidated by the UGTs. For example, bilirubin and estradiol-3-glucuronidations are selective for UGT1A1, propofol is glucuronidated by UGT1A9, and AZT and morphine glucuronidations occur through UGT2B7. Other endogenous substrates that undergo glucuronidation include bilirubin, estradiol, and other steroids, as well as some fatty acids [189]. UGT1A3 and UGT1A4 are expressed in the liver, intestine, and colon and are the important enzymes in the glucuronidation of many tertiary amine or aromatic heterocycles to form quaternary ammonium glucuronides. The formation of quaternary ammonium glucuronides appears to be highly species specific, with the highest activity being found in humans and monkeys. Rats and mice are generally incapable of forming quaternary ammonium glucuronides because the UGT1A3 and UGT1A4 isoforms are pseudogenes in these rodents. Lamotrigine is extensively glucuronidated at the 2-position of the triazine ring in humans, which represents >80% of the dose excreted in urine. Lamotrigine is not significantly glucuronidated in rats and dogs, while 60% of the dose is excreted in guinea pig urine as the 2-*N*-glucuronide [190]. UGT1A6 is the most important enzyme for the conjugation of planar phenols and amines. It displays high activity for a variety of aromatic alcohols, such as 1-naphthol, 4-nitrophenol, 4-methylumbelliferone, and in the detoxification of APAP. Cats are highly susceptible to APAP liver toxicity because UGT1A6 is a pseudogene in this species [191]. Afloqualone (AFQ) is a centrally acting muscle relaxant, and AFQ *N*-glucuronide is the most abundant metabolite in human urine when administered orally, whereas it was not detected in urine when administered to rats, dogs, and monkeys [192]. Species differences in AFQ *N*-glucuronidation were investigated with liver microsomes obtained from humans and experimental animals. The K_m and V_{max} values of glucuronidation in HLMs were 2.0 mM and 0.87 nmol/min/mg protein, respectively. The V_{max} and intrinsic clearance (CL_{int}) values of AFQ *N*-glucuronidation in human liver were approximately 4- to 10-fold and 2- to 4-fold higher, respectively, than those in rat, dog, and monkey. Both recombinant UGT1A4 and UGT1A3 exhibited high AFQ *N*-glucuronosyltransferase activities.

TABLE 5.9 Percentage Amino Acid Homology for UGT1A Subfamily in Various Species

	Human UGT- 1A1	Human UGT- 1A3	Human UGT- 1A4	Human UGT- 1A5	Human UGT- 1A6	Human UGT- 1A7	Human UGT- 1A8	Human UGT- 1A9	Human UGT- 1A10	Cyno- molgus Monkey UGT1A01	Cyno- molgus Monkey UGT1A06	Cyno- molgus Monkey UGT1A08	Dog UGT- 1A6	Rat Ugt- 1a1	Rat Ugt- 1a2	Rat Ugt- 1a3	Rat Ugt- 1a6	Rat Ugt- 1a8	Mouse Ugt1a1	Mouse Ugt1a2	Mouse Ugt1a6	Mouse Ugt1a9
Human UGT1A1	100	71	71	72	67	66	65	66	65	95	66	64	61	78	66	67	62	61	77	66	61	62
Human UGT1A3	71	100	93	94	66	66	67	67	66	69	65	65	60	64	75	75	61	60	64	75	60	62
Human UGT1A4	71	93	100	93	66	66	66	67	66	69	65	65	61	65	74	74	62	61	64	74	61	61
Human UGT1A5	72	94	93	100	66	67	67	67	66	70	64	66	60	64	75	74	61	60	64	74	61	62
Human UGT1A6	67	66	66	66	100	68	68	68	68	66	95	66	80	62	62	63	79	62	62	63	79	63
Human UGT1A7	66	66	66	67	68	100	94	93	93	65	66	92	61	62	61	60	62	77	62	62	63	78
Human UGT1A8	65	67	66	67	68	94	100	94	94	64	65	95	61	62	62	60	62	78	63	62	63	78
Human UGT1A9	66	67	67	67	68	93	94	100	93	65	66	93	61	63	63	61	63	78	63	62	64	78
Human UGT1A10	65	66	66	66	68	93	94	93	100	65	65	92	61	62	62	60	61	77	62	62	62	78
Cyno UGT1A01	95	69	69	70	66	65	64	65	65	100	68	66	62	78	66	67	62	61	78	66	62	62
Cyno UGT1A06	66	65	65	64	95	66	65	66	65	68	100	67	80	62	61	63	80	61	62	63	79	62
Cyno UGT1A08	64	65	65	66	66	92	95	93	92	66	67	100	62	62	62	60	61	78	63	62	62	79
Dog UGT1A6	61	60	61	60	80	61	61	61	61	62	80	62	100	63	61	63	78	60	62	61	78	60
Rat Ugt1a1	78	64	65	64	62	62	62	63	62	78	62	62	63	100	69	70	66	67	91	68	65	67
Rat Ugt1a2	66	75	74	75	62	61	62	63	62	66	61	62	61	69	100	83	66	66	69	91	65	66
Rat Ugt1a3	67	75	74	74	63	60	60	61	60	67	63	60	63	70	83	100	68	65	70	80	66	64
Rat Ugt1a6	62	61	62	61	79	62	62	63	61	62	80	61	78	66	66	68	100	66	66	65	93	65
Rat Ugt1a8	61	60	61	60	62	77	78	78	77	61	61	78	60	67	66	65	66	100	65	65	65	84
Mouse Ugt1a1	77	64	64	64	62	62	63	63	62	78	62	63	62	91	69	70	66	65	100	68	64	66
Mouse Ugt1a2	66	75	74	74	63	62	62	62	62	66	63	62	61	68	91	80	65	65	68	100	65	66
Mouse Ugt1a6	61	60	61	61	79	63	63	64	62	62	79	62	78	65	65	66	93	65	64	65	100	66
Mouse Ugt1a9	62	62	61	62	63	78	78	78	78	62	62	79	60	67	66	64	65	84	66	66	66	100

TABLE 5.10 Percentage Amino Acid Homology for UGT2A Subfamily in Various Species

	Human UGT2A1	Human UGT2A3	Rat Ugt2a1	Mouse Ugt2a1	Mouse Ugt2a3	Guinea Pig Ugt2a3
Human UGT2A1	100	61	87	88	72	65
Human UGT2A3	61	100	60	60	62	71
Rat Ugt2a1	87	60	100	95	76	65
Mouse Ugt2a1	88	60	95	100	78	64
Mouse Ugt2a3	72	62	76	78	100	66
Guinea Pig Ugt2a3	65	71	65	64	66	100

Expression of the UGT1A gene utilizes different promoters that yield transcripts containing alternative first exons and four identical downstream exons [193]. There is a 93–94% sequence homology in the first exon of human UGT1A7 to UGT1A10, two isoforms that show considerable variation in the level of tissue expression. UGT1A9 is expressed in human liver and kidney, whereas UGT1A7 (esophagus and gastric epithelium), UGT1A8 (intestine), and UGT1A10 (intestine) are expressed extrahepatically. In contrast, rat and rabbit Ugt1a7 are expressed in liver. The rabbit UGT1A7 shows high activity for a variety of small phenolic molecules and imipramine. Rat UGT1A7 catalyzes the glucuronidation of benzo[*a*]pyrene phenols and is inducible by 3-methylcholanthrene. Human UGT1A7 has high turnover for the glucuronidation of 7-ethyl-10-hydroxycamptothecin, an active metabolite of irinotecan, and therefore, may play a role in the gastrointestinal first-pass metabolism of the drug along with UGT1A8 and UGT1A10 [194]. UGT1A8 catalyzes reactions of several drugs including, opioids (buprenorphine, morphine, naloxone, and naltrexone), ciprofibrate, diflunisal, furosemide, phenolphthalein, propofol, raloxifene, 4-OH-tamoxifen, and tolcapone [194]. UGT1A9 is largely responsible for the glucuronidation of a variety of bulky phenols, for example, *tert*-butylphenols and propofol (a marker substrate for UGT1A8 and UGT1A9). UGT2B7 is expressed in the liver and involved in the glucuronidation of non-steroidal anti-inflammatory drugs (NSAIDs), morphine, 3-OH-benzodiazepines, and zidovudine. The glucuronidation of morphine to a pharmacologically active metabolite by human UGT2B7 has been well studied. Morphine-6-glucuronide is much more potent in receptor binding than morphine; however, it has poor permeability and is unlikely to cross the blood–brain barrier. Rats are unable to make morphine-6-glucuronide. UGT2B15 and UGT2B17 are the major forms in human prostate and other tissues such as liver, kidney, skin, brain, mammary gland, ovaries, adipose tissue, and uterus and are responsible for the conjugation of androgens and drugs such as racemic oxazepam [195]. Little has been reported on the species difference of these conjugation reactions.

5.6 SULFOTRANSFERASES (SULTs)

The cytosolic SULTs are derived from a large gene superfamily. SULT1 and SULT2 families are the most important for xenobiotic metabolism. SULTs catalyze the sulfation reactions of many endogenous substances and drugs in the presence of PAPS

TABLE 5.11 Percentage Amino Acid Homology for UGT2B Subfamily in Various Species

	Human UGT- 2B4	Human UGT- 2B7	Human UGT- 2B10	Human UGT- 2B11	Human UGT- 2B15	Human UGT- 2B17	Cyno- molgus Monkey UGT2B9	Cyno- molgus Monkey UGT2B18	Cyno- molgus Monkey UGT2B19	Cyno- molgus Monkey UGT2B20	Cyno- molgus Monkey UGT2B23	Cyno- molgus Monkey UGT2B30	Dog UGT- 2B31	Rat Ugt- 2b1	Rat Ugt- 2b2	Rat Ugt- 2b3	Rat Ugt- 2b4	Rat Ugt- 2b5	Mouse Ugt- 2b36	Mouse Ugt- 2b37	Mouse Ugt- 2b34
Human UGT2B4	100	85	85	85	78	78	85	86	88	76	83	88	77	71	65	65	66	64	68	66	70
Human UGT2B7	85	100	87	85	78	76	89	89	82	75	87	82	75	69	65	65	67	64	67	64	68
Human UGT2B10	85	87	100	90	77	77	87	87	81	75	84	80	74	67	62	62	64	62	62	67	68
Human UGT2B11	85	85	90	100	76	76	86	86	81	74	83	81	73	68	61	62	64	61	65	63	66
Human UGT2B15	78	78	77	76	100	94	78	78	76	92	77	77	76	71	65	65	67	65	69	66	67
Human UGT2B17	78	76	77	76	94	100	77	77	76	90	75	77	76	70	65	66	68	66	69	67	67
Cyno UGT2B9	85	89	87	86	78	77	100	95	82	75	94	82	75	68	64	64	66	63	66	65	69
Cyno UGT2B18	86	89	87	86	78	77	95	100	82	75	94	82	75	70	64	64	66	63	66	64	69
Cyno UGT2B19	88	82	81	81	76	76	82	82	100	75	80	94	75	68	63	63	66	63	67	64	67
Cyno UGT2B20	76	75	75	74	92	90	75	75	75	100	74	75	75	69	65	65	67	64	68	67	67
Cyno UGT2B23	83	87	84	83	77	75	94	94	80	74	100	79	73	67	62	62	65	62	65	63	67
Cyno UGT2B30	88	82	80	81	77	77	82	82	94	75	79	100	75	67	63	64	66	63	67	65	68
Dog UGT2B31	77	75	74	73	76	76	75	75	75	75	73	75	100	73	64	64	66	64	68	65	70

Rat Ugt2b1	71	69	67	68	71	70	68	70	68	69	67	67	73	100	61	61	63	60	65	61	68
Rat Ugt2b2	65	65	62	61	65	65	64	64	63	65	62	63	64	61	100	84	77	83	79	81	60
Rat Ugt2b3	65	65	62	62	65	66	64	64	63	65	62	64	64	61	84	100	79	92	81	85	62
Rat Ugt2b4	66	67	64	64	67	68	66	66	66	67	65	66	66	63	77	79	100	78	82	78	63
Rat Ugt2b5	64	64	62	61	65	66	63	63	63	64	62	63	64	60	83	92	78	100	80	85	62
Mouse Ugt2b36	68	67	62	65	69	69	66	66	67	68	65	67	68	65	79	81	82	80	100	82	63
Mouse Ugt2b37	66	64	67	63	66	67	65	64	64	67	63	65	65	61	81	85	78	85	82	100	61
Mouse Ugt2b34	70	68	68	66	67	67	69	69	67	67	67	68	70	68	60	62	63	62	63	61	100

(3'-phosphoadenosine 5'-phosphosulfate), providing the sulfuryl donor for the reactions. In humans, the SULT enzyme family comprises 11 isoforms of the SULT1, SULT2, and SULT4 families that code for 10 distinct genes. In humans, SULT1A1 is expressed in the liver and metabolizes sulfation of many known drugs, such as APAP, troglitazone, minoxidil, 4-OH-tamoxifen, and apomorphine. SULT1A3, a catecholamine sulfotransferase, is absent in the adult liver, with the major site of expression being the gastrointestinal tract. This has implications for the metabolism of a number of drugs; for example, salbutamol is almost exclusively sulfated in humans. Human SULT1C2 and SULT1C4 are almost exclusively expressed in fetal tissues, although the function of these enzymes remains unclear. Another important enzyme is estrogen sulfotransferase (SULT1E1), which has a very high affinity (low nanomolar) for its natural substrate 17 β -estradiol and for a major drug substrate 17 α -ethinylestradiol. SULT1E1 is important for modulating estrogen action in the endometrium [196,197]. SULT2A1 plays a selective role in the N-sulfation of quinolone drugs and other amine drugs (ciprofloxacin, moxifloxacin, garenoxacin, desipramine), as well as metoclopramide, pregnenolone, and dehydroepiandrosterone [198]. SULT4A1 cDNAs have been isolated so far from rats, mice, and humans, and the predicted proteins share a remarkable degree of similarity. The SULT4A1 proteins seem to be expressed only in the brain, and despite significant efforts, no natural or xenobiotic substrate has yet been identified [199,200]. Mouse Sult6b1 has been recently cloned and expressed, and it exhibited sulfation activity toward thyroxine and bithionol and a variety of other endogenous and xenobiotic compounds. While human SULT6B1 was specifically expressed in kidney and testis, mouse Sult6b1 was detected in brain, heart, kidney, thymus, lung, liver, and testis [201].

5.7 FLAVIN-CONTAINING MONOOXYGENASES (FMOs)

FMOs are a family of FAD-containing enzymes located in the endoplasmic reticulum. At least five gene products are known to exist in mammals (Table 5.12). The catalytic function of these enzymes involves the oxygenation of soft nucleophilic heteroatoms, including nitrogen, sulfur, phosphorus, and selenium. FMOs catalyze the biotransformation of a wide variety of xenobiotics and endogenous compounds. Human FMO1 has the broadest substrate specificity, particularly for bulky nonpolar lipophilic compounds such as imipramine and orphenadrine, which are selective markers for FMO1 in the kidney. Human FMO3, present in the liver, has a unique property to selectively metabolize small amines, such as trimethylamine. FMO5 is expressed in adult liver and has poor catalytic activity compared to other FMOs. It stereoselectively catalyzes sulfoxidation of short-chain arylalkyl sulfides. Little is reported about the species difference of their catalytic activity. However, the tissue distribution of FMOs in animal species has been reported in rabbits [202]. Rabbit Fmo1 mRNA expression is highest in liver and intestine, Fmo2 in lung, while Fmo3, Fmo4, and Fmo5 are present in lung and kidney. FMO1 seems to be the dominant form in pig and male rat livers. Hepatic Fmo3 expression levels in adult rat and rabbit livers show no gender difference but there is an age-dependent change. In dogs, FMO1 and FMO3 are expressed in liver and lung, with small amounts of FMO3 expression in kidney. There is also a significant gender difference in the hepatic expression of FMO isoforms that exist in mice.

TABLE 5.12 Percentage Amino Acid Homology for FMO Family in Various Species

	Human FMO1	Human FMO2	Human FMO3	Human FMO4	Human FMO5	Dog FMO1	Dog FMO3	Rat FMO1	Rat FMO2	Rat FMO3	Rat FMO4	Rat FMO5	Mouse FMO1	Mouse FMO2	Mouse FMO3	Mouse FMO4	Mouse FMO5	Rabbit FMO1	Rabbit FMO2	Rabbit FMO3	Rabbit FMO4	Rabbit FMO5	Guinea Pig FMO2	Guinea Pig FMO5
Human FMO1	100	57	54	50	51	88	55	82	56	53	51	50	83	56	53	51	50	86	58	54	51	51	56	50
Human FMO2	57	100	57	53	55	56	57	56	84	57	53	55	55	85	56	52	55	55	86	56	53	55	85	55
Human FMO3	54	57	100	52	52	55	83	54	56	80	52	52	54	56	79	51	53	54	57	83	53	53	56	54
Human FMO4	50	53	52	100	51	51	53	49	52	52	80	51	50	53	51	79	50	52	52	52	83	51	52	51
Human FMO5	51	55	52	51	100	51	51	50	56	50	48	84	49	56	51	48	84	51	56	51	49	84	55	87
Dog FMO1	88	56	55	51	51	100	56	84	56	54	52	50	84	56	55	51	50	87	57	55	51	51	56	51
Dog FMO3	55	57	83	53	51	56	100	55	56	81	52	53	55	56	80	51	53	55	58	84	54	53	57	54
Rat FMO1	82	56	54	49	50	84	55	100	56	53	50	50	94	55	53	49	50	83	56	54	49	50	54	51
Rat FMO2	56	84	56	52	56	56	56	56	100	56	53	56	55	94	56	52	55	55	86	55	53	55	83	56
Rat FMO3	53	57	80	52	50	54	81	53	56	100	51	52	53	57	90	50	52	53	57	84	52	52	57	52
Rat FMO4	51	53	52	80	48	52	52	50	53	51	100	49	50	54	51	90	49	51	53	51	78	48	51	50
Rat FMO5	50	55	52	51	84	50	53	50	56	52	49	100	49	55	52	49	92	50	55	53	51	83	55	83
Mouse FMO1	83	55	54	50	49	84	55	94	55	53	50	49	100	55	54	49	49	83	56	55	48	50	53	50
Mouse FMO2	56	85	56	53	56	56	56	55	94	57	54	55	55	100	56	52	55	55	87	55	53	56	84	56
Mouse FMO3	53	56	79	51	51	55	80	53	56	90	51	52	54	56	100	50	53	54	56	82	51	53	56	54
Mouse FMO4	51	52	51	79	48	51	51	49	52	50	90	49	49	52	50	100	49	50	52	50	77	48	51	49
Mouse FMO5	50	55	53	50	84	50	53	50	55	52	49	92	49	55	53	49	100	49	55	53	51	83	54	84
Rabbit FMO1	86	55	54	52	51	87	55	83	55	53	51	50	83	55	54	50	49	100	56	54	51	50	54	51
Rabbit FMO2	58	86	57	52	56	57	58	56	86	57	53	55	56	87	56	52	55	56	100	56	53	55	85	56
Rabbit FMO3	54	56	83	52	51	55	84	54	55	84	51	53	55	55	82	50	53	54	56	100	53	54	56	53
Rabbit FMO4	51	53	53	83	49	51	54	49	53	52	78	51	48	53	51	77	51	51	53	53	100	50	52	51
Rabbit FMO5	51	55	53	51	84	51	53	50	55	52	48	83	50	56	53	48	83	50	55	54	50	100	55	82
Guinea Pig FMO2	56	85	56	52	55	56	57	54	83	57	51	55	53	84	56	51	54	54	85	56	52	55	100	56
Guinea Pig FMO5	50	55	54	51	87	51	54	51	56	52	50	83	50	56	54	49	84	51	56	53	51	82	56	100

5.8 SUMMARY

In the pharmaceutical industry, the investigation of drug metabolism catalyzed by DMEs in various species is essential for drug development and helps us to understand whether the drug candidates will behave well in humans by demonstrating sufficient efficacy and little or no toxicological liability. Although it is generally believed that data from animal studies can be extrapolated to humans reasonably well with the application of appropriate pharmacokinetic principles, there are certainly some limitations. *In vitro* studies of the DMEs, such as reaction phenotyping, enzyme kinetics, induction, and inhibition, can provide information on the underlying mechanisms and possible prediction of the species differences between drugs and drug candidates in pharmacokinetics, DDIs, efficacy, and toxicity. In addition, the expression level of the enzymes among species responsible for the biotransformation of test drugs is also critical because the metabolic clearance of a drug in the body contributes not only to the catalytic activity of the enzyme(s) but also to the total concentration of the enzyme(s) responsible for the clearance. Therefore, species differences in drug metabolism are attributed to specific activity (product formed per unit time per unit enzyme) for a drug, expression level of the metabolizing enzyme(s), and, in some cases, differences between the inducible expression of the responsible enzyme(s).

The selection of the best animal model to be used in development of a new drug is difficult. Caution should be taken in extrapolation of animal data to humans; however, when *in vitro* metabolism studies with liver microsomes, hepatocytes, liver slices, and recombinant enzymes are available, the information can be incorporated into the decision-making process and can aid in the selection of the appropriate *in vivo* animal model. It is important to remember that it is highly unlikely that any animal model can appropriately model all aspects of drug metabolism for a given NCE, and in many cases, different species may be needed to answer specific questions. Further, as more and more *in vitro* tools are developed, more of these questions are likely to be answered without the use of *in vivo* models, further decreasing the number of animals used in the early drug development process.

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6 Structure and Function of Cytochrome P450 Enzymes

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6.1 Summary	1
6.2 Introduction	2
6.3 Cytochrome P450 nomenclature	5
6.4 Cytochrome P450 abundance, polymorphisms, and variability	6
6.5 Spectroscopic properties of cytochrome P450 enzymes	8
6.6 Structures of the human cytochrome P450 enzymes	9
6.7 Electron-donor partners: cytochrome P450 reductase and cytochrome <i>b</i> ₅	12
6.8 Cytochrome P450 catalytic cycle	13
6.9 Conclusions	15
References	16

6.1 SUMMARY

The cytochrome P450 (P450) enzymes are hemoproteins that normally insert one atom of molecular oxygen into their substrates. In mammalian systems, this catalytic process requires the transfer of electrons from NADPH to the P450 enzyme by NADPH-cytochrome P450 reductase (CPR) and/or cytochrome *b*₅. Many alleles exist of both the P450 enzymes and CPR, some of which give rise to major differences in the observed catalytic activity. The human genome contains a family of 57P450 proteins, of which approximately 15 are responsible for oxidative and reductive metabolism of drugs and xenobiotics. The other members of the human P450 family are devoted to the synthesis or processing of steroids, fatty acids, eicosanoids, and vitamins or have functions that are not yet understood. To uniquely identify each of the P450 enzymes, a systematic nomenclature system has been developed on the basis of their degree of protein sequence identity. This system facilitates recognition of the analogies between enzymes of the same or different species.

The crystal structures of several human P450 enzymes have been determined. The structures of these normally membrane-bound mammalian proteins have the same general fold as those of the soluble bacterial P450 enzymes. The spectroscopic properties of all P450 enzymes are similar and can be used to monitor the binding of substrates

to the enzymes. These structural and electronic analogies indicate that the same basic catalytic cycle is involved in substrate oxidation by all P450 enzymes. This consensus cycle involves the binding of molecular oxygen to the ferrous iron of the P450 enzyme followed by reductive cleavage of the dioxygen bond to give a highly reactive ferryl species that directly oxidizes the substrate.

6.2 INTRODUCTION

P450 enzymes are heme-containing monooxygenases that add one atom of molecular oxygen to their substrates while incorporating the other oxygen atom into a molecule of water [1,2]. The overall reaction catalyzed by P450 enzymes can be written as follows, where RH is a substrate and ROH is the product formed by addition of an oxygen atom to it:



A simple example is the conversion of toluene ($\text{C}_6\text{H}_5\text{CH}_3$) to benzyl alcohol ($\text{C}_6\text{H}_5\text{CH}_2\text{OH}$). NADPH is the reducing cofactor for mammalian P450 enzymes, but NADH is the relevant cofactor for most bacterial enzymes. The P450 enzyme in this process is responsible for converting molecular oxygen into a reactive, iron-bound species that can transfer one oxygen atom to the substrate within the enzyme active site. The activation of molecular oxygen and the insertion of an oxygen atom into the substrate are mediated by the heme iron atom. The catalytic cycle, however, requires the participation of auxiliary proteins whose role is to bind NAD(P)H, to uncouple the two electrons provided by this cofactor, and to deliver them singly to the P450 heme group. The auxiliary protein for the mammalian drug-metabolizing P450 enzymes is CPR [3], but two distinct proteins, a flavin reductase (e.g., adrenodoxin reductase) and a ferredoxin (e.g., adrenodoxin), are required for some of the mitochondrial and most of the bacterial P450 enzymes [3].

Genetic analysis has identified thousands of P450 enzymes, and scores of such proteins have been investigated at the biochemical level. This chapter focuses on the human P450 enzymes that are most relevant from the point of view of drug metabolism. Of the 57 P450 enzymes encoded in the human genome (Table 6.1), approximately 28 catalyze critical steps in the biosynthesis or removal of cholesterol, the sterol hormones, the bile acids, the eicosanoids, vitamins, and other physiologically important factors [4]. The P450 enzymes involved in these biosynthetic processes have narrow substrate specificities because evolution has tailored them to process specific endogenous substrates. The reactions catalyzed by such enzymes include removal of the 14α -methyl group of lanosterol in cholesterol biosynthesis [5], cleavage of the cholesterol side chain to generate the 17-keto sterols, progesterone and testosterone [6], and the 10-demethylation and aromatization of testosterone that produces estradiol [7] (Fig. 6.1). Relatively specific P450 enzymes are also involved in the degradation of fatty acids and elaboration of eicosanoids [8,9].

A second subset of approximately 16 human P450 enzymes are primarily involved in the oxidative, and occasionally reductive, metabolism of xenobiotics. This includes most drugs and the broad and variable range of compounds to which an individual is exposed. In most cases, the P450 system introduces an oxygen functionality such as

TABLE 6.1 The 57 Human Cytochrome P450 Enzymes

Drug Metabolism	Fatty Acids and Eicosanoids	Unknown (Orphan)	Sterols and Vitamins
1A1	2D6	2J2	2A7
1A2	2E1	4A11	20A1
1B1	2F1	4B1	2 S1
2A6	3A4	4F2	2U1
2A13	3A5	4F3	27 C1
2B6	3A7	4F8	2 W1
2 C8		4A22	8B1
2 C9		4F22	11A1
2 C18		4V2	11B1
2 C19		4X1	11B2
		4Z1	17A1
			19A1
			21A2

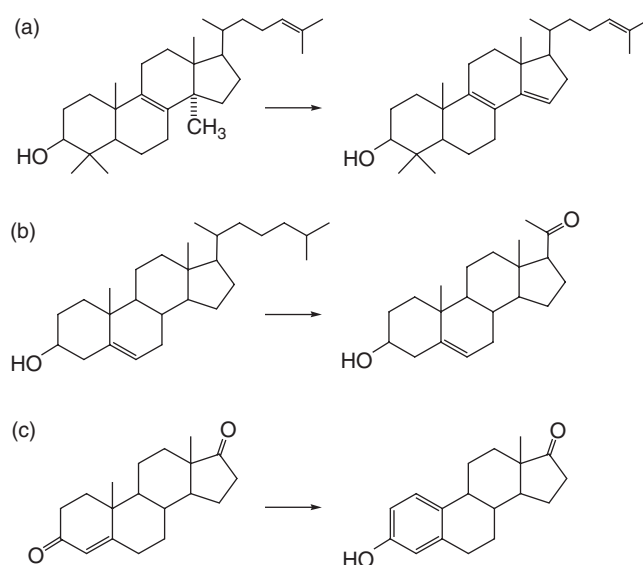


Figure 6.1 Biosynthetic transformations catalyzed by substrate-specific human P450 enzymes: (a) 14 α -demethylation of lanosterol (CYP51) [5], (b) cholesterol side chain cleavage (CYP11A1) [6], and (c) aromatization of testosterone (CYP19A1) [7].

a hydroxyl or an epoxide into its substrates and can do so even at positions that are chemically difficult to oxidize. A given substrate may undergo multiple P450 oxidations catalyzed by one or several P450 enzymes. The functionalities thus introduced often serve as anchor points for the attachment (conjugation) of sulfate, glucuronide, or other polar moieties. The net result is the conversion of a lipophilic, poorly excreted molecule into a more polar product and finally, after conjugation, into a highly polar, ionic compound that is readily eliminated.

The P450 enzymes primarily devoted to xenobiotic metabolism have broad specificities, in accord with the fact that a small number of enzymes must cope with a

potentially very large and mutable diversity of chemical structures. Their broad specificity means that the enzymes can only exert limited control over both the types of substrates and the sites on those substrates that are oxidized. It is therefore not surprising that P450 enzymes, in the process of oxidizing compounds to more polar and excretable metabolites, sometimes convert a relatively innocuous molecule into chemically reactive and potentially toxic products. Examples of this are the epoxidation of bromobenzene, hydroxylation of phenacetin to give a highly reactive iminoquinone, and hydroxylation of the side chain of chloramphenicol resulting in the formation of an acyl chloride that can either be hydrolyzed by water to the acid or acylate proteins (Fig. 6.2) [10–12].

Some of the sterol hormone biosynthetic enzymes are concentrated in the adrenals, testes, and other steroidogenic tissues [4]. However, the xenobiotic-metabolizing P450 enzymes are not only present in most tissues but also found at particularly high levels in the liver, kidneys, lungs, gut, and nasal passages that are portals for the entry of xenobiotics into the body [4]. All the mammalian P450 enzymes are membrane bound and, with the exception of the seven steroid biosynthetic enzymes that are located in the mitochondria (Table 6.2) [13], are found in the endoplasmic reticulum of the cell. The mitochondrial enzymes are located in the inner membrane on the inner matrix side. The early biochemical work on the mammalian P450 enzymes involved solubilization and purification of the enzymes from native tissues, a historically important approach but one that was ultimately unable to fully resolve and establish the size and nature of the human P450 family. The study of these enzymes has been greatly facilitated by two advances: (a) sequencing of the human genome, which clearly identified the individual members of the family, and (b) the development of *Escherichia*

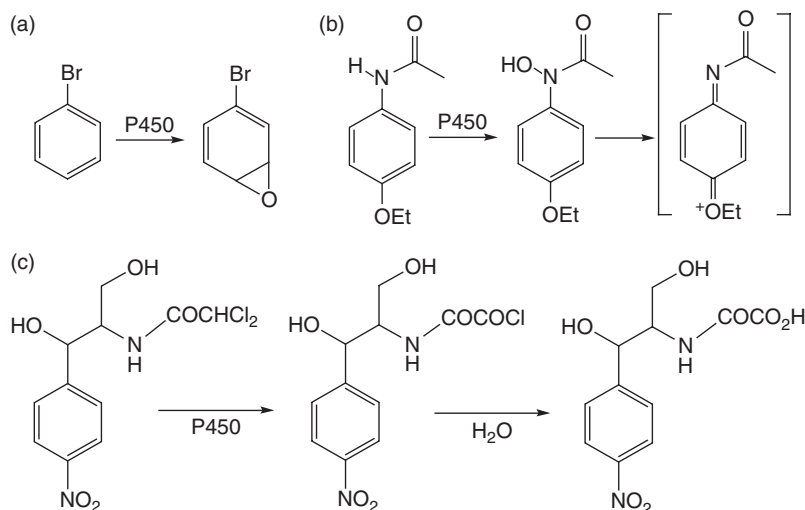


Figure 6.2 The oxidation of (a) bromobenzene to an epoxide derivative, (b) phenacetin to an iminoquinone that is responsible for the protein alkylation and toxicity associated with this drug, and (c) the oxidative dechlorination of chloramphenicol, which involves the formation of a reactive acyl chloride intermediate.

TABLE 6.2 The Mitochondrial Cytochrome P450 Enzymes and Their Location and Physiological Role

P450 Enzyme ^a	Function	Location
CYP11A (P450 _{scc})	Cleavage of cholesterol side chain to give pregnenolone	Adrenal cortex, gonads
CYP11B1 (P450 _{11β})	11β-Hydroxylation of 11-deoxycortisol to give cortisol	Adrenal cortex
CYP11B2 (P450 _{aldo})	Aldosterone synthesis from 11-deoxycorticosterone	Adrenal cortex
CYP24A1	24-Hydroxylation of 25-hydroxyvitamin D ₃	Kidneys
CYP27A1	27-Hydroxylation of cholesterol	Liver, kidneys
CYP27B1	1α-Hydroxylation of 25-hydroxyvitamin D ₃	Kidneys
CYP27C1	Unknown	—

^aThe historical, nonsystematic name is given in parentheses.

coli, [14], *Saccharomyces cerevisiae* [15], and baculovirus/insect cell systems [16] for heterologous expression of the individual enzymes [17].

6.3 CYTOCHROME P450 NOMENCLATURE

The human genome encodes 57P450 enzymes (Table 6.1), several of which are considered “orphan” enzymes in that little is yet known about their substrate specificities or physiological roles. The initial nomenclature of P450 enzymes was based on their catalytic and physical properties, including the turnover of specific substrates, the relative migration of the proteins on SDS-PAGE, and their UV–vis absorption maxima. However, this initial nomenclature became unsatisfactory as the number of distinct P450 enzymes increased and related but nonidentical enzymes were found in different tissues and species. A systematic nomenclature was therefore formulated, in which P450 enzymes were classified into families and subfamilies based on the extent of their sequence identity [18]. The hypothesis underlying this approach is that enzymes that are more closely related in sequence, and therefore in evolutionary terms, are likely to exhibit a higher similarity in their catalytic and physiological functions. The basic principle in the new nomenclature is that two enzymes that share 40% or more amino acid sequence identity are grouped within the same family and two enzymes that share more than 55% identity are placed into the same subfamily. The sequence identity values that define the family and subfamily borders are somewhat arbitrary and, to some extent, can be adjusted if required by other evidence. In practical terms, P450 enzymes are assigned a number designating the family, a letter indicating the subfamily, and a second number that identifies the individual protein (Table 6.1). The names can be presented in two formats. One is a historically derived format in which the term P450 precedes the identifying numbers and the second a more systematic name in which the term CYP is similarly placed. Thus, both P4503A4 and CYP3A4 refer to the fourth enzyme of family 3, subfamily A. The name of the gene coding for the protein is the same as the CYP form, except that it is given in italics, that is, *CYP3A4*. In addition

to these systematic names, some of the enzymes that have been studied for many years still have trivial names based on the reactions that they catalyze. Three examples of this are lanosterol 14 α -demethylase (CYP51A1), the cholesterol side chain cleavage enzyme P450_{scc} (CYP11A1), and aromatase (CYP19A1). The terms CYP and P450 are often used as shorthand notations for cytochrome P450.

6.4 CYTOCHROME P450 ABUNDANCE, POLYMORPHISMS, AND VARIABILITY

There are 57P450 enzymes in humans, but the metabolism of drugs and xenobiotics by this enzyme system is primarily mediated by members of the CYP1, CYP2, and CYP3 families (Tables 6.1 and 6.3). The importance of a given enzyme in drug metabolism depends on its distribution within the body, its concentration in specific tissues, and the extent to which its substrate tolerance encompasses the xenobiotics to which it is exposed. In one study of human liver, CYP3A4 (including CYP3A5) accounted for ~30%; CYP1A2, ~13%; CYP2A6, ~4%; CYP2C9, ~20%; CYP2D6, ~2%; and CYP2E1, ~7% of the total P450 content [19]. An immunoquantitation study of human intestinal P450 enzymes similarly found that CYP3A4/CYP3A5 accounted for 82%; CYP2C9, 14%; CYP2C19, 2%; and other forms, 2% of the total [20]. *In vivo*, CYP3A4, the most abundant hepatic and intestinal P450 enzyme, and the closely related enzyme CYP3A5, which cannot be immunochemically distinguished from CYP3A4, account for more than 50% of all P450-dependent drug and xenobiotic metabolism. CYP2C9 and CYP2D6 are each responsible for approximately 15% of xenobiotic metabolism in the liver, and CYP2E1, CYP2C19, CYP2C8, CYP2B6, CYP2A6, CYP1B1, CYP1A2, and CYP1A1 contribute most of the rest [21,22]. However, as summarized below,

TABLE 6.3 Principal Human Cytochrome P450 Enzymes Involved in Drug Metabolism and Examples of Their Substrates^a

Enzyme	Substrates
CYP1A1	<i>R</i> -warfarin, benzo[<i>a</i>]pyrene
CYP1A2	Acetaminophen, bufuralol, caffeine, clozapine, ethoxyresorufin, and theophylline
CYP2A6	Coumarin and nicotine
CYP2B6	7-Ethoxycoumarin and cyclophosphamide
CYP2C9	Benzphetamine, hexobarbital, phenytoin, tienilic acid, and tolbutamide
CYP2C19	Mephenytoin and omeprazole
CYP2D6	Bufuralol, debrisoquine, desipramine, dextromethorphan, propranolol, and sparteine
CYP2E1	Acetaminophen, aniline, caffeine, chlorzoxazone, and halothane
CYP3A4 and CYP3A5	Aldrin, Alfentanil, cortisol, cyclosporin A, dapsone, diltiazem, erythromycin, 17 β -estradiol, ethinyl estradiol, lidocaine, nifedipine, quinidine, sterigmatocystin, taxol, testosterone, and warfarin

^aThis substrate list is drawn from the extensive review of Rendic and Di Carlo [23].

interindividual variations due to genetics, gender, diet, concurrent and prior drug exposure, and environmental factors can alter the relative contributions of the individual P450 enzymes.

The P450 enzymes, to a lesser or greater degree, are polymorphically distributed in the human population (Table 6.4) [4,21,22]. For example, owing to genetic differences, the CYP2D6 activity is relatively low in approximately 7–10% of Caucasians, a difference that decreases the ability of these individuals to oxidize debrisoquine and other compounds that are largely metabolized by this P450 enzyme [24]. Likewise, the concentration of CYP2C19 is relatively low in 20% of the Asian population and 4% of Caucasians, giving rise to an impaired ability to oxidize mephenytoin and other CYP2C19 substrates [25]. Many allelic variants of the human P450 enzymes (>80 for CYP3A4 alone) are now known, some of which do not significantly influence catalytic function but others greatly attenuate, completely suppress, or even increase the activity of a given enzyme. P450 polymorphisms have important consequences for drug metabolism and drug use [26,27].

The majority of drug-metabolizing P450 enzymes are *inducible*, a term that indicates that their concentration can be elevated by exposure to specific chemicals or environmental conditions. Enzyme induction can be the result of a receptor-dependent increase in expression or maturation of the active protein, posttranslational processing of the protein, or a decrease in degradation of the functional enzyme (Table 6.5) [29,30]. For example, CYP3A4 is induced by many drugs including the antitubercular drug rifampicin and the barbiturates, CYP2E1 by ethanol and isoniazid, and CYP1A1 and CYP1A2 by smoking, polycyclic aromatic hydrocarbons, and omeprazole. In animals, most persistent lipophilic substrates cause the induction of one or more P450 enzymes, and this is also true for the human enzymes. Enzyme induction is a temporal

TABLE 6.4 Examples of Some Cytochrome P450 Allelic Variants and Their Frequency in Caucasian and Asian Populations^a

Enzyme	Allele	Activity	Allele Frequency	
			Caucasians	Asians
CYP2A6	*2	Inactive	3	0
	*4	Inactive	0.5	15.1
CYP2C9	*2	Decreased	10	0
	*3	Decreased	7	3
CYP2C19	*2	Inactive	13	25
	*3	Inactive	<1	8
CYP2D6	*2xN	Hyperactive	1.8	1
	*2	Decreased	32	—
	*3	Inactive	2	0
	*4	Inactive	21	1
	*5	Inactive	2	4.1
	*9	Decreased	2	—
CYP3A4	*2	Decreased	—	—
	*6	Decreased	—	—
CYP3A5	*7	Inactive	—	—

^aThe data in this table was taken from Ingelman-Sundberg *et al.* [21], Brockmöller *et al.* [22], and Kee and Goldstein [28].

TABLE 6.5 Human Cytochrome P450 Enzymes and Typical Inducers

Enzyme	Inducer
CYP1A1	2-Acetylaminofluorene, omeprazole
CYP1A2	Phenytoin, omeprazole
CYP2A6	Phenobarbital, ethinyl estradiol
CYP2B6	Phenobarbital, phenytoin
CYP2C9	Phenobarbital, rifampicin
CYP2C19	Phenobarbital, rifampicin
CYP2D6	Not clearly demonstrated
CYP2E1	Ethanol, isoniazid
CYP3A4	Rifampicin, dexamethasone
CYP3A5	Rifampicin

For original literature references see Pelkonen *et al.* [29].

process, as it takes hours to days to fully induce a P450 enzyme and, once the inducing stimulus is removed, results in a gradual return of the enzyme to its resting level. The levels of individual drug-metabolizing P450 enzymes are thus influenced by recent prior exposure to drugs and xenobiotics.

6.5 SPECTROSCOPIC PROPERTIES OF CYTOCHROME P450 ENZYMES

The spectroscopic properties of the heme group, which are sensitive to the nature of the heme iron ligands; the oxidation state of the iron atom; and the polar and hydrogen-bonding nature of the environment around the heme provide important information on the P450 active site [31,32]. The P450 family of enzymes is distinguished from nearly all other hemoproteins by the UV–vis spectrum of their ferrous–CO complexes. While the intense Soret absorption maximum of this complex appears at ~420 nm for most hemoproteins, in P450 enzymes, it is split into two peaks with maxima at ~380 and ~450 nm. The peak at ~450 nm is specifically taken as the hallmark of the P450 enzymes and is the source of the name for this family of proteins. This unique absorption spectrum is due to coordination of a thiolate (RS^-) rather than a histidine or tyrosinate to the heme iron atom [32]. One of the first indications that a P450 enzyme is denatured is a shift of the 450-nm ferrous–CO spectrum to ~420 nm, as this shift signals that the proximal thiolate ligand has been protonated to a thiol ligand or has been entirely displaced from the iron by another functionality [33].

The ferrous–CO spectrum of P450 enzymes defines their spectroscopic signature, but the UV–vis spectra of the ferric enzymes are also informative. In the presence of a distal axial water ligand, the P450 iron atom is in the low spin state and the protein has a Soret absorption maximum at ~416–419 nm. Displacement of this distal water ligand, leaving the sixth iron coordination site vacant, gives rise to a high spin iron with a Soret absorption maximum at 390–416 nm. P450 enzymes can exist as an equilibrium mixture of the high and low spin states, but generally favor the low spin state. The exception to this is provided by enzymes such as CYP1A2 and CYP2D6 that do not appear to have a distal water ligand [34,35]. If the distal water ligand is present, its displacement by a substrate causes a shift in the absorption maximum from

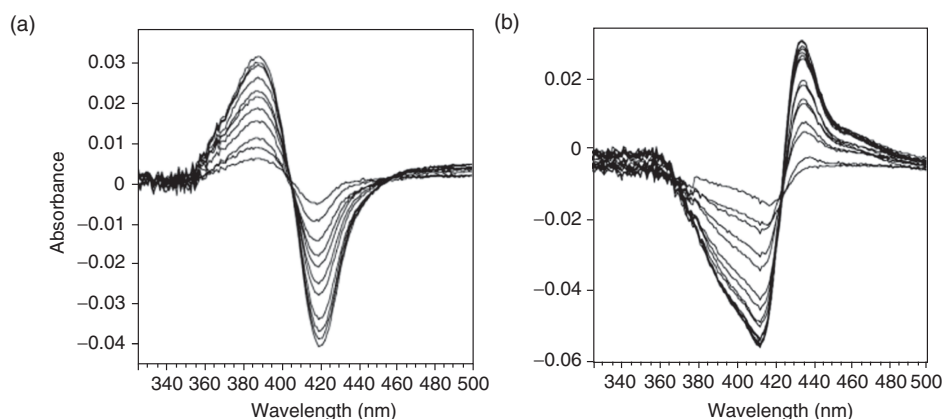


Figure 6.3 Typical difference spectra for the binding of ligands to a P450 enzyme. The spectra shown here are for the binding to bacterial CYP124 of (a) farnesyl pyrophosphate, giving a type I difference spectrum and (b) clotrimazole, giving a type II difference spectrum. In each panel, a spectrum was recorded after each incremental addition of the ligand to the sample cuvette. This figure, based on Ref. 36, was prepared by Dr. Jonathan Johnston.

~419 to 390 nm. This substrate-dependent spectroscopic shift is usually monitored by difference spectroscopy, a technique in which an exogenous ligand is only added to one of two otherwise identical enzyme-containing cuvettes (Fig. 6.3). The resulting difference spectrum, with a maximum at 385–390 nm and a trough at ~420 nm, is known as a *type I binding spectrum* [31]. A different spectroscopic shift is observed if the added ligand coordinates strongly to the heme iron atom, as this gives rise to a difference spectrum with a maximum at 425–435 nm and a trough at 390–405 nm. This so-called type II binding spectrum is observed, for example, when the nitrogen of an imidazole or triazole ring binds to the P450 heme iron atom. A type III difference spectrum with a maximum at 420 nm and a trough at 388–390 nm is obtained if the ligand only coordinates weakly to the heme iron atom. These spectroscopic changes are very useful, as they make it possible to spectroscopically monitor the binding of substrates and inhibitors to P450 enzymes. Exemptions to this approach occur if the resting enzyme does not have a distal water ligand or, occasionally, if the binding of a compound does not displace the distal water ligand.

6.6 STRUCTURES OF THE HUMAN CYTOCHROME P450 ENZYMES

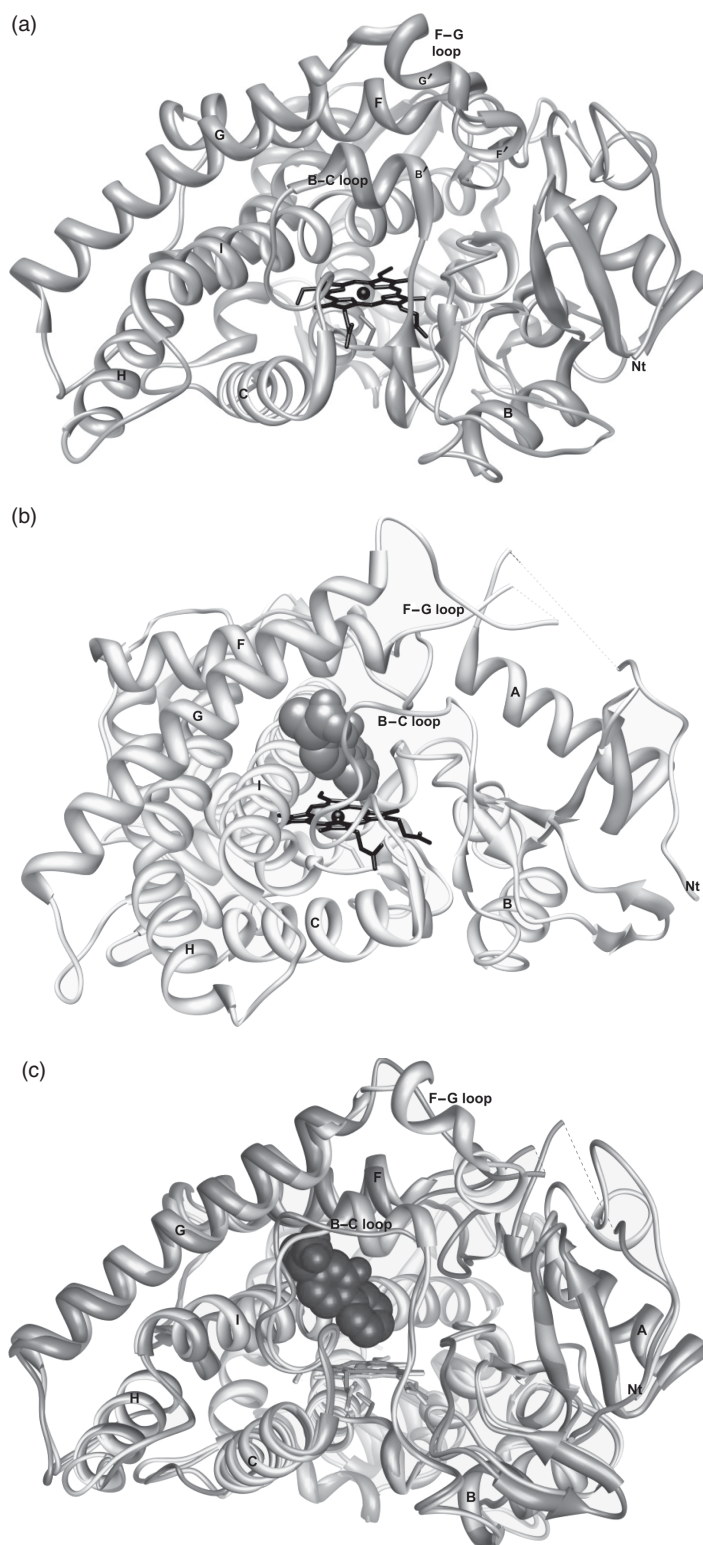
For many years, the only successful crystallizations and structure determinations of P450 enzymes were of the soluble bacterial enzymes. The structure of P450_{cam} (CYP101), the first to be determined, was reported in 1985 [37]. The structure of P450_{cam} and the subsequently determined structures of its complexes and other bacterial enzymes provided a wealth of information, much of which could be extrapolated to the membrane-bound mammalian P450 enzymes. However, 15 years passed before Johnson and coworkers [38] determined the structure of rabbit CYP2C5, the first of a mammalian, membrane-bound P450. The breakthrough represented by this structure has been followed at an increasing tempo by the determination of the

structures of human CYP1A2 [39], CYP2A6 [40], CYP2A13 [41], CYP2B4 [42], CYP2C8 [43], CYP2C9 [44,45], CYP2D6 [46], CYP2R1 [47], and CYP3A4 [48,49], often in the ligand-free state and in complexes with various ligands.

All the P450 enzymes have an approximately triangular prism shape that is dominated by 12 helices (Fig. 6.4) [38–49]. At the center of this structure is an iron protoporphyrin IX (heme) prosthetic group that is held in place by (i) the pincer action of the so-called I and J helices between which it is located, (ii) ion pairing and hydrogen bonds to the two carboxyl groups of the heme, and (iii) coordination of a cysteine thiolate to the proximal side of the heme iron atom. The catalytic action of P450 enzymes depends absolutely on coordination of this cysteine thiolate to the iron atom rather than a protonated cysteine thiol or some other ligand. As mentioned earlier, the ferrous–CO complex of the enzyme with a cysteine thiolate proximal ligand has a Soret maximum at ~450 nm, whereas this absorption maximum shifts to ~420 nm if the thiol is protonated [33]. The distal iron ligand, if one is present, is a water molecule. Given the complexity of the reactions catalyzed by P450 enzymes, there is surprisingly little in the way of catalytic machinery in the active site beyond the thiolate-coordinated heme group. The most important additional catalytic feature is a conserved threonine (or serine) residue on the distal side that (i) helps stabilize the ferrous–dioxy complex through hydrogen-bonding interactions and (ii) through similar interactions, promotes heterolytic cleavage of the dioxygen bond in the [Fe(III)–OOH] intermediate once it is formed. Replacement of this threonine by a non-hydrogen-bonding residue increases decoupling of substrate oxidation from oxygen utilization and results in a decrease or complete loss of substrate oxidation activity. This was first demonstrated for CYP101 (P450_{cam}) [50], but at approximately the same time was found with CYP1A2 to suppress oxidation of benzphetamine but not of 7-ethoxycoumarin [51]. Additional residues, exemplified by Asp251 in P450_{cam} [52], appear to be important in maintaining a hydrogen-bonded network that helps deliver protons to the active site.

On the basis of the crystal structure of CYP101 and an alignment of this P450 sequence with those of the mammalian CYP2 family of proteins, Gotoh proposed that six regions of the mammalian proteins would be involved in substrate binding and recognition [53]. These substrate recognition sequences (SRSs) proved useful in the analysis of substrate binding to the human enzymes before structures for these enzymes became available. The rapidly expanding list of crystallographically determined human P450 structures now provides a more precise structural basis for the analysis of substrate binding and specificity. However, it is increasingly clear that P450 enzymes can undergo both small and large conformational adjustments to optimize the binding of their diverse substrates and inhibitors. Thus, the ligand in the structure of CYP2C9 with warfarin bound in its active site is located at a distance of more than 10 Å from the iron atom, whereas the structure with flurbiprofen in the active site places the substrate much closer to the heme iron atom (Fig. 6.4) [44,45]. The structure of flurbiprofen also

Figure 6.4 The crystal structure of human CYP2C9 in (a) the ligand-free state, (b) with flurbiprofen bound in the active site, and (c) superposition of the ligand-free and flurbiprofen-bound structures. In (c), dark corresponds to the flurbiprofen-bound structure and light color to the ligand-free structure. Flurbiprofen is in very dark tint with the heme below it in dark sticks. Some of the key structural elements of the protein are labeled. The conformational change that occurs on substrate binding is most notable in the F–G loop region. This figure was prepared by Dr. Sylvie Kandel. (See color insert.)



differs from that of the protein without a bound ligand, a difference most clearly seen in the F–G loop region of the protein (Fig. 6.4). The CYP2C9 protein in these two crystal structures is found to have undergone substantial conformational alterations. A second example is provided by the unligated structure of CYP2B4, which exhibits an open access channel into the active site [42]. A second structure with the protein complexed with 4-(4-chlorophenyl)imidazole shows the ligand in the active site close to the heme, with the access channel closed by the shift of a domain of the protein [54]. The dynamic plasticity of the P450 active site confounds efforts to understand and predict substrate specificity based on the limited set of human P450 crystal structures that are currently available.

6.7 ELECTRON-DONOR PARTNERS: CYTOCHROME P450 REDUCTASE AND CYTOCHROME b_5

Two electrons are required for each successful oxidation of a substrate by a P450 enzyme. In the case of the human drug-metabolizing forms of P450, these electrons are provided by CPR and/or cytochrome b_5 (Fig. 6.5) [55]. CPR is a 78-kDa protein with a hydrophobic N-terminal sequence that anchors it to the endoplasmic reticulum membrane. In contrast to the multiplicity of P450 enzymes, there is only one form of CPR. As shown by the crystal structure [56], the protein contains one FMN and one FAD that are used to uncouple the electrons provided by NADPH in order to deliver them one at a time to the P450 heme group. The enzyme is specific for NADPH and is not significantly reduced by NADH. The FAD group, reduced by hydride transfer from

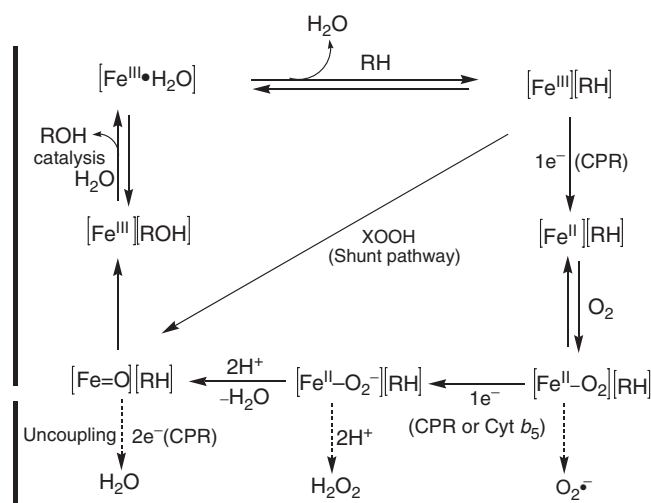


Figure 6.5 The catalytic cycle of most cytochrome P450 enzymes, including all of those involved in human drug metabolism. The iron in brackets represents the P450 heme iron atom; RH, a substrate; ROH, its oxidized product; and XOOH, a peroxide. The upper part of the cycle represents productive turnover in which the substrate RH is oxidized. In the lower part, turnover can also become uncoupled from substrate oxidation, producing superoxide, H_2O_2 , and/or a second molecule of water. CPR, cytochrome P450 reductase; Cyt b_5 , cytochrome b_5 .

NADPH, transfers electrons to the P450 heme group via the FMN prosthetic group. With two flavin groups, CPR can accept up to four electrons, two for each flavin, but catalytic turnover usually involves cycling between the one- and three-electron reduced forms. Although truncated forms of CPR obtained by limited digestion of the endogenous membrane-bound enzyme or heterologous expression are able to reduce cytochrome *c*, they are no longer able to effectively reduce P450 enzymes. CPR, like the P450 enzymes themselves, is subject to polymorphic expression [57]. These polymorphisms are associated with disorders such as Antley–Bixler syndrome, the result of a perturbation of sterol hormone synthesis, but also have implications for drug metabolism [27]. Interestingly, CPR variants can interact differently with different P450 enzymes, so that the influence of a given CPR allele on one P450 enzyme cannot be extrapolated to another P450 enzyme [27].

With many P450 enzymes and substrates, electron transfer from CPR alone is sufficient to obtain maximum P450 catalytic turnover. However, with some enzymes, including CYP3A4, substrate oxidation can be enhanced or even modified by electron donation from cytochrome *b*₅ [58,59]. Cytochrome *b*₅, which can itself be reduced by either NADH/cytochrome *b*₅ reductase or CPR, is generally unable to deliver the first electron required to initiate P450 catalytic turnover [59]. However, it can deliver the second electron required to complete the catalytic cycle. One effect of cytochrome *b*₅ is therefore to enhance coupling of the electrons for the formation of the P450-activated species, an improvement that is achieved by decreasing the futile, uncoupled reduction of O₂ to H₂O₂ or water. Cytochrome *b*₅ may also influence P450 catalysis by allosteric mechanisms independent of its own electron-transfer ability, as illustrated by the finding that CYP3A4-catalyzed testosterone 6 β -hydroxylation and nifedipine oxidation are stimulated by apocytochrome *b*₅ [58,60] and by the fact that cytochrome *b*₅ serves as a physiological control that helps determine which of the two possible products of sterol oxidation are formed by the sterol 17-hydroxylase/lyase (CYP17A1) [61].

6.8 CYTOCHROME P450 CATALYTIC CYCLE

The catalytic cycle of P450 appears to be the same regardless of whether the enzyme is from bacteria, plants, insects, or humans. Because the bacterial P450 enzymes are soluble and often more stable than their membrane-bound mammalian counterparts, much of the detailed work on the P450 catalytic cycle has been carried out with bacterial enzymes, particularly, CYP101 (P450_{cam}). However, a large body of the evidence obtained with the mammalian P450 enzymes confirms that the core mechanism of these enzymes is essentially invariant. As the high spin form of P450 enzymes is more easily reduced than the low spin form, catalytic turnover is initiated by the binding of a substrate, which displaces the distal water ligand if it is present (Fig. 6.5). In bacterial CYP101, the redox potential shifts from $E_{1/2} = -300$ to -170 mV upon binding of camphor, bringing the P450 within the range accessible to putidaredoxin, the electron-donor partner, which has a redox potential $E_{1/2} = -196$ mV [62]. Although a similar correlation of spin state with redox potential was initially not apparent for the mammalian enzymes reconstituted in artificial lipid mixtures [63], recent studies of CYP3A4 embedded in a more physiological nanodisc environment have given results consistent with those for CYP101 [64]. In these studies, substrate-free CYP3A4, which is 11%

high spin, was found to have an E° of -220 ± 10 mV, but the redox potential shifted on binding of erythromycin (22% high spin, $E^\circ = 210 \pm 10$ mV), testosterone (92% high spin, $E^\circ = -140 \pm 5$ mV), and bromocriptine (93% high spin, $E^\circ = -137 \pm 5$ mV). Electrons are transferred to these protein complexes by the FMN domain of one of the multiple oxidation states available to CPR [65].

Once the enzyme is reduced to the ferrous state by the first electron transfer, molecular oxygen binds to yield the ferrous–dioxy complex $[\text{Fe(II)}-\text{O}_2]$. This complex, unlike the analogous complex of myoglobin or hemoglobin, is not stable and, in the absence of further catalytic action, reverts to the ferric enzyme by the loss of superoxide. In the coupled catalytic cycle, however, the ferrous–dioxy complex is further reduced by a second electron from an electron-donor partner to the ferrous–peroxy complex $[\text{Fe(III)}-\text{OO}-]$. This intermediate is rapidly protonated to give the ferric hydroperoxide $[\text{Fe(III)}-\text{OOH}]$. The P450_{cam} peroxy anion and hydroperoxide complexes have been observed spectroscopically at low temperatures [66]. Heterolytic cleavage of the dioxygen bond in the ferric hydroperoxide, which requires the uptake of a second proton and loss of a water molecule, produces a ferryl intermediate [1,2]. This ferryl intermediate is two oxidation states higher than the resting ferric state. It can formally be written as $[\text{Fe(V)}=\text{O}]$, $[\text{Fe(IV)}=\text{O}]$ with a porphyrin radical cation, or $[\text{Fe(IV)}=\text{O}]$ with a protein radical, but the favored formulation appears to be $[\text{Fe(IV)}=\text{O}]$ coupled to a porphyrin radical cation. This formulation is the same as that for the “Compound I” activated species of horseradish peroxidase, although the proximal iron ligand in that enzyme is a histidine nitrogen [67]. Efforts to detect the P450 “Compound I” ferryl intermediate, including cryogenic X-ray studies [66], have been tantalizing but ambiguous. However, recent investigations at the low temperature in which the reactive intermediate is generated by photoirradiation of a chemically generated $[\text{Fe(IV)}=\text{O}]$ precursor provide strong support for its formulation as an $[\text{Fe(IV)}=\text{O}]$ /porphyrin radical cation [68,69]. This ferryl intermediate is held to be the species that is directly responsible for most substrate oxidations.

Although the ferryl intermediate mediates most of the oxidation reactions, in a few P450 reactions, the ferric peroxy anion $[\text{Fe(III)}-\text{OO}-]$ adds as a nucleophile to a carbonyl group, resulting in cleavage of the carbon–carbon bond between the carbonyl group and the parent structure [2]. A classic example is the 14α -demethylation of lanosterol, in which the enzyme (CYP51) oxidizes a methyl group first to an alcohol, then to an aldehyde, and in a final reaction involving the $[\text{Fe(III)}-\text{OO}-]$ intermediate removes the carbon atom as a molecule of formic acid (Fig. 6.6). It has also been proposed that the ferric hydroperoxy intermediate $[\text{Fe(III)}-\text{OOH}]$, in addition to serving as the precursor of the ferryl species, may itself oxidize some substrates. This intermediate, which is an electrophilic rather than nucleophilic oxidant, could conceivably oxidize thioethers, amines, olefins, and other easily oxidized functions. However, the evidence for a role of the $[\text{Fe(III)}-\text{OOH}]$ intermediate in substrate oxidation remains unclear, as most of the evidence in favor of such reactions is derived from studies of mutants designed to impair the ability to form the ferryl intermediate (e.g., 70). This compromises the evidence because the role of the ferric hydroperoxide intermediate will depend on the relative rates of its conversion to the ferryl species versus its direct reaction with the substrate. Nevertheless, although its participation in hydrocarbon C–H bond oxidation is highly unlikely, it may yet prove to contribute in some situations to the oxidation of more easily oxidized functionalities.

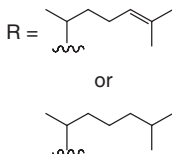


Figure 6.6 The three steps in the oxidative demethylation of lanosterol catalyzed by CYP51A1. [Fe(III)–OO–] represents the P450 ferrous peroxy anion intermediate.

An important parameter in P450 catalysis is the degree of uncoupling of oxygen consumption from substrate oxidation. As already indicated, the catalytic cycle traverses intermediates in which the heme iron atom is formally coordinated to a superoxide ion [$\text{Fe(II)}-\text{O}_2 \leftrightarrow \text{Fe(III)}-\text{O}_2^{\bullet-}$] or a hydrogen peroxide anion [$\text{Fe(III)}-\text{OOH}$]. Dissociation of these ligands can result in the formation of superoxide or H_2O_2 , respectively, as the aborted products of P450 catalysis. Furthermore, there is evidence that the ferryl intermediate can itself be reduced by two electrons to a water molecule, regenerating the resting ferric enzyme. The importance of these uncoupling reactions depends on the specific P450 enzyme, the specific substrate, and the intervention or not of cytochrome b_5 .

In vitro, many P450 enzymes can turn over their substrates with H₂O₂ rather than NADPH/CPR as the supporting system in what is termed the *shunt pathway*. In most cases, alkylhydroperoxides can be used instead of H₂O₂. In a sense, the mechanism of the P450 reaction with H₂O₂ is the reverse of autooxidation pathways, as the reaction is thought to generate the [Fe(III)–OOH] intermediate involved in normal catalytic turnover. The shortcoming of the “shunt” pathway is that peroxides readily damage the heme group and inactivate the enzyme, so that catalytic turnover can be fairly short-lived. It is unlikely that H₂O₂-dependent turnover of human P450 enzymes occurs to a significant extent *in vivo*, but the use of this approach *in vitro* has proven of value in investigating the mechanisms and specificities of the enzymes.

6.9 CONCLUSIONS

The P450 system is amazing in that by and large it can cope with the enormous variety of compounds to which mankind is exposed, particularly if one considers that only a dozen or so actual proteins are involved. A consequence of this metabolic efficiency is that highly reactive metabolites that are toxic or carcinogenic are not infrequently produced. The requirement for an efficient substrate oxidation system to enable the elimination of lipophilic xenobiotics thus carries with it the inherent potential to damage the host. This yin–yang balance is increasingly found in biology: one only

has to consider the cases of superoxide, nitric oxide, and carbon monoxide to find additional examples of biological two-edged swords.

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7 Mechanisms of Cytochrome P450-Mediated Reactions

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7.1 Introduction	1
7.2 Overview of P450 reactions	2
7.3 P450 reactions and mechanisms	4
7.4 Summary	16
Acknowledgments	17
References	17

7.1 INTRODUCTION

Most drugs are metabolized in the body, and understanding these processes is critical to the evaluation of pharmacokinetic suitability and their potential for toxicity and drug interactions. A generation ago, the lack of suitable human pharmacokinetic properties was the major cause of attrition of new chemical entities in the pharmaceutical industry [1]. Today, the fraction of failures due to poor pharmacokinetic properties is much lower (~8% [1]), and the change can be attributed in large part to our improved understanding of the human enzymes involved in drug metabolism, including the transporters.

The fraction of drug metabolism reactions (i.e., for all drugs) attributed to P450s is ~75% (Fig. 7.1) [2,3]. Of the 57 human P450 (*CYP*) genes, 5 account for 95% of drug metabolism [2,3]. In many cases, more than one P450 may be involved in the metabolism of a drug, even catalyzing the same reaction. The point should be made here that each P450 has the potential to catalyze many, if not all, of the types of reactions that discussed in this chapter because of the universality of the chemical mechanism.

The metabolism of a drug must be characterized in humans before it can be introduced into the market. The regulatory requirements have been made more stringent with the implementation of the Food and Drug Administration Guidance on Metabolites in Safety Testing in the United States [4], with similar International Conference on Harmonization guidelines now in place. Understanding metabolism, particularly, P450 reactions, is also important in considering and stratifying new chemical entities in pharmaceutical development, especially when there are issues regarding pharmacokinetics,

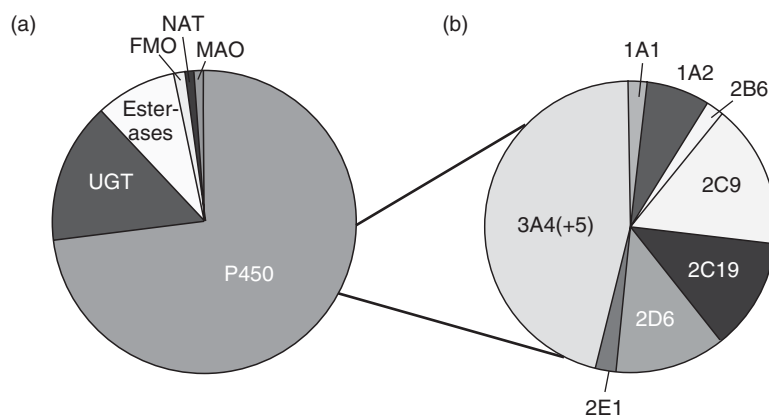


Figure 7.1 (a) Fractions of drug metabolism reactions catalyzed by each group of enzymes. (b) Fractions of P450 reactions catalyzed by the individual (human) P450s. UGT, uridine diphosphoglucuronosyl transferase; FMO, microsomal flavin-containing monooxygenase; NAT, N-acetyltransferase; MAO, monoamine oxidase. *Source:* Adapted from Williams *et al.* [2]. See also Wienkers and Heath [3].

drug interactions, and safety issues. Knowledge of P450 reactions may be critical in understanding the nature of glutathione (GSH) and macromolecular adducts and their implications.

7.2 OVERVIEW OF P450 REACTIONS

Before discussing the details of the P450 reactions, it is useful to consider the overall P450 cycle and which steps are most relevant to considerations in drug metabolism. A generally accepted scheme is shown in Fig. 7.2, although it must be emphasized that (i) the scheme shows only an electronic view of the reaction cycle (focused on the iron atom), without the changes in protein conformation that are known to be associated with various steps; (ii) some of the steps may have multiple steps within them (e.g., substrate binding); and (iii) this is not necessarily a linear cycle, for example, substrate may enter or come off at various states, not only at a single one.

The P450 cycle begins with the P450 iron in the ferric form. In step 1, the substrate binds. Such binding may or may not change the spin state or the oxidation–reduction potential. Step 2 involves the transfer of an electron from the flavoprotein NADPH-P450 reductase. This protein and the reaction have been studied extensively, and the system is not discussed here [5–7]. The ultimate source of the electrons is from NADPH, which transfers a hydride ion (two electrons) to the diflavin reductase, and the electrons flow through FAD to FMN to P450, necessarily one at a time. The rate of electron transfer to a P450 may or may not be enhanced by the presence of the substrate [8].

Step 3 involves the addition of O_2 to the ferrous iron. The resulting complex is unstable, and the rate of decomposition varies among P450s [9]. Step 4 involves the transfer of an electron to the ferrous–oxy complex ($Fe^{2+}O_2$). This can occur from NADPH-P450 reductase, although it has been much more difficult to study this reaction

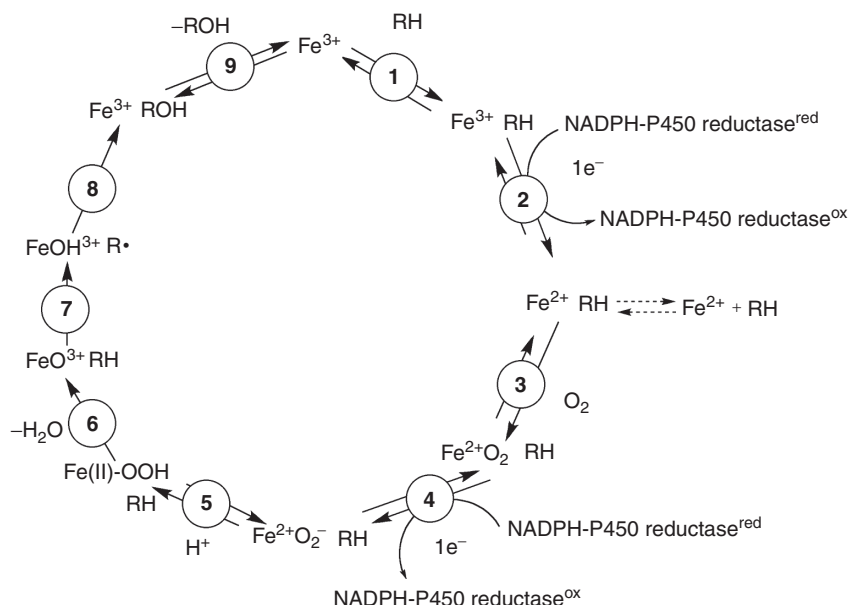


Figure 7.2 General catalytic cycle for P450 enzymes.

than step 2, so the nature of the electronic states is less secure. Cytochrome b_5 (b_5) can enhance some P450 reactions and has been shown to transfer an electron at this stage [10,11]. (Electron transfer from b_5 to P450 is possible at step 2—the difference in oxidation–reduction potential makes the rate slow [12].)

Following step 4, the reactions have been even harder to observe, and still much of our understanding of these steps is based largely on work with biomimetic models and, to some extent, theoretical considerations. The current understanding is summarized in Fig. 7.2. The FeO_2 complex can be depicted as $\text{Fe}^{2+}\text{O}_2^-$ and abstracts a proton; evidence exists that this is achieved via a Thr-based proton network in the I-helix [13,14]. As discussed later, there is speculation that the FeO^{2+} and FeOOH^{3+} complexes can be the oxygenating species. However, (in the author's opinion) the current bulk of evidence supports the view that homolytic scission of the O–O bond to yield the FeO^{3+} complex (step 6) is a critical step in most P450 reactions. The FeO^{3+} complex has a strong precedent in peroxidase chemistry as “Compound I”. However, attempts to characterize this intermediate in P450 have been difficult and controversy exists regarding exactly how stable it is [15–17]. In none of these reports has the intermediate been shown to form a product using unambiguous methods. Despite these experimental deficiencies, the FeO^{3+} complex is considered in all the mechanisms presented. The designation FeO^{3+} is used to avoid assigning electronic distribution to the iron atom, the oxygen, the porphyrin, and the Cys thiolate ligand (to the iron). The most common view of the electronic distribution is $\text{Fe}^{4+}\text{O}_2\text{-porphyrin radical}^+$.

Step 7 involves hydrogen abstraction (or one-electron oxidation) by the FeO^{3+} complex, yielding FeOH^{3+} (Compound II, electronically equivalent to FeO_2^{2+}) plus a radical. In general, these radicals do not accumulate in P450 reactions as they do in peroxidase reactions. The rate of “oxygen rebound” (step 8) is considered fast

and estimated to be $\sim 10^{10} \text{ s}^{-1}$ [18]. However, in some cases, rearrangements (of the substrate radical) are fast enough to occur and thus detect the steps.

The final step is release of the product to complete the reaction cycle. In general, most products seem to have affinities similar to the substrates.

7.3 P450 REACTIONS AND MECHANISMS

7.3.1 Substrate Binding

Substrate binding (step 1) is important in that the juxtaposition of the substrate in the P450 is critical to the reaction course, that is, the regio- and stereoselectivity, as well as the rate. Until recently, the nature of the P450 active sites was a matter of speculation or homology modeling based on a few bacterial P450s. Since 2003, structures have been obtained for the five human P450s most involved in drug metabolism—1A2, 2C9, 2C19, 2D6, and 3A4 [19–24] (2C19, Johnson EF, personal communication)—as well as for 2A6, 2A13, 2C8, 2E1, 2R1, 17A1, 19A1, 21A2, 46A1, and 51A1 [25–33]. The active sites vary considerably in size, from 190 to 1385 Å³ (for 2E1 to 3A4, respectively) [23,27–29,34]. Just as importantly, the shapes of the active sites and various binding forces contributed by individual amino acids influence the binding of substrates. To elaborate on this last point, the size of the P450 2C8 active site is as large as that of P450 3A4 [23,34] but the shape is less “open,” which may contribute to the narrower list of substrates. An example of how individual residues are involved is seen in P450 2D6 and the negatively charged amino acids Asp-301 and Glu-216 that interact with amine ligands [35–37]. The point should also be made that the shapes (and sizes) of the active sites are variable and change as the P450 binds different substrates, as judged from the crystal structures available to date. Thus, there is evidence that some aspect of induced fit occurs in substrate binding. However, the binding of ligands is clearly not so universal that every substrate can be bound by every P450.

One issue beyond the scope of this chapter is cooperativity. Cooperativity can be either homotropic or heterotropic. Homotropic cooperativity involves atypical interactions of the same substrate (or ligand), yielding positive or negative cooperativity for either binding or catalytic activity, for example, sigmoidal kinetics. Heterotropic cooperativity involves interaction of two different ligands and the ability of one to enhance the binding or catalytic activity of the other (*in vitro*, without any involvement of enzyme induction). These interactions are generally considered to involve two ligands within the active site of a P450, although few experiments have been done to eliminate the possibility of interactions due to binding at distant sites. In two cases, there is crystallographic evidence of multiple occupancy of a ligand in a human P450 [29,38], and some fluorescence evidence for direct interactions has also been reported [39].

Most P450s have the substrates imbedded in their interiors and must undergo conformational changes to open and close each time a ligand enters or exits. Some P450s can be described in a two-state system, either bound (with ligand) or unbound, and interchange is rapid. For instance, P450 2A6–coumarin binding can be characterized with a k_{on} of $2.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (in the range of diffusion-limited reactions of proteins) and k_{off} of 5.7 s^{-1} . Thus, P450 2A6 must open and close for more than five times every second (at 23°C). Similar kinetic parameters were measured with the product 7-hydroxycoumarin [10].

Studies with P450 3A4 [40,41] and (rabbit) P450 1A2 [42] have provided evidence for a different course. The initial rate of interaction of ligands with these P450s is fast, as demonstrated by the rate of decrease in the fluorescence of the ligand α -naphthoflavone (α NF). However, the changes in the heme iron spin state are one to two orders of magnitude slower and follow a biexponential course [40]. Our interpretation is that the substrate binds rapidly at a site of the periphery (explaining the α NF quenching) and then moves slowly into the active site of the heme, in a position for catalysis (Fig. 7.3). The course of the events is slow enough to overlap the oxygenation process [40] and then complicate the kinetic analysis.

One explanation for biexponential kinetics of substrate binding is the existence of two or more distinct structural populations of unbound P450, for example, one in a boundlike structure (poised for binding) and a second structure (“ground state”), which are in slow equilibrium and bind substrate at different rates. In the experiments with the P450s 1A2 and 3A4 [40–42], results from pulsed mixing and other techniques argue against this explanation. However, the possibility of such phenomena cannot be dismissed for other P450 systems.

When P450s open and close to allow ligands to enter and leave, or (as in the cases of P450s 1A2 and 3A4) to migrate within the enzyme, particular residues along the “access” channels may be involved in the interactions. At this time, postulates of these mechanisms are only speculative. Given the similarities of drug substrates and products of P450s (i.e., often the products are substrates for further oxidation), a strong case cannot be made for the existence of separate entrance and exit channels.

Finally, regarding practical significance of these issues, the point should be made that predicting metabolism is still difficult. Several bioinformatic strategies have been useful (e.g., MetaSite, Sporcalc, Glide, and Dock) [43], although most success has been achieved with systems based on prior experience with similar compounds as opposed to basic principles of chemistry and structure. Clearly it is more useful to have P450

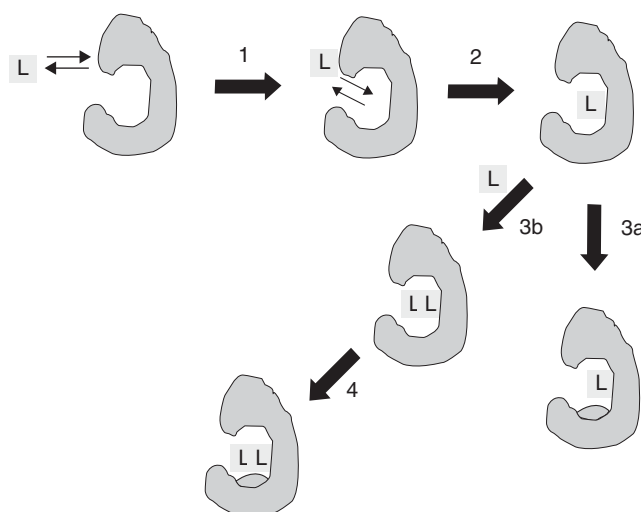


Figure 7.3 A proposed scheme for movement of a ligand into the active site of a P450, based on work with the P450s 1A2 and 3A4 [40–42]. L, ligand.

structures than not, but making predictions is still not trivial. As an example, the structure of the human P450 1A2- α NF complex [19] has the site of epoxidation positioned farthest from the iron [42]. Substrates can bind P450s in multiple ways, only some of which will be productive.

7.3.2 The Nature of the Active Oxygen

In early research on P450, the question of the nature of the active oxygenating species was a matter of interest. Much of the early discussion involved mobile elements such as superoxide anion ($O_2^{\cdot-}$) or some sort of an oxene species that could achieve the observed reactions. Such discussions were stimulated by evidence that NADPH-P450 reductase could generate $O_2^{\cdot-}$ [44]. While some findings supported this hypothesis [45], there were at least two major problems: (i) $O_2^{\cdot-}$ is nucleophilic but the nature of P450 reactions requires an electrophilic reagent and (ii) such a mechanism provides no control of regio- and stereoselectivity (or, in fact, even a role of the heme other than possibly reducing oxygen to a diffusible form).

Work in the area of biomimetic models suggested that a more appropriate model might be an electrophilic iron-oxygen complex [46], a view that developed further with metalloporphyrin models [47–49]. Another seminal study [50] showed that P450 reactions were associated with high intrinsic kinetic deuterium isotope effects and scrambling of stereochemistry, both of which could be interpreted as indicative of a reaction involving a radical intermediate. Further, typical P450 reactions occurred when P450 was mixed with single-oxygen donor “oxygen-surrogates” such as iodosylbenzene [51,52]. Thus, the view emerged that the P450 mechanism involves a course of events such as those shown in Fig. 7.4.

The P450- FeO^{3+} complex is electronically similar to peroxidase Compound I, which can catalyze some of the same reactions as P450s. The peroxidase Compound I is much more stable and has been extensively characterized, existing as an Fe(IV) ($=O$) complex with a one-electron deficiency in the porphyrin ring. The reduction of horseradish peroxidase Compound I occurs with $E_{m,7} \sim 1.0$ V, and the same $E_{m,7}$ has been estimated for the reduction of Compound II (of horseradish peroxidase) [54]. Similar values have been measured for biomimetic model metalloporphyrins [55,56]. A potential cannot be measured for the unstable P450- FeO^{3+} entity, but modeling and Marcus theory considerations suggest an even higher potential [57].

Although the perferryl FeO^{3+} species satisfies many criteria as the P450 oxygenating agent, alternate proposals have been developed and should be mentioned (Table 7.1). One unusual P450 reaction is the third step of the P450 19A1-catalyzed conversion of androgens to estrogens, involving the conversion of an exocyclic formyl group into formic acid and insertion of a double bond into a ring [58]. Model compounds have been shown to undergo similar deformylations [59–61]. One mechanism developed to explain this involves the $FeOO^-$ intermediate attack on the aldehyde [62]. Subsequent work with model P450 oxidations has led to the proposal that this reaction might be more general in P450 reactions [63]. Peracids are known to epoxidize olefins, and even flavin 4a-hydroperoxides are now known to be involved in some epoxidations [64]. The possibility exists that some of the more facile P450 oxidations can be catalyzed by the $Fe^{2+}O_2(H)$ entity (Table 7.1). However, three points should be kept in mind: (i) theoretical considerations have been used to argue against a role of $Fe^{2+}O_2^-$ or $Fe^{2+}OOH$ in C-hydroxylation and epoxidation [65];

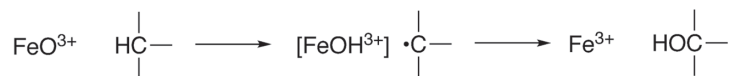
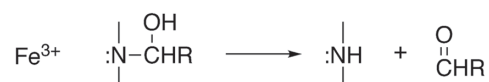
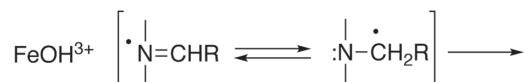
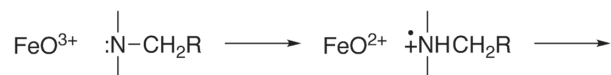
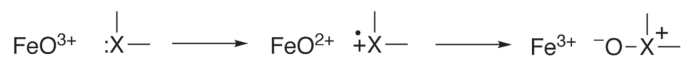
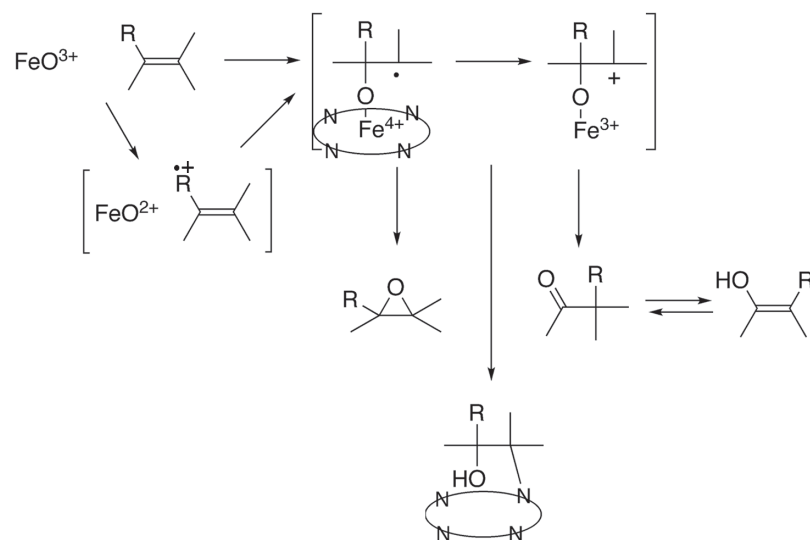
Carbon hydroxylation**Heteroatom release****Heteroatom release****Epoxidation and group migration**

Figure 7.4 Generalized mechanism of P450 oxidation, based on FeO^{3+} chemistry [53].

(ii) many of the arguments for a role of Fe^{2+}O_2 in reactions rely on mutants in which the active site Thr was converted to Ala to disrupt protonation of the $\text{Fe}^{2+}\text{O}_2^-$ entity, but the interpretation of these active site mutants, as is often the case, may not be unambiguous; and (iii) even the aromatase deformylation step (see above) can be rationalized by a radical mechanism, which has some favor on theoretical grounds [66].

Alternate mechanisms have been proposed to explain apparent inconsistencies in “radical clock” experiments, that is, the lack of rearrangement of some molecules before the oxygen rebound [73]. The lack of rearrangement leads to very short estimates of

TABLE 7.1 Potential Iron–Oxygen Complexes Involved in P450–Catalyzed Oxidations^a

Oxidation/Reaction Type	Some Potential Oxidations
FeO ³⁺ /low spin (no barrier) [68]	C-Oxidation, epoxidation, X-oxidation
FeO ³⁺ /high spin (no barrier) [68]	C-Oxidation, epoxidation, X-oxidation
FeO ³⁺ (agostic) [69]	C-Oxidation
FeO ³⁺ (1e [−] transfer) [70,71]	X-Oxidation
FeOO [−] [72]	Epoxidation
FeOOH [72]	Epoxidation, X-oxygenation

X, heteroatom.

^aRef. 67.

the lifetimes of intermediates (e.g., 10¹³ s^{−1} [74]), which are considered closer to transition-state lifetimes than intermediates. However, studies with other radical clocks have led to more consistent values for intermediate lifetimes [18]. The point should also be made that the strained cycloalkanes used as radical clocks may not necessarily undergo the same rates of ring opening inside the proteins (due to binding) as in solution and therefore lead to unusual values in some cases [75].

In part to explain some of the discrepancies in the radical clock results, Shaik has developed a two-state theory to explain alternate reactions that both proceed from a given substrate (Table 7.1) [68,76]. This is a theoretical system and has not been addressed in “wet” experiments. Details of the theory are beyond the scope of this chapter, but in short, the proposal has high and low spin configurations of the iron in the same FeO³⁺ entity, leading to different products. The proportions of high and low spin FeO³⁺ are a function of not only the P450 but also the substrate. Therefore, each system would have to be calculated (and the effort in calculation with a significant part of the protein is considerable in each case), so developing testable predictions is not trivial.

7.3.3 Considerations of Rate-Limiting Steps

In considering a particular P450 reaction, one question is which step (Fig. 7.2) is rate limiting. Associated with this is the even larger question of what really drives regioselectivity of reactions, beyond spatial and thermodynamic considerations. Some considerations are also in order regarding kinetic deuterium isotope effects, which may have new relevance in drug design [77].

In Fig. 7.2, step 1 has not historically been considered rate limiting, but the newer studies on the rates of movement into the active site with some P450s suggest that this aspect should be reevaluated in some cases [40]. Step 2, the reduction, has often been evaluated. It may be rate limiting in some cases [8], in that addition of extra reductase may stimulate some reactions [78,79]. Step 3 (O₂ binding) is not generally thought to be rate limiting, although in some cases P450s reduce substrates instead of oxidizing them, implying that the substrate competes effectively with O₂ for binding to ferrous iron.

Step 4 is often considered to be rate limiting because of the stimulatory effect of *b*₅. This appears not to be an artifact of the reconstituted system, in that anti-*b*₅ can also inhibit microsomal reactions [12]. However, electron transfer may not be the issue

[80–82]. If an electron is not transferred rapidly, the FeO_2^{2+} complex will dismute to ferric iron and $\text{O}_2^{\cdot-}$.

Little direct information is available on how rate-limiting step 5 is, in that this step cannot be measured. Site-directed mutagenesis can lower the rates of P450s, but these results do not provide an insight into rate limitation with wild-type P450s. Obviously, the complex will dismute if forward progress is attenuated. All of this discussion also applies to step 6.

Step 7 usually involves the breaking of a C–H bond and therefore is the only step amenable to interrogation by kinetic isotope experiments (except $^{18}\text{O}_2$ activation [83]). In numerous cases, kinetic deuterium isotope effects are seen and provide evidence that step 7 is the rate-limiting step, at least sometimes.

Step 8, oxygen rebound, has already been discussed. The rates are fast and do not limit the overall rate at which the P450 cycle (Fig. 7.2) operates, but the rate at which a radical intermediate rearranges will influence the distribution of products.

The final step (step 9) is product release. There are two ways of estimating this rate. One is to measure the apparent rate of product binding as a function of (product) concentration and extrapolate to zero concentration to obtain the off-rate. This experiment only works with a relatively low K_d . The other approach is to look for burst kinetics in a pre-steady-state kinetic experiment, with a burst indicating that the step following product formation is rate limiting [84]. Our own work has demonstrated the lack of rate limitation of step 9 in certain reactions catalyzed by the P450s 1A2 and 2A6 [10,85]. In the cases of oxidation of ethanol and acetaldehyde by P450 2E1, nearly quantitative bursts were observed [86,87]. However, considerations regarding the binding of acetaldehyde and acetic acid indicate that this slow step is probably a conformational change (undefined) instead of actual product release.

The point should be made that the concept of a rate-limiting step is too simplistic in a complex reaction cycle such as the P450 cycle (Fig. 7.2). The overall rate of product formation is limited not only by the forward rates of each step but also by the reverse rate constants, when reversible, and particularly by the rate of “side” pathways, for example, the loss of substrate at any of the several early points [10] and the formation of $\text{O}_2^{\cdot-}$, H_2O_2 , and H_2O [10,88].

Some more discussion of kinetic isotope effects is in order, given the number of published studies and the confusion often attached to them. Most P450 reactions involving direct C–H bond breaking show appreciable intrinsic isotope effects, in the range of 3 to as high as 20 or more [85,89]. The intrinsic isotope effects are usually estimated using noncompetitive intramolecular experiments (with consideration of possible secondary isotope effects) or indirect approaches such as Northrop’s method [90] or “branching analysis” [91]. Whether or not C–H bond breaking is rate limiting depends on the isotope effect measured in a noncompetitive intermolecular experiment, in which the (k_{cat}/K_m) parameter measured with a protiated substrate is divided by that measured with a deuterated substrate, with appropriate considerations (high fraction deuterium in deuterated substrate, no inhibitory impurities). This ratio is termed $^D(V/K)$, and the ratio of the two k_{cat} values is $^D V$ [90]. If these values approach the intrinsic isotope effect, then there is strong evidence for rate limitation. Obviously, if other steps are rate limiting then the isotope effect will not be expressed.

These considerations are important in light of recent interest in preparing deuterated versions of drugs to enhance pharmacokinetic properties [77]. Deuteration may or may not produce an isotope effect *in vivo*. Some drugs have produced *in vivo* isotope

effects [92]. One issue *in vivo* is which parameters to assess; the most useful are probably $C_{p, \max}$ (maximum extrapolated plasma concentration) and clearance, which is related directly to the area under the curve (AUC). N-Dealkylations (of amines, not amides) generally show low kinetic isotope effects, because of their mechanisms, and the strategy would be less useful. To date, there has been no clear distinction as to which P450s show isotope effects, so phenotyping of the reaction if of little guidance.

7.3.4 Mechanisms of Oxidation of Major Reactions

The groupings of reactions will be slightly different than in previous reviews from this laboratory. With P450 oxidations covering thousands of different drug substrates and reactions, memorizing them all is impossible and, as in teaching organic chemistry, the only solution is to teach these in logical sets. Using the approach of teaching from chemical mechanism also provides a sound basis for the learner to deal with rather unusual reactions that seem too complex to discern at first observation.

Drug metabolism is best taught—in the author's experience—like organic synthesis, in that one starts with the product and then considers ways to work backward at each step, considering what is known about P450 (and other) reactions. One must appreciate nonenzymatic changes such as carbinolamine breakdown and ring formations. Starting to solve a complex biotransformation pathway by working forward is like designing a synthesis this way from crude materials—there are too many possibilities—so it should be worked backward. Of course, drug metabolism scientists may be called upon to predict possible transformations, but the likelihood of certain reactions happening is always difficult and experience is useful in this regard (particularly experience with similar compounds).

7.3.4.1 Carbon Oxidations (sp^3). The basic paradigm was discussed earlier (Figs. 7.5 and 7.6). The FeO^{3+} abstracts a hydrogen atom, and then oxygen rebound completes the reaction. A few variations on this theme follow.

In O-dealkylation of ethers (Fig. 7.5a), a similar hydroxylation occurs to yield a hemiacetal, which hydrolyzes. The same type of reaction can occur with esters, yielding an aldehyde and carboxylic acid [93] (Fig. 7.5b).

Another mode of the C–H cleavage is desaturation (Fig. 7.5c). This process is agreed to start with hydrogen atom abstraction. Instead of direct oxygen rebound, either a subsequent abstraction of the vicinal C–H atom or a nonenzymic rearrangement could occur to generate the vicinal radical, and the $FeOH^{3+}$ entity could abstract ($H\cdot$) again at the original site. Either process would have the same stoichiometry and yield the desaturated product plus two molecules of H_2O . Desaturation reactions are usually accompanied by some alcohol formation, suggesting such a bifurcated pathway, but in at least one case desaturation appears to be extensive, without alcohol formation [94]. With ethyl carbamate, the subsequent epoxidation was much faster than desaturation and special methods were needed to quantify the olefin [95].

7.3.4.2 Heteroatom Oxidations (N, S, P, I). Most of this section applies to nitrogen, and the rest is applicable to sulfur and the other heteroatoms that often appear in drug heterocycles, and so on.

N-Dealkylations are considered to occur either by the hydrogen abstraction pathway described or, when the distance and oxidation potential are appropriate, by initial one-electron abstraction (Fig. 7.6). The aminium radical is unstable and, after α -proton

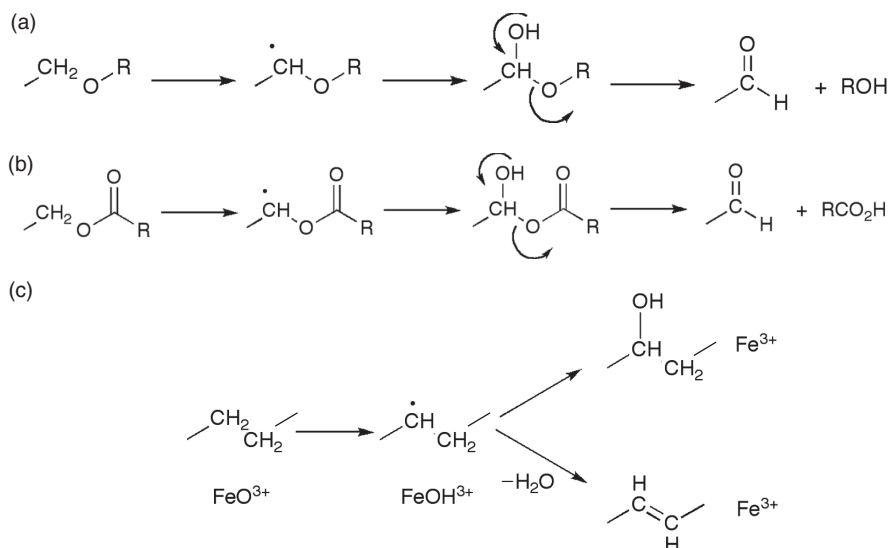


Figure 7.5 Carbon oxidation. (a) Oxidative cleavage of an ether, (b) oxidative cleavage of an ester, and (c) bifurcation between C-hydroxylation and desaturation reactions.

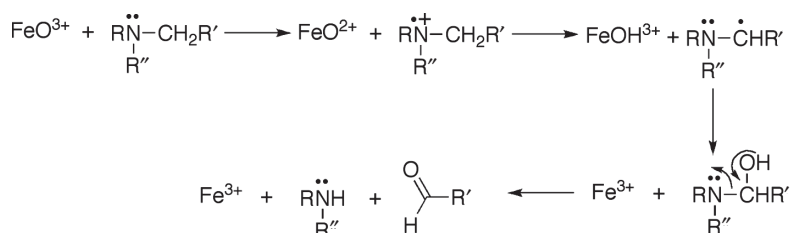


Figure 7.6 N-Dealkylation mechanism based on initial one-electron oxidation, and rearrangement of the resulting aminium radical.

abstraction, rearranges to a carbon radical for incipient oxygen addition, followed by breakdown of the carbinolamine. The FeO^{2+} is basic and helps abstract the α -proton, a mechanism not available in many peroxidases [96,97].

Several lines of evidence support the view that P450s N-dealkylate by the above pathway (although they are not excluded from hydrogen atom abstraction). (i) The low intrinsic kinetic deuterium isotope effects are consistent [97,98]. (ii) With dihydropyridines (which are vinylogous analogs), 4-alkyl groups are released and can be trapped as radicals (Fig. 7.7a) [99]. (iii) Other compounds with low oxidation potentials undergo characteristic rearrangements diagnostic for radical cations, for example, quadricyclane [100], or radical cations can be directly observed (e.g., 1,2,4,5-tetramethoxybenzene [101]). (iv) Rearrangements and mechanism-based inhibition with strained cycloalkylamines are observed and consistent with aminium radical formation [102–104]. (v) Analyses by Hammett and Marcus approaches are consistent with an aminium radical mechanism [57,105].

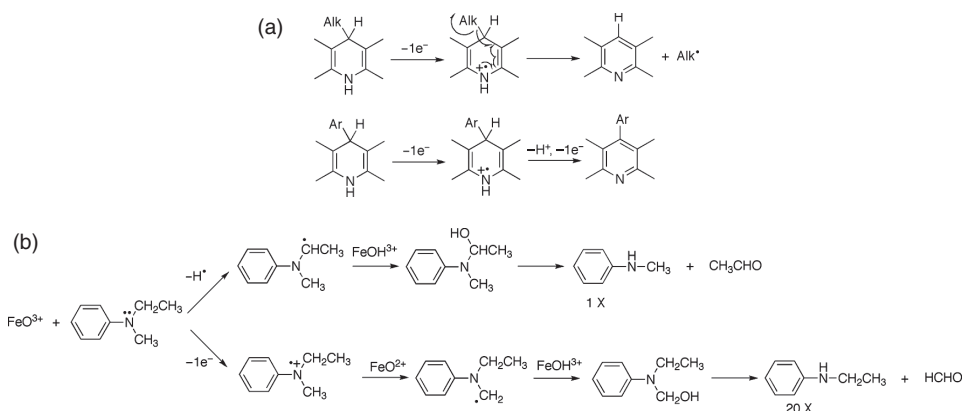


Figure 7.7 Two types of evidence in support of a one-electron transfer pathway (Fig. 7.6). (a) Oxidation of a 1,4-dihydropyridine with a 4-alkyl substituent leads to the extrusion of alkyl radical, which can be trapped, while oxidation of a 4-aryl analog yields to the retention of the 4-position moiety [99,106]. (b) Oxidation of *N*-ethyl-*N*-methylaniline by rat P450 2B1 yields a 20-fold excess of *N*-demethylation over the *N*-deethylation product [107].

Following abstraction of one electron, the resulting aminium radical rearranges to a carbon-centered radical. There is evidence that the FeO^{2+} (Compound II) entity acts as a base to facilitate this process [97]; most peroxidases only abstract electrons via the heme edge [96] and as a result are not capable of dissipating the aminium radicals and rebounding oxygen.

A similar process can be proposed for S- and P-dealkylations, in that the redox potentials are generally accessible. The point should be made that amides do not have low one-electron oxidation potentials, and therefore, N-dealkylation reactions of amides probably proceed largely via hydrogen abstraction. The higher kinetic deuterium isotope effects are consistent with this view [108].

Another point to be made is that the effective oxidation–reduction potential, $E_{(\text{app})}$, is a function of the intrinsic potential, $E_{(\text{int})}$, and the interatomic distance ($r_{1,2}$, in Å), as well as the dielectric constant (D).

$$E_{(\text{app})} = E_{(\text{int})} + \frac{14.4}{r_{1,2}D} \quad (7.1)$$

Thus, how far the nitrogen (or other heteroatom) is from the FeO^{3+} entity and the protein local dielectric constant influence the oxidation potential of the substrate.

Another experiment strongly implicating one-electron mechanisms is the N-dealkylation of *N*-ethyl-*N*-methylaniline by P450 2B1 [107], in which the ratio of N-demethylation to N-deethylation was 20:1, consistent with the higher stability of a methyl aminium radical but not with the inductive effect expected for methylene hydrogen atom abstraction [98] (Fig. 7.7b).

Oxygenation of heteroatoms is also observed with P450s. Two mechanisms can be considered. One is a one-electron oxidation, as discussed earlier (Fig. 7.8). This mechanism is intellectually satisfying in that it couples the different types of oxidations that occur with nitrogen and sulfur molecules and explains why oxygenation

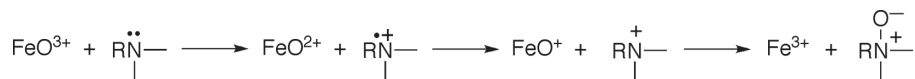


Figure 7.8 Proposed N-oxygenation proceeding via one-electron oxidation.

is usually a very minor reaction when dealkylation is possible [105]. Even when N-dealkylation is favored, some N-oxygenation occurs, and the generally greater tendency of sulfur atoms to undergo oxygenation (rather than S-dealkylation) is consistent with greater radical stability. Interestingly, N-dealkylation of a series of substituted *N,N*-dimethylanilines yields a Hammett plot with a negative ρ value, consistent with stabilization of an aminium radical intermediate [105] but no such relationship was seen for N-oxygenation [107]. An alternate mechanism involves a direct transfer of oxygen to the heteroatom, without one-electron chemistry. Conceivably, this might even happen with $\text{FeO}_2(\text{H})$, as proposed [109], instead of FeO^{3+} (Table 7.1). The point should be made that another microsomal monooxygenase, FMO, is prominent in the oxygenation of nitrogen and sulfur atoms [110], and care is needed to discern whether P450 or FMO is involved.

In heteroatom oxygenation, most of the focus is on nitrogen and sulfur, two atoms that often have low oxidation–reduction potentials. In general, the high oxidation potential of oxygen makes an electron-transfer pathway less likely, and most of these reactions proceed by the carbon hydroxylation pathway described earlier. Phosphorus oxygenation is known [111] and very feasible due to the low oxidation potential. Other heteroatom oxygenations involve halogens, as first proposed for dihaloethanes [112]. An iodosylbenzene-dependent oxygenation of iodobenzene was demonstrated by Burka *et al.* [113]. NADPH-dependent P450 oxygenation of aryl iodides and bromides was demonstrated later [114] using model compounds that trapped the resulting haloso products to yield stable entities. Although chlorine is harder to oxidize, He *et al.* [115] demonstrated that ω -chloro and ω -bromo fatty acids are oxygenated (on the halogen) by rat P450 4A1 (The products are unstable, and the analysis was based on the detection of isotopically labeled (^{18}O) alcohols, with the oxygen being derived from H_2O and not O_2 .)

7.3.4.3 Reaction of π -Bond Substrates. This class includes mainly olefins, acetylenes, and aryl compounds. The bulk of the evidence is consistent with a stepwise mechanism in which FeO^{3+} reacts with a double bond to generate an intermediate, with a radical localized at the carbon vicinal to the C–O bond formed (Fig. 7.9). An epoxide can be formed by a hemolytic mechanism. Alternatively, electron transfer can generate a carbocationic intermediate. This intermediate could also close to form an epoxide, in a heterolytic process, or undergo a 1,2-shift of an anion (e.g., halide or hydride ion [116]) to generate a rearranged carbonyl product. In the case of aryl compounds, the aryl ketone rearranges to generate a phenol. This process is a part of the so-called NIH Shift (of hydrogen or other atoms) [117]. The point should be made that the demonstration of an NIH shift in the formation of a phenol is not definite evidence for the existence of an epoxide intermediate in the process; the mechanism may occur without the epoxide (Fig. 7.10).

Similar mechanisms can be written for the oxidation of acetylenes, with the collapse of an intermediate via a heterolytic path to form a putative epoxide [118], hydrogen

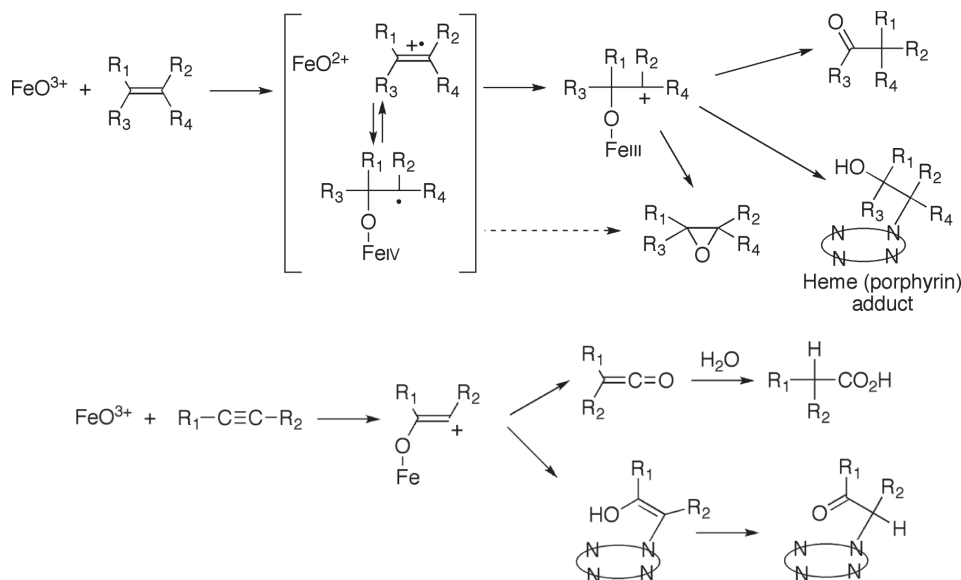


Figure 7.9 Some possible mechanisms of oxidation of olefins and acetylene.

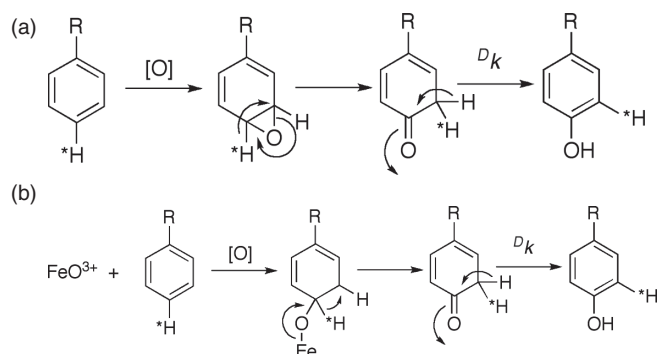


Figure 7.10 Aromatic hydroxylation and an NIH shift of a *para*-hydrogen [117] via two mechanisms: (a) concerted epoxidation followed by rearrangement and (b) stepwise electrophilic attack followed by rearrangement.

migration to generate a (reactive) ketene, or direct reaction with the heme porphyrin to generate an adduct (Fig. 7.9).

The view that a common mechanism drives these reactions is consistent with a number of lines of evidence and supported by kinetic hydrogen isotope effect results [119]. Thus, the mechanism can be considered to be nonconcerted. Another phenomenon that has been observed with some P450s and some of their substrates is proton exchange with water during epoxidation, which can only be explained with an intermediate involving a Fe–C bond [120]. However, an alternate point of view is that dual mechanisms are involved in the two-state system of Shaik [68]: a concerted low spin mechanism leads to epoxidation and a high spin nonconcerted reaction is involved in the formation of the other products [121].

7.3.5 More Complex Transformations of Drugs

Almost all P450 oxidation reactions can be explained with the basic mechanisms presented here, utilizing the perferryl FeO^{3+} entity and possibly $\text{FeO}_2(\text{H})$, as already mentioned (Table 7.1). Some examples of seemingly more complex reactions have been presented elsewhere and are not recapitulated in this chapter [67,122], including ring expansion [104], ring contraction [123], and ring formation [124] (Fig. 7.11).

C–C and C–O bond coupling reactions are not unusual in P450 reactions involved in biosynthetic reactions in plants [126]. Such coupling reactions have now been observed with the (human) P450s 2D6 and 3A4 in the biosynthesis of mammalian alkaloids [127] (Fig. 7.12), and there are also some examples in drug transformations [128].

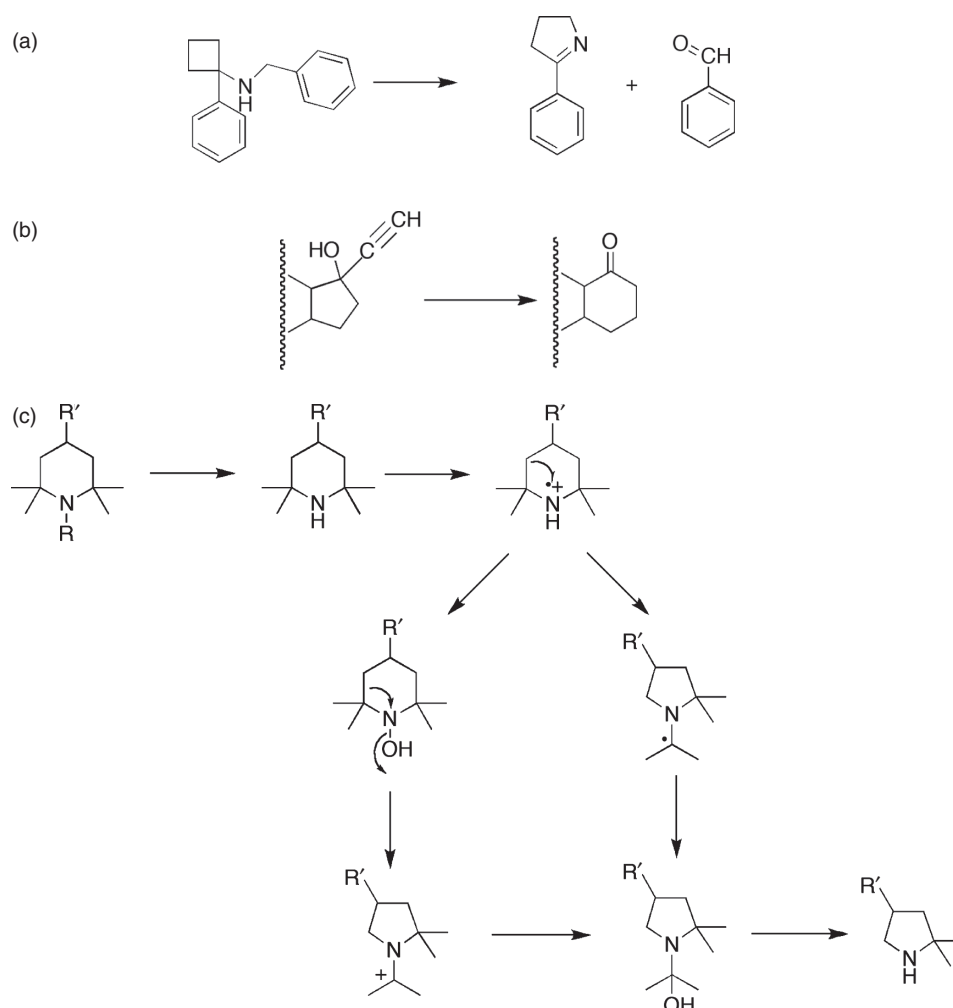


Figure 7.11 Some more “unusual” P450 reactions. (a,b) Ring expansions [104,125]. (c) Ring contraction [123]. (d) Ring formation [124].

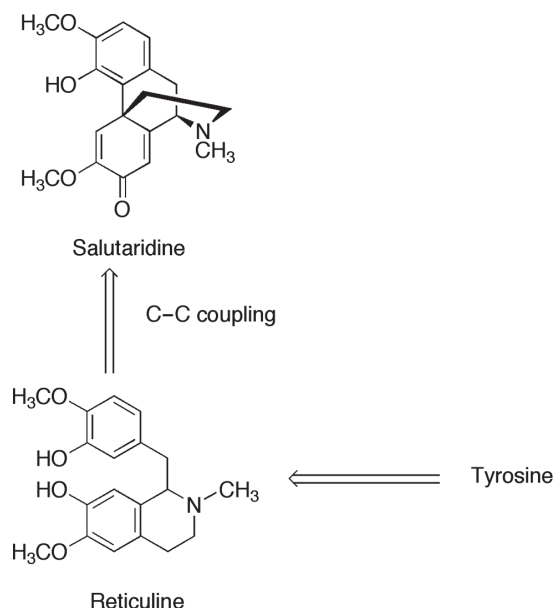


Figure 7.12 C–C bond coupling catalyzed by the P450s 2D6 and 3A4 [127].

7.4 SUMMARY

While reading about the details of P450 mechanisms, one can get the impression that some of the work is esoteric and unlikely to be of practical use. What is true is that some elements of P450 catalysis will be more practical than others. Following are several points that a drug metabolism scientist should remember and will be of use.

1. The basic sequence of events in the catalytic cycle is shown in Fig. 7.2. One should remember that the scheme is not necessarily ordered as such [10] and that which events are rate limiting can vary considerably among the P450s and substrates.
2. The general concepts of abstraction by FeO^{3+} and oxygen rebound will explain most P450s, so mastering this basic chemistry is important.
3. Rearrangements are to be expected, either before the oxygen rebound or after product formation, so these possibilities should be remembered in considering how a product might be formed.
4. P450 regioselectivity is driven by thermodynamic and steric aspects of possible P450 reactions, which are not independent of each other. The chemical reactivity at a given site can be predicted, but even with the available P450 crystal structures, predictions are difficult. Predictions about regioselectivity—and particularly rates—are most feasible in a series of compounds where data already exist for some of the members.

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8 Transcriptional Regulation of Cytochrome P450 Genes

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8.1 Introduction	1
8.2 Transcriptional regulation of cytochrome P450s	2
8.3 Other considerations for transcriptional regulation of cytochrome P450s	18
8.4 Conclusion	23
Abbreviations	23
References	24

8.1 INTRODUCTION

Our mechanistic understanding of the transcriptional regulation of cytochrome P450 (P450) expression has increased significantly in recent years, with the discovery and characterization of “orphan” nuclear receptors such as the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR). The role of these receptors as mediators of the inductive effects of xenobiotics continues to be the subject of intensive investigation and has added a further layer of complexity to an already burgeoning area of research.

It has become clear that significant variability exists at almost every level of the processes that contribute to the ultimate functionality of P450 proteins, and transcriptional regulation is no exception. Expression of these proteins is controlled by transcription factors that not only vary in their expression level and the degree to which they bind and respond to different classes of ligands but are also themselves subject to alternative splicing, producing variant receptor forms with different spatiotemporal expression and functionality. It is clear that only by gaining a detailed understanding of these receptors will we have a greater appreciation of the intricacies of transcriptional regulation of P450s.

This chapter concentrates on those transcription factors involved in xenobiotic-induced expression, giving an overview of regulatory interactions and raising issues that have a bearing on transcriptional regulation, such as polymorphisms, splice variants, and species specificity. However, it is important to note that other transcription

factors that are not covered in this review - including the peroxisome proliferator activated receptors α and γ , the vitamin D receptor, and the oestrogen receptors α and β - are also involved in regulation of endogenous and basal cytochrome P450 expression.

8.2 TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.2.1 Pregnane X Receptor (PXR)

The pregnane X receptor (PXR, also known as SXR, PAR, and NR1I2) is the most recently identified of the three main regulators of xenobiotic-induced CYP expression, with the mouse ortholog having been first identified by Kliewer *et al.* in 1998, closely followed by the identification of the human form by Blumberg *et al.* [1,2]. A ligand-activated orphan nuclear receptor was named the pregnane X receptor because of its activation by pregnenolone derivatives. Acting as a xenosensor, PXR is one of the key controllers of drug metabolism, in particular, for the expression of the CYP3A enzyme isoforms. In addition to the CYP3A enzymes, PXR, bound as a heterodimer to its partner, the retinoid X receptor (RXR), regulates a wide variety of P450s, as well as phase II enzymes and phase III drug transporters (Table 8.1). PXR is also promiscuous, being able to accommodate and be activated by a wide range of different ligands, both endogenous and exogenous (Table 8.2). This protein is expressed in many tissues, including those of heart, colon, stomach, and certain brain regions, although it is primarily expressed in the liver and small intestine [3]. Several splice variants have also been identified, which affect PXR-mediated transcriptional regulation (Section 8.3.3).

Flexibility in the ligand-binding domain (LBD) of PXR is the reason for its wide substrate specificity, with the binding pocket able to expand from its resting 1150 Å³ to more than 1600 Å³ when ligand bound. Large molecules, such as the macrolide antibiotic rifampicin (RIF), can therefore activate the receptor using an induced-fit

TABLE 8.1 Examples of Human Cytochrome P450s Regulated by Human PXR, CAR, and AHR

Gene	PXR	CAR	AHR	References
<i>CYP1A1</i>	—	—	↑ ^a	[4] ^a
<i>CYP1A2</i>	↑	↑ ^b	↑ ^a	[5] ^b and [4] ^a
<i>CYP1B1</i>	↑	↔	↑	[6,7]
<i>CYP2A6</i>	↑ ^b	↑	—	[8] ^b
<i>CYP2B6</i>	↑	↑	↔	—
<i>CYP2C8</i>	↑	↑	—	[9]
<i>CYP2C9</i>	↑	↔	↔ ^b	[10] and [4] ^b
<i>CYP2C19</i>	↑	↑	—	—
<i>CYP3A4</i>	↑	↑	↔	—
<i>CYP7A1</i>	↓ ^b	↓ ^a	↔ ^c	[11], ^b [12], ^a and [4] ^c
<i>CYP8B1</i>	↓ ^b	↓ ^p	—	[11] ^b and [13]
<i>CYP24A1</i>	↑	↑	—	[14]

Bold arrows indicate strong induction.

↑, induced; ↓, repressed; ↔, no change/basal expression; ^p, putative interaction in humans. a, b, or c on each line refers change in nuclear receptor expression to the corresponding citation.

Source: Unless otherwise stated, data was extracted from Ref. 15.

TABLE 8.2 Example Modulators of Human PXR, CAR, and AHR

Class	Compound	PXR	CAR	AHR	References
Classical	Rifampicin	++			PXR/CAR: [7,15–21] AHR: [6,22]
	CITCO		++		
	TCDD			++	
Drug	3-Methylcholanthrene			++	
	Omeprazole	+		+	
	Thiabendazole			+	
	Nicotine			+	
	Caffeine			+	
	Paclitaxel	+			
	Methadone	+	+		
	Clotrimazole		–		
	Ketoconazole	–	–		
	17 α -Ethinylestradiol	+			
	Mifepristone	+			
	SR12 813	+			
	Phenytoin	+			
	Primaquine	+			
	Spironolactone	+			
Herb	Hyperforin	++			
	Cryptotanshinone	+			
	Artemisinin	+	+		
	<i>Ginkgo biloba</i>	+	+		
	Schisandrin A-C	+			
Dietary	β -Carotene	+		+	
	Vitamin E	+			
	Flavonoids			$\pm$	
	Curcumin			+	
Endogenous	Androstanol		+		
	Corticosterone	+			
	17 β -Estradiol	+	+		
	Vitamin K ₂	+			
	Tryptophan metabolites			+	
	Bilirubin			+	
Environmental/ industrial	Benzyl butyl phthalate	+			
	Chlordane	+			
	Phthalic acid (DHEP)	+	+		
	Nonylphenol	+	+		
	Polychlorinated biphenyls	+			
	Toxaphene	+			
	Triclopyr	+			
	Benzo(a)pyrene			+	
	1-Methyl-1-phenylhydrazine			+	

Blank spaces indicate no data is available.

++, very strong agonist; +, agonist; –, antagonist (or inverse agonist, CAR only); $\pm$, antagonist/agonist, dependent on cell context.

For further information see reviews [6,15–17].

mechanism, which allows molecules to adopt various binding orientations before selecting the optimal configuration [23–25]. The LBD is predominantly hydrophobic in character, with Met243, Ser247, Gln285, Trp299, His407, and Phe420 commonly interacting with ligands [26,27]. Site-directed mutagenesis studies have indicated that even small changes in the LBD can have significant effects on the ligand-induced activation of PXR [25,26]. Interspecies variability in this region therefore has significant effects on the ligand activation profile (Section 8.3.2).

Nuclear translocation is a controlling factor in PXR-mediated P450 induction. However, because of difficulties with investigating translocation *in vitro*, namely, when PXR is overexpressed it spontaneously translocates to the nucleus [28], data regarding this mechanism remains limited. It has been reported that when inactive, PXR is bound in a complex with cytoplasmic CAR retention protein (CCRP) and HSP90, which retains the receptor in the cytoplasm [29,30]. Although the mechanism underlying PXR dissociation from the complex and translocation to the nucleus has not yet been elucidated, it is likely that a mechanism similar to that governing CAR dissociation from the retention complex is involved (Section 8.2.2). Once released from the cytoplasmic retention complex, PXR nuclear localization signals (NLSs) are targeted by importin- α proteins for nuclear import [29].

Upon entering the nucleus, PXR heterodimerizes with its binding partner, the RXR α , before binding to response elements in the promoter and enhancer regions of target genes (Fig. 8.1). PXR heterodimers preferentially bind direct repeat sequences possessing the half site AG(G/T)TCA separated by three nucleotides (DR3) and everted repeats of the half site separated by six nucleotides (ER6), that is, AG(G/T)TCAnnnAG(G/T)TCA and TGA(A/C)CTnnnnnnAG(G/T)TCA, respectively [15,16]. However, they are also able to bind other recognition sites, such as DR4, DR5, and ER8 motifs, although with lower affinity [31], thus accounting for the cross talk between receptors, which is an important contributor to P450 regulation.

A key component of transcriptional activation by nuclear receptors is the recruitment of coactivators and corepressors to promoter regions, following DNA binding. Coactivators of PXR, CAR, and AHR (aryl hydrocarbon receptor) are given in Table 8.3. As an example, PXR is a binding partner for the p160/SRC (steroid receptor coactivator) coactivator family, which recruits histone acetyltransferase complexes, such as cAMP response element binding protein/p300 (CREB), thus providing access to DNA strands for RNA-polymerase-catalyzed transcription [25]. These coactivators possess three LXXLL motifs that bind to the AF-2 helix, interacting with two charged residues on the receptor surface (Lys259 and Glu427) to form a charge clamp [24]. Crystal structures of SR12813 in complex with PXR and SRC-1 indicate that in the presence of coactivator, the ligand will bind in a single orientation. The combination of ligand and SRC-1 binding stabilizes the protein structure to restrict the flexibility of the LBD once an active ligand conformation has been achieved [25]. In contrast, corepressors are thought to bind in the absence of ligand, or in the presence of antagonists, retaining the AF-2 helix in a nonactive conformation [16]. Corepressors of PXR include the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT). SMRT possesses both ID1 and ID2 interacting domains, which consist of a CoRNR box (I/LXXI/VI) and the motif LXXXIXXXI/L, respectively [32]. PXR binds to the ID2 motif preferentially over the ID1, with the key interacting residues being Lys259, Gly270, and Pro423 of the PXR LBD and Arg2347, Lys2348, and Leu2350 of SMRT [32]. Site-directed mutagenesis studies show that mutation

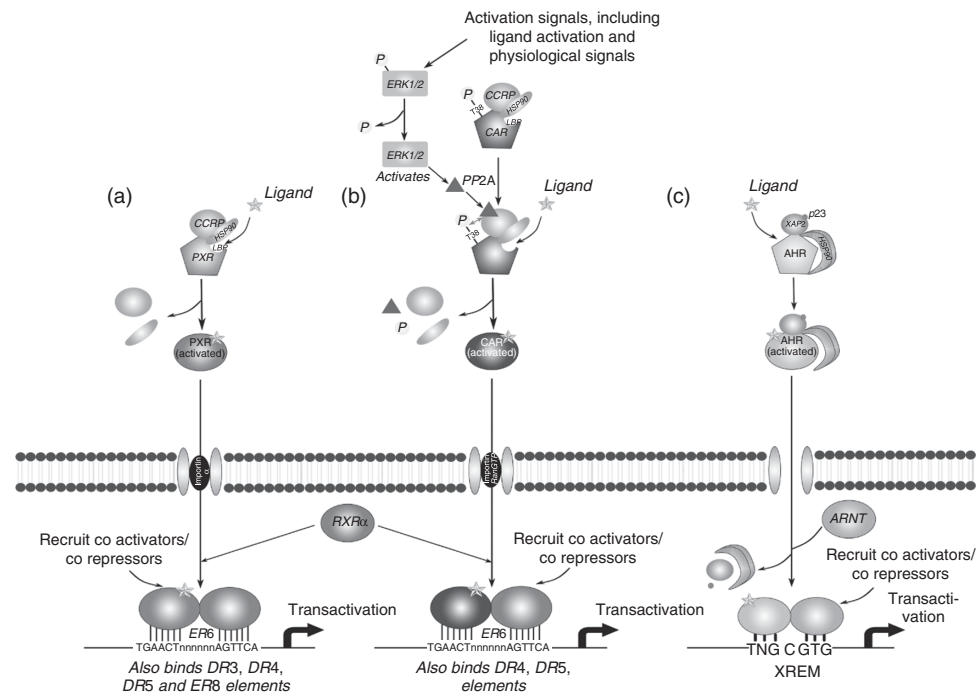


Figure 8.1 Activation mechanisms of (a) PXR, (b) CAR, and (c) AHR. (See color insert.)

TABLE 8.3 Coactivators and Corepressors Associated with PXR, CAR, and AHR

Coregulator Type	Coregulator	PXR	CAR	AHR	References
Coactivators	SRC-1	*	*	*	AHR: 33,34 PXR/CAR: 12, 15, 16, 35
	NCOA2 (GRIP1, SRC-2)	*	*	*	
	p/CIP (SRC-3)	*		*	
	P300			*	
	CBP			*	
	RIP 140	*		*	
	CARM1			*	
	PRMT1			*	
	PGC-1 α	*	*		
	PBP	*	*		
	ASC-2		*		
	SMC-1		*		
Corepressors	ANKRA2			*	
	SMRT	*		*	
	NCoR	*	*		
	SHP	*			

^aCorepression of AHR via the AHR repressor [36].

of charged residues within the PXR LBD (Arg410Asn, Asp205Ala, Glu321Ala, and Arg413Ala) increases the binding affinity for corepressors, indicating a core role for these residues in the maintenance of basal PXR activation [32]. Acting in concert, the recruitment of coregulators therefore controls the expression of target genes, such as P450s, by regulating access of RNA polymerases to the promoter regions.

A further aspect of PXR-mediated regulation of P450s is posttranslational modification of PXR by phosphorylation. This modification is known to be critical to the correct function of other nuclear receptors, including CAR and AHR, but, until recently, had not been subjected to a systematic analysis with respect to PXR. Research from the Staudinger group has indicated that protein kinase A (PKA) interacts with PXR, but in a species-specific manner, synergizing with ligand activation in mice but repressing ligand-induced activation in rats and humans [37]. Protein kinase C (PKC) and CDK2 signaling has also been implicated. A recent mutagenesis study, in which 18 serine and threonine residues identified by either *in silico* site prediction or comparison with other receptors, has shed more light on the role of phosphorylation in PXR activity. These studies have shown that the phosphorylation state of certain key residues (Ser8, Thr57, Ser208, Ser305, Ser350, and Thr408) is involved in the control of heterodimerization, DNA binding, corepressor recruitment, and nuclear translocation [38]. The phosphorylation state of PXR therefore appears to be important in controlling gene expression. However, these observations require further studies to fully understand the role of phosphorylation in PXR function.

In summary, PXR is a key controller of P450 regulation, being associated with a wide variety of target genes. It also has an important role in many endogenous processes, including control of glucose homeostasis and the cholesterol and bile acid metabolism [15,16]. However, most importantly from a pharmaceutical perspective

is its role as the key regulator of the CYP3A enzymes, which are responsible for the metabolism of a significant proportion of currently used pharmaceuticals, and as a consequence involved in a significant number of drug–drug interactions [39]. As a key regulator of P450s, xenobiotics that interact with PXR could have a profound effect on their own metabolism as well as on the metabolism of other drugs given concomitantly. The promiscuity of PXR in terms of its ligand interactions and the wide range of genes it transactivates can result in severe drug–drug interactions [40–43]. As a consequence, PXR transactivation is investigated as a part of preclinical drug development programs. Regulation of this signaling pathway is still incompletely understood, although it is clear that it involves a complex interaction between coregulators, protein structure, and phosphorylation state.

8.2.2 Constitutive Androstane Receptor (CAR)

Initially identified in humans in 1994 as MB67 [44], and in the mouse in 1997 [45], the constitutive androstane receptor (CAR, NR1I3) is well established as a key regulator of P450 expression [16,39,46–57]. The genes targeted by CAR are numerous and varied, encompassing P450s (Table 8.1), phase II enzymes such as UDP-glucuronyltransferases, and phase III drug transporters, with CYP2B6 being prototypically induced (Cyp2b10 in mice, CYP2B1/2 in rat) [58,59]. Lacking a physiological ligand, this protein was also classed as an orphan nuclear receptor. However, as a nuclear receptor, CAR has some unusual properties, having both a different protein structure and displaying constitutive transcriptional activity [60].

As a consequence of the constitutive activity of CAR, molecules such as androstanol and androstenol have been referred to as *inverse agonists* because they actively repress constitutive CAR activity instead of antagonizing in the classical sense [61]. While not as promiscuous as PXR, CAR can be activated by numerous compounds (Table 8.2), but few operate by binding directly to CAR. Its constitutive basal activity means that it can transactivate genes without ligand binding, in response to externally acting signals that induce nuclear translocation [62]. This indirect activation pathway is a key mechanism in CAR-mediated transactivation, with phenobarbital (PB) induction of CYP2B being the classic example, and is suggested to be the major activation pathway for this molecule [63,64]. These phenomena are dependent on several key structural features of CAR and the increasingly understood mechanism of nuclear translocation, which are considered below.

Structurally, a protein consists of three domains: (i) a highly conserved N-terminal DNA-binding domain, (ii) a hinge region, and (iii) a variable C-terminal domain associated with ligand binding (AF-2 domain), dimerization with the CAR-binding partner (RXR α), and transcriptional activation [54]. The ligand-binding pocket (LBP) is significantly smaller than that of PXR at approximately 675 Å³ in humans and is predominantly hydrophobic in nature. The conformation of the AF-2 domain within the LBP is thought to be vital to the constitutive activity of the protein, although it is not accessible for ligand binding, being protected by the side chains of Phe161 (also key in constitutive activity), Asn165, Phe234, and Tyr326, which are conserved across all mammalian orthologs of CAR [65,66]. It has also been suggested that disruption of the AF-2 conformation is responsible for switching CAR to its inactive form, such as is seen upon inverse agonist interaction [27].

Although CAR is described as constitutively active, it is unable to activate transcription of downstream genes in the absence of an external signal because of regulatory mechanisms controlling its translocation to the nucleus. In the absence of ligand or indirect activation, CAR is sequestered in the cytoplasm, bound to a complex consisting of CCRP and HSP90, along with other accessory proteins [67,68]. In response to direct or indirect ligand challenge, NLSs are activated, resulting in dissociation from this complex. Once the ligand is activated and released from the cytoplasmic retention complex, active CAR is translocated into the nucleus via an importin/Ran-GTP mediated process involving NLS binding to IPO13 [69].

The molecular trigger for, and underlying mechanism of, nuclear translocation is still unclear, although recent research has implied an essential role for cellular signaling pathways and phosphorylation status, especially in indirect control [28]. Although the role of phosphorylation state in CAR translocational control had been described previously, it was not until 2009 that the Thr38 amino acid was identified as the key residue in regulation of human constitutive androstane receptor (hCAR) translocation. Phosphorylation of Thr38 by PKC inactivates CAR by destabilizing the helix containing the C-terminal region of the first zinc finger, resulting in ablation of DNA binding [70]. However, on Thr38 dephosphorylation, the disrupted helix regains its stability, resulting in nuclear translocation and transactivation of downstream genes. It has been suggested that an essential process in PB-induced activation is the recruitment of protein phosphatase 2A (PP2A) to the retention protein complex in mouse hepatocytes, subsequently inducing translocation [71,72]. PP2A has therefore been suggested as a candidate for the dephosphorylation of this residue, acting in concert with other cofactors recruited to the CCRP/HSP90 complex. In addition, a recent report by Koike *et al.* identifies extracellular signal-regulated kinase (ERK) 1/2 as the endogenous signal controlling sequestration of CAR in the cytoplasm, with dephosphorylation of ERK 1/2 being sufficient to induce nuclear translocation [73]. AMP-activated protein kinase has also been suggested to influence CAR localization following PB treatment, although currently available data is conflicting [64,74–77]. Mutoh *et al.* suggest a model for both direct and indirect activators, in which PKC phosphorylates and PP2A dephosphorylates Thr38 to control nuclear translocation. The signal controlling the phosphorylation state of this residue appears to be extracellular signal-regulated kinase (ERK) 1/2 [70,73]. However, a membrane-bound regulatory subunit of protein phosphatase 1b, PPP1R16A, has recently been shown to interact with CAR, inducing translocation, which agrees with a report which localizes CAR at the cell surface in addition to the cytoplasm [78,79]. This suggests another layer of complexity in controlling nuclear translocation.

Once in the nucleus, CAR binds to RXR α in a head to tail arrangement and the resulting heterodimer binds to response elements in the promoter of target genes (Fig. 8.1). CAR will preferentially bind to three binding motifs: (i) DR4 (e.g., AGTTCAnnnnnAGTTCA), (ii) DR5 (e.g., AGTTCAnnnnnAGTTCA), and (iii) ER8 (e.g., TGAACtnnnnnnnnAGTTCA). However, CAR can bind other motifs, such as ER5–ER10 types, but with lower affinity [15,80]. Unusually, CAR is also able to bind to DNA as a monomer, having a preference for DR4 motifs [80]. The optimal binding site for monomeric CAR was found to be AGAGTTCA. Binding to these motifs as a monomer is as strong as that of the T₃ thyroid hormone receptor, and the preference for monomeric binding is more marked in humans than in mice. However, if the 5' nucleotide flanking sequences contain pyrimidine nucleotides, the binding

tendency of monomeric CAR is greatly reduced. All these binding motifs have been identified in the phenobarbital response element (PBREM) commonly found in CAR target genes [80]. Once bound, activated CAR will recruit coactivators/corepressors to modulate gene expression (Table 8.3).

Although this chapter has concentrated on the role(s) of CAR in xenobiotic metabolism, it is important to remember that CAR also has many endogenous functions. It is particularly important in control of lipid metabolism and energy homeostasis, appearing to act as an energy sensor, and influencing metabolism accordingly [17,81–86]. CAR is also involved in the control of bilirubin metabolism and heme biosynthesis, as well as bile acid and steroid/thyroid hormone homeostasis. For a recent review of this area, see di Masi *et al.* [15]. The range of endogenous roles with which CAR is involved means it is vital to consider the potential for functional cross talk between endogenous control and xenobiotic metabolism. For instance, the energy state of an organism can have significant effects on the uptake and pharmacokinetics of a drug because nutritional state can influence the induction of P450s by CAR [17]. Other considerations relating to CAR-induced xenobiotic metabolism include differential regulation due to species specificity, gender-specific induction, and the circadian control. Species specificity is discussed in more detail later in this chapter. Circadian control is a potentially important determinant of CAR expression and induction. CAR exhibits a circadian expression profile in mice because of its regulation by the PAR-domain basic leucine zipper transcription factors DBP (albumin D-box binding protein), TEF (thyrotroph embryonic factor), and HLF (hepatic leukemia factor), all of which are regulated by core components of the circadian machinery [87]. This results in a temporal induction of CAR target genes. Since downstream genes, such as P450s, are likely to display circadian expression profiles as a result, it is understandable that the clinical profile of certain drugs, such as cyclophosphamide and mitoxantrone, improves when dosing time in terms of circadian cycle is considered [87]. This feature should always be considered when investigating compounds that are likely CAR interactors, particularly in mouse models.

Overall, CAR regulation of P450 expression is complex, involving structural activation, regulation by signaling pathways, and interaction with various factors influenced downstream by CAR, including energy metabolism. It is, therefore, important when investigating compounds that appear to induce P450s via a CAR-dependent mechanism to consider that the endogenous control of CAR and the way in which it influences other homeostatic mechanisms, such as energy homeostasis, can have a significant effect on P450 expression profile and thus xenobiotic metabolism.

8.2.3 Aryl Hydrocarbon Receptor

The AHR has been known significantly longer than PXR and CAR, having been first identified in 1976 as the cytosolic receptor mediating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-dependent induction of aryl hydrocarbon hydroxylase activity [88]. However, unlike PXR and CAR, the AHR is not a nuclear receptor but is the only known ligand-activated member of the basic helix-loop-helix (bHLH)/period (PER)-aryl hydrocarbon receptor nuclear translocator (ARNT)-single minded (Sim) (bHLH-PAS) family of transcription factors. AHR ligands can be classified as synthetic, for example, dioxins and halogenated polycyclic aromatic hydrocarbons, and naturally occurring, such as flavonoids, indoles, and arachidonic acid metabolites [6,22,89]. As

a family, these proteins are implicated in the regulation of physiological processes, including organ development, metabolism, and stress and immune response [90]. However, one of the key roles of AHR is the control of xenobiotic metabolism.

AHR is responsible for the transactivational control of a variety of P450s (Table 8.1), most notably CYP1A1, CYP1A2, and CYP1B1, with CYP1A1 being considered a model enzyme for studying AHR-mediated gene activation. In order to modulate transcription, AHR heterodimerizes with its binding partner ARNT [33]. The transcriptional activation domains (TADs) of both molecules, once activated by ligands, activate transcription by inducing DNA binding via a subdomain N-terminal to the HLH domain. Both AHR and ARNT possess one TAD in the C-terminal region, with both containing glutamine and hydrophobic residues. However, the TAD of AHR also has acidic characteristics, whereas that of ARNT is rich in serine, threonine, and proline [91]. It is worth noting that while the N-terminal domain of AHR is well conserved across species, the C-terminal domain, and thus the TAD, is significantly divergent (58% identity between human and mouse forms) [92–94]. As a consequence, there are significant species differences in response to ligands (see Section 8.3.2 for details). Following activation, the AHR/ARNT heterodimer binds to the xenobiotic response element (XRE) in the promoter and/or enhancer regions of target genes (Fig. 8.1). Unlike classical bHLH proteins, the AHR/ARNT heterodimer binds to an asymmetric recognition site, binding to the core sequence of the XRE—*TNGCGTG* [92,95,96]. It has also been suggested that nucleotides adjacent to, but not part of, the core binding sequence are important for regulation of some genes [95]. Following DNA binding, AHR/ARNT recruits coactivators (Table 8.3) and other proteins, such as Mediator D and TFIID, resulting in chromatin remodeling and recruitment of RNA polymerases and in the initiation of gene expression (for a review of DNA binding, see Ref. 95).

Nuclear translocation is also a key step in the control of AHR-induced transcriptional regulation. AHR protein, which is inherently unstable in the absence of ligand, is stabilized in the cytoplasm by a complex consisting of two molecules of HSP90 and one each of hepatitis B virus X-associated protein 2 (XAP2) and the co-chaperone p23 [97–99]. The E3 ubiquitin ligases, C-terminal of HSP70 interacting protein (CHIP), and Cullin 4B also bind to AHR, which could explain the rapid degradation of free, monomeric AHR within cells [97]. In response to a molecular trigger, AHR dissociates from the core complex and localizes to the nucleus where it heterodimerizes with its binding partner to activate gene expression. At present, the signals regulating nuclear translocation are still being elucidated; however, there appears to be at least two separate mechanisms depending on whether the receptor is activated by ligand or an endogenous signal.

HSP90 has an important role in maintaining AHR stability and structure, with the central region binding to both the bHLH and PAS domains of AHR. HSP90 appears to be essential for maintaining AHR in its ligand-binding conformation, with AHR losing its dioxin-induced activity when HSP90 is disrupted *in vitro* [100–102]. Ligand binding appears to disrupt the binding of HSP90 to the PAS domain, which is adjacent to or just within the LBD, but not to the bHLH region, resulting in a conformational change that retains HSP90 binding during nuclear translocation. To explain this phenomenon, McGuire *et al.* [101] postulate that after ligand binding, HSP90 promotes a conformational change to accommodate the binding by AHR's dimerization partner, ARNT, following translocation to the nucleus, and therefore remains bound to AHR through the bHLH domain during nuclear shuttling. XAP2 is a 38-kDa protein with

significant homology to the immunophilins, such as FKBP52 [99]. Although not absolutely required for the structural integrity of the cytoplasmic core complex, XAP2 does appear to have a significant role in maintaining cytoplasmic localization [103]. In mice, nuclear localization appears to rely on importin β , which recognizes the bipartite NLS found in AHR. In the cytoplasm, XAP2 binding in the core complex masks the NLS, preventing importin binding and subsequent nuclear localization [103]. Ligand binding induces a conformational change in XAP2, resulting in dissociation of XAP2 and HSP90 from AHR. This unmaskes the bipartite NLS, making it available for importin binding and thus nuclear import [98,104]. However, XAP2 does not seem to fulfill the same role in humans, as it appears to shuttle into the nucleus still bound to AHR [105,106]. This mechanism also involves PKC-mediated phosphorylation of two serine residues adjacent to the NLS—Ser12 and Ser36—which inhibits nuclear translocation by masking the NLS from importins [107].

Although ligand-dependent nuclear translocation seems to rely on ligand-induced conformational change of XAP2 and HSP90, evidence for another pathway involving cyclic-nucleotide-dependent signaling has been reported [97,98,108,109]. cAMP has been shown to interact with the AHR core complex and induce nuclear translocation [109]. However, the conformational change induced by this interaction reduced the ability of AHR to dimerize with ARNT, changed the DNA recognition sequence of AHR, and reduced dioxin-induced target expression [98,109].

Following nuclear translocation, it has been reported that phosphorylation of AHR and ARNT is essential for heterodimerization and thus DNA-binding activity [22]. The kinases involved were PKC and members of the ERK/MEK signaling pathway. This once again illustrates the importance of cooperative control with endogenous signaling pathways in xenobiotic metabolism. A novel nuclear binding partner has also been identified, which acts through the cAMP-related signaling pathway: the RelB subunit of NF- κ B [110–112]. This heterodimer behaves in a PKA phosphorylation-dependent manner, with AHR DNA binding identified in the promoter region of interleukin-8. Although this alternative binding partner has a role in coregulation of inflammatory genes, acting in concert with the NF- κ B pathway, any possible role in P450 regulation has yet to be investigated.

In summary, AHR is a promiscuous receptor that has a key role in metabolic response to environmental and dietary toxins. The mechanisms regulating AHR-induced P450 regulation are complex, requiring cross talk between many signaling and physiological processes. It is worthy to note that the majority of AHR research to date has been conducted *in vitro*, especially using murine cell lines. This has a number of limitations, including, in particular, AHR function is linked with a number of physiological processes, therefore important interactions will be missed and there are significant species differences in the structure and function of AHR. The application of AHR-humanized mice could be of use to further establish the *in vivo* regulation of this signaling pathway [113].

8.2.4 Receptor Cross Talk

In order to maintain a robust response to chemical challenge, multiple transcription factors have evolved to modulate P450 expression. This has created a complex interacting network of gene regulation [114]. The cross talk between the transcription factors, both in the ligands with which they interact and the downstream genes that they regulate,

allows the systems to be fine-tuned for the detoxification of specific compounds and the interactions between endogenous and exogenous pathways to be controlled. It is, therefore, imperative to understand the degree to which this phenomenon controls both *in vitro* and *in vivo* drug-metabolizing enzymes. This section discusses the cross talk between the three main transcription factors and the regulation of certain key P450s involved in drug metabolism.

8.2.4.1 CYP1A1/2. The CYP1A enzymes are associated with the metabolism of environmental toxins and aromatic hydrocarbons, providing protection against acute dioxin-induced hepatocellular necrosis and hepatic inflammation [4,5,115,116]. They are primarily under the control of AHR, with *Cyp1a1* and *Cyp1a2* possessing conserved XRE clusters in their proximal promoters [116]. However, other response elements have been located in the promoters of these genes, suggesting regulatory roles for other transcription factors. Also, PXR ligands can induce AHR target genes through the transcriptional regulation of AHR [4]. Yoshinari *et al.* [5] recently provided evidence that binding of the hCAR/RXR α heterodimer to a conserved ER8 motif within the proximal promoter of the CYP1A1 and CYP1A2 genes increases their expression. This finding suggests that drugs that activate CAR could alter the metabolism of CYP1A1 and CYP1A2 substrates and therefore be a source of drug–drug interactions. Glucocorticoid response elements (GREs) have also been identified in the promoter region of the rat *CYP1A1* gene, which, once activated, interact with the initiation complex to enhance AHR-induced transcription [54]. However, regulation by this mechanism appears to be species specific and does not occur in humans.

Inflammation has been reported to negatively regulate CYP1A1 expression, with exposure to UV-B repressing the induction of CYP1A1 immediately following irradiation, although CYP1A1 is induced in an AHR-dependent manner several hours following irradiation [115,117]. This was attributed to direct interaction between AHR and the NF κ B RelA (p65) subunit, interfering with cofactor recruitment to the promoter. The repression of *Cyp1a1* expression in response to oxidative stress had been reported by Morel and Barouki, who identified nuclear factor 1 (NF1) as the mediator of this effect [118]. Reciprocal cross talk between AHR and Nrf2 has also been recently reported, with XRE sequences being located in the Nrf2 promoter [12], and ARE sequences in the AHR promoter [119]. It has been suggested that a functional protein interaction may also exist between these molecules, although no data is currently available [120]. The reciprocal cross talk between these two systems further highlights the key role of AHR in the protection of the body from damage caused by electrophilic metabolites.

Regulation of CYP1A expression is therefore controlled by a number of core interacting transcription factors. However, because of the many endogenous roles played by the AHR, they are also subject to regulatory control by diverse signaling pathways, including those involved with inflammation and oxidative stress response, playing a key role in protecting the body from xenobiotic challenge.

8.2.4.2 CYP2B6. CYP2B6 metabolizes a wide range of drugs, including cyclophosphamide, valproic acid, ketamine, aminopyrine, methadone, and bupropion [59]. Its overall role in drug metabolism in man, however, has been considered minor [59,121]. CYP2B6 is induced by activators of hCAR, with the activated hCAR/RXR α heterodimer binding to the DR4 motif in the phenobarbital-responsive-element module

(PBREM) in the promoter [54,59]. However, cross talk with human pregnane X receptor (hPXR) has also been implicated in CYP2B6 regulation, through binding to the DR4 motif, all be it with lower affinity. A response element termed the *distal xenobiotic responsive enhancer module* (XREM) has also been identified as activated by either hCAR/RXR α or hPXR/RXR α [122]. This element works synergistically with the activated PBREM [59,122].

Although cross talk with PXR is reported to be part of the mechanism in the control of CYP2B6, other receptors are also implicated. For instance, in rodents, the glucocorticoid receptor (GR) directly activates CYP2B/Cyp2b expression [123,124]. Although the GR does not directly activate CYP2B6 in humans, it can affect CYP2B6 expression indirectly through induction of CAR and PXR [59]. Recent studies from the Negishi group have suggested that for maximal activity, the CYP2B6 promoter can be synergistically activated by the PBREM and a novel 52-bp response element, known as the *okadaic acid response element* (OARE_{KI}) (Fig. 8.2) [125]. The OARE_{KI} contains a DR1 motif, which possesses an HNF4 α binding site, a CACCC motif, which binds early growth response 1 (EGR1) protein, and an E box motif. EGR1 binding is required for maximal CAR-induced CYP2B6 activity, facilitating CAR/HNF4 α cross talk by inducing DNA looping to bring the proximal OARE_{KI} into proximity with the distal PBREM [126]. A recent study has demonstrated that the CCAAT/enhancer binding protein α (C/EBP α) and HNF4 α cooperate with CAR to control CYP2B6 expression [121]. HNF4 α binding to a proximal element located in the OARE_{KI} (–217 bp) favors

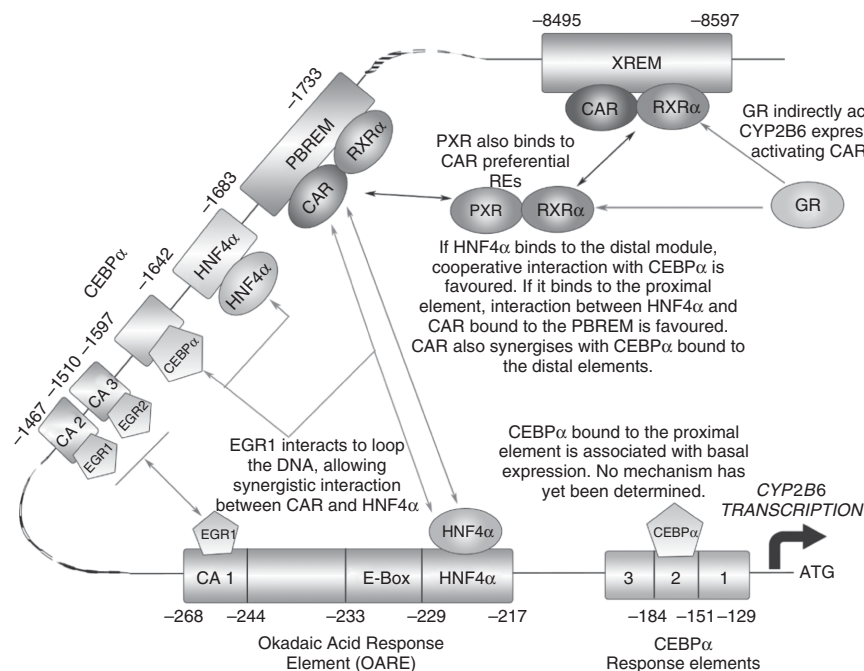


Figure 8.2 Regulatory network controlling CYP2B6 expression. *Source:* Adapted from Benet *et al.* [121], with additional information from Inoue and Negishi [126] and Wang and Tompkins [59]. (See color insert.)

CAR interaction, whereas binding to the distal region (–1642 bp) favors interaction with C/EBP α (Fig. 8.2). Variation in expression and binding of HNF4 α and C/EBP α to the CYP2B6 promoter has been correlated to interindividual variations in CYP2B6 expression and activity, suggesting their importance in the regulation of this gene [121]. It is clear that CAR-induced transcriptional regulation of CYP2B6 involves intricate cross talk between CAR, HNF4 α , EGR1, and C/EBP α as a minimum. However, the role of PXR in this mechanism remains to be clarified.

8.2.4.3 CYP2C8. CYP2C8 is the most inducible gene in the CYP2C family, with a role in the metabolism of drugs such as rosiglitazone, paclitaxel, cerivastatin, and chloroquine, in addition to endogenous compounds, such as arachidonic acid [9]. The regulation of this protein remains poorly characterized, although DR4 binding at –8.8 kb is needed for CAR and PXR-mediated CYP2C8 induction [9]. Both CAR and PXR can bind to this element in response to ligands. However, Ferguson *et al.* [9] found that this response is not apparent when the reporter gene assay is performed in the HepG2 cell line but is seen when using metabolically competent primary human hepatocytes, demonstrating the limitations of HepG2 cells for drug induction studies. As with CYP2B6, basal expression of CYP2C8 involves HNF4 α binding to a DR1 motif in the basal promoter region. A second functional HNF4 α binding site has also been identified in the proximal promoter region [127]. Site-directed mutagenesis of the CYP2C8 promoter has shown that both HNF4 α sites are important for RIF-induced PXR-mediated expression of CYP2C8, with that in the proximal promoter region being essential. Also, silencing of HNF4 α using RNA interference (RNAi) decreased basal CYP2C8 reporter activity by 48% and abolished RIF-mediated induction. However, this treatment also repressed expression of CAR and PXR by 60% and 40%, respectively [127]. In light of the current limited information, further studies on the role of CAR in CYP2C8 control are required.

In addition to the above regulatory pathways, the GR also appears to be involved in the induction of members of the CYP2C gene family, with a functional GRE being located in the proximal promoter region in many CYP2C genes [9,54]. Ferguson *et al.* [9] demonstrated that CYP2C8 induction by the prototypical GR activator, dexamethasone, occurs by direct interaction with the GRE located in the promoter region of the gene, and not by interaction with PXR or CAR. A recent study in HepG2 cells identified retinoic-acid-receptor-related orphan receptor (ROR) response elements (ROREs) in the promoter of CYP2C8, which are activated by ROR α 1, ROR α 4, and ROR γ 1 isoforms [128]. The importance of RORs in the control of CYP2C8 was further supported by the finding that knockdown of the three isoforms resulted in a 50% decrease in CYP2C8 mRNA. Since natural agonists of these receptors include cholesterol and its metabolites, CYP2C8 expression is likely to be influenced by normal physiological processes. These receptors are also linked to the control of circadian rhythm and therefore could be associated with circadian expression of the CYP2C genes. In summary, the regulatory control of CYP2C8 is still poorly understood, although certain key factors have been identified. More research is therefore required to integrate these signals into an understandable network and to more fully characterize regulation by CAR and PXR.

8.2.4.4 CYP2C9. CYP2C9 is the most highly expressed of the CYP2C family [129], with identified substrates including tolbutamide, diclofenac, and *S*-warfarin [130]. In

human hepatocytes, CYP2C9 could be regulated with prototypical PXR, CAR, and GR activators (RIF, PB, and dexamethasone, respectively), indicating that all three receptors have a role in the regulation of this enzyme [131]. Subsequent studies suggested that CAR plays a role in the basal expression of CYP2C9, with constitutive expression in HepG2 cells increasing with stable transfection of mouse or hCAR [131,132]. Two DR5 response elements, in a configuration similar to that of the PBREM and XREM modules, are located in the proximal promoter at -2899 bp and preferentially bind CAR over PXR [132]. A DR4 CAR/PXR binding motif at -1839 bp has also been identified [131]. Mutagenesis of these binding sites provided evidence that both are sensitive to CAR activation [132]. The induction of CYP2C9 by treatment with RIF, hyperforin, and PB has been shown to be mediated by hPXR binding to the DR4 response element [10,133]. The proximal DR4 binding site is therefore essential for activation, with the distal site acting in a cooperative capacity.

HNF4 α plays an essential central role in the regulation of CYP2C9, with HNF4 α binding sites located at -211, -185, and -152 bp. The site at -185 bp plays a central role in HNF4 α -induced CYP2C9 expression and CAR/PXR synergism [127,134]. The HNF4 α binding sites cooperate with the CAR/PXR responsive DR4 motif for maximal gene expression [127]. The underlying mechanism appears to be as a result of recruitment of a number of coactivators to the promoter, including cAMP response element binding protein (CBP), PGC-1 α , PRIP-interacting protein with interacting methyltransferase (PIMT) domain, and nuclear receptor coactivator 6 (NCOA6) [135]. Current evidence suggests that NCOA6 forms a bridge between CAR and HNF4 α bound to their respective response elements by direct interaction with the LXXLL motifs of each. NCOA6 therefore acts as a mediator promoting synergistic cross talk between CAR and HNF4 α . It is not yet clear whether the same mechanism applies to hPXR-mediated control.

Other pathways that do not appear to directly interact with CAR/PXR-induced CYP2C9 expression have also been detailed. An imperfect GRE sequence at -1675 bp allows direct activation of the promoter by the GR, which appears to act synergistically with CAR/PXR [132]. HNF3 γ , also known as FOXA3, has been implicated in the regulation of CYP2C genes, being found to bind to HNF3 γ response elements in the promoter region, although a mechanism has yet to be elucidated [136]. A study by Drocourt *et al.* [137] suggested that the VDR can also regulate CYP2C9 expression. However, they point out that the concentrations of 1 α ,25-dihydroxyvitamin D₃ required for this interaction are above the levels seen physiologically, and therefore, the relevance of this interaction is questionable. A recent study has also shown the transcription factors GATA-2, -4, and -6 induce CYP2C9 through direct interaction with response elements in the promoter [138]. Again, any potential cross talk with other motifs has yet to be investigated, but the tissue-specific nature of GATA transcription factors could make this an interesting pathway when considering gene expression in extrahepatic tissues.

The regulation of CYP2C9 appears unusual in comparison to the other commonly inducible P450s. Although, HNF4 α is again central to promoter activation, regulation by CAR and PXR is such that constitutive expression appears to be mediated through a CAR-mediated pathway, whereas ligand induction occurs through a PXR-mediated pathway, showing a more physiologically cooperative cross talk between these receptors than is often seen. Cross talk mediated through cofactors is also apparent in the mechanism underlying cooperation of CAR and HNF4 α through mediation by NCOA6.

8.2.4.5 CYP3A4. CYP3A4 is the major P450 expressed in human liver and is responsible for the metabolism of approximately 60% of current drugs. The variation in CYP3A4 expression due to enzyme induction is therefore a major determinant of drug–drug interactions [39]. The key regulator of CYP3A4 expression is PXR, with the hPXR/RXR α heterodimer binding to an ER6 PXRE motif located at –172 bp relative to the transcription start site in the proximal promoter region [139–141]. Mutation of this element led to a 50% reduction in RIF-mediated response, indicating that this motif acts cooperatively with other regulatory elements [140]. A distal XREM enhancer has been identified at approximately –7800 bp, consisting of three nuclear-receptor-binding sites, dNR1-3. Disruption of the PXRE in the proximal promoter and the dNR1 imperfect DR3 binding motif in the distal XREM represses xenobiotic response by approximately 85%, indicating that both elements are essential for CYP3A4 expression. The dNR3 site is also required for maximal gene expression [140]. A further polymorphic enhancer module, known as the *constitutive liver enhancer module of CYP3A4* (CLEM4), has been identified at –11.4 kbp [142]. This is also required for maximal gene expression. This module contains multiple cis-acting elements, including binding sites for HNF-1, USF-1, AP-1, and HNF4 α , as well as a perfect ER6 PXR binding motif. This enhancer region acts synergistically with the distal XREM and proximal promoter in PXR-mediated *CYP3A4* induction [143]. This region also appears to be involved in constitutive CYP3A4 expression [142]. However, the motif located in this region has been found to be significantly less competitive for PXR/RXR α binding relative to motifs found in the distal XREM and proximal promoter, instead the motif has a higher affinity for a PXR homodimer *in vitro* [143]. This homodimer has yet to be described *in vivo*, but potentially, this could result in a previously uncharted regulatory mechanism associated with CYP3A4 control.

All PXR binding motifs described above have also been shown to bind hCAR, although binding to the proximal promoter region alone is insufficient for CAR-induced expression. Therefore, evidence suggests that CYP3A4 is coordinately regulated by hCAR and hPXR [144,145]. Although PXR/CAR cooperation appears to be the main regulatory pathway connected to ligand-induced control of CYP3A4, neither are essential for constitutive expression, with individual disruption of these genes *in vivo* having no influence on basal CYP3A4 expression [142]. Istrate *et al.* [146] have used gel shift and LS174T-based reporter gene assays to show that a number of transcription factors can bind the ER6 and DR3 motifs, thus decreasing PXR-mediated gene induction through competition. For instance, the thyroid receptor (TR) α 1 can bind to the response elements to repress CYP3A4 basal and ligand-induced expression. The ratio of PXR:TR α 1 was moreover found to be correlated to basal expression level, suggesting a role in endogenous control. The VDR has also been found to compete with PXR for ER6, DR3, and DR4 binding sites to induce CYP3A4 expression in rat and human liver and intestinal slices, all with lower activity than PXR [137,147]. However, a recent study has shown that VDR and PXR can act synergistically in intestinal cell lines, apparently through VDR binding to the PXRE located in CLEM4 [148].

HNF4 α also has a significant role in controlling CYP3A4 expression. Tirona *et al.* have identified a DR1, HNF4 α binding site immediately upstream of the dNR1 and dNR2 binding sites in the XREM (–7783 bp). This was shown to be essential for ligand-induced promoter activity. *In vitro* reporter transactivation assays in HepG2, Caco-2 and HeLa cell lines indicated that basal and induced promoter activities are mediated through PXR/CAR, with the activity increasing synergistically in the presence of

HNF4 α [149]. In contrast, the PXRE located in CLEM4 is flanked by two downstream HNF4 α binding motifs [143], which suppress rather than facilitate PXR/CAR-induced gene expression as a consequence of HNF4 α interference with PXR DNA binding. Liu *et al.* also reported that while disruption of the ER6 motif in the CLEM4 domain has no effect on basal expression, disruption of the DR3 motif in the XREM results in a decrease in basal activity. Other regulators of CYP3A4 expression have also been suggested. These include STAT1, HNF1, HNF3 β (FOXA2), HNF3 γ (FOXA3), and C/EBP α [150]. A systematic *in vitro* analysis in HepG2 and HuH7 cell lines has been performed by Bombail *et al.*, in which putative binding sites were disrupted to identify functional interactions [151]. This study identified C/EBP α as a regulator of CYP3A4 expression, with mutation of the C/EBP α binding site at -132 bp causing a 60% decrease in basal expression and a decrease in $I_{\max}$ associated with RIF-, PB-, and metyrapone-mediated activation. In a previous work, two distal C/EBP α sites (located at -1402 and -1668 bp) were identified in the activation of CYP3A4 expression. HNF3 γ is also a putative regulator, with a binding site located at -1730 bp. However, this receptor does not activate CYP3A4, but rather synergizes with C/EBP α , promoting gene expression by chromatin remodeling [151,152]. In addition to these findings, Bombail *et al.* reported that disruption of a putative Sp1 site at -104 to -97 bp resulted in a 50% decrease in promoter activity following PB treatment. Despite the presence of a putative GRE in the proximal promoter region, it is likely that GR agonists influence CYP3A4 expression by inducing PXR expression rather than interacting directly with the promoter [147,153]. CYP3A4 expression is also influenced by various physiological processes, as demonstrated by its regulation by FOXA2 and FOXA3, which have numerous endogenous roles, including embryonic development, organogenesis, and glucose homeostasis [154]. Other endogenous modulators include the HIF-1 α transcription factor, which is upregulated in response to oxidative stress, and interacts to downregulate drug-metabolizing enzymes, including CYP3A4, in order to prevent the production of reactive oxygen species [155]. This seems to act through an indirect mechanism, such as downregulation of PXR and CAR, rather than through direct promoter binding. It is also important to remember that these transcription factors are subject to control by endogenous signaling molecules. This is highlighted by a recent report linking CYP3A4 expression to the cell cycle via phosphorylation of PXR by cyclin-dependent kinase 2 [156].

As this section has shown, although each of the enzymes discussed is preferentially controlled by a given transcription factor or nuclear receptor, a large variety of signaling pathways and coregulating molecules interact to control gene expression. The redundancy inherent in this system could be explained by the need to adapt to a wide range of environmental demands and endogenous stimuli. Our understanding of the interactions underlying transcriptional control is rapidly increasing. However, the networks we have currently identified are simplistic, omitting key endogenous signaling information that can have dramatic consequences for drug metabolism. While this section has aimed to give a broad overview of interaction at the level of P450 promoter regulation, it has of necessity excluded another level of functional cross talk, which exists between different nuclear receptors and transcription factors. Several reviews are now available, which discuss functional cross talk between nuclear receptors and certain transcription factors [15,16,114]. This interaction can significantly affect downstream regulation of target genes and thus the overall P450 expression profile.

8.3 OTHER CONSIDERATIONS FOR TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.3.1 Methodological Challenges

Understanding P450 regulation is a vital step toward understanding drug interactions. Although the mechanisms underlying P450 transcriptional regulation are becoming clearer, methodological difficulties have introduced other issues that must be considered when analyzing current evidence.

Although *in vitro* methods, such as reporter gene assays, have been used to identify P450-inducing agents and mechanisms of transcriptional control, cell-based systems are unable to fully model transcriptional activation. Traditional overexpression systems have limitations because PXR and CAR will spontaneously translocate to the nucleus, probably because the members of the cytoplasmic retention complex become saturated [28,157]. This removes one of the key mechanisms underlying transcriptional regulation. A further problem is that with the exception of primary hepatocytes and a handful of immortalized cell lines, such as the HepaRG model [158–160], the majority of cell lines do not express physiologically relevant levels of both the P450-related transcription factors or P450s themselves. Cells also have the disadvantage that they cannot model systemic interactions, a significant problem when transcriptional regulators are influenced by systemic cues, such as hormonal signaling and energy state.

The use of conventional animal models to study ligand induction of P450s is therefore common, being able to model induction in a more physiologically relevant manner. However, because of the divergence of the metabolic system between species, models do not accurately predict P450 induction in humans [161]. The consequences of this can be profound. For instance, from a pharmaceutical perspective, failing to consider potential differences in drug metabolism could result in drugs reaching clinical trials that are therapeutically ineffective or, much more importantly, toxic to humans [162]. Species specificity is therefore a major issue to be addressed when considering results from animal models.

8.3.2 Species Specificity

The mechanisms underlying species specificity in drug induction are complex. However, species differences in P450 induction can often be ascribed to differences in the amino acid sequence of the LBD of the transcription factor involved [7,163–168]. Changes in the activity of NLS sequences have also been associated with alterations in nuclear localization, resulting in aberrant gene transcription [92,105,106,169,170]. Lichti-Kaiser *et al.* [37] have also identified species specificity in the response of hPXR and mPXR (mouse pregnane X receptor) to the cAMP nucleotide signaling pathway. CYP3A4 expression was found to be synergistically increased on treatment with RIF and the PKA inhibitor H89, whereas it decreased following treatment with H89 and pregnenolone-16 α -carbonitrile (PCN). However, the study compared data from HepG2 cells transduced with an adenoviral expression vector containing hPXR with that from primary mouse hepatocytes, which may influence the findings. In the case of AHR, species-specific differences in receptor protein stability (hAHR < mAHR) and histone deacetylase complex recruitment have been implicated in differential response [170]. Improved understanding of species differences in nucleocytoplasmic shuttling,

TABLE 8.4 Examples of Species-Specific Ligands for PXR and CAR

Drug	Interacts with	Species			
		Mouse EC ₅₀ (nM)	Action	Human EC ₅₀ (nM)	Action
PCN	PXR	200–700	+	>10,000	N/A
Rifampicin		Weak		200–3000	+
SR12813		4100	+	120–200	+
Hyperforin		NR		23	+
5 β -Cholestan-3 α ,7 α , 12 α -triol		2500	+	5000	+
TCPOBOP	CAR	20–100	+	Weak	N/A
CITCO		Weak		25–304	+
5 α -Androstan-3 α -ol		250–1500	–	1000–>10,000	–
Meclizine		25	+	500–1000	–
5 β -Pregnane-3,20-dione		670–3000	+	>10,000	Weak+
				$\gg$ 10,000	–
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	AHR	0.6	+	0.4–9	+

Includes EC₅₀ (nM) where data is available.

NR, not reported; +, agonist; –, antagonist (inverse agonist, CAR only).

PXR and CAR data adapted from Refs 15 and 16. AHR data adapted from [177] (mouse), [178] (human), and [179].

cell signaling pathways and subsequent activation mechanisms is therefore of central importance. The availability of mouse models humanized for both the receptors which mediate drug induction as well as the P450's involved can help circumvent many of these problems.

Although the precise mechanisms have yet to be fully elucidated, numerous P450 inducers have been identified which interact with transcription factors in a species-dependent manner. A selection of these ligands has been detailed in Table 8.4. These include numerous drugs, endogenous compounds, environmental contaminants and certain herbal medicines. Most of this data has been generated using *in vitro* reporter gene assays, which are a useful model for identifying species-dependent ligand effects on transactivation activity of transcription factors. However, there are certain ligands that have proved of particular importance in the study of receptor activation. The macrolide antibiotic RIF and PCN have been shown to be species-specific activators of PXR, with RIF activating hPXR and PCN activating mPXR [15,171–173]. In the same way, 6-(4-chlorophenyl)imidazo-[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) are species-specific activators of CAR, with CITCO preferentially activating hCAR and TCPOBOP activating mCAR [15,171,174]. All four compounds are high affinity ligands for their preferred receptor, showing significantly lower or no affinity for the other species receptor. They are therefore commonly used as prototypical inducers of CYP3A4 (hPXR), Cyp3a11 (mPXR), CYP2B6 (hCAR), and cyp2b10 (mCAR), respectively, to validate models of P450 transactivation. With regard to AHR, a study by Westerink *et al.* identified differential CYP1A inducers between rat and human cell lines [175], while Boutros *et al.* also suggested that TCDD, although not affecting core AHR genes such as the CYP1 family, does exert species-specific effects, having a binding affinity for hAHR 10-fold less than that of mAHR, which could significantly

affect downstream regulation of target genes [176]. In view of the marked differences that can exist between ligand-induced regulation of drug metabolism, understanding how emerging drugs interact with human receptors before clinical testing is vital.

In order to address this issue, a number of new animal models have been created, which are humanized for the metabolic enzymes and transcription factors involved in drug metabolism [174,180,181]. Several approaches have been used in the construction of such models. Katoh *et al.* [181] created chimeric mice in which the innate hepatocytes of an immunocompromised mouse (uPA^{+/+}/SCID) were replaced with >90% human hepatocytes *in vivo*, resulting in an artificially humanized liver. However, the drawback of this model is that because these models must be produced as required, it requires a ready supply of human hepatocytes, which are both costly and hard to source as well as having the innate interindividual variability seen in the human population. The technique may also be restricted under legislation regulating the use of animals in experiments in some countries. A more common approach is to introduce the human gene into the mouse genome, either under the control of its own promoter by random integration or by replacing the endogenous mouse gene [174,180,182]. This has the advantage that the mouse lines can be continuously bred as well as potentially crossed to other mouse lines carrying linked genes of interest. This potentially expands the number of criteria that can be studied in a single model and helps overcome potential ethical and sourcing issues encountered with the chimeric models.

Many humanized models are now available, including a range of humanized P450 models, such as CYP2D6 and CYP3A4, to enable drug metabolism to be characterized. However, the key models from the perspective of P450 transcriptional regulation are those in which the transcription factors are humanized. Some of these models are reviewed by Stanley *et al.* [16] and Gonzalez and Shah [180]. A battery of PXR and CAR models have now been developed to allow PXR- and CAR-mediated P450 induction to be dissected [174,183,184]. These include a model in which both PXR and CAR have been replaced with the human genes under the control of the corresponding endogenous promoters, as well as models in which one receptor is humanized while the other is deleted. Together with more traditional single humanized and knockout models, these models have the potential to allow species specificity and the role of receptor cross talk in P450 induction to be fully analyzed. A further advantage of these models is that their design allows expression of PXR and CAR splice variants at physiologically relevant levels, allowing interindividual variability to be studied [183] (Section 8.8.3). These models therefore provide a powerful tool for use in the analysis of mechanisms underlying species-specific regulation of P450s. Although not perfect, with receptors still under the influence of the mouse physiological environment, the data that can be gained from these humanized models is more relevant to humans than traditional animal models, especially since the majority of species-specific activity is thought to depend on the sequence of the LBD. As these models become more complex, with multiple genes being humanized in one model, data quality is likely to improve.

8.3.3 Splice Variants

Alternative splicing of mRNA is ubiquitous in eukaryotes, and many human genes are subject to the process, with 86% having a minor isoform frequency of 15% or greater, resulting in the production of different mRNAs, thus potentially generating multiple proteins from a single gene and greatly increasing genomic diversity [185]. There are

a number of different mechanisms involved in alternative splicing of mRNA, including exon skipping, intron retention, and the use of alternative splice donor and acceptor sites [186,187].

Alternative splicing of human P450 mRNAs was first described in the 1980s [188]. However, the identification of splice variants of hCAR and hPXR was more recent. Different-sized hPXR mRNAs were first identified in the late 1990s [189,190], with Fukuen *et al.* describing a total of 9 hPXR SVs whose expression levels varied significantly across a panel of 15 liver samples [191]. Further work to define the expression of hPXR SVs found the most common variants to be PXR.2 (lacking 111 nt) and PXR.3 (lacking 123 nt), representing approximately 6.7% and 0.32%, respectively, of hPXR mRNA in human liver and also found in a number of other tissues, including brain, heart, colon, and bone marrow [3]. PXR.2 was subsequently reported not to function in a transactivation assay because of the failure of ligands to bind to the LBD in a productive manner, probably as a result of a change in protein structure and the continued binding of corepressors [192].

Choi *et al.* in describing murine CAR, also reported the existence of a variant mRNA (mCAR2), which lacked exon 8 and was unable to function in a transactivation assay [45]. The first SVs of the hCAR gene were reported in 2003 when Savkur *et al.* described an mRNA for hCAR with a 4-amino-acid insert between exons 6 and 7 and a 5-amino-acid insert between exons 7 and 8 resulting from the use of alternative splice acceptor sites, and another with a complete deletion of exon 7 and loss of 39 amino acids [193]. In addition to these variants, Auerbach *et al.* [194] reported further hCAR SVs with one or other of the insertions between exons 6/7 and 7/8 and confirmed that such variants had compromised function. The range of hCAR SVs was extended dramatically when Lamba *et al.* reported 22 unique mRNAs from a panel of human liver and other tissues [195]. Several of these SVs had premature termination codons and failed to produce protein, but many had significant deletions or insertions, yielding proteins significantly different in size from the wild-type, or reference, form and which had altered N- and/or C-terminal amino acids. The hCAR SVs were differentially expressed, with only the hCAR reference form found in the intestine, whereas spleen, heart, and prostate expressed only the SVs, and were nonfunctional in transactivation assays, although the authors speculated that such SVs might possess biological function(s) yet to be identified [195]. A biological function was demonstrated for the hCAR SV with a 5-amino-acid insertion between exons 7 and 8, termed CAR3 by Auerbach *et al.*, who found activity in a transactivation assay with an optimized 3× DR4 reporter using CITCO, thus defining CAR3 as a ligand-activated receptor in contrast to the reference form (CAR1) [196]. The same group further demonstrated that CAR2, with a 4-amino-acid insertion between exons 6 and 7, displayed a regulatory response distinct from that of CAR1, speculating that the ligand-binding pocket may be modified by the additional residues found in CAR2 [197]. Subsequently, CAR2 was also shown to be a ligand-activated receptor present in approximately 30% of total CAR transcripts in human hepatocytes and uniquely responsive to the plasticizer di(2-ethylhexyl) phthalate (DEHP) [198]. These latter findings—that a nuclear receptor present at significant levels in human liver (but absent in other animal models) is highly responsive to a chemical agent commonly found in a wide range of everyday materials, including medical devices—have potentially significant implications for safety testing in general and predictive human toxicity in particular.

To address these concerns, a unique panel of transgenic mice humanized for CAR and/or PXR have been generated, which are nulled for murine CAR and PXR and express the reference forms and two major SVs of hCAR (CAR2 and CAR3) and hPXR (PXR2 and PXR3) under the control of the endogenous mouse promotor [174]. Expression levels of the CAR and PXR SVs in these mouse models were comparable to that found in a panel of human livers, with the exception of hPXR2 in which the expression was approximately fourfold higher in the transgenic mouse [183]. The expression profile of the PXR SVs in the hPXR mouse found that PXR1 was expressed in a range of tissues at varying levels—liver > small intestine > kidney > lung > gonads > brain—while PXR2 was expressed, as a % of total PXR mRNA, at approximately the same level in all tissues, and PXR3 at highest levels in the kidney, gonads, and brain (Bower, CCM, unpublished observations). These mouse models provide a useful tool to investigate the role of CAR and PXR in drug metabolism, disposition, and efficacy in humans.

8.3.4 Genetic Polymorphisms

Interindividual variation in human drug metabolism is a significant cause of variability in drug response [199]; a main contributor is genetic polymorphism [200]. P450s are a highly polymorphic group of enzymes, with more than 350 different functional alleles so far identified in the genes, accounting for 40–50% of P450-dependent metabolism in humans [201,202]. Most of the characterized polymorphisms are variations in the coding sequence, such as SNPs, deletions, or gene amplifications [203,204]. They can, however, also be in gene promoters or intronic regions, which interfere with transcription factor binding or cause alternative or aberrant mRNA splicing [202,204]. Many of these polymorphisms manifest themselves as enzymes possessing altered activity [201]. One example of this is seen in the *CYP2B6**6 polymorphic isoform, which possesses two SNPs, 516Gly > Thr and 785Ala > Gly [59]. These substitutions result in alternative gene splicing, yielding a product with a deletion of exons 4–6 and that displays a significant reduction in catalytic activity and protein expression relative to the reference *CYP2B6**1. Presence of these polymorphic variants can therefore significantly affect downstream drug metabolism.

Another source of interindividual variability in P450 expression is the polymorphisms in their regulators. One such polymorphic regulator is PXR, with 373 SNPs having been identified, and many of these proving to be functional [205,206]. As an example of the effect of PXR polymorphism on downstream gene expression, a study by Wang *et al.* [205] has examined the effect of two haplotypes of PXR commonly found in the Han Chinese population, H1 (TCAGGGGCCACC) and H2 (CCGAAAAC-TAAT), on P450 expression, using CYP3A4 activity as a marker. They found that those with the haplotype pair H1/H1 had much higher inducible CYP3A4 metabolic activity than those subjects with the H1/H2 and H2/H2 pairings following treatment with St Johns' Wort, as indicated by the significant differences in AUC_{0-t} and $AUC_{0-\infty}$ for the CYP3A4 probe drug nifedipine and its metabolite, dehydronifedipine. This phenomenon has been further confirmed by identification of functional polymorphisms in the FOXA2 transcription factor, which affect *CYP3A4* transcription and are also linked to diseases, such as type II diabetes [207]. Polymorphisms in P450s and the transcription factors, which control their expression, can therefore play a significant role in interindividual variation in xenobiotic metabolism. Understanding the genetic differences in P450 regulation remains an important subject for future study.

8.4 CONCLUSION

The adaptive response system that has been evolved by organisms to protect the cell from the many forms of toxic insult plays a central role in the metabolism and disposition of drugs. The importance of this system is underlined by its robust nature and built-in functional redundancy that allows at least a partial response in the absence or failure of a component part. The species differences observed in the regulation of P450 expression underline the importance of developing relevant models to test for human drug safety and efficacy, and in which factors such as gender, circadian rhythm, diet may also be taken into account. It is thus clear that an understanding of how drug metabolism is regulated, and the multitude of variable factors involved, is crucial to the optimal application of drug treatments and minimization of unwanted side effects and drug–drug interactions.

ABBREVIATIONS

AF-2	Activation Function 2
AHR	Aryl Hydrocarbon Receptor
AP-1	Activator Protein 1
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
bHLH	Basic Helix-Loop-Helix
bp	Base Pair
cAMP	cyclic AMP
CAR	Constitutive Androstane Receptor
CCRP	Cytoplasmic CAR Retention Protein
Cdk2	Cyclin-Dependent Kinase 2
C/EBP α	CCAAT/Enhancer Binding Protein α
ChIP	Chromatin Immunoprecipitation
CHIP	C-terminal of HSP70 interacting protein
CITCO	6-(4-Chlorophenyl)imidazo[2,1- <i>b</i>]thiazole-5-carbaldehyde <i>O</i> -(3,4-dichlorobenzyl)oxime
CLEM4	Constitutive Liver Enhancer Module of CYP3A4
CREB	cAMP Response Element Binding Protein
CYP	Cytochrome P450
DBP	Albumin D-Box Binding Protein
DEHP	Di(2-ethylhexyl) Phthalate
DR	Direct Repeat
EGR1	Early Growth Response 1
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
HIF1 α	Hypoxia Inducible Factor 1 α
HLF	Hepatic Leukemia Factor
HNF1	Hepatic Nuclear Factor 1
HNF3 β	Hepatic Nuclear Factor 3 β
HNF3 γ	Hepatic Nuclear Factor 3 γ
HNF4 α	Hepatic Nuclear Factor 4 α
LBD	Ligand-Binding Domain

LBP	Ligand-Binding Pocket
NCOA6	Nuclear Receptor Coactivator 6
NCoR	Nuclear Receptor Corepressor
NF1	Nuclear Factor 1
NLS	Nuclear Localization Signal
nt	Nucleotide
OARE _{KI}	Okadaic Acid Response Element
P450	Cytochrome P450
PAS	PER-ARNT-Sim
PB	Phenobarbital
PBREM	Phenobarbital-Responsive Enhancer Module
PCN	Pregnenolone-16 α -carbonitrile
PDE4A5	Phosphodiesterase 4A5
PGC1 α	Peroxisome-Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PIMT	PRIP-Interacting Protein with Interacting Methyltransferase Domain
PKA	Protein Kinase A
PP2A	Protein Phosphatase 2A
PRIP	Peroxisome-Proliferator-Activated Receptor (PPAR)-Interacting Protein
PXR	Pregnane X Receptor
PXRE	PXR-Response Element
RIF	Rifampicin
ROR	Retinoic-Acid-Receptor-Related Orphan Receptor
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SMRT	Silencing Mediator of Retinoid and Thyroid Hormone Receptor
SNP	Single Nucleotide Polymorphism
Sp1	Specificity Protein 1
SRC	Steroid Receptor Coactivator
STAT1	Signal Transducers and Activators of Transcription 1
SV	Splice Variant
TAD	Transcription Activation Domain
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TEF	Thyrotroph Embryonic Factor
TR	Thyroid Receptor
USF-1	Upstream Stimulatory Factor 1
VDR	Vitamin D Receptor
XAP2	X-Associated Protein 2
XRE	Xenobiotic Response Element
XREM	Xenobiotic Responsive Enhancer Module

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9 Cytochrome P450 Polymorphisms

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9.1 Summary	1
9.2 Introduction—significance of P450 polymorphisms and pharmacogenetics	2
9.3 Human drug-metabolizing cytochrome P450s	3
9.4 Conclusions and future perspectives	23
References	24

9.1 SUMMARY

The majority of drugs in clinical use undergo oxidative biotransformations catalyzed by cytochrome P450 (CYP) enzymes. Approximately one dozen isoforms of the CYP families 1, 2, and 3 are mainly responsible for the metabolism of drugs and other xenobiotics. The typically large interindividual variability of these enzymes is an important determinant of drug pharmacokinetics and drug response. Both genetic and nongenetic factors contribute to this variability, but the degree to which genetic polymorphism plays a role in determining expression and function is different for each individual P450 gene. This chapter summarizes the enormous body of experimental evidence that has been gathered on the functional, clinical, and toxicological relevance of P450 genetic polymorphism. In the family CYP1, the *CYP1A1*, *CYP1A2*, and *CYP1B1* genes encode enzymes with preference for polycyclic and heterocyclic aromatic compounds. *CYP1A2* is expressed at higher levels in liver, whereas CYPs 1A1 and 1B1 are mostly extrahepatic enzymes. While *CYP1B1* mutations are causally involved in the development of congenital glaucoma, genetic polymorphism in the CYP1 family is of inferior significance for drug metabolism compared to nongenetic factors. In the largest human family CYP2, five subfamilies are of particular importance. Of the CYP2A genes *CYP2A6*, *CYP2A7*, and *CYP2A13*, the *CYP2A6* polymorphism is an established determinant of nicotine metabolism with influence on addictive behavior. *CYP2B6*, the only functional enzyme encoded in the CYP2B subfamily, is highly polymorphic, and slow metabolizer genotypes are frequent among all major ethnic groups. The *CYP2B6* polymorphism is important for HIV therapy with the antiretroviral drug efavirenz. The CYP2C subfamily comprises the four expressed genes *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*. Despite their high homology, these genes differ tremendously with respect to expression, regulation, substrate selectivity, and genetic

polymorphism. Clear genotype–phenotype correlations are established for CYP2C19 and CYP2C9. The historical polymorphism of *S*-mephenytoin hydroxylation, which is caused by loss-of-function alleles more prevalent among Asians than in other ethnic groups, recently gained importance for additional drugs. The polymorphism of CYP2C9 comprises amino acid variants that can have differential effects depending on the substrate and is of high clinical relevance. The strong genotype–phenotype correlation of CYP2D6 is in part due to the lack of gene regulation such as induction and on the existence of numerous single nucleotide polymorphisms (SNPs) and larger structural variations, including gene deletions, duplications, and recombinations. This results primarily in different expression levels leading to the ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM), and poor metabolizer (PM) phenotypes, the frequencies of which depend on ethnic background. The enzyme has a broad substrate selectivity comprising up to 20% of all drugs on the market. In contrast, CYP2E1, an ethanol-inducible P450, has a preference for low molecular weight compounds especially relevant for toxicological processes, which are only marginally influenced by genetic polymorphism. Often cited as the most important P450 subfamily, CYP3A comprises four functional genes, the most abundant and highly drug-inducible *CYP3A4* and the minor forms *CYP3A5*, *CYP3A7*, and *CYP3A43*. For *CYP3A4*, only few polymorphic markers of controversial value exist. *CYP3A5* and *CYP3A7* are polymorphically expressed but are of limited clinical importance because of their lower expression.

9.2 INTRODUCTION—SIGNIFICANCE OF P450 POLYMORPHISMS AND PHARMACOGENETICS

The human genome comprises 57 putatively functional, protein-coding CYP genes and 58 pseudogenes compared to 102 putatively functional genes and 88 pseudogenes in the mouse [1]. The human genes are grouped according to their sequence similarity into 18 families and 44 subfamilies (<http://drnelson.utmem.edu/human.P450.table.html>). The CYP1, 2, and 3 family isozymes have broad and overlapping substrate specificities, which usually provides for a robust elimination of lipophilic xenobiotics, including most drugs in clinical use [2–4]. In contrast, the CYPs of families CYP4 to CYP51 are mainly involved in endogenous metabolic pathways of steroids, fatty acids, prostaglandins, retinoids, and others [5]. The CYP1–CYP3 family isozymes show extremely variable expression and function, typically exceeding 100-fold within a given population sample, which leads to unforeseen drug responses including overreaction, toxicity, or lack of response in a considerable fraction of patients who have been treated with drug substrates of these enzymes. The reasons for this high interindividual and intraindividual variability include environmental influences such as drug–drug interactions, biological factors including sex and circadian rhythm, physiological determinants such as disease and hormonal status, and genetic polymorphisms in CYP genes and their regulators [3,4,6]. Genetic polymorphisms, although present in practically all human genes, affect only some CYP isoforms to a functionally relevant extent, including, in particular, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and the minor CYP3A forms CYP3A5 and CYP3A7. On the contrary, the highly abundant liver enzymes CYP1A2 and CYP3A4 are not functionally polymorphic because the known sequence variants only marginally affect their expression or function. The focus of this chapter is

to provide a summary of the medical relevance of the most important drug-metabolizing human CYPs of families CYP1, CYP2, and CYP3, with special emphasis on functionally relevant polymorphisms and their role in the metabolism of clinically relevant drugs. The numerous *in vitro* and *in vivo* pharmacogenetic studies that have been published on the subject illustrate the complex biology of these polymorphic genes at the level of basic mechanisms (*null* alleles, partially functional alleles, substrate-dependent effects, linkage disequilibrium, etc.) as well as their pharmacological, toxicological, and clinical relevance (PD (pharmacodynamics) vs PK (pharmacokinetics), adverse reactions, prodrugs, etc.). The website of the cypallele nomenclature committee served as the basis for naming variant alleles, and the reader is referred to this site for more detailed coverage of literature regarding individual allele variants ([7]; available at <http://www.cypalleles.ki.se>; consulted April 2010).

9.3 HUMAN DRUG-METABOLIZING CYTOCHROME P450s

9.3.1 Family *CYP1* — Overview

The human *CYP1A* family contains three functional genes in two subfamilies *CYP1A* and *CYP1B*. The highly conserved *CYP1A1* and *CYP1A2* genes, both of which consist of seven exons and six introns, are located on chromosome 15q24.1, whereas *CYP1B1* is located on chromosome 2p22.2 and contains three exons and two introns [1]. Many of the CYP1 substrates are ligands to the aromatic hydrocarbon receptor (AhR) that positively regulates the expression of *CYP1* and a battery of other genes in a coordinated manner at the transcriptional level [8,9]. The *CYP1A1* and *CYP1A2* genes are oriented head to head with a shared bidirectional promoter in between, which contains at least 13 AhR response elements [10]. Despite their common AhR-dependent regulation, the ontogenic patterns differ substantially, in that *CYP1A1* is expressed more during embryogenesis, whereas *CYP1A2* only appears around birth [11]. Pharmacologically most relevant is *CYP1A2*, which is constitutively expressed in liver, where it contributes on average ~10–15% to the total microsomal P450 pool, but it is also expressed in intestine, pancreas, lung, and brain. *CYP1A2* appears to display higher activity in men than in women [12,13]. Constitutive expression of *CYP1A1* in liver is low, but inducible expression triggered by environmental inducers such as cigarette smoke, dioxin, and polycyclic hydrocarbons occurs ubiquitously in all tissues including lung and breast [14]. *CYP1B1* is an extrahepatic enzyme expressed predominantly in various tumors and a useful tumor marker because of its absence in the corresponding normal tissues [15]. Notably, *CYP1B1* is also expressed in various cell types of the human and mouse eye, where it has a yet undefined developmental role [16].

The substrates of the CYP1 enzymes include many polycyclic and other aromatic substances including diverse procarcinogens such as arylarenes, nitroarenes, and arylamines to reactive metabolites such as epoxide intermediates, which are carcinogenic in experimental animals and humans because they can cause DNA damage. Activation of the carcinogen benzo[*a*]pyrene is an important example of this type of metabolic activation [14]. *CYP1A2* shows a preference for aromatic amines and heterocyclic compounds, including the two prototypic substrates theophylline and caffeine. Endogenous substrates are also known, for example, prostaglandins, estrogens, melatonin, and retinoic acid, some of which appear to affect the phenotype of *Ahr*^{-/-} knockout mice

[17]. Only CYP1A2, because of its high expression in liver, metabolizes a significant number of clinically important drugs, including acetaminophen, phenacetin, lidocaine [18], and the CNS drugs olanzapine, clozapine, and duloxetine, among others [19,20]. Also of clinical importance is the fact that CYP1A2 is sensitive to drug interactions because of reversible and/or irreversible inhibition and induction by numerous drugs and several other xenobiotics. The structure of human CYP1A2 in complex with the inhibitor α -naphthoflavone was recently presented at a resolution of 1.95 Å, revealing a compact active site cavity highly adapted for the positioning and oxidation of relatively large, planar substrates [21]. CYP1B1 has catalytic activities overlapping with CYP1A1 and CYP1A2 and also catalyzes activation of diverse procarcinogens, but the catalytic role depends on the substrate and the expression level in each tissue [22].

9.3.1.1 Subfamily *CYP1A*—*CYP1A1* and *CYP1A2*.

9.3.1.1.1 *CYP1A1*. The *CYPAllele* website lists 10 variant alleles and a number of subvariants, as well as additional SNPs, where the haplotype has not yet been determined. Four early identified, more common variants were originally termed m1–m4. The m1 variant (*CYP1A1**2A), which harbors a 3698 T>C transition creating a new MspI site downstream of the polyadenylation site, and the m2 variant (*CYP1A1**2C) with the key mutation Ile462Val near the heme-binding region were associated with greater enzymatic activity in some but not all studies [14,23]. These variants are common among Asians (~35% and 20%, respectively, Table 9.1) and have been proposed as genetic susceptibility factors for lung cancer in the Japanese [24,25], but this finding was not confirmed in other populations, possibly because of the much lower allele frequencies [26]. The m3 variant (*CYP1A1**3 creating an MspI site in the 3' noncoding region) appears to be an African-specific mutation that does not occur in the other large ethnicities. The m4 variant is located near the m2 mutation site and codes for an amino acid change (Thr461Asn) near the heme-binding region. Its frequency is quite low (~3%) in Caucasians, and no association with lung cancer was found [26]. Besides these variants, other coding polymorphisms (in total to 16 amino acid variants) were described but functional data is scarce.

Because many *CYP1A* substrates are procarcinogens occurring in industrial combustion products, cigarette smoke, and charred food, polymorphisms in the human *CYP1A* family genes have been investigated mostly as risk factors for various forms of cancer including breast and lung cancer. The evidence for association of *CYP1A1* polymorphisms with cancer is controversial, as some studies reported increased susceptibility, whereas others have reported no relationship, although most experimental data support positive associations for the more common variants [27–33]. Further investigation is required with larger clinical studies, consideration of environmental and other non-genetic factors, more detailed haplotype analysis, as well as a more rigorous functional assessment of the variants.

9.3.1.1.2 *CYP1A2*. Like other drug-metabolizing enzymes, *CYP1A2* expression in human liver is highly variable between individuals, which is certainly due to several factors including genetic, epigenetic, and environmental factors (e.g., smoking [34]). By measuring the caffeine metabolic ratio as a *CYP1A2* activity marker in a large cohort ($n = 378$) of mono- and dizygotic twins selected to exclude the influence of smoking, oral contraceptives, and gender, Brøsen and colleagues found a strong overall heritability of 0.72 [35]. To date, more than 15 variant alleles and numerous haplotype variants

TABLE 9.1 Genetic Polymorphisms of Human cytochrome P450s with Functional Significance

CYP Allele Designation ^a	Key Mutation(s) ^b ; rs Number	Location; Protein Effect	Allele Frequencies ^c	Functional Effect
<i>CYP1A1</i> *2A	3698T>C; rs4646903	3' untranslated region; MspI site created	0.05–0.07 Ca ~0.30–0.40 As 0.82 In 0.04–0.28 SA	Unclear
<i>CYP1A1</i> *2C	2454A>G; rs1048943	I462V	0.03 Ca ~0.20 As 0.82 In 0.09–0.9 SA	Unclear
<i>CYP1A2</i> *1C	–3860G>A; rs2069514	Promoter	0.26–40 AA, Af 0.01–0.08 Ca 0.21–0.27 As 0.20–0.30 Hs 0.24 Pc	↓ Inducibility (smokers)
<i>CYP1A2</i> *1F	–163C>A; rs762551	Intron 1	0.5–0.8 all ethnicities	↑ Inducibility (smokers, omeprazole)
<i>CYP1B1</i> *6	142C>G; rs10012	R48G	0.5 Af	↑ K_m , ↓ V_{max}
	355G>T; rs1056827	A119S	0.4 Ca	(17β-estradiol, recombinant)
	4326C>G; rs1056836	L432V	0.12 As	
<i>CYP1B1</i> *7	*6 + 4360C>G	A443G	0.07 Af	↑ K_m , ↓ V_{max}
			0.026 Ca	(17β-estradiol, recombinant)
<i>CYP2A6</i> *2	1799T>A; rs56844942	L160H	0.01–0.05 Ca 0.01 AA 0.00 As	No activity
<i>CYP2A6</i> *4A/D	Recombination	CYP2A6 deleted	0.01–0.04 Ca 0.01–0.02 AA 0.05–0.24 As	No activity

(continued overleaf)

TABLE 9.1 (*continued*)

CYP Allele Designation ^a	Key Mutation(s) ^b ; rs Number	Location; Protein Effect	Allele Frequencies ^c	Functional Effect
<i>CYP2A6</i> *7	6558T>C; rs5 031 016	I471T	0.00 Ca 0.00 AA	Decreased activity
<i>CYP2A6</i> *9	−48T>G; rs28 399 433	TATA box	0.06–0.13 As 0.05–0.08 Ca 0.08 AA	Decreased activity
<i>CYP2A6</i> *17	5065G>A; rs28 399 454	V365M	0.16–0.20 As 0.00 Ca 0.10 AA	Decreased activity
<i>CYP2B6</i> *4	18053A>G; rs2 279 343	K262R	0.00 As 0.00 AA, Af 0.04 Ca	↑ Expression and activity
<i>CYP2B6</i> *6	15631G>T; rs3 745 274 18053A>G; rs2 279 343	Q172H K262R	0.07 As 0.33–0.5 AA, Af 0.17 As 0.25 Ca	↓ Expression and activity
<i>CYP2B6</i> *18	21011T>C; rs28 399 499	I328T	0.37 Hs 0.04–0.07 AA, Af 0.00 As, Ca, Hs	↓ Expression and activity
<i>CYP2C8</i> *2	1 1054A>T; rs11 572 103	I269F	0.10–0.21 AA, Af 0.00 As, Ca	↓ Activity
<i>CYP2C8</i> *3	2130G>A; rs11 572 080 3 0411A>G; rs10 509 681	R139K K399R	0.13 Ca 0.00 AA, Af, As	↓ Activity (paclitaxel) ↑ Activity (antidiabetics)
<i>CYP2C9</i> *2	3608 C>T; rs1 799 853	R144C	0.07 AA, Hs 0.00 Af 0.13–0.17 Ca 0.02 Pc	↓ Activity

<i>CYP2C9*3</i>	4 2614A>C; rs1 057 910	I359L	0.00–0.01 Af, AA 0.04 As 0.06 Ca	↓↓ Activity
<i>CYP2C19*2</i>	19154G>A; rs4 244 285	Splicing defect	0.10–0.16 AA, Af 0.22–0.32 As 0.15 Ca	<i>Null</i> allele
<i>CYP2C19*3</i>	17948G>A; rs4 986 893	W212X	0.00–0.01 Af, Ca 0.03–0.07 As, Pc	<i>Null</i> allele
<i>CYP2C19*17</i>	–806C>T; rs12 248 560	Promoter	0.15–0.25 AA, Af, Ca 0.04 As	↑ Expression and activity
<i>CYP2D6*3</i>	2549delA; rs35 742 686	Frameshift	0.00–0.01 all ethnicities	<i>Null</i> allele
<i>CYP2D6*4</i>	1846G>A; rs3 892 097	Splicing defect	0.15–0.25 Ca <0.01 Most others	<i>Null</i> allele
<i>CYP2D6*5</i>	Recombination	Deletion	0.03–0.06 All ethnicities	<i>Null</i> allele
<i>CYP2D6*6</i>	1707delT; rs5 030 655	Frameshift	0.00–0.01 All ethnicities	<i>Null</i> allele
<i>CYP2D6*10</i>	100 C>T; rs1 065 852	P34S	0.02 Ca 0.40–0.50 As	↓ Expression and activity
<i>CYP2D6*17</i>	1023C>T; rs28 371 706 2850C>T; rs16 947	T107I R296C	0.34 Af 0.00 As, Ca	↓ Expression and activity
<i>CYP2D6*41</i>	2988G>A; rs28 371 725	Splicing defect	0.085 Ca < 0.01 All others	↓ Expression and activity
<i>CYP2D6*Nxn</i>	Recombination	Copy number variations	0.01–0.09 Ca up to 0.30 Af, Ar	↑ Expression and activity
<i>CYP3A4*1B</i>	–392A>G; rs2 740 574	Promoter	0.68–0.82 AA, Af 0.00 As 0.03–0.05 Ca, Hs	Probably effect on transcription

(continued overleaf)

TABLE 9.1 (*continued*)

CYP Allele Designation ^a	Key Mutation(s) ^b ; rs Number	Location; Protein Effect	Allele Frequencies ^c	Functional Effect
<i>CYP3A5</i> *3	6986A>G; rs776 746	Splicing defect	0.37 AA 0.12–0.15 Af 0.66–0.75 As, Hs 0.94 Ca	↓ Expression and activity
<i>CYP3A7</i> *1C	Several SNPs	Functional ER6 element created in the promoter region	0.06 AA 0.04 Ca 0.00 As	Increased expression

^a According to the CYPallele nomenclature homepage (<http://www.cypalleles.ki.se>).

^b Genomic positions are given.

^c Frequencies of the indicated variant alleles were obtained for different ethnicities (AA, African-American; Af African; As, Asian; Ar, Arab; Ca, Caucasian; Hs, Hispanic; In, Indian; Pc, Pacific; SA, South American) from dbSNP at <http://www.ncbi.nlm.nih.gov/SNP>, from the Allele Frequency Database (ALFRED) at <http://alfred.med.yale.edu/alfred/index.asp>, and from the references indicated in the text.

of the *CYP1A2* gene have been defined, and some of them have been associated with altered drug clearance and response and with disease susceptibility. However, it appears that the basis for inheritable CYP1A2-dependent phenotypes has not been satisfactorily elucidated. In a recent study, the human *CYP1A1-CYP1A2* locus was resequenced in 5 major ethnicities phenotyped, revealing a total of 85 SNPs, 57 of which occurred more than once [36]. Attempts to relate the most common of these SNPs to phenotype in genotyped volunteers were disappointing, and it was concluded that no SNP or haplotype in the *CYP1A2* gene has a clear predictive value [37]. Among the polymorphic sites in the coding and noncoding regions in the *CYP1A2* gene, only few common SNPs are currently considered to be of potential predictive value, including the 5'-upstream variant -3860G>A (*CYP1A2*1C*) and the intron 1 polymorphism -163C>A (*CYP1A2*1F*) located downstream of the untranslated first exon. Functional evidence for the *CYP1A2*1C* variant suggests decreased inducibility by smoking on the basis of promoter analyses and its association with decreased caffeine 3-demethylation in Japanese smokers [38]. In contrast to the **1C* variant, the intron 1 variant, *CYP1A2*1F*, was associated with increased enzyme inducibility in German [39] and Swedish [34] smokers and with "ultrarapid" caffeine metabolism and nonresponse to clozapine in smoking patients with schizophrenia [40]. The opposite effect of the two variants is consistent with the findings that carriers of the combined genotype *CYP1A2*1C/*1F* were not induced by omeprazole [41]. The *CYP1A2* genotype also modifies the risk of hypertension in coffee drinkers, who were at increased risk when they carried the slow **1F* allele [42]. Although slower metabolism of caffeine was confirmed in Chinese breast cancer patients, the variant was not related to disease risk [43]. However, some data with different substrates are contradictory, as no influence was found for clozapine [44], haloperidol [45], and trazodone [46]. These discrepancies may be due to ethnically diverse haplotype patterns.

CYP1A2 polymorphisms that lead to amino acid changes are generally rare, but for some of them, decreased activity could be shown in recombinant expression systems. The Arg431Trp substitution (*CYP1A2*6*), which is located in the "meander" peptide, a region critical for maintenance of protein tertiary structure, was shown to interfere with binding and to result in nonfunctional protein [47]. However, because of their rare occurrence, this and other amino acid variants [7] are of limited clinical use. In Chinese subjects phenotyped with caffeine, a common haplotype containing the novel SNP -3113G>A was associated with significantly lower metabolic activity. This SNP is located in a proposed regulatory element, but it remained unclear whether it is causally responsible for the effect [48].

In conclusion, the currently known polymorphisms in the *CYP1A2* gene explain only a small fraction of the *CYP1A2* expression and functional variability. Although novel SNPs may still be discovered, genetic determinants in other genes that contribute to the regulation of *CYP1A2* expression, including the AhR pathway, inflammation signaling pathway, or other signaling pathways, may be involved. Although AhR polymorphisms were reported [49,50], systematic investigations and an evaluation of their effects on *CYP1A2* phenotypes are scarce.

9.3.1.2 Subfamily *CYP1B*—*CYP1B1*. *CYP1B1* is the first CYP that was shown to be involved in a primary developmental defect if mutated. It was identified by genetic linkage analysis and mutation studies as a causative gene in primary congenital glaucoma, an inheritable neurodegenerative disease leading to blindness [51].

Because CYP1B1 may be involved in the metabolism of steroids, arachidonic acid, vitamin A, and melatonin, the enzyme is believed to be responsible for the generation of an as yet undefined signaling molecule(s) that is somehow involved in the development and pathogenesis of glaucoma. Over 80 mutations have been identified in patients with various forms of glaucoma [51]. Most of them are missense or nonsense mutations, or deletions, insertions, and/or duplications. Some of them are predicted to interrupt the open reading frame, and some were shown to lead to severely compromised enzyme function [51–54]. Because these mutations are rare in the general population, their value as tumor markers or markers of altered metabolism of drugs may be limited.

At least six more common polymorphic alleles, which contain five amino acid changes in different combinations, also exist in the *CYP1B1* gene. Functional analysis of four variant proteins coexpressed with P450 reductase in *Escherichia coli* revealed little influence of the Arg48Gly, Ala119Ser, and Asn453Ser variants on kinetic properties toward 17 β -estradiol as substrate, but a threefold increase in K_m for the Leu432Val (*CYP1B1**3) variant [55]. An expression study in yeast found contradictory data regarding the Leu432Val variant, in that no change was seen for the individual variant using the same substrate but decreased V_{max} and increased K_m values toward 17 β -estradiol when additional amino acid changes were present in the CYP1B1.6 and CYP1B1.7 alleles [56]. The CYP1B1.7 variant was also shown to have decreased capacity to hydroxylate benzo[*a*]pyrene. The Leu432Val variation was also correlated to changed urinary estrogen metabolites, indicating *in vivo* contribution of CYP1B1 to catabolism of estrogen [57]. These functional *in vitro* studies suggest that these common amino acid changes in the *CYP2B6* gene lead to little or moderate but potentially substrate-dependent changes in the catalytic properties of the enzyme.

Association of *CYP1B1* genotype with various forms of cancer was reported including a possible effect on breast cancer risk in Caucasians but not in Asians [58]. Regarding three further *CYP1B1* polymorphisms resulting in amino acid changes Arg48Gly, Ala119Ser, and Asn453Ser, a recent meta-analysis concluded that these polymorphisms are not associated with breast cancer risk [59]. In a large study that included more than 10,000 patients, *CYP1B1* variants *3 [Leu432Val] and *4 [Asn453Ser], which influence the metabolism of polycyclic aromatic hydrocarbons of tobacco smoke, were also not associated with the risk of various diseases including chronic obstructive pulmonary disease and tobacco-related lung cancer [60].

9.3.2 Family CYP2—Overview

The *CYP2* family is the largest family in humans. It contains 16 full-length genes and all have 9 exons and 8 introns. Among them are most of the important drug-metabolizing CYPs expressed in liver as well as several extrahepatic enzymes, for some of which the function has yet to be elucidated (“orphan” CYPs [61]). The genes are organized in a number of multigene clusters that can contain one or more subfamilies and are spread over different chromosomes. The three largest gene clusters are the *CYP2ABFGST* cluster on chromosome 19q13.2, the *CYP2C* cluster on chromosome 10q23.33, and the *CYP2D* cluster on chromosome 22q13.1–2. In the evolution of rodents, many of the *CYP2* subfamilies expanded tremendously, making direct comparisons between mouse and human P450s especially challenging in this family [1].

Almost all *CYP2* genes are highly polymorphic. This chapter describes those genes that are of highest importance for xenobiotic metabolism.

9.3.2.1 Subfamily *CYP2A*—*CYP2A6*, *CYP2A7*, and *CYP2A13*.

9.3.2.1.1 Molecular Genetics and Pharmacological Properties. The human *CYP2A* genes are part of a 350-kb *CYP2ABFGST* gene cluster on chromosome 19q13.2 that contains genes and pseudogenes of the *CYP2A*, *2B*, *2F*, *2G*, *2S*, and *2T* subfamilies [1]. It contains the four *CYP2A* members *CYP2A6*, *CYP2A7*, *CYP2A13*, and a split pseudogene *CYP2A18P*. In humans, *CYP2A6* is mainly expressed in the liver where it represents on average about 2% of the total liver P450 protein, whereas in extrahepatic tissues, it was found only in trace amounts. *CYP2A7* is a nonfunctional enzyme that is unable to incorporate and exhibit catalytic activity in various heterologous systems [62]. *CYP2A13* codes for a catalytically active protein and is predominantly expressed in the respiratory tract, where expression levels decrease from nasal mucosa to peripheral lung tissues [63,64].

Human *CYP2A6* has been recognized as the major isoform involved in the oxidative metabolism of the psychoactive tobacco ingredient nicotine to the inactive cotinine [65,66]. A rather specific marker activity for *CYP2A6* is the 7-hydroxylation of coumarin [67,68]. Up to 300-fold interindividual differences in *CYP2A6*-mediated coumarin 7-hydroxylase activity were observed, which is influenced by ethnicity, as only 1% of white subjects are PMs compared to up to 20% of Asians [69,70]. *CYP2A6* is also the main isoenzyme responsible for the minor 7-hydroxylation of efavirenz [71]. Furthermore, *CYP2A6* contributes to the metabolism of a number of clinically used drugs such as disulfiram, fadrozole, halothane, letrozole, losigamone, methoxyflurane, and valproic acid [72]. *CYP2A13* has similar substrate specificity and also metabolizes coumarin and nicotine, but it has been shown to be the most efficient metabolic activator of nicotine-derived nitrosamine ketone (NNK), a major tobacco procarcinogen [73].

9.3.2.1.2 Genetic Polymorphisms and Pharmacogenetics. Genetic variation in the *CYP2A6* gene can both increase or decrease enzyme activity because it includes alleles with deleted or duplicated genes, gene conversions, nucleotide deletions and insertions, as well as numerous coding and noncoding SNPs. These variants may change mRNA and/or protein expression levels or affect the structure and function of the protein [74,75]. The CYPAllele website currently lists 37 distinct alleles, that is, variants that cause amino acid changes or with proven functional effect. Many of these variants occur in various combinations, giving rise to complex haplotypes that occur at distinct ethnic frequencies [76]. A detailed compilation of the known structural changes and their functional impact was presented by Mwenifumbo and Tyndale [74]. The prevalence of functionally relevant *CYP2A6* alleles was recently estimated to be ~9% in Caucasians, ~22% in Africans, and up to 50% in Asians [77].

The *CYP2A6**2 [Leu160His] and the *4A-*H* deletion alleles have been shown to dramatically reduce enzyme activity *in vivo* in homozygous or hemizygous combination, resulting in the PM phenotype in affected individuals (Table 9.1). The most frequent of these presumable *null* alleles in Asians are the *4A-*H* hybrid-deleted alleles that consist of a *CYP2A7*-derived 5' part and a 3' part of *CYP2A6* origin. The *4A deletion variant is rare in Caucasians and Africans but present with up to ~20% frequency in Asian populations [76]. The *CYP2A6**2 [Leu160His] codes for an unstable

protein that fails to incorporate heme, but its frequency is only ~1% to 5% in Caucasians and is lower in Africans and Asians [76]. In a study involving 156 liver samples from Caucasians, resequencing revealed 33 haplotypes of which two (*9B, containing a -48T>G TATA-box polymorphism, and the 2A7/2A6 recombination allele *12B) were major genetic determinants associated with decreased hepatic expression [78].

A number of additional alleles including *CYP2A6**7, *10, and *17 severely reduce enzyme activity in homozygous or hemizygous individuals. The *7 [Ile471Thr] and *10 [Ile471Thr; Arg485Leu] alleles are Asian specific, and *17 [Val365Met] was only found in African-Americans. The *CYP2A6**5, *6, *11, *19, and *20 alleles were shown to have reduced activity by heterologous expression, but their low frequencies did, so far, not allow to confirm this *in vivo*. A further number of variants were shown to affect expression or function less dramatically, including several promoter variants that result in decreased expression, and some variants have not been characterized yet [74]. The *CYP2A6* gene was found in a duplicated variant in at least two different allele variants (*1X2A and *1X2B), which appear to be correlated to increased activity [79,80]. A decreased gene copy number is associated with gene deletions of the *CYP2A6**4A-4H alleles.

Most pharmacogenetic studies involving *CYP2A6* were carried out to study the effect of genotype on nicotine metabolism, smoking behavior, or lung cancer risk.

An *in vivo* study in 278 healthy Caucasian volunteers investigated the relationship between variant *CYP2A6* genotypes and the disposition and metabolism of intravenously administered nicotine [81]. Genotype-dependent differences in nicotine and cotinine plasma clearance were observed with the group, including the normal activity alleles having the largest capacity for nicotine C-oxidation. In a nicotine-replacement clinical trial, the group with slow metabolizer alleles had 50% reduced *CYP2A6* activity before treatment and reached 44% higher steady-state plasma levels of nicotine [82]. A large interethnic variability study compared nicotine metabolism and *CYP2A6* genotype in white, black, Korean, and Japanese subjects [76]. Large interindividual differences were observed in the cotinine/nicotine ratios in plasma. While no difference was found in the metabolic ratio of white, black, and Korean subjects, Japanese subjects showed a significantly lower metabolic ratio. The influence of low activity alleles on nicotine metabolism was confirmed in this study.

In contrast to the influence on pharmacokinetics, association of genetically variant *CYP2A6* with smoking behavior or lung cancer risk is still debated. The reader is referred to recent specialized reviews on these topics [74,75,83].

CYP2A6 polymorphism also plays a role for other substrates. A recent study emphasized the role of *CYP2A6* for efavirenz, an antiretroviral drug usually metabolized by *CYP2B6* to the 8-hydroxyderivative. As initially shown in human liver microsomes, the minor 7-hydroxy-efavirenz metabolite is mainly formed by *CYP2A6* [71]. The wide interindividual variability in efavirenz plasma exposure is largely explained by *CYP2B6* polymorphisms (see below). However, in patients with genetically impaired *CYP2B6*, substrate flux via the *CYP2A6*-dependent pathway is increased. The accessory metabolic pathway was shown to be critical in limiting drug accumulation in individuals characterized as *CYP2B6* slow metabolizers [77]. As combined *CYP2B6* and *CYP2A6* genetic deficiency occurs at significant frequency in various human populations, the *CYP2A6* polymorphism may be a clinically relevant determinant of extremely high efavirenz exposure in HIV patients.

9.3.2.2 Subfamily CYP2B—CYP2B6.

9.3.2.2.1 Molecular Genetics and Pharmacological Properties. The human *CYP2B* subfamily consists of the functional *CYP2B6* gene and the nonfunctional pseudogene *CYP2B7P*, which are located in a tandem head-to-tail arrangement within the large *CYP2ABFGST* gene cluster on chromosome 19 [1]. Expression of human CYP2B6 was initially underestimated [84], but despite higher estimations of more recent studies, it is one of the weaker expressed P450s, contributing on average ~2% to the total hepatic P450 pool [85]. CYP2B6 is also detectably expressed in human brain, kidney, heart, intestine, uterine endometrium, skin, and various tissues of the respiratory tract, including lung and nasal mucosa [64,86,87].

The CYP2B6 enzyme metabolizes diverse chemicals including several clinically used drugs as well as a large number of environmental chemicals. Therapeutically important drug substrates include the prodrug cyclophosphamide, which is activated by CYP2B6 to the cytotoxic 4-hydroxy-metabolite; the antiretroviral drugs efavirenz and nevirapine; the antidepressant and smoking cessation agent bupropion; the benzodiazepine diazepam; the antimalarial artemisinin; the anesthetics propofol and ketamine; and the synthetic opioid methadone [88–90]. Several suitable probe drugs for CYP2B6 have been discussed, but bupropion hydroxylation appears to be the most selective one [91] and is now most often used. However, because bupropion hydroxylation is not a sensitive marker for pharmacogenetic differences *in vivo* [92], other biotransformations may be more suitable markers. Liver microsomal efavirenz 8-hydroxylation has been shown to be correlated to CYP2B6 protein amount and is highly dependent on genotype *in vivo* (see below). Its usefulness as a suitable probe drug for CYP2B6 has, however, not yet been formally shown. CYP2B6 also metabolizes the N-demethylation of the recreational drug ecstasy (MDMA), which leads to potentially neurotoxic metabolites [93]. Nondrug xenobiotics metabolized mainly by CYP2B6 include the organophosphorus insecticide chlorpyrifos, which is metabolized to the more toxic oxon metabolite; the insecticide and endocrine disruptor methoxychlor; and the extensively used insect repellent DEET (*N,N*-diethyl-*meta*-toluamide) (for review, see Ref. 94).

Hepatic CYP2B6 is highly variable. In our study of 235 human liver samples, CYP2B6 protein varied ~360-fold, and all samples expressed at least a very low amount of the protein [85,95]. In this and other studies [96], no significant sex difference in CYP2B6 expression was found, but the issue remains controversial as other studies reported gender differences with higher expression in females versus males, which were more pronounced among Hispanics than Caucasians [97]. Several other mechanisms have been shown to contribute to inter- and intraindividual variability in hepatic CYP2B6 function, including induction of transcription by xenobiotics, [98] reversible and irreversible inhibition by diverse substances, [89] and genetic polymorphism [95]. As the human ortholog to the rodent phenobarbital-inducible CYP2b9/2b10 enzymes, CYP2B6 induction by drugs and other xenobiotics is due to similar mechanisms involving the xenosensors constitutive androstane receptor (CAR) and/or the pregnane X receptor (PXR) [98–100]. Known inducers are, for example, rifampin, barbiturates, cyclophosphamide, artemisinin, carbamazepine, efavirenz, and nevirapine, as well as metamizole [99,101] and statins [102]. Although this environmentally triggered variability should be expected to mask pharmacogenetic phenotypes, *CYP2B6* polymorphisms turned out to be highly significant for pharmacokinetics and possibly response to certain drugs, in particular, the anti-HIV drug efavirenz.

9.3.2.2.2 Genetic Polymorphisms and Pharmacogenetics. The CYPallele website currently lists 29 distinct alleles, that is, variants that cause amino acid changes or with a proven functional effect. More than 30 SNPs code for amino acid changes, which occur in numerous complex haplotypes and with distinct ethnic frequencies [90]. The most common variant *CYP2B6**6 harbors two amino acid changes Gln172His and Lys262Arg, which occur in various combinations with other changes that are apparently of minor functional significance. The *6 allele occurs with frequencies between 15% and 60% across different populations (Table 9.1), and it is associated with 50–75% decreased hepatic protein expression [71,95,97]. The causal variant for decreased expression was identified as the c.516G>T [Gln172His] polymorphism, which prevents correct splicing of the *CYP2B6* pre-mRNA and leads to a shorter mRNA that lacks exons 4–6 [85]. The second most important functionally deficient allele is *CYP2B6**18 (c.983 C>T [Ile328Thr]), which did not form a functional protein in transfected mammalian cells and may thus be a *null* allele [103]. This allele was not found in Caucasians. In African populations, it occurs with a frequency of up to 7% (Table 9.1). Most other functional polymorphisms occur at lower frequencies [95,103–106]. An allele associated with increased transcription *in vitro* was also identified (*CYP2B6**22, [105]). The –82T>C change was shown to alter the TATA box into a functional CCAAT/enhancer-binding protein binding site that causes increased transcription at an alternative downstream initiation site [105].

The major clinical role of *CYP2B6* polymorphism was elucidated after the *in vitro* studies identified *CYP2B6* as the enzyme responsible for the conversion of efavirenz into its major 8-hydroxylated and 8-, 14-dihydroxylated metabolites [107]. Efavirenz is a potent agent recommended for initial therapy in regimens with two nucleoside reverse transcriptase inhibitors (NRTIs), but patients with subtherapeutic plasma concentrations can develop resistance and treatment failure, whereas those with too high plasma concentrations are at increased risk of CNS side effects. Several clinical studies found that HIV-infected patients homozygous for the 516 T allele develop several-fold higher median AUC values compared to those with only one or no T-allele [108]. The 516 T variant was also associated with increased CNS side effects [109] and treatment discontinuation [110]. In a prospective, genotype-based dose adjustment study, the therapeutic dose of efavirenz could be successfully reduced and CNS-related side effects decreased [111]. Furthermore, it was shown that analysis of rare loss-of-function alleles can improve prediction of high efavirenz plasma levels [106].

CYP2B6 genotype also affects plasma levels of the antiretroviral drug nevirapine [112]. However, the common *CYP2B6* polymorphisms appear to be less penetrant for some other substrates, probably because the amino acid changes can cause additional effects. A single-dose pharmacokinetic study in volunteers revealed no change in total bupropion clearance, although $C_{\max}$, AUC, and metabolic ratio for hydroxybupropion were lower in *6/*6 and *1/*6 compared to *1/*1 [92]. In this study, a higher clearance was found for allele *4, consistent with higher activity of the K262R variant in an *in vitro* system [113]. Cyclophosphamide, one of the most widely used anticancer and immunosuppressant drug, requires bioactivation by P450 enzymes to yield the active 4-hydroxy-metabolite, and *CYP2B6* was shown to be the major enzyme for this step [114]. Studies reported that cyclophosphamide bioactivation may be enhanced by the c.516G>T allele in white subjects, further emphasizing the possibility of substrate-dependent effects of these amino acid variants [115]. *CYP2B6* allele variants were also investigated in the context of the synthetic μ -opioid receptor agonist, methadone,

which is used as a maintenance treatment for opioid addiction, and in $*6/*6$ carriers, (S)-methadone plasma levels were increased, leading to potentially higher risk of severe cardiac arrhythmias and sudden death [116,117].

In conclusion, the clinical impact of *CYP2B6* pharmacogenetics has only recently been investigated. *CYP2B6* genotyping predicts elevated plasma concentrations of efavirenz and nevirapine in HIV-infected individuals. This is in agreement with basic *in vitro* studies showing lower expression as a consequence of erroneous splicing of the most common $*6$ variant. Substrate-dependent effects have to be expected because of the presence of amino acid changes in the polymorphic alleles.

9.3.2.3 Subfamily CYP2C—CYP2C8, CYP2C9, CYP2C18, and CYP2C19.

9.3.2.3.1 Molecular Genetics and Pharmacological Properties. The human *CYP2C* subfamily consists of the four genes *CYP2C18*, *2C19*, *2C9*, and *2C8*, which are localized in this order in a gene cluster on chromosome 10q24. Although *CYP2C18* mRNA is highly expressed in liver, the transcript is not efficiently translated into protein and, therefore, does not make significant contributions to drug metabolism. The other three *CYP2C* members together constitute on average between 30% and 50% of the microsomal hepatic P450 pool, with *CYP2C8* and *CYP2C9* being expressed roughly 10-fold higher than *CYP2C19*. At lower levels, functional *CYP2C* enzymes were also detected [63], for example, in human small intestine, where they are independently regulated, and in cardiovascular tissues [118]. All three expressed *CYP2C* enzymes are inducible by ligands of the PXR/CAR and glucocorticoid (GR) nuclear receptor pathways through different response elements in their upstream regulatory regions [119]. However, the relative inducibilities are different for the three genes. All four *CYP2C* genes are genetically polymorphic. The *CYP2C19* or “S-mephenytoin” polymorphism is one of the early examples of P450 polymorphisms [120]. *CYP2C19* null alleles are common enough among Caucasians and Asians to produce a homozygous PM phenotype. In contrast, the common *CYP2C8* and *CYP2C9* variants code for full-length proteins with amino acid changes that lead to more moderate and substrate-dependent functional changes.

9.3.2.3.2 CYP2C8. *CYP2C8* is mainly responsible for the metabolism of the antidiabetics rosiglitazone and pioglitazone, the antiarrhythmic amiodarone, as well as the retinoic acid drugs used in acne and cancer treatment. Some overlap in substrate specificity with *CYP2C9* occurs, for example, as in the case of ibuprofen [4,121]. Additional important drug oxidations catalyzed primarily by *CYP2C8* are the 6 α -hydroxylation of the natural anticancer drug paclitaxel and the deethylation of the antimalarial amodiaquine, both useful probe drugs for *CYP2C8* phenotyping. In addition to amodiaquine, *CYP2C8* has a major role in metabolizing other antimalarials such as chloroquine and dapsone [122]. The clinical significance of *CYP2C8* became apparent after its involvement in fatal drug interactions was described, which were in part due to its potent inhibition by gemfibrozil acyl-glucuronide, ultimately leading to cerivastatin-induced rhabdomyolysis [123,124]. Many more potent inhibitors at clinically relevant concentrations have been described [125].

Apart from rare variants with no ($*5$, $*7$), reduced ($*8$), or unknown activity, there are three more common alleles *CYP2C8* $*2$, $*3$, and $*4$, which code for amino acid variants (Table 9.1). The $*2$ allele [Ile269Phe] most frequently (15–20%) occurs in individuals of African origin, and much lower to no occurrence is observed in

Caucasians and Asians [126–128]. The heterologously expressed variant had twofold increased intrinsic clearance for paclitaxel because of increased K_m . The *3 allele [Arg139Lys + Lys399Arg] occurs more frequently in white subjects but is almost absent in Africans and Asians. The CYP2C8.3 variant was initially shown to reduce metabolism of arachidonic acid and paclitaxel hydroxylation *in vitro* by ~40% and ~80%, respectively [126]. The evidence for altered pharmacokinetics, toxicity, or efficacy of these drugs in carriers of any of these alleles is however controversial. Owing to the close distance, the CYP2C8*3 variant is in partial linkage disequilibrium to the CYP2C9*2 allele, which may be of particular importance for substrates metabolized by both enzymes such as ibuprofen. Indeed, CYP2C8*3 genotypes were associated, together with CYP2C9*2 and *3 alleles, with reduced clearance of ibuprofen [129]. Some functional effects of CYP2C8*3 are substrate dependent, for example, for repaglinide and rosiglitazone, higher metabolic capacity of this variant was observed. The functionally less-well-investigated *4 allele occurs in Caucasians (~8%) and is practically absent from all other major races. A recent review on CYP2C8 pharmacogenetic studies can be found in Ref. 130.

In conclusion, the most relevant CYP2C8 variants are the alleles CYP2C8*2 and CYP2C8 *3 that code for amino acid variants whose frequencies vary between 7.5% and 20% depending on ethnicity. Their functional effects appear to be substrate dependent.

9.3.2.3.3 CYP2C9. In liver, CYP2C9 is one of the most abundantly expressed P450s. It accepts weakly acidic substances such as the anticoagulant warfarin, the anticonvulsants phenytoin and valproic acid, cardiovascular drugs such as rosuvastatin and losartan, and several nonsteroidal anti-inflammatory drugs (NSAIDs, [131]). Many of these drugs have a narrow therapeutic index, and variations in CYP2C9 activity are thus among the recognized factors for adverse drug reactions. The CYP2C9 enzyme also metabolizes endogenous substances, in particular, arachidonic acid and some steroids, and the fact that it is expressed in endothelial cells implicates it in the regulation of vascular tone [132].

Among the 34 distinct alleles listed on the CYPallele website, the initially discovered alleles, *2 [Arg144Cys] and *3 [Ile359Leu], have been investigated more thoroughly than the more recently identified and less frequent ones. These two variants are present in up to 20% and 15% in Caucasians, respectively, but much less common in Africans and Asians (Table 9.1). According to *in vitro* experiments with expressed mutant enzymes, the CYP2C9.2 and CYP2C9.3 proteins have reduced intrinsic clearance, although the degree of activity reduction appears to depend on the particular substrate. The *2 allele causes reductions in *in vitro* activity of ~20–40%, whereas the *3 allele is more severely affected and its activity reduction can be 70–90% for some substrates. In vivo, this results in clearance reductions of more than 70% in *3 homozygotes and in about half of the clearance for heterozygotes. Other alleles with decreased function but rare occurrence are *5, *6, *8, *11, as well as several promoter variants with unclear significance.

Numerous studies demonstrated the clinical significance of the CYP2C9*2 and *3 polymorphisms for most drug substrates mentioned above. Owing to their common occurrence in Caucasians, there are about 1–2% of homozygous (*2/*2 and *3/*3) and hemizygous (*2/*3) carriers that are at risk to experience more dramatic effects, but even for the heterozygous carriers, higher incidence of adverse drug reactions was

reported. This includes hypoglycemia as a result of treatment with hypoglycemic drugs [133], gastrointestinal bleeding from NSAIDs [134], and serious bleedings from warfarin treatment, where anticoagulant response also depends on variants of vitamin K epoxide reductase. Further examples of the clinical relevance of CYP2C9 polymorphisms can be found in recent reviews [131,135].

9.3.2.3.4 CYP2C19. Compared to CYP2C8 and CYP2C9, the CYP2C19 isozyme has a much lower expression in liver. However, it was the first CYP2C enzyme to be discovered by its marked genetic polymorphism resulting in the *S*-mephenytoin PM and EM phenotypes [120]. The CYP2C19 enzyme was later shown to be the major enzyme for the inactivating metabolism of proton pump inhibitors (PPI), including omeprazole and pantoprazole, and for the metabolic activation of the anticoagulant clopidogrel [136]. CYP2C19 has also a prominent role in the metabolism of several antidepressants of the first and second generation [137].

The PM phenotype results from two null alleles, that is, alleles that are unable to produce any functional protein, whereas EMs carry at least one functional allele. About 3–5% of white and black populations but up to 20% of Asians are CYP2C19 PMs, [6]. The two most common null alleles are *CYP2C19**2, which occurs almost exclusively in Caucasians, and *CYP2C19**3, which occurs primarily in Asians (Table 9.1). A further variant with potential clinical significance is a promoter variant *CYP2C19**17 that appears to be related to increased substrate turnover [138].

The effect of the *CYP2C19* polymorphism on *Helicobacter pylori* eradication therapy is a particularly intriguing example of a clinical application of pharmacogenetics. The common eradication strategy involves application of two antibiotics, for example, amoxicillin and clarithromycin, together with the PPI, which contributes to accelerated ulcer healing and increases effectiveness of the antibiotics. The PPI-induced increase in intragastric pH depends on the *CYP2C19* polymorphism. In several studies in Asia and Europe, it was shown that PM subjects benefit from their lower metabolism rate because their drug levels stay higher for a longer time [139].

Pharmacokinetic effects associated with *CYP2C19* genotype have also been reported for several antidepressants, including clomipramine [140], citalopram [141], and amitriptyline [142]. These were sometimes related to adverse side effects, but an influence on pharmacodynamic outcome remains so far controversial.

Several recent studies investigated *CYP2C19* as a genetic determinant of the efficacy of the platelet-aggregation-inhibiting thienopyridine, clopidogrel (Plavix). This is important in the management of patients with coronary artery disease because a significant proportion of patients may be resistant and may experience insufficient platelet inhibition. The activation of clopidogrel by P450-dependent oxidations has been investigated, and several P450 enzymes, including the CYPs 2B6, 2C9, 2C19, and 3A4, have been found to contribute to the formation of the active 2-oxo metabolite [143]. Numerous clinical studies have confirmed that CYP2C19 PMs have significantly lower anticoagulation effect of clopidogrel, which is associated with an increased risk of major adverse cardiovascular events [144].

In conclusion, the CYP2C subfamily contains three active proteins CYP2C8, CYP2C9, and CYP2C19. Despite the number of common and functionally important variants in each gene being low (one or two), the clinical significance of these polymorphisms has been firmly established for a number of frequently used drugs. Given their strong relatedness in DNA and protein sequence (>82%) and common

mechanisms of transcriptional regulation, it is surprising how unique each enzyme is in terms of substrate specificity and clinical significance.

9.3.2.4 Subfamily CYP2D—CYP2D6.

9.3.2.4.1 Molecular Genetics and Pharmacological Properties. The *CYP2D* locus on chromosome 22q13.1 consists of the functional *CYP2D6* gene and two pseudogenes *CYP2D7* and *CYP2D8P*. While *CYP2D7* is expressed as mRNA in liver, only *CYP2D6* leads to functional protein and constitutes on average about 5% of the total P450 pool. Expression levels vary dramatically from person to person. A recent quantitative comparison of hepatic CYP2D6 protein by Western blot and mass spectrometric analyses revealed comparable results from undetectable in PMs up to 70 pmol/mg of microsomal protein and more in UMs [145]. At the RNA level, CYP2D6 expression is characterized by the occurrence of numerous splice variants. However, with the exception of the effect of polymorphic splice variants (see below), the functional significance of alternative splicing of CYP2D6 pre-mRNA has not been clarified. In fetal liver, CYP2D6 is virtually undetectable, but expression surges within hours after birth [146]. CYP2D6 is also expressed in several extrahepatic tissues, most notably in the gastrointestinal tract and in different areas of the human brain [147–149].

CYP2D6 metabolizes a large number of drugs from virtually all therapeutic classes, including analgesics, antiarrhythmics, antidepressants, antipsychotics, β -blockers, and anticancer drugs. Several highly selective test drugs have been used to determine the CYP2D6 drug oxidation phenotype, including debrisoquine, dextromethorphan, metoprolol, sparteine, and tramadol [150]. Endogenous biotransformations include 5-methoxyindolethylamine *O*-demethylase [151] and regeneration of serotonin from 5-methoxytryptamine [152]. The structure–activity relationships for CYP2D6 substrates and inhibitors were used to develop pharmacophore models [153]. Recently, the crystal structure of the CYP2D6 protein has been resolved, yielding further insights into the active site and the chemical requirements for binding and catalysis [154].

9.3.2.4.2 Genetic Polymorphisms and Pharmacogenetics. Among the human drug-metabolizing CYP1, 2, and 3 families, CYP2D6 shows the greatest impact of genetic polymorphism on expression and function and comparably little influence by environmental and nongenetic factors (except for drug–drug interactions). Initial evidence for *CYP2D6* genetic polymorphism came from population and family pharmacokinetic studies in the 1970s, which showed that deficient debrisoquine 4-hydroxylation [155] and sparteine N-oxidation [156] occur in 5–10% of European Caucasians as a monogenic recessive trait but at much lower frequencies in other ethnicities [157]. The molecular studies that followed have been reviewed in detail previously [6,148,158]. Currently, 78 distinct alleles and a large number of allele variants have been described, and the most relevant ones have been functionally assessed and shown to lead to absent or nonfunctional protein, or to decreased or increased expression (Table 9.1). The most frequent null alleles in Caucasians are haplotype variants of *CYP2D6**4, which all harbor a consensus splice site mutation (1846G>A) that leads to absence of a detectable protein in the liver [159]. Its frequency among Caucasians is about 20–25%, and in white populations, it is responsible for 70–90% of all PMs [160,161]. The low frequency of the PM phenotype in Oriental and African populations is due to the virtual absence of the *4 allele, whereas in African-American populations, it is present with intermediate frequencies. The *CYP2D6* gene deletion allele *5 is present at a

frequency of 3–5% in most populations. The null alleles *3 and *6 are present at frequencies slightly above 1% in Caucasians, whereas the other null alleles are very rare [160,161]. In 10–15% of white individuals, the IM phenotype was shown to be the result of inheritance of the partially defective allele *41 in combination with another partially or fully defective allele. The mechanism involves an intron 6 SNP that leads to erroneous splicing resulting in only a fraction of correctly spliced mRNA [162].

In addition to the SNPs, a large number of structural variations exist at the *CYP2D* locus. Unequal crossing-over between the highly homologous genes involving a certain repetitive sequence also present in the *c-myc* gene leads to variants with deleted, duplicated, or recombined genes. *CYP2D6* gene duplications were identified in combination with various alleles including *1, *2, *4, *6, *10, *17, *29, *35, *41, *43, and *45, but most commonly, it occurs with the functional amino acid variant *2. The overall frequency of the gene duplications in white Europeans is between 1% and 5%, whereas in some Arabian, Eastern African, and Pacific populations, it varies between 10% and over 50%, giving rise to speculations about possible selection processes, which might be related to the striking preference of CYP2D6 for plant alkaloids [158].

While PM alleles are very rare in Africans and Asians, other partially defective alleles termed *17 and *10, respectively, are prevalent. The *17 allele is present at frequencies of up to 30% in Africans [163], and the *10 variant occurs at a frequency of up to 50% in Asians [164]. The partial activity conferred by these alleles leads to a shift in the metabolic drug oxidation capacity toward lower values, which has clinical relevance [165].

The presence of the pseudogenes, structural variants, and numerous SNPs at the *CYP2D* locus requires particular cautiousness in the design of genotyping assays. Coamplification of pseudogenes, unexpected recombination events, and failure to account for important variants, for example, due to ethnic variation, can lead to erroneous interpretation of genotype. Numerous genotyping assays and strategies were developed, which, because of the complexity of variants, usually identify only one functionally dominant key mutation per allele. This possibly reduces the predictive power of genotyping for certain haplotypes. The most comprehensive commercially available platform for CYP2D6 genotyping is the AmpliChip CYP450 test from Roche. This microarray has probes to identify 33 CYP2D6 alleles, including most confirmed variants responsible for absent or impaired enzyme activity and 7 gene duplications, as well as 2 *CYP2C19* variant alleles [166–168].

The *CYP2D6* polymorphism is one of the most intensely studied polymorphisms in man. It has been studied not only in relation to drug response for many CYP2D6 substrates but also as a risk factor for numerous diseases. Most of the epidemiological studies revealed conflicting results, and the reader is referred to specialized articles on the association of polymorphic *CYP2D6* alleles with, for example, Parkinson's disease [169], schizophrenia [170], Alzheimer's disease [171], and several forms of cancer [172–174].

In contrast to the largely inconsistent epidemiological studies, numerous clinical studies have shown profound effects of CYP2D6 polymorphism on pharmacokinetics, therapeutic effect, and associated toxicity of drugs that are either inactivated or activated by this enzyme. The limited scope of this chapter allows to mention only some of the newer studies, for example, on the impact of *CYP2D6* genotype on drug response following treatment with metoprolol [175,176], propafenone and other antiarrhythmics [177,178], and amitriptyline and other antidepressants [142,179–181]. An

important prodrug converted by CYP2D6 to the pharmacologically active substance is codeine, which is O-demethylated to morphine. Increased effectiveness of codeine with sometimes life-threatening opioid intoxication was observed in patients with multiple *CYP2D6* gene copies consistent with higher rates of conversion to morphine in patients with UM phenotype [182–184]. Recently, this scenario has also been the subject of a computerized quantitative modeling study [185].

CYP2D6 pharmacogenetics appears to have a major impact in the adjuvant treatment of breast cancer with tamoxifen, an antiestrogen extensively metabolized in the liver to several primary and secondary metabolites [186]. Formation of at least two metabolites, 4-hydroxytamoxifen and the secondary metabolite endoxifen, thought to be mainly responsible for the antiestrogenic effect because of their high affinity to the estrogen receptor depends mainly on the catalytic action of CYP2D6 [187]. Recent retrospective and prospective studies demonstrated that the CYP2D6 genotype significantly influences the plasma concentrations of these active tamoxifen metabolites, thus influencing treatment outcome [188]. According to these studies, patients with PM or IM phenotype produce lower levels of active metabolites and profit less from the treatment [77]. Nevertheless, it must be realized that the studies so far were limited by size and by the fact that confounding factors have not been taken into account systematically [189–191].

In conclusion, CYP2D6 is one of the cornerstones of pharmacogenetics and its translation into personalized medicine. The clinical significance of the *CYP2D6* polymorphism, which includes variants with completely deficient, decreased, and increased activity, has been firmly established for numerous frequently used drugs.

9.3.2.5 Subfamily CYP2E—CYP2E1. Among the human drug-metabolizing CYPs, CYP2E1 displays a substrate preference for low molecular weight molecules, including ethanol, acetone, pyrazole, acetaminophen, and many other toxicants and carcinogens, and thus is of primary toxicological importance, for example, for the metabolic activation of acetaminophen and many other chemicals [192]. The enzyme is inducible by many of its substrates, and in contrast to the transcriptional induction mechanisms, which play a role for most other drug-metabolizing P450s, CYP2E1 is induced post-translationally by substrate-induced stabilization. A number of studies also established the role of CYP2E1 in oxidative stress and alcoholic liver disease [193,194].

CYP2E1 is the only functional gene of the *CYP2E* subfamily, which is located at chromosome 10q26.3. Relatively few polymorphisms were described compared to other *CYP2* genes [7], but conclusive functional assessment of their phenotypic effects is lacking for most variants, and only moderate associations were described in studies with human liver [195–197]. Association with expression changes was reported for a polymorphic Rsa I site in the 5'-regulatory region of *CYP2E1**5 in one study [196] but not in another [195]. A recent study analyzed 11 polymorphisms and their haplotypes in over 2600 individuals from population samples representing the major geographical regions of the world [198]. As observed in studies on the CYP3A locus (see below), haplotype diversity was much higher in Africa (6–10 common haplotypes) than in other parts of the world (about 1–6 common haplotypes). This study also eluded on the difficulties in relating the haplotypes and alleles with different information content.

Because CYP2E1 is of particular relevance for metabolic activation of procarcinogens and chemical carcinogenesis, numerous pharmacogenetic studies were carried out

toward identifying variants associated with various cancers. As these studies are complex and often contradictory results were obtained, the reader is referred to some recent specialized reviews and meta-analyses on CYP2E1 and the risk of chemically induced cancers [199], its association with lung cancer risk [200], and alcohol-related cancers [201].

9.3.3 Family CYP3

9.3.3.1 Subfamily CYP3A—CYP3A4, CYP3A5, CYP3A7, and CYP3A43.

9.3.3.1.1 Molecular Genetics and Pharmacological Properties. The CYP3A cluster on human chromosome 7q22.1 has a size of 231 kb and comprises the four genes 3A4, 3A5, 3A7, and 3A43. The mouse cluster consists of twice as many genes, but there are no orthologous pairs between mouse and human, suggesting that a single CYP3A gene present in the common ancestor existed, which independently expanded during the last 75 million years [1]. CYP3A enzymes, in particular, CYP3A4, are responsible for the metabolism of ~30–40% of the clinically used drugs from all therapeutic categories. In part, this is explained by the structure of the enzymes. The active site of CYP3A4 is large and flexible enough to bind and metabolize many preferentially lipophilic compounds with comparatively large structures [202]. Typical substrates are, for example, the immunosuppressants cyclosporin A and tacrolimus; macrolide antibiotics, such as erythromycin; anticancer drugs, including taxol, cyclophosphamide, ifosfamide, and tamoxifen; benzodiazepines; the HMG-CoA reductase inhibitors simvastatin and atorvastatin; antidepressants; and opioids [203,204]. CYP3A4 is also an efficient steroid hydroxylase with an important role in the catabolism of several endogenous steroids including testosterone, progesterone, androstenedione, cortisol, and bile acids. The high sequence similarity between the CYP3A isozymes leads to highly similar substrate selectivity, so that no really selective probe substrates could be identified to date. Although several probe drugs that measure general CYP3A activity are available, including midazolam, erythromycin, alprazolam, and dextromethorphan [204], phenotyping for CYP3A is not a trivial task, as results obtained with different probe drugs are not generally well correlated to each other, which is a CYP3A4-specific feature that may be related to allosteric regulation of enzyme activity [205].

In the majority of individuals, CYP3A4 is the most abundantly expressed P450 in liver and intestinal enterocytes, as well as in colon and breast [63,84,206,207]. Expression of the three minor isoforms, CYP3A5, CYP3A7, and CYP3A43, is generally much lower than that of CYP3A4, although their exact contribution to the total CYP3A pool remained controversial [207–211]. CYP3A7 is more abundantly expressed in fetal liver than in adult liver, but the mechanism for this has not been studied in detail [146,212], whereas expression of CYP3A43 is negligible in liver [207]. Different signaling pathways contribute to the complex regulation of the CYP3A genes at the transcriptional level [203]. The xenosensor nuclear receptors PXR and CAR, on binding to structurally diverse drug ligands, including barbiturates, glucocorticoids, and rifampicin, are translocated to the nucleus, where they heterodimerize with RXR and enhance transcription from several PXR and CAR response elements located in the proximal promoter and in the xenobiotic-responsive enhancer module (XREM) region located ~8 kb upstream [213,214]. By contrast, downregulation of CYP3A4 occurs in the course of inflammation [215]. Most CYP3A4 substrates show about 20–50% higher

in vivo clearance in females than males, and this difference has been explained by a higher hepatic expression at the mRNA, protein, and activity levels [216].

9.3.3.1.2 Genetic Polymorphisms and Pharmacogenetics.

CYP3A4. On the basis of a comparison of between- and within-person variances in CYP3A4 catalytic function, Ozdemir *et al.* [217] estimated a high inheritability of CYP3A4 catalytic function that warrants investigation into genetic polymorphism. Nevertheless, it should be noted that expression distribution was found to be unimodal, thus not indicating monogenic control. The CYPallele website currently lists 22 non-synonymous variants, 3 SNPs resulting in frameshift, and a number of noncoding variants. Although several of the amino acid variants were shown to have an impact on catalytic function, their frequency in the general population is low so that homo- or hemizygotes are extremely rare, and therefore, only modest impact on *in vivo* pharmacokinetics can be expected. A more common and frequently studied polymorphism is the proximal promoter variant *CYP3A4*1B* [−392A>G], which occurs in white populations at ~2–9% but at higher frequencies in subjects of African origin (Table 9.1). *CYP3A4*1B* was initially found to be associated with a higher tumor grade and stage in prostate cancer and showed higher nifedipine oxidase activity in human livers [218]. Association of *CYP3A4*1B* with markers of advanced disease was confirmed by some but not all further studies [219,220]. Despite additional *in vitro* studies with different substrates, the functional effect of this variant remained controversial [221–223]. Several systematic resequencing and haplotype tagging studies at the CYP3A locus carried out in ethnically diverse population samples essentially failed to discover any further variants of significant predictive power for CYP3A4 expression and/or function [208,209,220,224–226].

There are several possibilities to explain the apparent discrepancy between the expected high inheritability of CYP3A4 phenotype and the lack of predictive polymorphism in the *CYP3A4* gene. The importance of haplotype structures in the *CYP3A* locus is one possibility, but haplotypes have so far only been considered in few studies [225,227]. In one study, 37 tag SNPs distributed over the entire *CYP3A* locus in 5 ethnic groups were analyzed, and it was concluded that in European Caucasians, the *CYP3A* locus consists of the largest LD (linkage disequilibrium) blocks compared to other ethnic groups and that only 4 haplotypes account for over 80% of common European Caucasian haplotypes [228]. Haplotype diversity may therefore be more important in other ethnic groups, particularly of African origin. It is also possible that some important predictive variants have not been identified yet, as suggested by the recent discovery of an intronic SNP that appears to affect hepatic expression and response to statins [229]. Furthermore, the influence of genetic polymorphism may be masked or influenced by nongenetic effects. Evidence for the latter possibility was provided by an analysis that took the sex-dependent expression of CYP3A4 into account [227]. Finally, the genetic variants that determine CYP3A4 phenotype need not necessarily be located within the *CYP3A* locus. The various regulatory and signaling pathways that influence CYP3A4 expression may harbor variants that modulate constitutive or inducible transcription; for example, in nuclear receptors or their coactivators [230], drug transporters have been suggested to modulate CYP3A4-inducible expression by controlling the intracellular concentration of nuclear receptor ligands [231] and variants of the monooxygenase redox genes NADPH-CYP oxidoreductase and cytochrome *b5* may affect enzymatic activity [232]. Pathway-oriented gene approaches with a broad

coverage of genes in phenotypically well-defined tissues or volunteers may help to advance the pharmacogenomics of CYP3A4.

CYP3A5. In contrast to CYP3A4, expression of CYP3A5 in liver is polymorphic, and only a fraction of about 5–10% of Caucasians but 60% or more of Africans or African-Americans express this minor form. These ethnic differences are largely explained by two alleles that result in aberrant splicing and deficient expression. The most common allele is *CYP3A5*3*, which harbors an intron 3 mutation that leads to aberrant splicing and a truncated protein and occurs in all ethnic groups studied [211]. *CYP3A5*6* with an exon 7 mutation that also leads to an aberrantly spliced mRNA lacking exon 7 was only detected in populations of African origin. The high frequency of the *3A5*3* allele in Caucasians, compared to the lower frequency in Asian and African populations, explains the corresponding opposite frequency pattern of the functional *CYP3A5*1* reference allele that determines expression.

Pharmacogenetic studies addressing the CYP3A5 polymorphism have generally resulted in no or only moderate effects. This lack of penetrance is mainly due to the overlap in substrate selectivity among the CYP3A enzymes and to the minor expression of CYP3A5 compared to CYP3A4. Nevertheless, association with the *CYP3A5* genotype was reported, for example, for the immunosuppressant tacrolimus [233,234], the antihypertensive verapamil [235], and the HIV protease inhibitor saquinavir [236]. Further references on this subject and on the numerous studies on evaluation of CYP3A polymorphisms and cancer risk can be found in specialized reviews [211,237].

CYP3A7. The fetal predominant form CYP3A7 accounts for up to 50% of total P450 content in fetal livers [146,212]. Although expression shifts after birth from CYP3A7 to CYP3A4, it remains polymorphically expressed in some adult livers and in intestine [238]. Most of the CYP3A7 mRNA high expressor phenotypes could be explained by the *CYP3A7*1C* allele, which is more effectively transcribed due to several SNPs in the promoter region that lead to increased binding of and transactivation by PXR/RXR and CAR/RXR heterodimers to a polymorphic ER6 motif also found in the CYP3A4 promoter [238]. Further studies with a specific antibody estimated the relative content of CYP3A7 to the total CYP3A pool to be between 9% and 36% for the ~10% of high expressors and about 10-fold lower in the low expressors [239]. Additional alleles of the *CYP3A7* gene can be found on the CYPallele website [7]. The clinical significance of CYP3A7 polymorphism has not been well studied.

9.4 CONCLUSIONS AND FUTURE PERSPECTIVES

Among the 200 most frequently sold drugs in the United States, about 80% are cleared primarily by CYPs of the CYP1, CYP2, and CYP3 families [4]. The CYP1A2, CYP2C8, and CYP3A4 isoforms, which lack major functional polymorphisms, are responsible for the metabolism of about half of these drugs, whereas metabolism of the other half is by the polymorphic enzymes CYP2B6, CYP2C9, CYP2C19, and CYP2D6, which have an established role in pharmacogenetics [240]. An analysis of adverse drug reaction studies published between 1995 and 2000 identified 27 drugs frequently cited in connection with adverse drug reaction. While 59% of these drugs are metabolized

by at least one polymorphic enzyme, compared to only 7% of randomly selected top-selling drugs, emphasizing the clinical significance of pharmacokinetic polymorphisms [241]. For the major polymorphic human P450s, the most important functional variants have been identified and functionally characterized, although in some ethnically isolated populations, undiscovered important alleles may still exist. Impressive examples of genotype–phenotype or genotype–clinical outcome relationships have been elucidated that suggest advantageous clinical application. The less well predictable genes, especially CYP1A2 and CYP3A4, still pose a big challenge. Future studies need to consider pathways involved in their regulation and concepts for the inclusion of gene–gene and gene–environment interactions, as well as epigenetic aspects. Drug toxicity and treatment outcome depend on many additional genetic and nongenetic factors, and multigene and multifactorial approaches have to be considered more seriously in the future for successful application of the CYP polymorphisms in personalized drug therapy. Integration of such diverse data into comprehensive models of metabolic and gene regulatory networks poses future challenges for pharmacogenetics.

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10 Flavin-Containing Monooxygenase: The Role of Flavin-Containing Monooxygenase in Lead Design and Selection

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10.1	Summary	1
10.2	Introduction	2
10.3	Overview of the drug discovery process	5
10.4	Academic drug development using admet	7
10.5	Enhancing drug candidate selection	10
10.6	FMO in drug candidate selection	11
10.7	FMO-mediated metabolism: strong nucleophiles	13
10.8	Non-nucleophiles	24
10.9	Drug optimization	24
10.10	Conclusions and further perspectives	25
	References	26
	Further Reading	30

10.1 SUMMARY

The flavin-containing monooxygenase (FMO) is an important human drug-metabolizing enzyme because it oxygenates sulfides and amines, which are two functional groups very prevalent in drugs and drug candidates. FMO also oxygenates many other nucleophile-containing compounds to more polar, readily excreted metabolites. Generally, FMO-mediated metabolism constitutes a detoxication process, but sometimes it produces electrophilic metabolites that inhibit other enzyme systems because FMO is quite recalcitrant to inhibition. FMO is also not readily induced and the properties of lack of inhibition and induction contribute to FMO being a detoxication catalyst. Because FMO is not readily inhibited or induced, most of its functional variability is not due to environmental factors but due to genetic polymorphisms. Compared with cytochrome P450 (P450), drugs and drug candidates that are metabolized by FMO possess less potential for toxicity and adverse drug–drug interactions.

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Highly nucleophilic drugs and drug candidates are oxygenated by FMO, and some of these oxygenated metabolites can be readily retroreduced to the parent compound quite efficiently. Retroreduction of drug candidate metabolites formed by FMO confounds the evaluation of the role of FMO in drug candidates and probably underestimates the amount of metabolism that FMO contributes to new chemical entities. One class of highly nucleophilic agents that are not oxygenated by FMO is the endogenous physiological nucleophiles such as glutathione or cysteine. This likely cytoprotective process probably spares the cell from unnecessary consumption of reductive equivalents of NADPH used in FMO-mediated oxygenation as well as from the loss of thiophile cytoprotective agents such as glutathione.

Introducing a functional group into drug candidates that are metabolized by FMO may be beneficial in drug development. This might distribute the metabolism of the drug candidate over a wider set of enzymes as well as potentially provide FMO metabolites that are generally polar, nontoxic, and readily excreted. By avoiding metabolism (and bioactivation) by P450, the prospects for increasing druglike properties of a drug candidate are likely increased. Because so many drugs are metabolized by P450 3A4, decreasing the amount of metabolism through this enzyme system is likely to decrease adverse drug–drug interactions. Distributing the metabolism of drugs over a wide range of metabolic processes including FMO might decrease reliance on one metabolic pathway and decrease toxicity and adverse drug–drug interactions.

10.2 INTRODUCTION

The FMO (E.C.1.14.12.8) is a microsomal, NADPH-dependent, chemical- and drug-metabolizing enzyme that possesses broad substrate specificity [1,2]. Compared to biosynthetic enzymes with great substrate specificity, monooxygenases such as FMO appear to trade robust rates of metabolism for the ability to accept a wide variety of nucleophilic heteroatom-containing chemicals and endogenous materials [3,4]. In this regard, FMO resembles P450, although its specific rates of metabolism appear to be greater than those of P450. Generally, FMO converts lipophilic nucleophilic compounds to more polar metabolites that are more readily excreted; for example, tertiary amines are converted to their *N*-oxides, and often, the *N*-oxides possess significantly less pharmacological activity. FMO can bioactivate compounds to more reactive metabolites; however, these reactive products generally leave the product domain of FMO but can inactivate other proteins (including hemoproteins such as P450). This aspect of FMO function underscores an underlying mechanism: somehow the product-binding domain of the enzyme precludes some very reactive metabolites from inactivation of the enzyme. Amino acid residues surrounding the product-binding domain presumably are non-nucleophilic and prevent covalent binding by electrophilic metabolites. Accordingly, FMO is not easily inhibited. Lack of ready inhibition of FMO is in contrast to the relatively easily inhibited P450 class of enzymes and could suggest that FMO would have less adverse drug–drug interactions via FMO-dependent metabolism [5].

There are five forms of FMO (i.e., FMO1–5) that show 50–58% amino acid identity across species lines [1]. Additional pseudogenes (i.e., FMO6–11) have been reported in human tissue [6,7]. Because FMO pseudogene products (i.e., FMO6–11) lack functional activity, they are not discussed further in this summary. FMO utilizes a

peroxyflavin cofactor as the source of molecular oxygen, and for human FMOs, much of the FMO metabolism literature published to date suggests FMO3 as having a prominent role in FMO-dependent drug and chemical metabolism [8]. It should be pointed out, however, that FMO5 is the most prevalent form of FMO in adult human liver but its functional role is not clear, possibly because of the limited number of substrates reported [3,9]. In contrast to many small animal models of hepatic FMO where FMO1 is often a prominent hepatic form in adult human liver, very little FMO1, FMO2, and FMO4 are present [3,10]. Relatively few reports in the literature related to human FMO-mediated metabolism exist, and this is surprising in view of the fact that FMO3 is present in adult human liver to a surprisingly great extent. For a normal liver, FMO3 is present in ~65% of the amount of protein that is typically observed for P450 3A4 [10]. Adult human liver FMO3 is a prominent enzyme that likely plays a larger role in the metabolism of chemicals and drugs than has previously been reported. There are several possible reasons for this. First, FMO is thermally labile and the order of addition of cofactor (i.e., NADPH) is very important to preserve maximum enzyme activity. As much as 80% of FMO activity can be lost by treating microsomal or other sources of FMO3 with heat (even preincubation at 37°C) for as little as one minute preincubation in the absence of NADPH [11]. Accordingly, incubations initiated by the addition of NADPH to prewarmed preparations of microsomes or FMO considerably decrease the functional activity. Traditionally, initiation of microsomal incubations by the addition of NADPH has been done to decrease autooxidation of N- and S-containing substrates that are particularly prone to autooxidation. However, autooxidation of susceptible substrates can be precluded by addition of an antioxidant such as diethylaminepentacetic acid (DETAPAC). Addition of substrate to a chilled incubation containing a source of FMO, NADPH, and an antioxidant and initiation of the incubation at 37°C is an optimal means of preserving FMO functional activity. Second, the nature of the metabolite formed by FMO (i.e., *N*-oxides, *S*-oxides, and hydroxylamines) is sometimes difficult to quantify from an analytical standpoint. In addition (and this point is discussed in greater detail below), metabolites of FMO can undergo retroreduction and reform the parent drug or chemical. Thus, no apparent FMO-dependent metabolism is present when in fact, considerable metabolites are formed, but they are rapidly retroreduced. Another aspect is that sometimes, initially formed FMO-dependent metabolites (e.g., hydroxylamines) are more nucleophilic than the starting amine and are more extensively metabolized than the parent amine. Moreover, the ultimate metabolite may not be readily recognized as arising from the starting material. In rare cases, the metabolite (e.g., a tertiary amine *N*-oxide or an *S*-oxide) may be chemically unstable and may undergo a Cope-type elimination to provide a product that is also not readily recognizable as derived from a typical FMO-mediated transformation. In fact, sometimes, FMO-dependent metabolites resemble those that might arise from P450. Finally, the efficiency of FMO-mediated oxygenation is also highly dependent on the quality of the microsomes or other source of FMO. Compared to P450, for example, there are some significant differences to the approach to preparing microsomes highly enriched with FMO functional activity, but most approaches involve assuring that the microsome sample is kept at 4°C or less during preparation [11]. To further illustrate ways to characterize FMO and distinguish functional enzyme activity from P450, a few simple methodological approaches are listed below that can be used to distinguish FMO-dependent from P450 dependent metabolic processes [12].

While the dogma that FMO oxygenates highly nucleophilic compounds has been described above, there are a number of important exceptions to the rule. For example, some unusual examples of FMO-mediated carbon-centered oxygenation (e.g., 6-methylhydroxylation of the anticancer agent 5,6-dimethylxanthenone-4-acetic acid [13] and carbon oxygenation and defluorination of 4-fluoro-*N*-methylaniline [14]) have appeared in the literature. The latter example is particularly intriguing because FMO1-mediated carbon oxygenation of 4-fluoro-*N*-methylaniline is coupled with defluorination to afford 4-*N*-methylaminophenol via an electrophilic quinoneimine intermediate. Another important class of nucleophiles not oxygenated by FMO includes biological nucleophiles (e.g., cysteine or glutathione). Examined from the standpoint of biology, FMO presumably does not accept cysteine or glutathione because S-oxygenation of such nucleophiles would rapidly deplete cysteine or glutathione, putting defense mechanisms of the cell at risk. S-Oxygenation of biological thiols would also deplete NADPH, and this would be biologically wasteful and also put the cell at risk from biological electrophiles because of the inability of NADPH to otherwise participate in endogenous cellular defense mechanisms. Apparently, key amino acids in the mouth of the FMO substrate-binding channel are specifically in place to ward off biological nucleophiles. The fact that FMO is not prone to inhibition by electrophilic oxygenated metabolites (e.g., even highly reactive sulfenic acids) suggests that FMO may have evolved to detoxicate lipophilic nucleophilic N- and S-containing compounds in the primordial biota. Inhibition of FMO would have brought this vital function to a halt and predisposed any developing biology to risk. Likewise, FMO apparently evolved critical amino acids in the substrate-binding channel to disavow the metabolism of cellular protective agents (e.g., glutathione oxidized to glutathione disulfide). By using both these mechanisms, FMO presumably provides a strong detoxication presence to the cell. It is not clear that this is the physiological role of FMO, although, to date, the physiological role of FMO has eluded identification [2,15,16]. Certain physiologically important N-containing compounds are also efficiently N-oxygenated. For example, the neurotransmitters tyramine and phenethylamine are sequentially N-oxygenated by FMO3 to the oxime [17,18]. N-Oxygenation of these and other biologically relevant amines may serve as a detoxication pathway to decrease the accumulation of excess and deleterious amines by converting them into metabolites that possess considerably less pharmacological activity than the parent amine [19].

The prediction is that FMO will oxygenate any nucleophilic heteroatom-containing compound that can be oxidized by hydrogen peroxide or peracids [20]. This is one distinction for this class of monooxygenase made on the basis of products derived from FMO: compared to P450, the structures of products are relatively easy to predict with a great deal of certainty. However, FMO does not appear to oxygenate biological nucleophiles (e.g., glutathione and cysteine), although there are some apparent exceptions (e.g., biotin [2]).

This chapter summarizes some of the general considerations of the role of FMO in preclinical drug lead selection and development. The idea that concepts of FMO drug metabolism can be used effectively to guide preclinical drug lead development is not new. Previously, some of these ideas were summarized [5]. Now, a few examples are emerging that underscore the importance of FMO3 in human drug development. The idea is that building drug lead design strategies for a wider range of metabolic pathways of a molecule to decrease reliance (or dominance) on any one metabolic pathway (e.g., P450 3A4) could have significant advantages. Engineering soft nucleophilic centers

into molecules could afford a greater amount of metabolism to go through the FMO3 detoxication route. This may improve detrimental metabolism, toxicity, and bioavailability issues that sometimes beset drug candidates and result in failure of candidates to advance to clinical drug status. Because FMO3 is not readily inhibited or induced, drug candidates that are metabolized by FMO3 may offer advantages by way of decreasing adverse drug–drug interactions that have been observed for drugs prominently metabolized by metabolic enzymes that are readily induced or inhibited. This is not to say that rapid metabolism should be built into a drug candidate. On the contrary, as we shall see with a number of examples, it is likely that FMO3-related metabolism also enables futile metabolic cycles of oxygenation and retroreduction (e.g., for *N*-oxides and *S*-oxides), thus enabling potentially greater cellular accumulation of the parent drug, greater therapeutic efficacy, and more favorable bioavailability. It is possible that the overall process of FMO3-mediated oxygenation and consequent retroreduction are a much greater dynamic than previously recognized. Of course, in addition to FMO-mediated processes, there are many other metabolic processes that can contribute to the overall biotransformation and distribution of a drug candidate. In this chapter, a summary of some of the differences between FMO and other metabolic processes is provided. To stimulate further discussion, the author takes the position that judicious use of soft nucleophiles incorporated into lead candidates and incorporation of FMO-dependent metabolism into drug design paradigms may be useful to improve the overall drugability of candidates and may decrease potential adverse drug–drug interactions.

10.3 OVERVIEW OF THE DRUG DISCOVERY PROCESS

The drug discovery process has changed dramatically over the past 20 years [21]. The drug discovery pipeline has expanded with the use of combinatorial chemistry, rapid synthetic methods, and high throughput screening. However, the success rate of moving a new chemical entity (NCE) forward to an approved drug status is low and the expense is considerable [22]. In the 1960s and 1970s, the observation that many drug candidate failures in clinical development was due to inappropriate pharmacokinetics ushered in a new approach that incorporated a greater reliance on ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties earlier in the preclinical setting [23]. Drug metabolism and pharmacokinetic studies in small animals planned and carried out earlier in the preclinical drug development paradigm and used to make predictions about the way drug candidates could be metabolized and distributed in humans have become a more accepted strategy. While predictions about the way small molecules are handled in small animals compared to humans is an inexact science, utilization of ADMET studies early in the drug development pipeline has decreased the failure rate due to inappropriate pharmacokinetic properties [22,24]. The reported success rate for drug candidates that entered clinical trials was reported as improved for the period between 1981 and 1992 because of improved pharmacokinetic properties and a greater understanding of chemical toxicology [25,26]. This may have had something to do with the improved practice of high throughput synthesis and screening coupled with *in vitro* ADMET studies that improved the metabolic properties and bioavailability for preclinical drug development during this period. However, the success rate reported for this period (i.e., 20.9%) is still unacceptably low and more recent data do not point to

an improvement [27]. The high attrition rate for drug development that still persists is likely due to unfavorable toxicologic properties of new drug candidates. Less progress has been made in understanding the fundamental chemical and biological basis underlying the reason that certain compounds cause toxicity. Compounding the problem is the uncertain nature that small animal studies extrapolate to the human situation [28]. While there is general agreement that production of metabolic reactive metabolites and intermediates is linked to impairment of cell function and cell death, it is not completely clear how small animal toxicology studies relate to human toxicology other than to follow the general idea that it is prudent to divert metabolism pathways from those that produce reactive electrophilic metabolites [29]. Metabolic bioactivation of small molecules to toxic materials as an underlying principle responsible for toxicity is not a new concept, and the idea of linking protein and DNA covalent modification with drug toxicity in *in vitro* and *in vivo* systems is becoming more and more widely accepted. New molecular and genetic approaches have emerged to identify the potential for metabolic bioactivation. Today, many departments responsible for drug safety and ADMET in the pharmaceutical industry have established protocols to identify potentially problematic compounds by a process including (i) reaction phenotyping (to identify the prominent enzymes involved in the metabolism of the drug candidate), (ii) induction or inhibition of P450 (the enzyme system that accounts for about 65% of human drug metabolism), (iii) covalent binding studies (that indicate if reactive electrophiles are being created *in vitro* or *in vivo*), and (iv) pharmacogenetic studies that point to a metabolic potential for adverse drug–drug interactions. With this information in hand, a more complete understanding of the potential for drug toxicity and adverse drug–drug interactions of a drug candidate can be assessed. However, it is likely the situation is much more complex because the biology and molecular genetics are more complex than we currently understand. To underscore this point, paradoxically, drugs of significant clinical utility that possess undesirable properties (e.g., covalently modify proteins or induce or inhibit P450) are still on the market. It is possible that the delicate and complex balance between drug candidate metabolic bioactivation and detoxication is at play under this situation in ways that we still do not fully appreciate.

While the use of combinatorial chemistry and large library screening for identifying “hits” of a pharmaceutical target in the early stages of a drug screening campaign has led to an increase in the number of compounds evaluated, many times the “hits” are of low quality from the standpoint of pharmaceutical properties (i.e., solubility, permeability, bioavailability, and chemical and metabolic stability). A key aspect of drug development is to identify compounds with ADMET and physiochemical property deficiencies as early as possible in the drug development pipeline to advance the candidates possessing the properties leading to the greatest likelihood of success. Two key developments that have spurred advances in the area of ADMET and physiochemical or pharmacokinetic properties have been in the area of genetics (e.g., genotyping to decrease idiosyncrasies) and advances in bioanalytical chemistry (e.g., LC-MS/MS to detect and quantify metabolites), respectively. However, even extensive knowledge of the enzymes involved and the types and quantities of metabolites formed do not completely address deficiencies in understanding their role in toxicity. In addition to the parent drug, the issue of the role of metabolites in the toxicity of drug candidates has emerged in the discussion to explain possible adverse drug-induced toxicities [30]. Generally, the type of metabolite under consideration is a stable drug metabolite that

can be isolated or synthesized as opposed to chemically reactive metabolites or short-lived metabolites that cannot be readily isolated or synthesized. Part of the challenge to characterize the relevance of a metabolite stems from the question as to the level of metabolite formed (i.e., should the metabolite studied be formed at a level of 5%, 10%, or 20% or more of the parent?) and how much effort should be invested in evaluating the toxicity of the metabolite and which metabolites should be studied (i.e., should just human drug metabolites or both small animal and human drug metabolites be studied?) [31]. Nevertheless, for drug approval in the United States, it is likely that increased evaluation of human drug metabolites that are also particularly prevalent in small animals administered the parent drug will be required by the US Food and Drug Administration [32]. Thus, synthesis, characterization, and quantification of drug metabolites will take on an even larger role in drug development and safety evaluation in the future. Because often metabolite synthesis is a nontrivial undertaking, in the future, it may be that use of enzyme-based or semisynthetic approaches will play a larger role in producing difficult-to-obtain metabolites.

Improving the ADMET properties earlier in the drug development paradigm will continue to play a prominent role in preclinical drug discovery. It has been suggested that medicinal chemists would prefer to work in a lead compound series that possesses tractable pharmaceutical and physiochemical properties with lower potency than the other way around [33]. Said in another way, it is more difficult to reengineer or rescue a compound with inherently poor pharmaceutical, chemical, or ADMET properties than to start with a series that possesses good druglike properties and work to improve potency [34]. Another way to express this idea is by the concept of identifying candidates with undesirable properties early in the discovery process and discarding those candidates for the ones with more favorable ADMET and physiochemical properties [35]. Thus, greater resources can be expended on drug candidates with favorable properties and considerably less time and money on candidates with poor prognosis for success. Today, considerable progress has been made to attempt to explain the mechanistic basis for metabolism in the toxicity of drugs and carcinogens. Most of the effort has been directed at P450, and this is understandable in view of the large percentage of chemicals that are prominently metabolized by P450. However, the information is not complete and is lacking in non-P450 drug-metabolizing enzymes such as FMO. A more thorough knowledge of FMO- and other non-CYP-mediated metabolism could provide additional understanding concerning predictions about metabolism, toxicity, and human drug development [36]. Accordingly, this chapter summarizes recent advances related to FMO and drug development with the hope that some of the information will be useful to solve problems and develop new approaches in the use of drug metabolism in drug development.

10.4 ACADEMIC DRUG DEVELOPMENT USING ADMET

With the advent of the NIH Roadmap and funding of university and research institute high throughput molecular probe discovery (<http://pubchem.ncbi.nlm.nih.gov/>), considerably greater attention has been focused on probe and drug discovery in the nonprofit setting. However, in academia, application of ADMET concepts early in the probe or medications development approach has not been as widely utilized. The author suspects that as more and more academic laboratories begin to work on probe and

drug discovery and drug repurposing (i.e., drug rescue to improve a known drug that possesses a weakness or the improvement of a clinical candidate that needs removal of a deficiency to become a drug), application of ADMET concepts early in the drug discovery paradigm may come into vogue and result in more druglike materials from the academic setting. Out of economic necessity and because of the expense of large chemical libraries, the Human BioMolecular Research Institute (HBRI) has embraced using concepts of dynamic medicinal chemistry in its molecular probe and medications development programs [37]. This is because typical academic laboratories including HBRI do not have the financial or other resources for extremely large-scale library screening and pharmacological compound evaluation throughput that a typical for-profit entity has at its disposal. Dynamic medicinal chemistry is an iterative process of recursive chemical synthesis, testing, and optimization by utilizing ADMET principles early in the discovery phase to focus on relatively modest-sized “smart libraries” of druglike compounds that have a much greater chance of surviving and possessing suitable properties for *in vivo* efficacy testing. At HBRI, progress has been made turning high throughput screening “hits” into drug leads with a very modest budget [38,39]. Synthesizing “smart libraries” of druglike materials early in the preclinical drug development paradigm could in principle lead to more favorable candidates with fewer liabilities. The goal of this approach is not to predict the toxicity of a series of compounds but rather to avoid compounds that could afford toxicity and develop more promising candidates in the first place. Such candidates would be more likely to be useful in preclinical animal model efficacy studies because they are designed on the basis of ADMET properties to possess appropriate pharmacophores that are associated with greater bioavailability and other favorable pharmaceutical properties. To illustrate this point, an example from one of our in-house NIH Roadmap Projects is considered. Figure 10.1 shows the structure of a potent nuclear factor-kappa B (NF- κ B) inhibitor.

NF- κ B is a member of the family of transcription factors that play a critical role in many pathways including host defense, immune responses, inflammation, and cancer. The NF- κ B pathway activated by antigen receptors is critical for acquired (as opposed to innate) immunity and contributes to T- and B-lymphocyte activation, proliferation, survival, and effector functions. Dysregulated NF- κ B activation in lymphocytes can contribute to the development of autoimmunity, chronic inflammation, and lymphoid malignancy [40,41]. The NF- κ B activation pathway linked to antigen receptors involves a cascade of adapter and signal-transducing proteins, which minimally includes Carma1, Bcl-10, Paracaspase (MALT1), TRAF6, and Ubc13. In T and B cells, the NF- κ B pathway is initiated by protein kinase C (PKC)- θ and PKC- β , respectively, that induces phosphorylation of components of this signaling pathway,

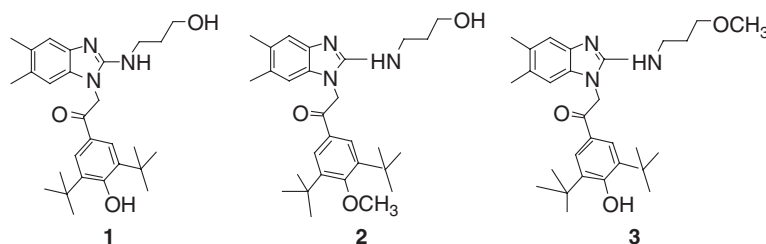


Figure 10.1 Structure of NF- κ B inhibitor **1** and analogs **2** and **3**.

leading ultimately to inhibitor of $\kappa\beta$ Kinases (IKKs) activation through a mechanism involving IKK- γ [42]. Dysregulated antigen-receptor-mediated NF- κ B activation can contribute to the development of autoimmunity, chronic inflammation, and malignancy. A chemical biologic screening strategy for identification of chemical compounds that selectively inhibit antigen-receptor-mediated NF- κ B activation was devised and implemented [39]. Chemical libraries comprising a total of 110,000 compounds (http://mlsmr.glpig.com/MLSMR_HomePage/) were screened for inhibitors of antigen-receptor-mediated NF- κ B activation. Compounds with an $IC_{50} < 3 \mu M$ were further characterized by 12 additional counter-screens that helped determine inhibition selectivity. From the initial screens, three hits with IC_{50} values $< 2 \mu M$ were identified and confirmed as selective for the antigen receptor activation pathway by the counter-screen assays. Of the three hits, benzimidazole (**1**) (Fig. 10.1) was found to be a highly potent and selective inhibitor of antigen-receptor-mediated NF- κ B activation.

Because the next goal was to test the NF- κ B inhibitor *in vivo*, and because a few sites of **1** could be prone to metabolism and decrease *in vivo* potency, we examined **1** and a few analogs for metabolic stability. As is standard in our dynamic medicinal chemistry approach, *in vitro* metabolic stability studies in the presence of both human liver microsomes (HLMs) and S9 and a reduced NADPH generating system was done with compounds **1**, **2**, and **3**.

In vitro metabolic stability studies showed compounds **1–3** were quite stable to hepatic metabolism. Generally, if a compound has a half-life of > 60 min in these types of *in vitro* metabolism studies, it is judged sufficiently stable to move the compound into *in vivo* studies. Of course, if Phase II conjugation metabolites could be formed, addition of the appropriate cofactor is necessary to observe optimal phase II metabolism. Another possible explanation for metabolic stability of **1–3** was that **1**, **2**, or **3** inhibited its own metabolism. This was a distinct possibility because imidazole-containing compounds are known to inhibit P450. However, significant inhibition of P450 3A4-mediated microsomal hydroxylation of testosterone by **1**, **2**, or **3** was not observed [38], suggesting that inhibition of its own metabolism was not occurring. However, it is possible that some of the metabolic stability of compounds **1–3** was due at least in part to inhibition of other P450s that are more prominent in the metabolism of **1–3**.

That metabolic deficiencies do not appear to be present in **1–3** have thus encouraged more extensive biological studies of these compounds [39]. Metabolic liabilities have significantly contributed to the failure rate of NCEs as clinical candidates and because P450-mediated bioactivation has been associated with P450 inhibition and induction (two features that can confound clinical candidate advancement), improving the metabolism by distributing the metabolism of a drug candidate over a wider range of metabolic enzymes may have significant advantages. As described above for the NF- κ B inhibitor **1**, during lead evaluation and optimization, several steps were taken to improve the druglike properties of **1**, including testing for metabolic stability and inhibition of P450. Appropriate ADMET properties for **1** have afforded a compound that can be tested for efficacy *in vivo* and can possibly lead to a candidate with a favorable bioavailability profile. Even in an academic setting, employing concepts in drug metabolism and applying informed approaches for acceptable physiochemical drug properties can lead to elaborating more druglike materials with decreased potential for adverse drug–drug interactions. This can ultimately lead to biological probes or drug candidates with more favorable properties and to increased success rate for clinical candidates.

10.5 ENHANCING DRUG CANDIDATE SELECTION

Because many examples of drugs with adverse drug–drug interactions have been reported for drugs that are prominently metabolized by one enzyme system (e.g., P450 3A4), one potential strategy to avoid this situation is to distribute the metabolism of a drug over a wide variety of monooxygenases including FMO. In principle, this could also lead to a decrease in potential toxicity, if toxicity was related to one major metabolite. One of the purposes of this chapter is to point out examples where optimization of the role of FMO in drug metabolism and drug design could lead to advantages in drug development owing to decreased toxicity and decreased adverse drug–drug interactions. One recent example comes from the development of a multitargeted Src kinase inhibitor called TG100435 by TarGen, Inc., (San Diego, CA). Src is dysregulated in several types of cancers and is involved in tumor progression and metastases [43]. Anticancer activity was observed in the parent drug, TG100435, **4**, and its tertiary amine *N*-oxide metabolite, **5** (Fig. 10.2).

The tertiary amine *N*-oxide metabolite was formed by FMO in preclinical species including human, dog, rat, and mouse liver. FMO-mediated formation of tertiary amine *N*-oxides is not that unusual, but it is unusual that the *N*-oxide possessed potent anti-cancer potency. Generally, polar, readily excreted *N*- or *S*-oxide metabolites arising from the action of FMO possess less biological activity than the parent compound. This is because *N*- or *S*-oxides, being polar metabolites, tend to part away from lipophilic centers that are often associated with molecular drug targets. However, *N*- or *S*-oxides can be retroreduced to their parent compounds, and this complicates matters somewhat insofar as determining which compound is the active species. In the

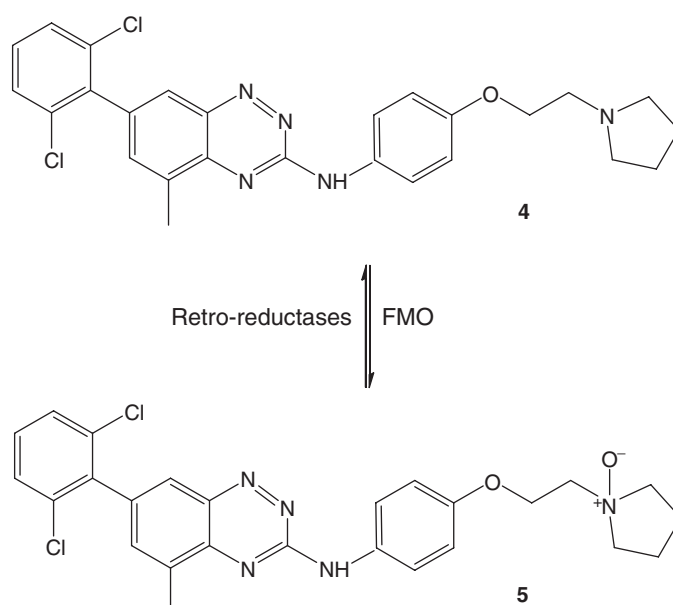


Figure 10.2 Chemical structure of the Src kinase inhibitor, TG100435 (compound **4**), and its tertiary amine *N*-oxide, TG100855 (compound **5**).

case of TG100435, *N*-oxygenation and *N*-oxide retroreduction plays a large part in its metabolism. FMO, and not P450, is apparently reported to be solely responsible for formation of the *N*-oxide, TG100855, although P450 3A4 contributed to other parent compound metabolism [44]. This conclusion was based on studies using some of the standard methods currently used to distinguish FMO from P450-mediated metabolism, including (i) use of microsome preparations enriched in P450 and/or FMO, (ii) incubation with selective inhibitors of P450 or alternate competitive substrate inhibitors of FMO, (iii) thermal inactivation studies in the presence or absence of NADPH under conditions that largely abrogate FMO activity and retain P450 functional activity, and (iv) use of highly purified recombinant P450 and FMOs [12]. That the *N*-oxide TG100855 was observed as a prominent metabolite followed from investigations of the kinetics of *N*-oxygenation versus *N*-oxide retroreduction back to the parent amine. In microsomes, *N*-oxide retroreduction was 15-fold slower than FMO-mediated *N*-oxygenation.

To examine the metabolic retroreduction of TG100855, compound **5**, to its parent, (i.e., TG100435, compound **4**), several approaches were tried. The experimental approaches included (i) synthesizing the authentic *N*-oxide metabolite and incubating it with liver microsomes in the presence or absence of NADPH, (ii) incubating the *N*-oxide in heat-inactivated liver microsomes to show that FMO was not responsible for *N*-oxide retroreduction, (iii) incubating the *N*-oxide in the presence of recombinant P450 with or without P450 inhibitors to show that P450 was not responsible for *N*-oxide retroreduction (in fact, the P450 inhibitor 1-aminobenzotriazole (ABT) actually increased retroreduction from three- to fourfold), and (iv) varying the amount of P450 reductase present in the microsomal and P450 incubations, showing that retroreduction was tracked with the P450 reductase amount (although recombinant P450 reductase in phosphate buffer alone did not cause retroreduction of **5** to **4**). Because P450 reductase alone did not retroreduce **5** to **4**, additional factors, presumably involving other cofactors or microsomal or lipidoidal factors, are involved in retroreduction of **5**. In addition, the results did not indicate whether other enzymes associated with retroreduction (i.e., aldehyde oxidase, quinone reductase, or other hemoproteins) contribute to *N*-oxide retroreduction of **5** [44]. Regardless, this is the example of a futile metabolic cycle involving retroreduction of an FMO metabolite that may be very useful from a clinical standpoint.

10.6 FMO IN DRUG CANDIDATE SELECTION

To date and to the author's knowledge, there have been no published reports specifically utilizing FMO in a drug candidate selection process. However, there have been several drugs that have been recognized as having FMO as a prominent contributor to their metabolic profile and more often than not, this has resulted in a useful outcome or at least an advantage from the standpoint of more favorable metabolism or decreased adverse drug–drug interactions. Utilizing functional groups in drug development candidates that are metabolized by FMO may have some advantages compared to a molecule predominantly metabolized by P450 (and, in particular, P450 3A4). This is because in contrast to P450, FMO is not readily inhibited or induced. Accordingly, drug candidates that avoid prominent P450 metabolism may possess more attractive pharmacokinetics and have a better safety profile. Generally, FMO is not readily inactivated but can undergo alternate substrate competitive inhibition by chemicals that

contain functional groups with low K_m values and are readily oxygenated by FMO (i.e., thiones, thioureas, thioamides, and thiols), but these functional groups are not commonly present in drugs. True competitive inhibition of FMO is rare. In contrast to P450, no reports of a time-dependent irreversible inactivator of FMO have appeared in the literature. In rare cases, apparent FMO inhibition is actually the result of interference with the NADPH cofactor-binding site. One example of this is inhibition of FMO by indomethacin [45]. For nitrogen-containing functional groups that are widely present in drugs, frequent alternate substrate competitive inhibition is not observed probably because the commonly present nitrogen-containing functional groups in drugs (i.e., tertiary and secondary amines) generally do not possess K_m values that are low enough for FMO, which would afford apparent inhibition. In contrast to P450, the lack of apparent inhibition of FMO may contribute to decreased adverse drug–drug interactions for drugs prominently metabolized by FMO because a detoxication role of FMO is unaffected.

A few reports of FMO induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment have been noted in mice [46,47]. In addition, in experimental small animal models of diabetes, significant increases in FMO have been observed [8], and in one study, elevated levels of FMO were restored to normal after treatment with insulin in the diabetic rat [48]. In another study, significant downregulation of FMO was observed in different mouse models of inflammation [49]. However, the general lack of extensive examples of induction of FMO also suggests that compared with inducible forms of P450, adverse drug–drug interactions will also be less for drugs mainly metabolized by FMO. Because human FMO is not readily induced or inhibited, it is likely that most of the variation in metabolism due to human FMO is not dependent on environmental factors but on genetic polymorphisms. Because the pharmacogenetic basis for FMO variability has been described in recent reviews [8,50], it is not discussed further in this summary, except to highlight some of the functional consequences of drug metabolism.

There are several examples of currently used drugs that are extensively metabolized by FMO and show significant differences in efficacy or bioavailability apparently due to FMO genetic polymorphisms. For example, the prodrug sulfoxide (sulindac) is retroreduced to sulindac sulfide, which is an anti-inflammatory agent and sulfide substrate of FMO [51]. Retro-S-oxygenation of sulindac sulfide decreases the efficacy of the anti-inflammatory agent in familial adenomatous polyposis (FAP) [52]. Individuals with a genetic polymorphism of FMO3 have a defective ability to convert the active sulfide to the inactive *S*-oxide prodrug, resulting in greater concentrations of the active agent. Those individuals with FMO3 genetic polymorphisms show greater efficacy in the treatment of FAP [53].

Two examples of an FMO3 genetic polymorphism that decreases metabolism and increases the efficacy of drug administration stem from the N-oxygenation of ranitidine [54] and the antiulcer agent and benzydamine [55]. Tamoxifen is an example of a tertiary amine substrate of FMO3 that shows an interesting relationship between tissue-selective metabolism and toxicity [56]. In the adult human liver, where P450 is primarily responsible for tamoxifen hydroxylation, a sulfate metabolite that is produced covalently labels hepatic DNA and produces cellular toxicity. Because FMO1 preferentially N-oxygenates tamoxifen and is present in the kidney but not in the adult liver, detoxication by FMO1 protects the renal tissue from covalent binding of tamoxifen metabolites. The lack of FMO1 in the adult human liver predisposes the adult liver to P450-mediated bioactivation and covalent binding.

Itopride is another example of a second-generation drug that was reengineered to decrease a deficiency and optimize its drug efficacy [57]. Itopride is a gastrokinetic agent that is related to mosapride. In contrast to mosapride that is predominantly metabolized by P450 3A4, itopride is primarily converted to the tertiary amine *N*-oxide by FMO3. Because itopride is not metabolized by P450 3A4, it does not engage in adverse drug–drug interactions that have been described for mosapride [58].

Finally, a considerable number of reports have shown a relationship between FMO3 genetic polymorphisms and defective trimethylamine *N*-oxygenation [59–61]. Conversion of trimethylamine (TMA) to its *N*-oxide is a detoxication and deodoration process resulting in the formation of a polar, readily excreted metabolite. For certain individuals with causative FMO3 mutations, trimethylamine is not *N*-oxygenated and accumulates and causes the individual to possess an offensive odor. It is notable that no apparent retroreduction of trimethylamine *N*-oxide occurs. This may have been an important evolutionary advantage to increase the clearance of trimethylamine from the body. What is more likely is that the various reductases that retroreduce tertiary amine *N*-oxides to the tertiary amine do not accept a polar material such as trimethylamine *N*-oxide (TMA *N*-oxide). Regardless, defective TMA metabolism has provided a keen insight into the functional aspects of FMO because the naturally occurring genetic mutations have shed light on amino acids in FMO3, which are critical for function.

10.7 FMO-MEDIATED METABOLISM: STRONG NUCLEOPHILES

10.7.1 Thiones

Electron-rich highly polarizable nucleophilic functional groups are generally accepted as substrates for FMO [62]. Barring steric effects that limit approach to the substrate-binding domain of FMO, highly nucleophilic thiones, thioamides, thioureas, thiocarbamides, thiocarbamates, and the like are the substrates most efficiently *S*-oxygenated by FMO. The same is also likely for nucleophilic phosphorus- and selenium-atom-containing counterparts, but this has not been exhaustively examined. This substrate selectivity can be most easily understood in terms of FMO-accepting lipophilic highly polarizable nucleophilic compounds as substrates. A distinction between endogenous substrates and dietary substrates should be made for both *N*- and *S*-containing compounds metabolized by FMO. As described previously [2], an endogenous substrate (e.g., tyramine) or a substrate that results from a conjugate (e.g., cysteine conjugate) is synthesized in the body. Dietary constituents (e.g., alkaloids or plant-derived sulfides) are not synthesized in the body but derived from external sources. Sometimes an FMO substrate could come from both sources (e.g., phenethylamine or trimethylamine), and the definition of endogenous and dietary substances is somewhat ambiguous. Previously, the role of the metabolism of endogenous sulfur-containing substrates by FMO has been summarized [2]. Highly nucleophilic *S*-containing compounds can be substrates for FMO, including *S*-cysteine conjugates (i.e., mercapturic acids). Generally, however, mercapturates of xenobiotics are sufficiently polar to be excreted *per se*. It may be that highly lipophilic *S*-cysteine conjugates require additional metabolism by FMO to increase metabolite polarity for excretion [63]. Because the FMO oxygenation mechanism utilizes a peroxyflavin intermediate, if a sulfur-containing compound is treated with hydrogen peroxide in an enzyme model condition and produces an *S*-oxide

product, it is likely (barring steric limitations) that the compound will be a substrate for FMO. From the standpoint of drug development, such S-oxygenated thione products also have potentially interesting pharmaceutical and metabolic properties. Once formed, an S-oxide often possesses sufficient nucleophilicity to be S-oxygenated a second time, thus forming a thione S,S-dioxide. Generally, thione S,S-dioxides are more electrophilic than a thione S-oxide and can undergo reaction with nucleophiles, although thione S-oxides sometimes possess sufficient electrophilicity to be attacked by nucleophiles at the thione carbon atom. This has implications for predicting the toxicity of thione-containing compounds and their S-oxygenated metabolites. Often, if toxicity is observed with thione-containing compounds, it is associated with nucleophilic attack on electrophilic S-oxygenated metabolites and not necessarily with the parent compound. Neighboring atoms can control the electrophilicity of the oxygenated sulfur atom by either electron donation or electron withdrawal. For example, primary or secondary thioamides are more likely to form S,S-dioxides than simple thioketones because the neighboring nitrogen atom increases the nucleophilicity of the sulfur atom on the basis of resonance properties [64]. The thioamide nitrogen atom also helps stabilize the incipient S,S-dioxide by hydrogen-bonding interactions. Evidence for certain thione S-oxides (e.g., carbon disulfide) to form cyclic structures with the extrusion of elemental sulfur has been reported via a three-membered intermediate [65].

One important consideration in the overall metabolism, disposition, and toxicity of sulfur-containing compounds is the retroreduction of the S-oxide metabolite. An initial S-oxide metabolite of a thione is also electrophilic and can be attacked on the sulfur atom by thiophiles (Fig. 10.3).

An attack by a thiol generally results in the formation of a disulfide and liberation of water, and often the disulfide is converted to the parent compound (Fig. 10.3). Thioamide S-oxides can be retroreduced, and this process is also apparently dependent on the electron-donating versus electron-withdrawing nature of the neighboring groups. For example, the electron-deficient *para*-trifluoromethyl thiobenzamide S-oxide is metabolically retroreduced to its thioamide more rapidly than the electron-rich *para*-methoxy thiobenzamide S-oxide [64]. The oxygenation–reduction dynamic plays an important role in the potential for thione-mediated toxicity. For thione S-oxides that are efficiently retroreduced, less metabolite is available to proceed to the more electrophilic (and hence potentially more toxic) S,S-dioxide. A generality often observed is that decreased S,S-dioxide metabolite formation likely results in less potential for toxicity. Thioketone-containing compounds as well as other sulfur-containing compounds are also subject to autooxidation and from an analytical standpoint, this can be very challenging. In any case, it is important to clearly distinguish enzymatic from autooxidative processes in any metabolic incubation or biotransformation process.

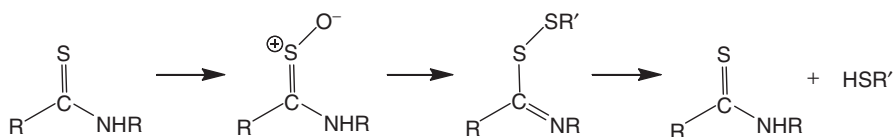


Figure 10.3 S-Oxygenation of a thioamide to the sulfoxide, and thiophile attack by HSR' to ultimately reform the thioamide.

10.7.2 Thioamides

Thioamides are among the most efficiently metabolized substrates of FMO because of their pronounced nucleophilicity. But thioamide functionality generally does not appear in drugs to a great extent because of the potential for toxicity. Oxidative bioactivation of thioamides represents an example of a functional group that can produce toxicity, but this is dependent on a number of factors including steric constraints and electronic properties. S-Oxygenation of thioamides involves two sequential steps that afford *S*-oxide and subsequently *S,S*-dioxide as major metabolites (although the latter is generally too unstable to isolate and characterize). Thioamide S-oxygenation may be mediated by both FMO and P450s. An initially formed thioamide *S*-oxide metabolite is often relatively stable and in many cases isolable. Thioamide *S*-oxides possess capacity for toxicity because they are electrophilic but more likely because they are one step closer to a subsequent metabolite (i.e., thioamide *S,S*-dioxide) that is extremely chemically reactive. Thioamide *S*-oxides possess some potential toxicity in their own right because they are electrophilic enough to be attacked by thiophiles to form an imino disulfide (Fig. 10.3).

Reduction of the imino disulfide by glutathione would result in the formation of the parent thioamide but is wasteful to the cell because of the consumption of NADPH. In addition, the cell could become susceptible to toxicity under conditions where glutathione depletion was to occur. However, not all thioamide *S*-oxides are toxic likely owing to steric factors that prevent thiophile attack or decrease formation of *S,S*-dioxide formation [64]. Another factor that comes into play concerns the nucleophilicity of the *S*-oxide sulfur atom determined by neighboring electronic effects. If an *S*-oxide is relatively non-nucleophilic or electron deficient, the *S*-oxide sulfur atom can be the site for reduction. As discussed earlier, thioamides are generally not present in drugs to a great extent. However, some examples of second-line antitubercular drugs are available, including ethionamide and thiacetazone (a hydrazinecarbothioamide), that are sequentially S-oxygenated by the mycobacterial enzyme EtaA [66] as well as mammalian FMOs [67]. Like other thioamide *S*-oxides, ethionamide *S*-oxide has greater biological activity than ethionamide, but the observation of hepatotoxicity has limited its use clinically.

10.7.3 Thiols

Thiols are highly nucleophilic functional groups that probably do not occur extensively in drugs because of rapid metabolism. Thiols are substrates for FMO and are initially converted to the sulfenic acid (RSOH) [68]. Once formed, sulfenic acids can be attacked by thiophiles such as glutathione and rapidly converted to disulfides in the presence of thiols. Sometimes the disulfides are S-oxygenated one or more times to produce materials that can undergo additional transformations, both oxidative and reductive. In the case where the thiol that reacts with a sulfenic acid metabolite is glutathione, a mixed disulfide is formed, representing a detoxication process. The corresponding *S*-glutathione conjugate that is formed can be metabolized to a mercapturic acid. However, in the case where thiol S-oxygenation and glutathione attack are extensive, glutathione depletion can occur, and this may have toxicological consequences because glutathione depletion allows other electrophilic metabolites to accumulate. In the presence of depleted glutathione, additional metabolites that are sufficiently electrophilic could covalently modify important nucleophiles and lead to cell damage and



Figure 10.4 S-Oxygenation of thiol RSH to sulfenic acid, and retroreduction to the thiol via a disulfide.

eventually cellular necrosis. As discussed above, disulfide metabolites can be further oxygenated by FMO or other oxidases. In addition, disulfides can be reduced. This is another example of a futile metabolic cycle because the parent thiol is regenerated (Fig. 10.4).

In the case of FMO-mediated thiol S-oxygenation and subsequent reduction of the disulfide, the overall process consumes NADPH and potentially leads to loss of glutathione. Under such cellular conditions, glutathione needs to be biologically resynthesized, and hence, this overall process is physiologically wasteful. Disulfide S-oxygenation could also produce an electrophilic center (i.e., R-S-(O)-S-R) that is susceptible to further S-oxygenation and/or reduction. In addition, S-oxygenation of either a thiol or a disulfide affords susceptibility to displacement by another thiol (i.e., disulfide exchange). If the attacking thiol is a protein thiol, this could lead to protein disulfide formation and potentially could result in toxicological (or immunological) consequences. Disulfides arising from oxidation of thiol-containing xenobiotics that are recognized as foreign can elicit immune reactions and immunotoxicity. However, disulfides can be utilized as prodrugs in drug development. Because disulfide reduction in the body is relatively facile, thiol drugs can be liberated relatively efficiently. However, disulfide prodrugs have not been utilized that extensively, quite possibly because of the downstream metabolism outlined above. Thiols can be enzymatically S-methylated by an S-methyltransferase, and this tends to prolong the lifetime of a thiol-containing drug because of the types of metabolism available to the S-methyl functional group described below. An S-methyl metabolite is a thioether and, as discussed below, has attractive physiochemical and drug metabolism properties compared to the corresponding thiol drug. There are a few examples of endogenous thiol-containing substrates for FMO. Cysteamine is S-oxygenated to a sulfenic acid that is attacked by another molecule of cysteamine to produce the disulfide cystamine [69]. Currently, the physiological role of cysteamine S-oxygenation by FMO is not known but it could be involved in protein disulfide isomerization or FMO could have evolved as a general cellular protectant to ward the cell from reactive oxygen species by forming mixed disulfides [70]. It is interesting to speculate that cysteine conjugates of S-oxidative electrophiles produced by FMO (e.g., cysteamine) could serve as the necessary precursor for biosynthesizing glutathione because cysteine is an essential precursor that is transported into the cell to elevate cellular glutathione levels [2], and glutathione requires de novo synthesis after it is exported from the cell.

10.7.4 Thioethers

Thioethers possess many favorable druglike properties, and it is for this reason that the thioether functional group appears in many drugs. Replacement of a thiol with a thioether moiety in a drug candidate generally increases the lipophilicity of the parent drug, and because thioethers are typically not exceptional hydrogen-bond participants, this feature also contributes to improved cell permeability and bioavailability compared to the corresponding thiol. In addition to favorable physiochemical properties,

thioethers have beneficial metabolic properties. Barring steric constraints, lipophilic thioethers are generally good substrates for FMO, and S-oxygenation affords sulfoxides. When the two substituents adjacent to the sulfide are not the same, an enzymatically prepared *S*-oxide metabolite can possess a center of chirality [71]. Often, FMO catalyzes *S*-oxide formation with significant stereoselectivity. Of course, nonenzymatic oxidation (or autooxidation) of a sulfide with two different substituents can produce a racemic sulfoxide. In some cases, use of prochirality of a sulfide that produces an *S*-oxide with a center of chirality can provide information about the enzymatic process because often P450 and FMO work with opposite stereoselectivity [71]. Enzymatically formed *S*-oxides are zwitterionic in character and tend not to penetrate cells as efficiently as their parent sulfides. Consequently, sulfoxide metabolites can accumulate in cells and tend to have favorable cellular distribution if the target of the parent sulfide is inside the cell. This is because retroreduction of a sulfoxide inside the cell can elevate the relative concentration of a drug inside the cell. *S*-Oxides can be retroreduced to their sulfides, and this constitutes another example of a futile metabolic cycle. Aldehyde oxidase has often been implicated as the reductase for this process [72]. After retroreduction of an *S*-oxide to a sulfide, enzymatic reoxygenation completes the cycle and again tends to facilitate accumulation inside the cell, thus prolonging the action of the drug within the cell. In addition to retroreduction, *S*-oxide metabolites can be further oxidized to sulfones. Sulfone formation has been associated with FMO, P450, and other oxidases probably because the nucleophilicity is not always sufficient for FMO *S*-oxygenation. For FMO, the propensity for *S*-oxygenation of an *S*-oxide to a sulfone depends on the nucleophilicity of the *S*-oxide sulfur atom. In the case of P450, formation of sulfones from *S*-oxides involves additional considerations that are not easy to predict.

Sulfones tend to be relatively benign metabolites and are not reduced to their parent sulfides. However, there are examples of sulfones of aryl sulfides that participate in additional chemical and enzymatic transformations including displacement reactions. Unlike sulfoxides, sulfones are not charged and do not accumulate in cells to as great an extent compared with their corresponding sulfoxides. Sulfones do participate in extensive hydrogen bonding and thus possess slightly different permeability properties than sulfides.

Where a center of chirality can be formed on *S*-oxygenation, an investigation of the stereochemistry of the *S*-oxide itself should take place to rule out the role of autooxidation. In this regard, a chemical or biochemical method to produce sufficient amounts of the chiral *S*-oxide is desirable so that the chiral *S*-oxide can be incubated with the metabolic system to examine retroreduction and serve as a control for possible racemization. In the case of the sulfide cimetidine, the issue of enzymatic versus nonenzymatic oxidation was examined in some detail [73]. Cimetidine *S*-oxide is a prominent metabolite formed by FMO in humans. Although not explicitly studied, there is some evidence that cimetidine *S*-oxide is retroreduced to the sulfide or undergoes interhepatic recycling because pharmacokinetic studies in some humans show a biphasic profile [73]. It is notable that even HPLC grade solvents contain oxidizing agents, and solvents used in analytical work related to chiral *S*-oxide analysis and the like should be scrupulously treated to remove oxidizing agents, so that the analytical procedure does not contribute to confounding the determination of stereochemistry of the metabolite examined. As a practical matter, chiral HPLC and similar approaches

can be used to examine the contribution of enzymatic versus nonenzymatic oxidation as well as the stability of the *S*-oxide chiral center [71].

10.7.5 Sulfoxides

As discussed above, both P450 and FMO contribute to further oxidation of sulfoxides. One sulfoxide-containing chemical widely used in the drug discovery industry is dimethylsulfoxide (DMSO) and is worthwhile to discuss in some detail. With the advent of high throughput screening, DMSO has become a widely used vehicle to administer compounds for both *in vitro* and *in vivo* evaluation. This is because many compounds or closely related congeners from high throughput screening campaigns do not possess good solubility properties but are soluble enough in DMSO for *in vitro* evaluation or *in vivo* administration. The issue with *in vivo* administration of DMSO is that it is rapidly retroreduced to dimethylsulfide (DMS), which is an excellent substrate for FMO and is efficiently *S*-oxygenated by FMO. This sets up a metabolic futile cycle [74]. In addition, at the doses typically used as a vehicle for *in vivo* studies, DMS saturates FMO and effectively inhibits the enzyme as an alternate substrate competitive inhibitor. Thus, after administration to an animal, DMSO sets up a futile cycle, but because DMS is such a good FMO substrate, it effectively abrogates contribution to metabolism of most compounds coadministered with DMSO because (as discussed below) many of these compounds are amines. Thus, administration of DMSO (e.g., 1 mL/kg) to a small animal serves as a chemical knockdown that is similar to the more eloquent molecular knockout of FMO recently reported [75]. Generally, but not always, amines are poorer FMO substrates than DMS. From the perspective of evaluating the contribution of FMO in the metabolism of an amine drug candidate coadministered with DMSO, this represents quite a confounding situation. Likely, coadministration of an amine with DMSO will significantly decrease FMO-dependent metabolism of the amine. In addition, futile metabolic cycling of DMSO/DMS may also influence other NADPH-requiring metabolic pathways because of the consumption of NADPH in the reoxygenation of DMS. However, to my knowledge, this has not been extensively examined.

10.7.6 Amines

As a generality, nitrogen-containing functional groups are less nucleophilic than sulfur-containing ones, and while amines are well-recognized substrates for FMO, they tend to be *N*-oxygenated less efficiently than sulfur-containing substrates. Hence, avidity of an amine to FMO tends to be less, and, as a generality (although there are notable exceptions), K_m values tend to be greater for tertiary amines than for sulfur-containing FMO substrates. There are notable exceptions where amines are excellent substrates for FMO. In fact, the original name for FMO was *N,N*-dimethylaminoaniline-*N*-oxidase because *N,N*-dimethylaminoaniline was such a good substrate for the originally isolated FMO from pig liver [76]. Today, we recognize that FMOs *N*-oxygenate primary, secondary, and tertiary amines, depending on the form of the enzyme. Because many drugs employ amine functionality as an intrinsic part of their pharmacophoric composition, evaluation of the role of FMO in the metabolism of amines constitutes an important endeavor.

10.7.7 Hydrazines

Hydrazines comprise a group of highly nucleophilic nitrogen-containing compounds that are substrates for FMO. Metabolism of hydrazines by FMO illustrates a number of important points. Because of the lone pair on the adjacent nitrogen atom, the nitrogen atom that is oxygenated possesses increased nucleophilicity. This is the so-called alpha effect of the hydrazine nitrogen atom to oxygenation via a through-space lone pair–lone pair assisted mechanism. The alpha effect is also present in other molecules that have heteroatoms alpha to a nucleophilic center (i.e., hydroxylamines). Of course, the relative alpha effect for substituted hydrazines is dependent on the substituent pattern and the degree of electron-withdrawing or electron-donating properties of the neighboring substituents. For FMO-mediated N-oxygenation of substituted hydrazines, the extent of N-oxygenation may include some steric considerations. Generally, the tertiary nitrogen atom of a hydrazine is the site of N-oxygenation by FMO [77]. However, hydrazines are among the most toxic substances known, and extensive studies examining the role of human FMO in their metabolism have not been reported. Nevertheless, depending on the substitution pattern of the hydrazine, N-oxygenation could lead to several unusual azo metabolites and some of the metabolites could contribute to the toxicity of these compounds.

10.7.8 Hydroxylamines

Primary and secondary aliphatic amines are converted to hydroxylamines by FMO [17–19]. Generally, aliphatic hydroxylamines are more nucleophilic than the parent amine (because of the alpha effect), and often, they are N-oxygenated a second time to produce an *N,N*-dihydroxy intermediate that is not stable (Fig. 10.5).

In the case of primary amines, elimination of the elements of water produces an oxime. Both *cis*- and *trans*-oximes are capable of being formed from FMO, but often considerable stereoselectivity is observed [19]. Because loss of water from the *N,N*-dihydroxy intermediate is a spontaneous reaction and not strictly speaking enzyme

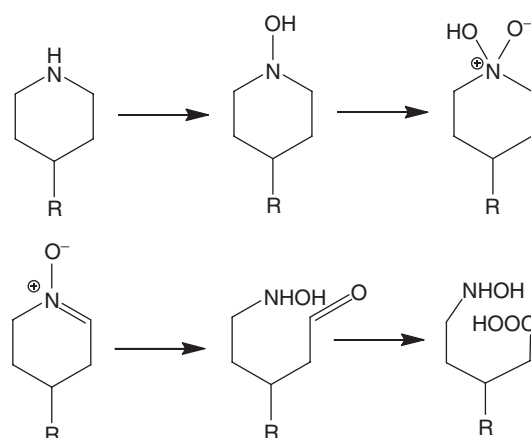


Figure 10.5 Sequential N-oxygenation of a cyclic secondary amine, with eventual ring opening and oxidation to the carboxylic acid.

catalyzed, it is somewhat surprising that very often a great degree of stereoselectivity in the FMO-derived product is observed. The large degree of stereoselectivity observed for oxime formation has been interpreted as an FMO enzyme “template effect” on product formation because the final oxime-forming reaction is not apparently FMO catalyzed [17,18]. It is also somewhat surprising that in the formation and disposition of aliphatic hydroxylamines, this transformation can go on in the presence of the strongly reducing environment of the cell. Like tertiary amine *N*-oxides, hydroxylamines can be efficiently retroreduced to the parent amine [78]. While the enzymatic basis for retroreduction of hydroxylamines is somewhat controversial (i.e., some favor a system of cytochrome *b*₅, NADPH-cytochrome *b*₅ reductase, and a P450, while others favor a hydroxylamine reductase), the fact that oxime formation can out-compete retroreduction suggests that FMO-mediated formation of oxime is quite robust. Similarly, formation of nitrones from aliphatic secondary hydroxylamines suggests that N,N-dihydroxylation of secondary hydroxylamines by FMO can effectively compete with hydroxylamine retroreduction (Fig. 10.5). An exhaustive examination of primary and secondary hydroxylamine retroreduction has not been reported. It is likely that different retroreduction systems are at work for different hydroxylamines (and for different tertiary amine *N*-oxides). But obviously, N-oxygenation may play a much larger role in primary and secondary aliphatic amine metabolism than previously recognized, if retroreduction is a facile process. It may be that considerable futile cycling occurs in many cycles of amine oxygenation and reduction. Because in some cases, the ultimate metabolite arising from FMO-mediated processes is quite distinct from the starting amine, careful evaluation of the metabolic pathway should be considered before the relative contribution of FMO in the metabolism of an amine is adjudged. For example, after formation of a nitron from a secondary amine, unless the nitron is highly conjugated or stabilized in some manner, it is prone to hydrolysis to the corresponding aldehyde and hydroxylamine [79]. Generally, because aldehydes are converted to carboxylic acids that are conjugated and excreted, this type of metabolism presents quite a distinct product metabolite profile compared to the starting amine. In the case of cyclic secondary amines, even more complex metabolites can be formed because of the propensity for cyclization and intramolecular reactions of the metabolites that are formed (Fig. 10.6).

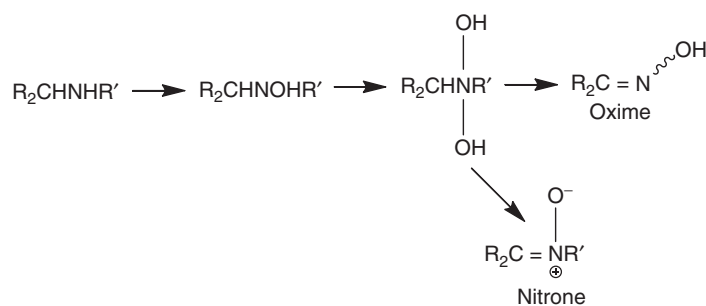


Figure 10.6 N-Oxygenation of primary ($\text{R}' = \text{H}$) and secondary ($\text{R}' = \text{substituent}$) amine to *cis*- or *trans*-oximes and a nitron, respectively, via the intermediacy of a *N,N*-dihydroxy intermediate.

Regardless, and in agreement with tertiary amine metabolism, the role of FMO in primary and secondary aliphatic amine metabolism is potentially quite complex. Further confounding the metabolism of primary and secondary aliphatic amines is the role of retro-reductase systems in hydroxylamine (or tertiary amine *N*-oxide) retroreduction. Additional studies are needed to clarify the oxidative and retroreductive nature of primary and secondary hydroxylamine metabolism.

10.7.9 Cyclic Amines

Certain cyclic tertiary amines such as piperidines, piperazines, morpholines, and tetrahydropyridines are commonly found in biologically active drugs and drug candidates. Cyclic amines such as these are often found in drugs and drug candidates because of their favorable pharmaceutical properties and the propensity of pharmacological targets to bind these types of tertiary amines with high avidity. It is a common drug design strategy to decrease the metabolism of aliphatic secondary and tertiary amines and increase their bioavailability by incorporating the secondary and tertiary amines as a cyclic amine. In addition, other proximal substituents appended to the nitrogen atom or ortho to the nitrogen atom in the ring often further decrease metabolism and increase bioavailability. Sometimes, by decreasing metabolism via *N*-demethylation (in the case of aliphatic tertiary amines), *N*-oxygenation mediated by FMO (in the case of cyclic tertiary amines) becomes a more prominent metabolic pathway. One of the reasons for this is that cyclic tertiary amines such as piperidines are preferentially *N*-oxygenated by FMO because of the increased nucleophilicity of the tertiary amine. In the case of piperidines, for example, the through-space influence of the neighboring nitrogen atom lone pair increases the nucleophilicity of the tertiary amine. This is analogous to a through-space α effect discussed earlier for hydrazines and hydroxylamines. However, cyclic tertiary amines are not impervious to further metabolism, including *N*-demethylation. Cyclic tertiary amine *N*-oxides can also enter into further metabolism after retroreduction. After *N*-dealkylation, a des-alkyl cyclic amine metabolite can also, in principle, be a substrate for FMO (although it tends to be a less avid substrate than cyclic tertiary amines), but cyclic secondary amines can sometimes participate more in extensive metabolism than cyclic tertiary amines. This is particularly true in comparison to cyclic tertiary amine *N*-oxides (that, once formed *in vivo*, often possess sufficient water solubility to be directly excreted in the urine). For cyclic secondary amines, the initial FMO-dependent metabolism produces a hydroxylamine that often undergoes *N*-oxygenation a second time to afford an *N,N*-dihydroxy metabolite because the hydroxylamine is more nucleophilic than the parent cyclic secondary amine (Fig. 10.6).

Of course, this assumes that facile retroreduction of the cyclic secondary hydroxylamine does not occur to provide the parent amine. Nevertheless, the *N,N*-dioxxygenated metabolite is not indefinitely stable and eliminates the elements of water to afford a cyclic nitron. Generally, unless highly conjugated, nitrones are unstable to hydrolysis and result in ring opening to produce an aldehyde and a hydroxylamine (Fig. 10.6). Typically, the aldehyde and hydroxylamine portions of the metabolite undergo further metabolism. Sometimes, the ultimate metabolites are quite distinct from the structures of the initial metabolites and are not obviously related to classical FMO-mediated products. Considerable care needs to go into deciphering some of these more complex metabolic profiles.

10.7.10 Pyrrolidines

Cyclic tertiary amines such as pyrrolidines are also often N-oxygenated by FMO. Like some of the six-membered heteroatom-containing compounds discussed earlier, the compact structure of a pyrrolidine creates a pinned-back nitrogen atom lone pair that markedly increases the nucleophilic nature of the tertiary amine (e.g., N-oxygenation of nicotine is an example of a probe substrate of human FMO3 [80]). Similar to piperidine *N*-oxides, pyrrolidine *N*-oxides are relatively stable and in the *in vivo* setting are often excreted unchanged because of the polarity of the molecule. In principle, pyrrolidine *N*-oxides can be metabolically retroreduced in animals but this may be dependent on the route of administration; for example, a pyrrolidine *N*-oxide (e.g., nicotine-*N*-1'-oxide) administered i.v. is excreted unchanged [81] in humans but nicotine *N*-oxide administered orally to small animals is retroreduced [82]. One possible explanation to this phenomenon is that bacterial reductases of the gut reduce nicotine-*N*-1'-oxide after oral administration. The relative polarity and stability of cyclic tertiary amine *N*-oxides contribute to the lack of toxicity in these types of metabolites. While no evidence was observed for retroreduction of nicotine-*N*-1'-oxide after administration to humans [81], this is likely not the case for other cyclic tertiary amine *N*-oxides *in vivo*. However, the polarity of the *N*-oxide generally decreases pharmacological activity observed for the parent amine, and these types of metabolites are generally not potent and are excreted in the urine. Although there are some notable exceptions that tertiary amine *N*-oxides possess pharmacologic activity, it is unclear whether this is due to retroreduction to the parent amine or functional activity of the tertiary amine *N*-oxide in its own right.

10.7.11 Aliphatic Tertiary Amines

Aliphatic tertiary amines have long been associated as the prototypical FMO substrate. Conversion of tertiary amines to their *N*-oxide results in polar, readily excreted metabolites that are usually devoid of pharmacological potency. However, the zwitterionic nature of tertiary amine *N*-oxides can in some cases bestow pharmacological function. Although not extensively examined, aliphatic tertiary amine *N*-oxides can be retroreduced to the parent tertiary amine [5]. This is another example of a futile metabolic cycle. As described, the overall process consumes NADPH and leads to the regeneration of the tertiary amine. It is likely that many more examples of retroreduction of aliphatic tertiary amine *N*-oxides to tertiary amines have not been reported or observed due to analytical challenges or lack of direct evaluation. To date, a number of retro-reductases have been reported for tertiary amine *N*-oxides [83], and examples for tamoxifen *N*-oxide include aldehyde oxidase, cytochrome reductase, P450, and other various hemoproteins (e.g., hemoglobin and catalase) [84]. The importance of aldehyde oxidase as a general tertiary amine *N*-oxide retro-reductase is notable [85] because its presence in small animals is gender specific (i.e., males $\gg$ females) and estimates of the relative amount of tertiary amine *N*-oxides can be confounded as a consequence of gender [86]. Thus, comparison of metabolism between both genders can provide circumstantial evidence for the formation of tertiary amine *N*-oxides as well as evidence for retroreduction. Further confounding the situation for retroreduction of tertiary amine *N*-oxides is the observation that many commonly used drugs inhibit aldehyde oxidase [87].

Certain tertiary amines are prone to nonenzymatic autooxidation, and this can confound analysis of products formed and contribution of enzymes to the process. In the

case where *N*-oxide metabolites can produce a stereochemical outcome (e.g., in the case of *cis*- vs *trans*-nicotine-*N*-1'-oxide), ruling out the involvement of autooxidation is important for correct stereoselectivity determination. To prevent autooxidation in *in vitro* metabolic experiments, antioxidants such as DETAPAC can be added to the incubation [11]. DETAPAC is superior to EDTA because EDTA can also serve as a prooxidant under certain conditions and exacerbate the autooxidation situation.

10.7.12 Aliphatic Secondary Amines

Aliphatic secondary amines can be *N*-oxygenated by FMO to produce the hydroxylamine. The hydroxylamine metabolite, being more nucleophilic, is often *N*-oxygenated again by FMO to produce a nitron (after loss of water) because the hydroxylamine is generally a better substrate for FMO than the parent amine. Generally, aliphatic nitrones are unstable to hydrolysis and afford the aldehyde that can be further metabolized to a carboxylic acid and other metabolites. Hydroxylamines are also efficiently retroreduced to the parent amine. In the case of FMO-mediated *N*-oxygenation, *N,N*-dioxygenation affords a metabolite (i.e., a nitron after elimination of water) that is not subject to ready retroreduction. However, the initially formed hydroxylamine is efficiently retroreduced. For FMO substrates that do not desorb from the enzyme surface, substrates can be converted to nitrones without the intermediacy of retroreduction because the second *N*-oxygenation is kinetically faster than the initial one. In cases where steric inhibition to hydroxylamine *N,N*-dioxygenation is apparent, hydroxylamine retroreduction can be observed and little net *N*-oxygenation observed. To experimentally examine the role of retroreduction, saturating amounts of another hydroxylamine (e.g., dimethylamine hydroxylamine) can be added to the incubation to enable study of the test agent hydroxylamine formation. Other approaches to examining the role of retroreduction in secondary amine metabolism include varying the NADH/NADPH cofactor because certain retro-reductases preferentially require NADH to NADPH [88].

10.7.13 Primary Amines

Aliphatic primary amines are *N*-oxygenated by FMO. To date, compared with FMO1, FMO3 has shown much greater activity as a primary amine *N*-oxygenase. The initially formed hydroxylamine is more nucleophilic than the parent amine and is *N*-oxygenated a second time to an *N,N*-dioxygenated material. The *N,N*-dioxide is not indefinitely stable and eliminates the elements of water to produce an oxime as a stable metabolite (Fig. 10.5).

For sterically unhindered aliphatic primary amines, generally, the hydroxylamines are not observed because the second *N*-oxygenation is more efficient than the initial hydroxylamine formation. It is possible that an enzyme template effect is in operation for FMO-mediated oxime formation where the intermediate does not desorb from the enzyme surface until the oxime is formed. Because dehydration is fast and nonenzymatic for primary amine *N,N*-dioxygenated intermediates, the relative stereochemistry of the oxime formed is probably determined by a template effect and not via true enzyme catalysis [17,18]. Somewhat surprising to the nonenzymatic nature of the oxime formation process is that some primary oximes are formed with great stereoselectivity. In the case where this has been studied in detail, considerable stereoselectivity has been observed for a number of primary amine substrates [17,18].

In other cases, much lower stereoselectivity has been observed [19]. It is not clear as to the precise substrate structural features required that affect great stereoselectivity, but it underscores the utility of FMO in forming oximes from primary amines with sometimes considerable stereoselectivity.

10.8 NON-NUCLEOPHILES

In a few cases, relatively non-nucleophilic substrates have been reported to be oxygenated by FMO. Generally, non-nucleophilic substrate oxygenation by FMO is very rare, and considerable care needs to be applied to rule out nonenzymatic autooxidation. Another possible origin for FMO-dependent oxygenation of a non-nucleophilic substrate involves uncoupling of FMO (i.e., where the normal mechanism employing the peroxyflavin becomes uncoupled from the enzyme catalytic cycle and acts as a hydrogen peroxide generator to oxidize substrate). In the case where a center of chirality can be generated, a check of the stereochemistry of the product can indicate if uncoupling has occurred for FMO because if hydrogen peroxide or lipid peroxides are the oxidants, a racemic product will result. On the other hand, if a center of chirality can be formed, FMO produces products often with marked stereoselectivity [71]. If a center of chirality cannot be formed, standard incubation of FMO and comparison with the presence or absence of 3–5% hydrogen peroxide and product analysis can indicate if nonenzymatic hydrogen peroxide is responsible for product formation because racemic product will form in the case of the nonenzymatic process. Incubations in the presence of hydrogen peroxide alone will provide an indication of the nonenzymatic rate of oxidation. Of course, a true control incubation without hydrogen peroxide (and without NADPH) must also be run because certain hepatic microsome preparations (i.e., those that are prepared without antioxidants or at elevated temperatures) contain significant quantities of lipid peroxides that can readily autooxidize nucleophilic organic compounds. More complex experiments involving the use of $^{18}\text{O}_2/^{16}\text{O}_2$ and labeled water can help distinguish the role of FMO in substrate oxidation because FMO incorporates molecular oxygen and not water into enzyme-derived products. Thus, examination of a non-nucleophilic substrate in the presence of $^{18}\text{O}_2$ should show incorporation of 18-oxygen into the product if the product is formed in an NADPH- and FMO-dependent process. An oxidation product arising from aldehyde oxidase would incorporate oxygen from water and not molecular oxygen. Using commercially available recombinant FMO (e.g., BD Gentest, Woburn, MA) in these types of experiments would get around any potential confounding reaction with microsomal monooxygenases.

10.9 DRUG OPTIMIZATION

While functional groups primarily metabolized by FMO generally are not associated with apparent toxicity, there are a few such functional groups (e.g., thiones, thioureas, thioamides) that if present in a drug candidate likely should be replaced because of their propensity to cause toxicity via oxidative bioactivation. Generally, the corresponding oxygen-containing compound possesses less potential for toxicity. In principle, replacement of functional groups with pharmacophorically similar moieties that can be

metabolized to reactive metabolites by FMO should take place early in the development paradigm. Another strategy is to purposely introduce functional groups into drug candidates that are selectively metabolized by FMO. This approach would accomplish the objective of distributing the metabolism of the drug over a wider set of enzymes as well as potentially affording a variety of metabolites including FMO metabolites that are generally polar, nontoxic, and readily excreted. By avoiding metabolism (and bioactivation) by P450, the prospects for increasing the druglike properties of a drug candidate is likely increased. Because so many drugs are metabolized by P450 3A4, decreasing the amount of metabolism through this enzyme system is likely to decrease adverse drug–drug interactions. Enhancing the metabolism of a drug by FMO could take a few different forms. One approach might be to increase the propensity for FMO-mediated oxygenation, and another approach might be to decrease the reductive pathways that tend to decrease FMO product formation. Yet another method is to use a prodrug approach to fundamentally change the nature of the drug properties to improve some aspect of ADMET or bioavailability. A concept that has not been discussed herein could be to target drugs to different tissues by taking advantage of the distribution of FMOs. Because FMO1, for example, is prevalent in kidney and not present in adult human liver, developing drugs with FMO1 selectivity could potentially and preferentially target drugs to the kidney.

10.10 CONCLUSIONS AND FURTHER PERSPECTIVES

With the introduction of greater and greater numbers of preclinical drug candidates because of high throughput synthesis and screening, the bottleneck in drug discovery has shifted to include safety considerations. Applying metabolism and drug safety concepts earlier in the drug discovery and development process is likely to yield greater numbers of druglike materials that have a chance to become clinically useful agents.

One of the concepts presented herein is the idea that FMO can be utilized in the preclinical paradigm to facilitate the process of improved “hit” rate in drug discovery. FMO possesses some attractive features as a metabolic system: FMO is neither readily inhibited nor induced and the likelihood for adverse drug–drug interactions is likely decreased. It is likely that most of the variability of FMO is due to pharmacogenetics and genetic polymorphisms. As more clinical studies are done with drugs prominently metabolized by FMO, more information about the variability of FMO in the human setting will be revealed.

While not discussed to any great depth in this chapter, the structural biology of FMO has begun to show advances. Several crystal structures of FMO-like proteins have been reported, including the FMO from *Schizosaccharomyces pombe* [89], the FMO from *Methylophaga* sp. strain SK1 [90], and a Baeyer–Villiger oxidation catalyst called *cyclohexanone monooxygenase* from bacteria [91,92]. These FMO-like proteins are considerably smaller than mammalian FMOs; nevertheless, considerable new information has been forthcoming that can be related to the mammalian FMO structure and mechanism of action. In the future, additional progress with the structural basis of mammalian FMOs is anticipated, which will undoubtedly aid in understanding this important monooxygenase and designing new and more efficacious drug candidates.

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FURTHER READING

The Scripps Research Institute from the NIH compound collection (http://mlsmr.glpig.com/MLSMR_HomePage/).

The Sanford-Burnham Medical Research Institute's small molecule collection (<http://bccg.burnham.org/Assays/ScreeningCompoundCollection.aspx>).

11 Molybdenum-Containing Hydroxylases

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11.1	Summary	1
11.2	Structure and function of molybdenum-containing hydroxylases	2
11.3	Genetics of molybdenum-containing hydroxylases	8
11.4	Biotransformations of xenobiotics by XOR and AO	11
11.5	Inhibitors of molybdenum-containing hydroxylases	27
11.6	Distribution of molybdenum-containing hydroxylases in humans	31
11.7	XOR and AO activity variation	34
11.8	<i>In vitro</i> – <i>in vivo</i> correlation (IVIVC)	40
11.9	Clinical implications	41
11.10	Future perspectives	45
	References	46

11.1 SUMMARY

Molybdenum-containing enzymes represent a large and growing class of enzymes, which incorporate molybdenum, a transition metal as a biologically active cofactor. Nitrate reductase, sulfite oxidase, and the dimethylsulfoxide (DMSO) reductase family are of the most prominent in this class of enzymes. Of interest to drug research are the molybdo-flavoenzymes or molybdenum-containing hydroxylases, which not only need the molybdenum cofactor (MoCo) but also require a flavin cofactor for catalytic activity. These molybdenum-containing hydroxylases consist of mainly two enzymes from this class: aldehyde oxidase (AO) EC 1.2.3.1 and xanthine oxidoreductase (XOR) EC 1.2.3.2 [1], both enzymes being present in the cytosol of the cell and found in all vertebrates. AO and XOR catalyze both oxidation and reduction reactions, but the oxidation reaction has a higher degree of prevalence *in vivo*. The substrates for oxidation

by AO are N-heterocycles, iminium ions, and aldehydes, while XOR primarily oxidizes N-heterocycles. The reduction reactions include reductions of *N*-oxides, sulfoxides, hydroxamic acids, as well as, ring opening due to reductive cleavage. XOR is quite conserved among species and has consistent enzyme kinetics among species for a specific XOR substrate, xanthine. This may be because of its critical role in the catabolism of purines, putatively essential in the metabolism of xanthine to the terminal catabolite uric acid, its potential influence on the development of the mammary gland, and XOR's role in the longevity of lactation. AO, on the other hand, has multiple homologs, a broad substrate pool, varying kinetic parameters with the same substrate among species, and no pathophysiology has been associated with its absence. Recently, emphasis has been put on AO in drug research, due in part to two apparent reasons. The first reason is a perceived need during the early drug design phase of drug research to mitigate cytochrome P450 (CYP) metabolism in new compounds being considered for testing in clinical trials in an effort to achieve the required exposure in humans. Replacement of an aromatic substituent that can be rapidly metabolized by P450 (e.g., phenyl or naphthyl) with an azaaromatic (e.g., pyridyl, pyrimidyl, quinoliny) can lead to a decrease in P450 catalyzed metabolism but potentially introduce oxidation catalyzed by AO [2,3]. The second reason relates to the necessary use of azaaromatic chemical scaffolds in drug design, which happen to be AO substrates, but are required to achieve specific binding interactions at the site of a therapeutic target, such as specific kinases [4–6]. A switch in emphasis from P450 to AO moves drug researchers from a drug-metabolizing enzyme family for which there is much understood and numerous experiments and strategies already established to aid in drug design, cross-species correlations, predictions of human pharmacokinetics, drug–drug interactions, and so on to an enzyme family for which there is much less understood. For AO and XOR, there is a lesser understanding of abundance in tissues, little to no understanding of polymorphisms, no species that consistently translates to humans, and sheer complexity of the assembly of the enzymes. As science helps evolve our ability to incorporate these enzymes into drug research, strategies will become easier. In this chapter, a comprehensive summary of what is known from the peer-reviewed literature about these enzymes will be provided, with emphasis on the key understandings around the molybdenum-containing hydroxylases.

11.2 STRUCTURE AND FUNCTION OF MOLYBDENUM-CONTAINING HYDROXYLASES

Molybdenum-containing hydroxylases perform both oxidation and reduction reactions, but the oxidation reaction has a higher prevalence *in vivo*. Molybdenum is an indispensable component for this enzyme family and is necessary for catalysis along with flavin adenine dinucleotide. The oxidative hydroxylation catalyzed by the molybdenum is different from monooxygenases, in that, the molybdenum hydroxylase generates reducing equivalents during the hydroxylation process (e.g. not consume them), and the final source of the oxygen added to the substrate is from water, not molecular oxygen [7]. The reaction mechanism is discussed later in detail.

The primary physiological function of XOR has been understood for quite some time. XOR has a key role in the catabolism of purines, in which it oxidizes xanthine to hypoxanthine, then into the terminal catabolite uric acid [8,9]. The high kinetic efficiency of XOR to xanthine and hypoxanthine *in vitro* and *in vivo* provides evidence

that XOR would have significant activity toward drug-containing purine moieties. In mammals, XOR exists in two interconvertible forms, xanthine dehydrogenase and xanthine oxidase, the former predominates *in vivo*, although, the likely major role of XOR is purine catabolism. Of late, evidence has been growing that XOR has a broader biological role. One possible function stems from some observations that mice that are heterozygous for a loss of function in the XOR gene are not able to maintain lactation because of membrane defects in the milk fat droplets and mammary epithelial cell disruption [10]. This loss of function indicates that XOR may have a structural role in the development of the mammary gland. Higher levels of XOR have been observed in human colostrums, the fluid that is produced by mammals during the first two to three days post partum. This observation provided a hypothesis that newborn infants have a need for maternal XOR in the first few days of life, which may affect the absorption of dietary iron from the still developing gut of the infant [11,12]. Another possible function of XOR is antimicrobial properties by inhibiting bacterial growth *in vitro* via a nitric-oxide-dependent manner [13]. This antimicrobial role has extended to *in vivo*, where again infants that receive breast milk rich in XOR have less of a chance to develop gastroenteritis than those who are fed formula [14]. XOR has also been reported to generate superoxide radicals, which may also act beneficially as an antimicrobial agent [15]. The same beneficial aspect of superoxide radical formation has also been implicated in influencing the pathophysiology of alcohol-induced liver injury, ischemia reperfusion, endothelial dysfunction, hypertension, and heart failure [16,17]. However, the relevance to pathophysiology is somewhat speculative due to the large abundance of enzyme oxygen radical scavengers, such as catalase and superoxide dismutase (SOD), which should minimize the clinical effect.

The physiological function of AO is not completely understood. What is most known about AO is that it is involved in the metabolism of xenobiotics—specifically those containing aldehydes, iminium ions, and N-heterocycles are typical substrates in the oxidation reaction; and nitro-containing compounds, N-oxides, sulfoxides, hydroxamic acids, 1,2-benzisoxazole, and 1,2-benzisothiazole are typical substrates of AO reduction. Therefore, while it is not known, it is reasonable to assume that potential endogenous substrates may be similar in structural nature. One possible path to understand the function of AO is to recognize the tissue distribution of AO and infer mechanism. Putting this thought into practice, it has been reported that both AO and XOR are found in the skin. The function of these two enzymes in skin is still unknown, but possible functions could include elimination of biogenic waste or break down products formed by ultraviolet radiation or environmental stress and homeostatic control of vitamins [1,18]. Another possible function of AO is lipid homeostasis and lipid deposition. These links were observed through silencing the *Aox1* gene using siRNA in a 3T3-L1 mouse cell line. An arrest of the differentiation of the adipocyte was observed and a sequential diminished lipid accumulation resulted in the mice [19]. This observation was further supported in humans with a high level of AOX1 mRNA in human adipose tissue [19]. This proadipogenic link with AOX1 has been hypothesized to be a protein–protein interaction with the ABCA-1 lipid transporter [20,21] or, perhaps affecting the enzyme activity of PPAR γ and reducing the hepatic levels of AO [22].

AO has been suggested to influence the pathophysiology of numerous clinical disorders, although no direct link has been drawn. Renal toxicity is one disease that has been reported to be affected by AO oxidation due to AO generated toxic metabolites [23].

Two other clinical disorders hypothesized to be impacted by AO are amyotrophic lateral sclerosis (ALS) and alcohol-induced liver injury. It has been reported that the cause of the pathogenesis in these diseases is similar to what is believed for XOR, where the formation of oxygen radicals contribute to the manifestation of the disease [16,24,25]. Similar to the shortcomings of XORs link to disease pathophysiology, the linking of AO and oxygen radical formation to disease may also suffer from the vast abundance of oxygen radical scavengers in the liver, as well as other tissues in the body.

11.2.1 Classification and Structural Features

Mammalian AO and XOR are putatively similar in structure and considerable extrapolation of the biochemical properties of XOR has been done for AO. Overall similarity between AOX1 and XOR has been stated to be ~50% based on amino acid sequence [26]. Furthermore, the other AO forms that were discovered in mouse are even more homologous to AOX1 [27–29]. The enzymes exist as homodimers of subunits of ~150 kDa. Each monomer consists of three main domains: an 85-kDa portion containing the MoCo and substrate-binding sites on the C-terminal end, a 40-kDa FAD-containing domain in the middle, and a 20-kDa domain at the N-terminus that contains the two Fe–S clusters [1,30] (Fig. 11.1). However, it should be noted that during catalysis the passage of electrons occurs from MoCo to Fe–S to FAD, indicating that the protein must assume a tertiary structure that facilitates this order of transfer. The FAD-containing domain is where the transfer of electrons to oxygen occurs; however, for XOR when it is in the dehydrogenase form, this transfer occurs to NAD^+ .

Exploration of specific amino acids within the protein that contribute remarkably to enzyme function has been done. For example, AO activity has been shown to possess considerable variability across rat strains and among individual rats. These findings stimulated investigations into the amino acid sequence differences and revealed, for example, that conversion of glycine 110 to a serine was critical for high catalytic activity [31]. The glycine residue in the catalytically active enzyme is proximal to one of the Fe–S clusters, but its replacement with serine results in an inability to form a dimer, which is essential for catalytic activity [32]. Mutagenesis studies have yielded information regarding the roles of specific amino acid residues in substrate specificity. Mutation of human XOR to convert XOR to a protein that oxidizes AO substrates has been accomplished. Mutants in which either arginine 881 was altered to a methionine or glutamic acid 803 was altered to valine, diminished oxidation of hypoxanthine and xanthine, but activity toward benzaldehyde or cinnamaldehyde, was introduced [33]. Crystallography of the mutant XOR proteins revealed that large changes in conformation were not observed, and it was postulated that the arginine and glutamic acid side chains served in the formation of hydrogen bonds with the nitrogen and carbonyl oxygen atoms of purine substrates. The hydrophobic groups in the mutants no longer offer that H-bonding potential but instead permit the binding of hydrophobic aldehydes as substrates. Studies have also been done to compare substrate specificities among AO enzymes from different species. An investigation of rabbit/monkey chimeric proteins narrowed down the region of residues from 993 to 1088 in rabbit as important in cinchonidine oxidation. Replacement of individual amino acids of this region in monkey AO, which ordinarily metabolizes cinchonidine slowly, showed that a simple conversion of valine to alanine at position 1085 imparted activity, whereas the reverse for the rabbit enzyme (conversion of alanine 1081 to valine) removed the activity [34].

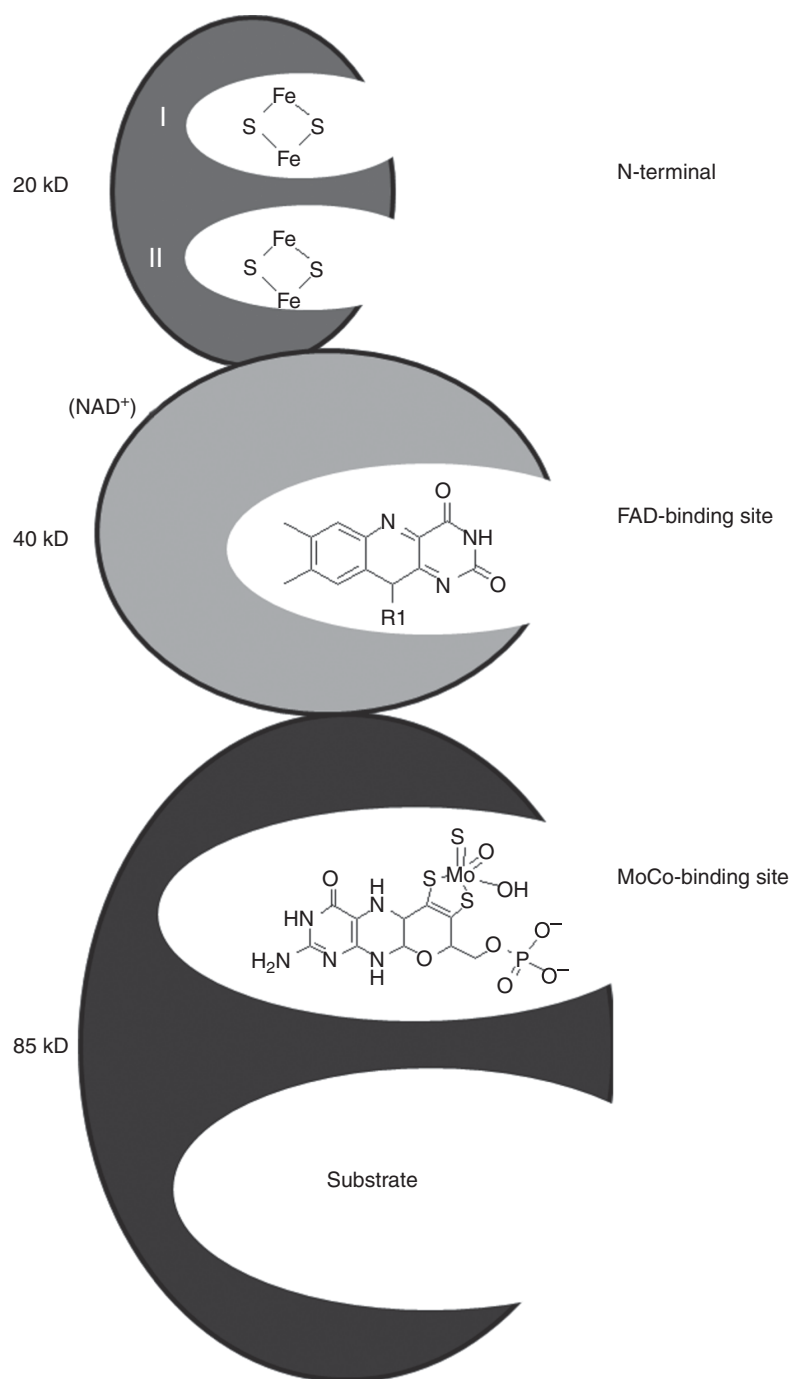


Figure 11.1 Monomeric unit of molybdenum-containing hydroxylases.

It is clear that the relatively recently acquired ability to express active AO to permit directed mutagenesis, coupled with the crystal structure of XOR (and perhaps AO in the future), will yield a much greater understanding of the roles of specific amino acids in the structure and activity of these enzymes [35–37].

While FAD and Fe–S clusters are common throughout enzymes, the MoCo is more unusual and merits a brief description. The structure of molybdopterin is shown in Fig. 11.2, and its elucidation was challenging because of its inherent instability on extraction from purified MoCo enzymes. Nevertheless, its structure elucidation represents a fascinating series of experiments and clever organic chemistry reasoning to solve this puzzle [38] (for a summary, see Leimkuehler *et al.* [39]). Much of the efforts to understand the biosynthesis of molybdopterin cofactors was obtained from bacteria as model systems, and thus must be extrapolated to mammalian systems. GTP is the common precursor. The synthesis of molybdopterin requires several enzymes catalyzing some unusual reactions, including those to add the two sulfur atoms to form the ethenedithiolate (which occurs via a thiocarboxylate on a terminal glycine of the enzyme itself), insert the Mo from molybdate, as well as sulfurase that adds the last sulfur (Fig. 11.2). The cofactor that is conjugated with cytosine monophosphate is putatively the one present in XOR and possibly AO, where as other organisms utilize other structures along this biosynthetic pathway in their respective MoCo enzymes. A detailed description of MoCo biosynthesis is described in Leimkuehler's work [39].

11.2.2 Mechanism of Catalysis

The reaction mechanism of molybdenum-containing hydroxylases has been studied extensively in XOR, and in general can be applied to AO. The reaction mediated by XOR consists of coupled reductive and oxidative half-reactions, therefore either oxidation or reduction of substrate has been observed in XOR/AO reactions. Figure 11.3 shows the redox state changes of XOR during substrate oxidation. Hydroxylation of a XOR substrate at the Mo binding site reduces Mo from VI to IV. Mo(IV) is then oxidized back to Mo(VI) via sequential electron transfer to the two Fe–S complexes. Further, electron transfer to FAD reduces FAD to FADH₂, which is oxidized back to FAD by O₂ to restore the enzyme to its original redox state. Kinetic studies have suggested that the different redox states are in rapid equilibrium [40–42]. The two Fe–S centers act as electron sinks to maintain Mo as VI and flavin in FADH₂ state for efficient reduction and oxidation.

Monooxygenases such as P450s oxidize substrate by incorporating oxygen atom from O₂ to the product. In contrast, the molybdenum hydroxylases use water as the ultimate source of the oxygen atom incorporated into the product [9,43], and generate reducing equivalents in the course of substrate hydroxylation (Fig. 11.3). Hydroxylation of XOR/AO substrates happens at the Mo center. Mo adopts a distorted square pyramidal coordination geometry, where the apical position is occupied by a Mo=O group and the four equatorial ligands are a terminal Mo=S group, two sulfurs from a pterin cofactor, and a ligand proposed to be hydroxide from water (Mo–OH) [44–48]. Previous studies have demonstrated that Mo–OH rather than the Mo=O is catalytically labile [7,49]. Oxidation of xanthine involves base-assisted nucleophilic attack on xanthine by the Mo–OH group with concomitant hydride transfer to the Mo=S group (Fig. 11.4). The concerted mechanism has been supported by both experimental evidence and computational studies [50–53]. A glutamate in the active site plays a central

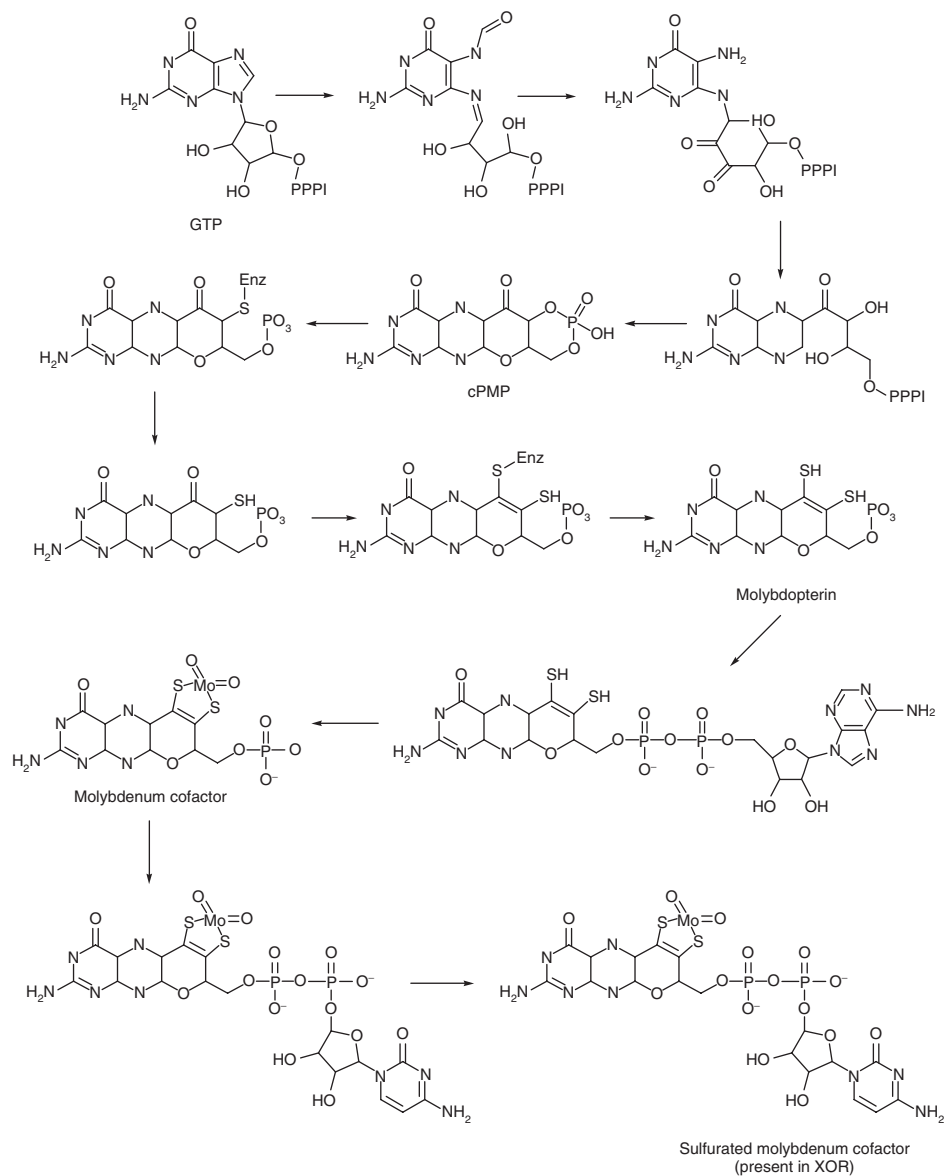


Figure 11.2 Structure of molybdenum cofactors and the pathway of their biosynthesis.

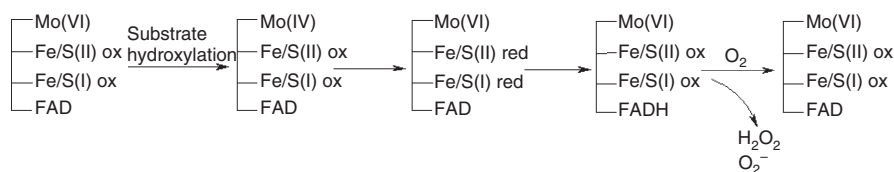


Figure 11.3 Redox state changes of XOR during substrate oxidation.

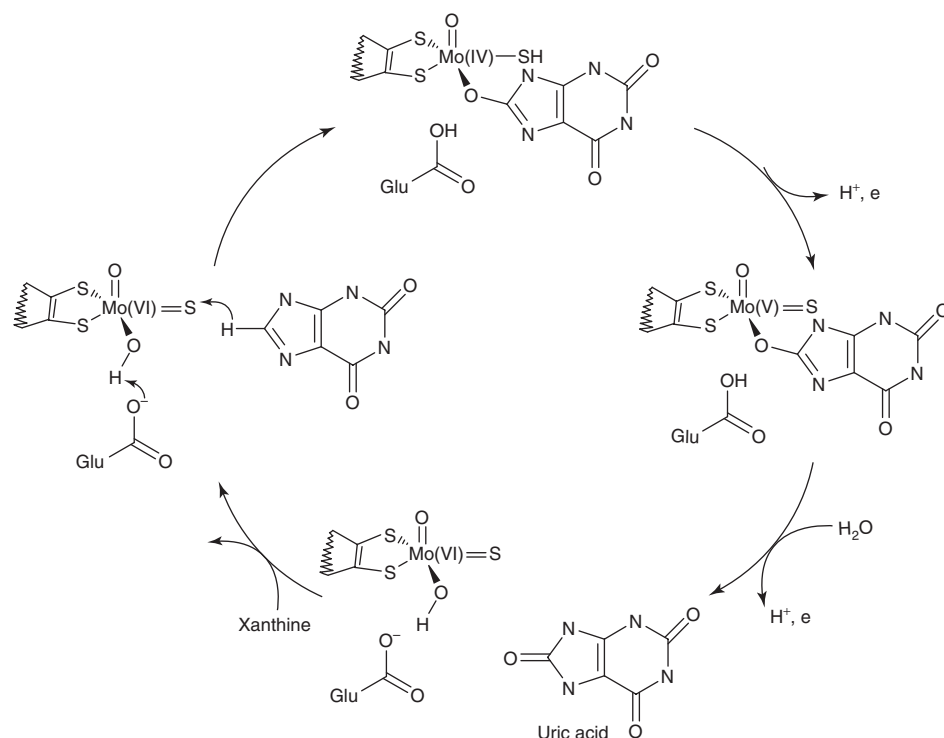


Figure 11.4 A proposed reaction mechanism for the oxidation of xanthine by XOR.

role in catalysis. Mutation of this group to alanine decreases the enzyme reduction rate by a factor of 10^7 as compared with wild-type enzyme [51]. This glutamate is also preserved in AO. Both AO and XOR oxidize a wide range of N-heterocycles and aldehydes; however, XOR shows narrower substrate specificity than AO.

11.3 GENETICS OF MOLYBDENUM-CONTAINING HYDROXYLASES

As of 2011, there had been five known molybdenum-containing hydroxylases identified in mammals. Two of the five have been previously introduced in this chapter, human XOR and AO (AOX1). The other three were identified as related mouse proteins; AO homolog 1 (AOH1), AO homolog 2 (AOH2), and AO homolog 3 (AOH3) [1,54], currently known as AOX3, AOX4, and AOX3L1, respectively [1,55,56]. The solution of multiple molybdenum-containing enzyme crystal structures [45–47,54,57] has provided an understanding of protein folding and amino acid homology, and thus made tracing the evolution of molybdenum-containing enzymes easier.

In humans, only one expressed AOX gene (AOX1) is present, which differs in other mammals where multiple AOX homologs have been identified [55]. The AOX isoforms are expressed in specific tissues in different organisms, and are believed to recognize specific substrates and carry out different physiological responsibilities [54]. When AOX1 was first sequenced, it was apparent that the primary structure of the protein was quite similar to many mammalian XORs [26]. The amino acid sequences of AOX1

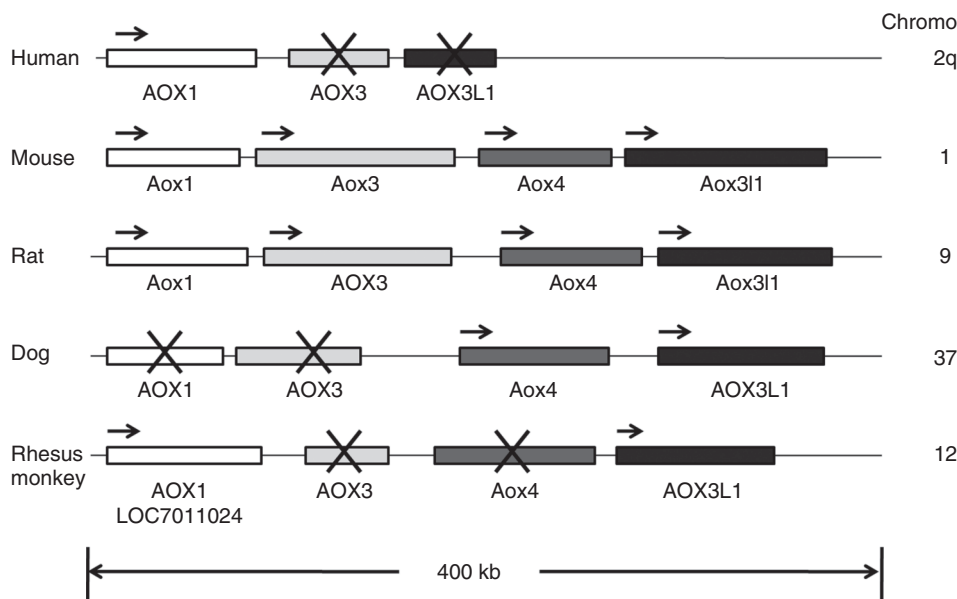


Figure 11.5 A comparison of aldehyde oxidase genes and pseudogenes present in the genomes of humans and commonly utilized animal models in pharmacokinetic and drug-metabolism studies. Orthologous genes are indicated in boxes of the same shade. Pseudogenes are indicated with an “x” through the box, and the species difference in chromosomal location is indicated on the right. *Source:* Adapted from the work of Garrattini and Terao [55,59].

and XOR from many different animal species can be easily aligned along the entire length [1]. As previously mentioned in Section 11.2.1, the similarity of AOX1 and XOR reaches ~50%, which indicates a common ancestral precursor [26,28,29]. When comparing the three mouse identified homologs of AO, a 63% amino acid homology is observed [1].

Since it is widely held that the eukaryote XOR coding region is the progenitor of all AOs, Rodriguez-Trelles *et al.* [58] provided a hypothesis of how these AOs came about. They hypothesize that AOs were created from two independent duplications of the progenitor XOR and further expressed, or not, because of species-specific evolutionary suppression or deletion events. It is also hypothesized that the duplication events occurred before species divergence, due to the high similarity among vertebrate orthologous proteins, and at the gene level, with the conserved exons of the three homologs of AOX observed in many mammals (Fig. 11.5) [1,55,59].

XOR has been identified in a very wide range of mammalia, and have been purified from liver and milk in bovine, human, rat, mouse, and rabbit. Regardless of the species and the source used for isolation, there is relatively little observed variability in the kinetic parameters (K_M or V_{max}) when measured using hypoxanthine or xanthine as a substrate [1]. Furthermore, allopurinol, a very selective tight-binding XOR inhibitor, inhibits all mammalian XORs, providing further evidence of extensive homology from species to species.

The human locus for XOR maps to chromosome 2p22 [60], which in mouse maps to chromosome 17 [61]. Other animal species of interest are rat, dog, and rhesus

monkey, which map to chromosomes 6, 17, and 13, respectively. The XOR genes of both human and mouse consist of 36 short exons and are 80 and 85 kb, respectively, in length. All of the exon/intron junctions of these two orthologs are completely conserved in both position and type. In comparison with the other organism, orthologs of XOR (i.e., *Aspergillus nidulans*, *Caenorhabditis elegans*, and insects such as *Drosophila melanogaster*, *Bombyx mori*, and *Calliphora vicina*, the number of exons in mammalian XOR genes is much larger, but the exon/intron junctions are concordant in the various species. This suggests a preserved code from a common origin. A few functional studies to define the regulatory elements of XOR in mammals provide that the 5'-flanking regions of the two genes are lacking a canonical TATA box, which is substituted by an initiator element [62,63]. These DNA regions contain most of the elements responsible for the constitutive expression of XOR, but do not seem to contain coding for tissue and cell specificity.

The gene structures of mammalian AO are quite similar to that of reptiles and birds but tend to diverge from that of insects and amphibians [27]. The disparate number of genes of AO in different mammalian species, as mentioned previously, is thought to be due to asynchronous duplication from a precursor XOR gene, which is further influenced by subsequent suppression and deletion events [58]. Evidence of this suppression is observed in humans. The complete Human *AOX1* is mapped to chromosome 2q32.3–2q33.1 [24]. Interestingly, the XOR gene is mapped to the same chromosome in humans, although this trait is uncommon in other mammalian species, except a close relative the Chimpanzee [59]. The human *AOX1* gene spans ~80 kb and contains 35 exons. Two pseudogenes, homologous to mouse *AOX3* and *AOX3L1*, have been identified in the same genomic region of chromosome 2 (Fig. 11.5). These pseudogenes named *duplication 1* (*dupl1*) and *dupl2* are the vestiges of *Aox3* and *Aox3L1*, which are transcribed to mRNAs that do not seem to code for protein products [59,64]. The observed suppression in humans is also observed in many preclinical species but to varying degrees among species (Fig. 11.5). With this variation, the prediction of human pharmacokinetics from preclinical species has proven challenging [55,59].

In humans, classical xanthinuria is a rare autosomal recessive metabolic disorder characterized by impairment of the final steps in the catabolism of purines; for example oxidation of hypoxanthine to xanthine and xanthine to uric acid by xanthine dehydrogenase. Consequently, classical xanthinuria manifests with diminished levels of uric acid and increased amounts of xanthine and hypoxanthine in the serum and urine. This displays itself clinically as a tendency to form xanthine stones in the urinary tract and xanthine precipitates in the muscles. There are two types of classical xanthinuria: type I that results from an isolated deficiency of XDH due to the autosomal recessive gene and type II that results from combined XDH and AO deficiency [65]. Xanthinuria type II has been connected with at least three individual single amino acid changes in the human molybdenum cofactor sulfurase (HMCS) gene [66–68], which when expressed catalyzes the conversion of the oxo- to the sulfido form of the MoCo required for the activity of XOR and AO. These mutations have obvious implications on XOR, because of the connection made between the HMCS expression and the lack of function. Since genetic deficiencies in the HMCS gene have not yet been associated with the pathophysiology associated with an AO related mechanism, it is hypothesized that AO is not essential for normal human function.

AO genes in other animal species' cluster are similar to what is observed in humans. In mouse, the clustering is close on chromosome 1 band c1, *Aox1* is on the most 5' end, followed by *Aox3*, 5 kb after, and then 15 kb *Aox4*, and 9 kb after *Aox311* [59,64] (Fig. 11.5). In rat, the same cluster of active genes is located on chromosome 9 [69], which has similar synteny to mouse. In dogs, there are two active genes in a cluster of four on chromosome 37. The two active genes are the orthologs of mouse *Aox4* and *Aox311*, and the other two stretches of DNA have homology to rodent *Aox1* and *Aox3*, but these sequences do not code for active protein [1,55,56]. In a review of AO by Garattini and Terao [55,59], some unpublished work had been reported, which claims that the rhesus monkey AO cluster is located on chromosome 12. Garattini and Terao's review also claims that the two genes that code for active protein are orthologous to those of mouse *Aox1* and *Aox311*, and the two nonexpressing pseudogenes are similar in DNA sequence to *Aox3* and *Aox4*.

11.4 BIOTRANSFORMATIONS OF XENOBIOTICS BY XOR AND AO

Oxidation by XOR/AO involves nucleophilic attack at an electron deficient carbon, which in turn provides the insertion point of an oxygen atom to generate a carboxylic acid from aldehydes or a lactam from aromatic N-heterocycles. Both AO and XOR reduction occur at either N- or S-functional groups under anaerobic or hypoxic conditions, providing that an appropriate electron donor is present. In regard to AO, the appropriate electron donor is either benzaldehyde or 2-pyrimidinone, or other oxidized substrate; and for XOR, the electron donor is xanthine. Reduction rates are dependent on both the concentration and availability of the electron donor. Some known reduction reactions include reductions of *N*-oxides, sulfoxides, nitro-containing compounds, hydroxamic acids, as well as reductive ring opening of heterocycles. These metabolic routes attributed to XOR and AO will be elaborated on in the following sections.

11.4.1 Oxidation of Aromatic N-Heterocycles

Oxidation of N-heterocycles accounts for a majority of AO/XOR-mediated reactions. A recent survey of several current compound collections showed that 35–56% of the compounds in these collections are potential AO/XOR substrates based on the presence of an unsubstituted aromatic carbon atom adjacent to the nitrogen [2]. Figure 11.6 is a summary of heterocyclic cores that are reported to be oxidized by AO/XOR. Simple unsubstituted N-heteroaromatics such as imidazole, pyrazole, pyridine, pyrazine, and pyrimidine are not readily oxidized by XOR/AO. Substituted pyridines are more easily oxidized by AO (Fig. 11.7). Both metyrapone [70] and 1,2-bis(nicotinamideo)propane were oxidized to pyridone at the α -position [71]. A methylpyridine group in a TLR7 agonist has also been shown to be oxidized to α -pyridone by AO [72].

Substituted pyrimidines such as 2- and 4-hydroxypyrimidine were readily oxidized by AO to form uracil, the former being widely used as an electron donor for AO reduction studies *in vitro*. The conversion of hydroxypyrimidine to uracil by AO has been utilized to oxidize several 5-substituted hydroxypyrimidine (5-fluoro- and 5-ethynyl-2-pyrimidinone, Fig. 11.8) to the corresponding pyrimidine nucleoside bases to overcome low and/or inconsistent oral bioavailability of this compound class

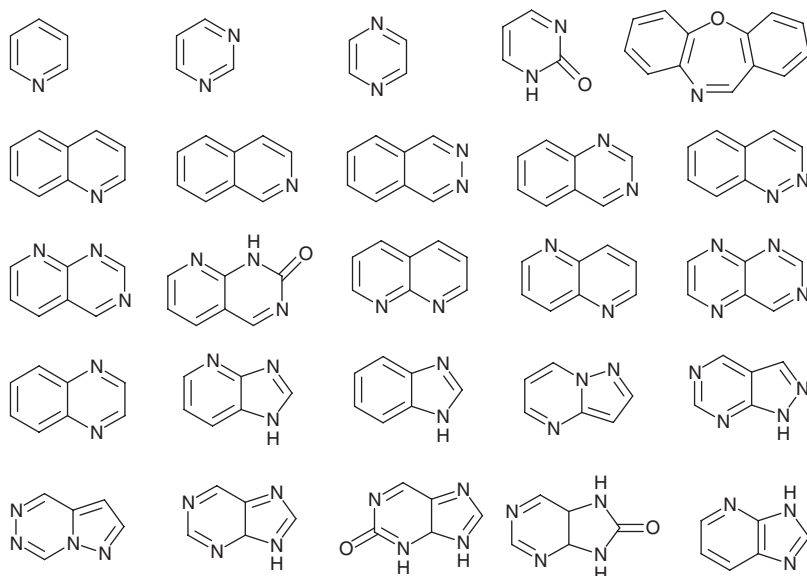


Figure 11.6 Typical aromatic heterocyclic cores in AO/XOR substrates.

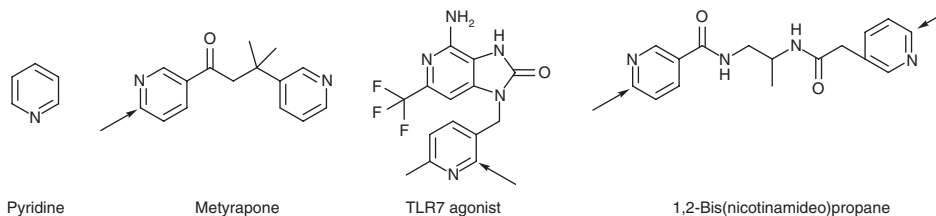


Figure 11.7 Pyridine-containing substrates of AO/XOR (arrow indicates the site(s) of oxidation).

[73,74]. This prodrug approach has also been employed in the bioactivation of 5-iodo-2-pyrimidinone-2'-deoxyribose (IPdR) to 5-iodo-2'-deoxyuridine (IUdR) [75,76] and zebularine to uridine [77]. FK3453 is an adenosine A1/2 dual inhibitor for the treatment of Parkinson's disease. Despite its satisfactory absolute bioavailability and total body clearance in animals, the plasma concentrations of FK3453 in humans were extremely low. This was attributed to AO metabolism where an oxidative metabolite of the 2-aminopyrimidine moiety was identified as a major metabolite [78]. RS-8359 is a selective and reversible MAO inhibitor containing 4-aminopyrimidine. Itoh *et al.* [79] showed that AO exhibited stereoselectivity for the S-enantiomer in rat *in vitro* (Fig. 11.9). The stereoselective AO metabolism was also observed *in vivo*. After oral administration of racemic RS-8359 to rats, mice, dogs, monkey, and humans, plasma concentrations of the R-enantiomer were substantially higher than those of the S-enantiomer [80].

The fused-ring analogs are typical targets for oxidation by AO/XOR. Fusion of benzene rings to pyridine such as quinoline, isoquinoline, and phenanthridine increases

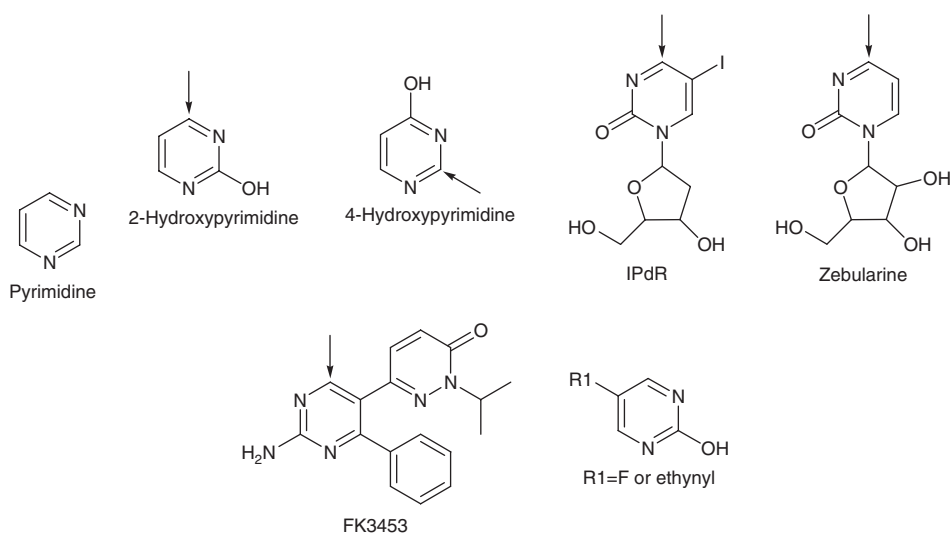


Figure 11.8 Pyrimidine substrates of AO/XOR (arrow indicates the site(s) of oxidation).

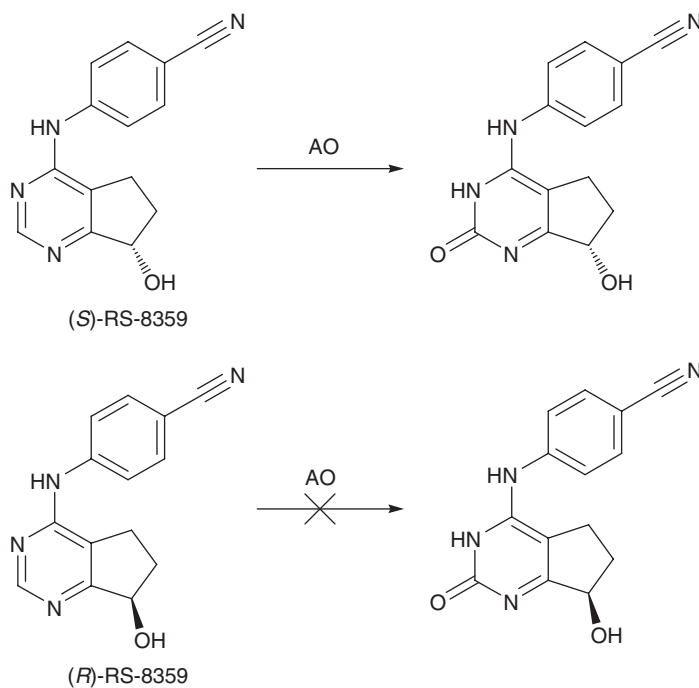


Figure 11.9 Stereoselective oxidation of RS-8359 by AO.

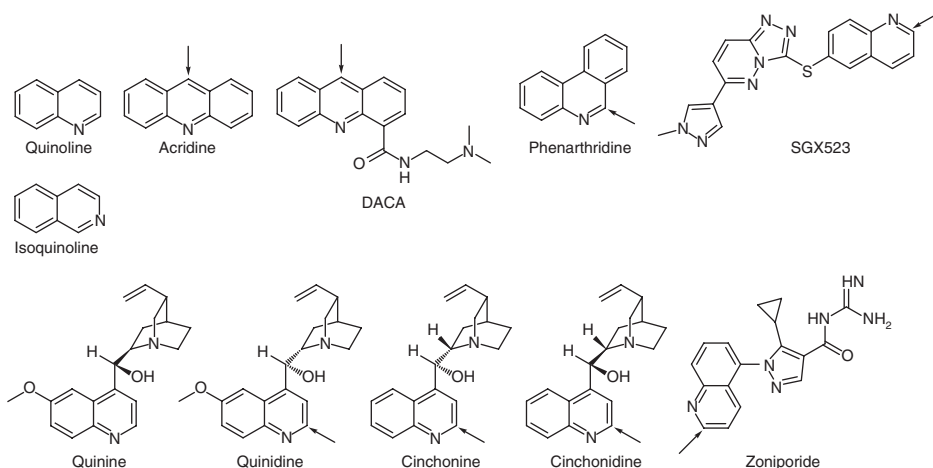


Figure 11.10 Quinoline and isoquinoline substrates of AO/XOR (arrow indicates the site(s) of oxidation).

binding to rabbit liver AO [81] (Fig. 11.10). Quinoline and isoquinoline have K_m values of 3 and 0.2 mM, respectively, while phenanthridine has higher binding affinity toward AO (K_m , 2.3 μ M), which could be due to the increased lipophilicity that facilitates substrate binding to the active site. Interestingly, although phenanthridine, 3,4-benzoquinoline, is a good AO substrate, 5,6- and 7,8-benzoquinoline do not bind to the enzyme [82]. The AO oxidation of acridine is unusual in that hydroxylation takes place at carbon para position rather than ortho to the nitrogen atom [83]. The normal oxidation position, ortho to the nitrogen, is not available for enzyme attack. Similarly, the acridine anticancer agent *N*-[(2'-dimethylamino)ethyl]acridine-4-carboxamide (DACA) is rapidly converted to the acridone by human, rat, and guinea pig AO [84]. Quinine, quinidine, cinchonine and cinchonidine, four quinoline-based antimalarials are all oxidized at the quinoline ring by AO [85]. The oxidation rates of quinine and quinidine were lower than those of cinchonine and cinchonidine, probably because of the electron-donating 6'-methoxy group. Stereoselective metabolism by AO was observed for the two pairs of stereoisomers: cinchonidine and quinidine were oxidized at much higher rates than their corresponding stereoisomers, cinchonine and quinine [86]. SGX523, a quinoline-containing c-MET inhibitor in clinical development for the treatment of solid tumors, was oxidized by AO to a 2-hydroxyquinoline metabolite [23]. This metabolite has low solubility and was excreted in urine, and was suspected to form crystal deposits in renal tubules to compromise renal function in patients. The selective dopamine D3 receptor antagonist SB-277011 is another quinoline-containing compound. SB-277011 was predominantly metabolized by AO and its clearance value in human liver homogenates was 35-fold higher than that in microsomes [87]. Zoniporide, a quinoline-containing inhibitor of the sodium/hydrogen exchanger, was mainly cleared via metabolism, and the major excretory and circulating metabolite in humans was 2-oxozoniporide formed by AO [88].

Quinazoline and phthalazine, cinnoline, and quinoxaline (benzene-fused pyrimidine, pyrazine, and pyridazine) are oxidized by AO to the lactam metabolites (4-hydroxyquinazoline, 2,4-di-hydroxyquinazoline, 1-hydroxyphthalazine, 4-hydroxy-

cinnoline, 2-hydroxyquinoxaline, and 2,3-dihydroxyquinoxaline) [81] (Fig. 11.11). These compounds are weak XOR substrates. Ghafourian *et al.* [89,90] have studied the quantitative structure–activity relationship (QSAR) of phthalazine and quinazoline derivatives and the influence of substituents on AO-mediated oxidation, utilizing kinetic data reported previously. Their studies suggest that polarity of phthalazines had a negative effect on the enzyme activity, leading to the reduction of $V_{\max}$ and increase of K_m . Electron-withdrawing substituents at 2- or 4-position of quinazoline favored the AO oxidation. Carbazeran, a phthalazine-containing inotropic agent, is primarily metabolized by AO in humans to the phthalazinone. The oral bioavailability in man was too low to be measurable, although oral bioavailability in dog was ~68% because of low AO activity in dogs [91,92]. Bromidine, a quinoxaline-containing potent ocular hypotensive agent, has been shown to be metabolized by AO to quinoxaline-2,3-dione and two isomeric quinoxalinone in both ocular tissues and liver fractions [93,94]. A potential new anticancer agent XK-469 underwent extensive metabolism in human hepatocytes and in a phase 1 patient study. An oxidized product at C-3 of quinoxaline formed by AO was the predominant metabolite both *in vitro* and *in vivo* [95]. Bicyclic heteroaromatic moieties in molecules as big as macrolides can be metabolized by AO. The 1,8-naphthyridine ring system in compound **1** was shown to be vulnerable to AO in human liver cytosol. Addition of 3-hydroxyl group (compound **2**) failed to improve AO metabolic stability. However, the 1,5-naphthyridine from rearrangement of nitrogen in the ring (compound **3**) was stable in human liver cytosol [96].

Pteridines are readily oxidized by AO/XOR. Of the four carbons adjacent to the nitrogens, 2, 4, 7, but not 6 are oxidizable. Multiple oxidations can happen in pteridine (Fig. 11.11) [97,98]. However, XOR is more active in carrying out the sequential oxidation. For example, 2- and 7-hydroxypteridine were oxidized by XOR but not AO. *In vitro* studies suggest that XOR may predominate pteridine metabolism; however, methotrexate, a potent inhibitor of dihydrofolate reductase for the treatment of leukemia, was metabolized by AO [99,100]. A major metabolite of methotrexate is its 7-hydroxylated derivative.

Purine was rapidly oxidized by XOR and AO (Fig. 11.12). Purine is sequentially oxidized via hypoxanthine and xanthine to uric acid [8]. XOR is more efficient in catalyzing this sequential reaction than AO. 6-Mercaptopurine is oxidized to 6-thiouric acid via 6-thioxanthine. The first and rate limiting step in this biotransformation is catalyzed solely by XOR, whereas both XOR and AO are involved in the oxidation of 6-thioxanthine to 6-thiouric acid [101]. 6-Mercaptopurine is subject to a large drug–drug interaction when coadministered with allopurinol, a specific XOR inhibitor [102]. AO shows broader substrate specificity and oxidizes a wide variety of drugs containing purine. Both famciclovir and its deacetylated metabolite 6-deoxypenciclovir are efficient substrates of AO; however, they show little activity with XOR [103,104]. AO appears to preferentially oxidize the 8-carbon (Figure 11.13). When dosed intravenously, which bypassed intestinal XOR, both 6-mercaptopurine and 6-thioguanine were oxidized to 8-oxo metabolites, presumably catalyzed by AO [105,106]. Similarly, *O*⁶-benzylguanine is oxidized at 8-carbon by human AO [107]. Krenitsky *et al.* [98] studied a series of substituted purines. While an electron-withdrawing (CONH₂, CN) or neutral (CH₃) substituent at the 6-position of purine maintained the rate of AO metabolism, an electron-donating group (NH₂, OH, SH) rendered the purine significantly less reactive (Table 11.1). Electron-donating 8-substituents also decreased AO metabolism; however, the same effect was not observed for electron-donating groups at

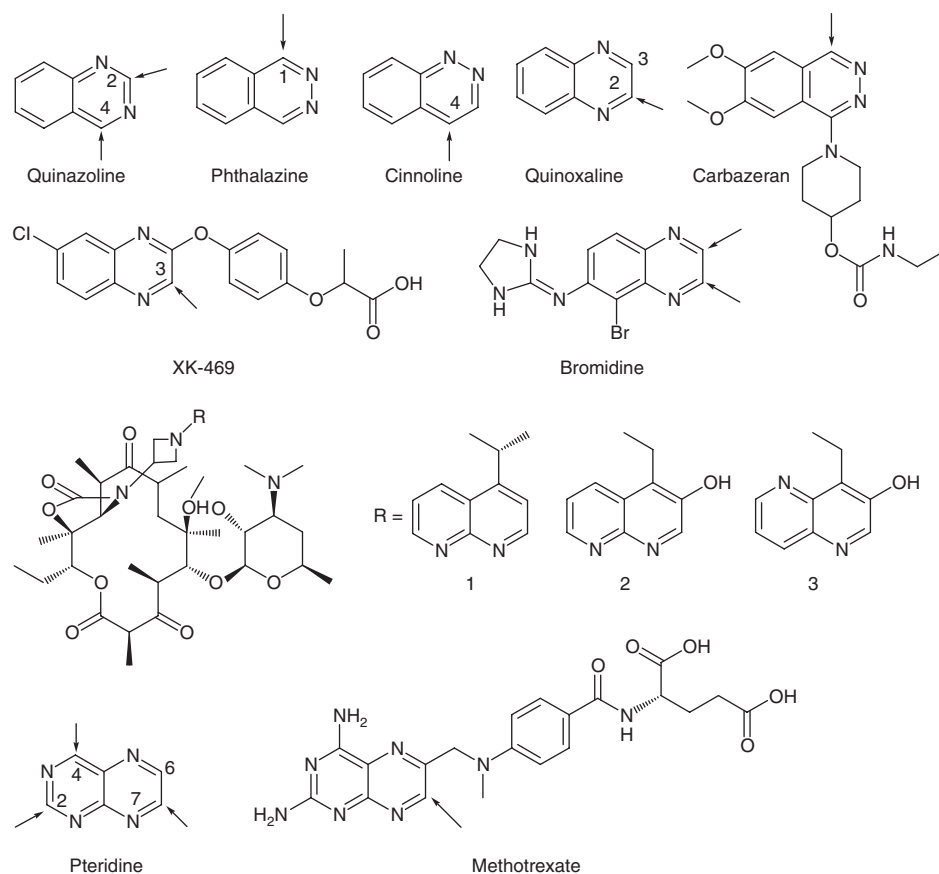


Figure 11.11 Quinazoline, phthalazine, cinnoline, quinoxaline, and pteridine substrates of AO/XOR (arrow indicates the site(s) of oxidation).

the 2-position of purine. The trend is less clear for XOR metabolism of purine analogs (Table 11.1).

In addition to the cores described above, there are other heterocyclic AO substrates reported in the literature (Fig. 11.14): pyrolopyrimidine (zaleplon) [108], dibenz[*b, f*]-1,4-oxazepine [109], azabenzimidazole (a gp120-CD4 inhibitors) [110], pyrazolo-triazine (a GABA_A 5 inverse agonists) [111], and 8-azaquinazolinone (a CXCR3 antagonists) [112].

11.4.2 Aldehyde Oxidation

AO/XOR catalyzes the oxidation of aldehydes to carboxylic acids. Generally, drugs rarely contain an aldehyde moiety; however, aldehydes can be intermediate metabolites of alcohols. Hence AO/XOR may be involved in sequential metabolism of alcohol (the same reaction can be catalyzed by aldehyde dehydrogenase). Reactive oxygen species (ROS) such as H₂O₂ and superoxide are the byproducts of AO oxidation (Fig. 11.1). Increased generation of ROS during ethanol metabolism is proposed to

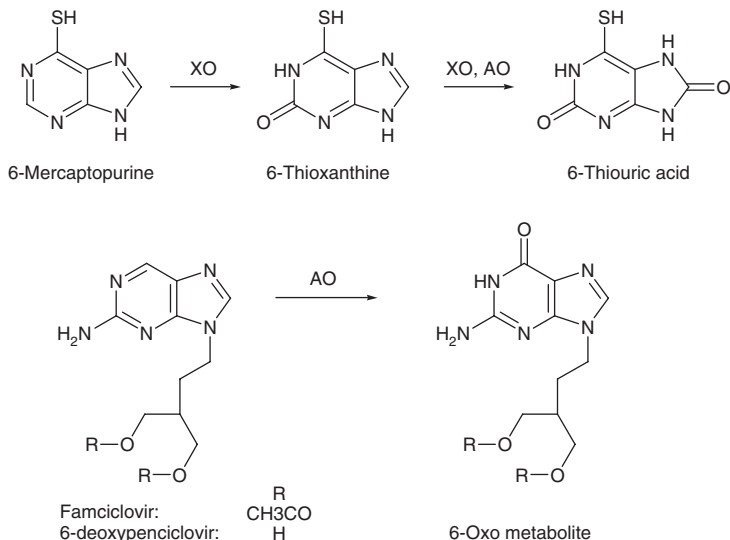


Figure 11.12 Purine substrates of AO/XOR.

contribute to alcohol-related oxidative injury to liver and pancreas [113,114]. Both aliphatic (i.e., retinal) [115] and aromatic aldehydes are oxidized by AO/XOR to the corresponding carboxylic acids. Lipophilicity appears to enhance aldehyde binding to AO, as a result, many aromatic aldehydes are excellent substrate for AO (Fig. 11.15), such as benzaldehyde, indole-3-aldehyde, vanillin, and pyridoxal [116–118]. However, these aromatic aldehydes are poor substrates of XOR.

Acid metabolites of drugs were reported to be formed by AO (Fig. 11.16). For example, tolbutamide is hydroxylated to the benzyl alcohol, by P450, further oxidized into an aldehyde by alcohol dehydrogenase and finally oxidized to the carboxytolbutamide metabolite by AO [119]. Tamoxifen is oxidized first by monoamine oxidase (MAO) to an aldehyde intermediate and subsequently oxidized to the corresponding carboxylic acid by AO [120]. Similarly, the antidepressant citalopram is oxidized by MAO to an aldehyde and further oxidized by AO to a carboxylic acid metabolite [121].

11.4.3 Oxidation of Iminium Ions

Iminium ions, with a positive charge on the nitrogen atom of C=N, facilitates nucleophilic attack to the C=N bond, therefore, they are excellent substrates for AO. For example, *N*¹-methylnicotinamide, which is formed by the N-methylation of nicotinamide by methyltransferase, can be oxidized by AO [122,123]. Both 2- and 4-carbons

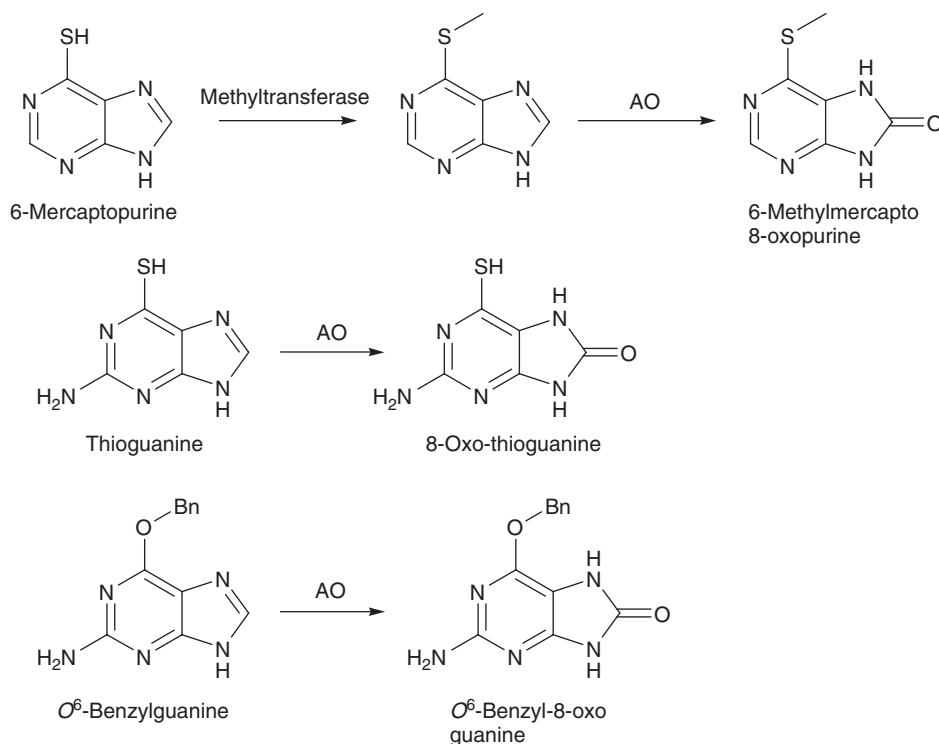


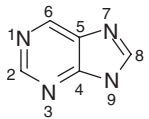
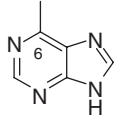
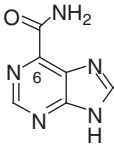
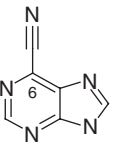
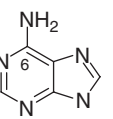
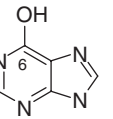
Figure 11.13 8-Oxidation of purine analogs by AO.

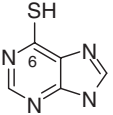
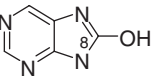
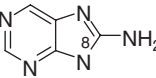
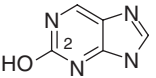
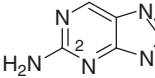
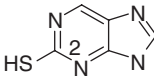
have been shown to be oxidized to form 2- and 4-pyridone, respectively, however, the ratio of pyridone metabolites varies among species [124,125]. Human AO oxidizes *N*-methylnicotinamide mainly to 2-pyridone, whereas mouse AO generates equal amounts of 2- and 4-pyridone (Fig. 11.17) [126].

Iminium ions can be generated as intermediates during the metabolism of cyclic amines such as pyrrolidines, piperidines, indoles, and dihydropyridines via CYP450 or monoamine oxidase. These iminium ions are highly reactive species and can cause toxicity [127]. AO oxidizes these iminium intermediates to lactams, which represents an important detoxification pathway for these compounds. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an exogenous neurotoxin whose neurotoxicity is believed to be mainly because of 1-methyl-4-phenylpyridinium (MPP). MPP is generated through sequential oxidation via an intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP). AO is one of the major enzymes, which catalyzes conversion of MPDP to MPTP lactam and therefore decreases the formation of MPP (Fig. 11.18) [128–130].

AO is involved in the formation of a number of lactam drug metabolites. For example, nicotine iminium, which is formed from P450-mediated oxidation of nicotine, is converted to cotinine by AO (Fig. 11.19) [131]. Similarly, ABT-418, prolintane, and azapetine undergo initial P450-mediated oxidation to iminium ions and further AO-mediated oxidation to lactams [132].

TABLE 11.1 Relative AO/XOR Activities of Some Purine Analogs

Purine						
AO activity	100	82	94	280	2	3
XO activity	100	<3	164	8	6	130

Purine						
AO activity	16	<1	<1	140	38	43
XO activity	17	24	<3	53	80	110

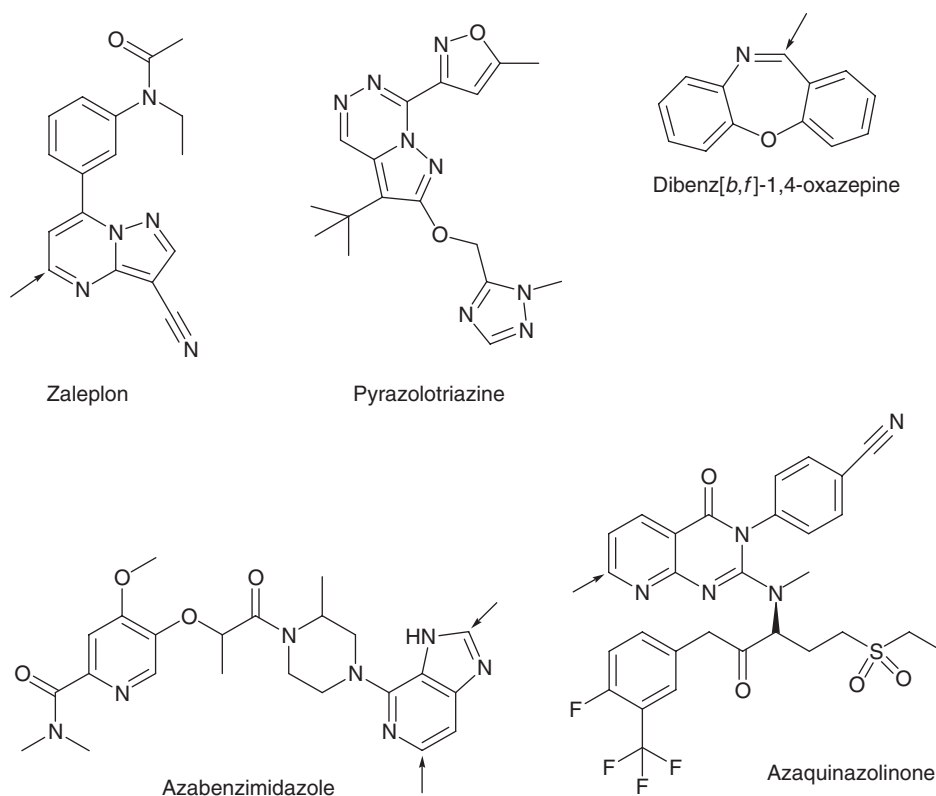


Figure 11.14 Other heterocyclic AO substrates (arrow indicates the site(s) of oxidation).

11.4.4 Reduction

AO/XOR-mediated reductions of nitro groups, *N*-oxide, nitrate, hydroxamic acids, oximes, isoxazoles, and isothiazoles have been demonstrated *in vitro*. However, the extent to which these reductions are actually carried out by AO *in vivo* is difficult to assess. This is because of several reasons: (i) *in vitro*, the incubations must be done under anaerobic conditions, as the substrate is taking the place of oxygen, (ii) electron donors such as *N*¹-methylnicotinamide are required *in vitro*, however, such cofactors are not identified *in vivo*, (iii) often other reducing enzymes such as P450 reductase, NADPH-benzoquinone reductase, and a recently identified molybdenum-containing mitochondrial amidoxime reducing component (mARC) can catalyze the same reduction.

11.4.5 Nitroreduction

AO and XOR are involved in nitroreduction of nitro-containing compounds (nitrated polycyclic aromatic hydrocarbons, nitrofurans, and nitroimidazoles). These nitro-containing compounds are generally mutagenic and nitroreduction is responsible for their mutagenicity. Nitroreduction is a sequential one- and/or two-electron reduction process, which generates reactive intermediates as well as ROS (Fig. 11.20). Both

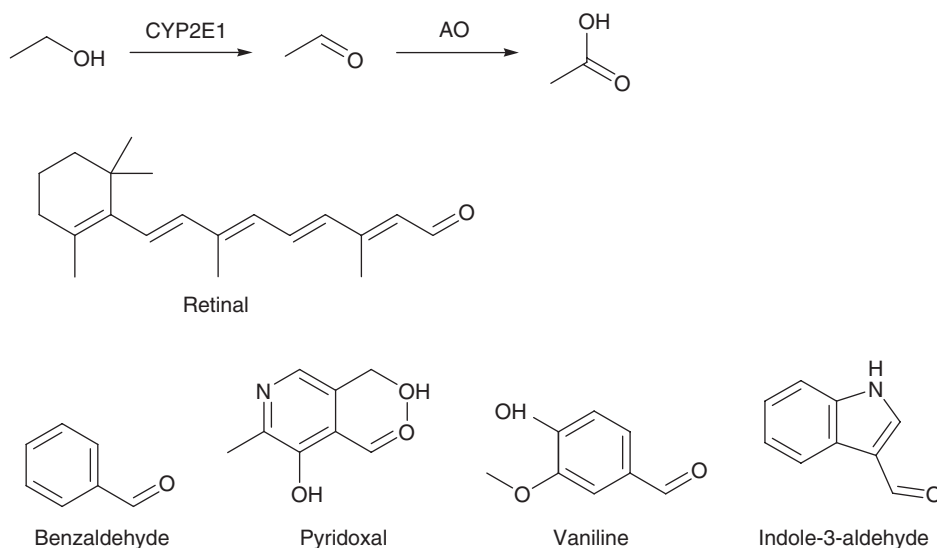


Figure 11.15 Aliphatic and aromatic aldehyde substrates of AO/XOR.

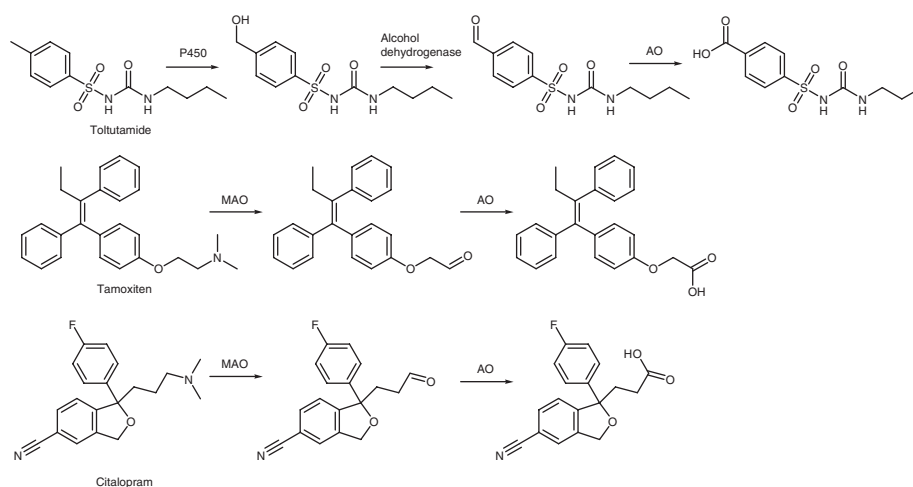


Figure 11.16 Examples of drugs that undergo sequential aldehyde oxidation by AO.

nitroso and *N*-hydroxyamino metabolites are potential electrophiles; however, only the latter derivative appears to modify DNA [133].

Nitroreduction of nitrated polycyclic aromatic hydrocarbons (2-nitrofluorene, 1-nitropyrene, and 4-nitrobisphenyl, Fig. 11.21) in animal livers has been investigated extensively, and it was reported that the reductive metabolism of these compounds is catalyzed by cytochrome P450, P450 reductase, and AO/XOR in mammalian liver [134]. Nitroheterocycles such as nitrofurazone, metronidazole, and benznidazole (Fig. 11.21) underwent XOR/AO-mediated nitroreduction under anaerobic condition

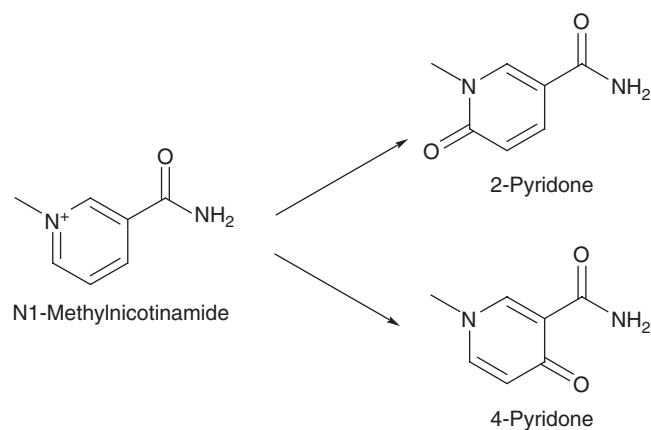


Figure 11.17 Oxidation of *N*¹-methylnicotinamide by AO.

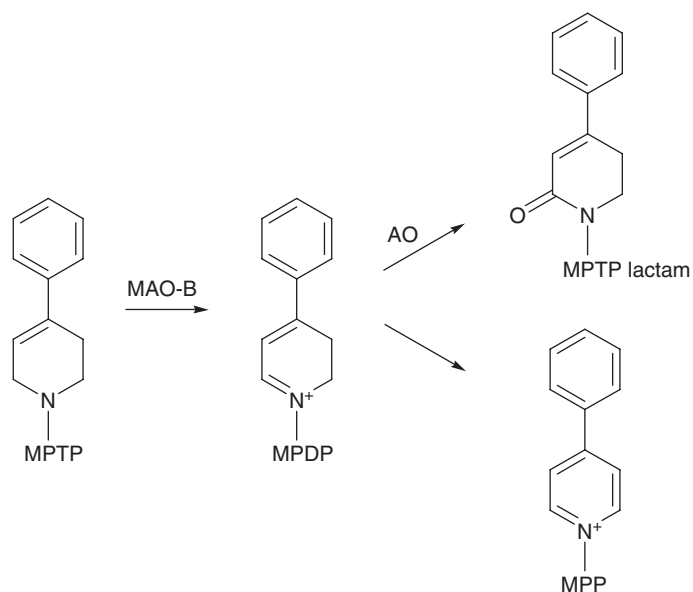


Figure 11.18 Detoxification of MPTP by AO.

[135]. P450 reductase also participated in the reaction. A number of nitroguanidine and nitromethylene insecticides have been reported to undergo nitro group reduction by rabbit liver AO. The nitro groups of clothianidin and imidacloprid was reduced by rabbit AO to nitroso and amino groups [136,137].

11.4.6 Reduction of Nitrates and Nitrites by XOR

XOR catalyzes reduction of organic and inorganic nitrates and nitrites to nitric oxide (NO). It is suggested that XOR may be an important enzyme system that produces

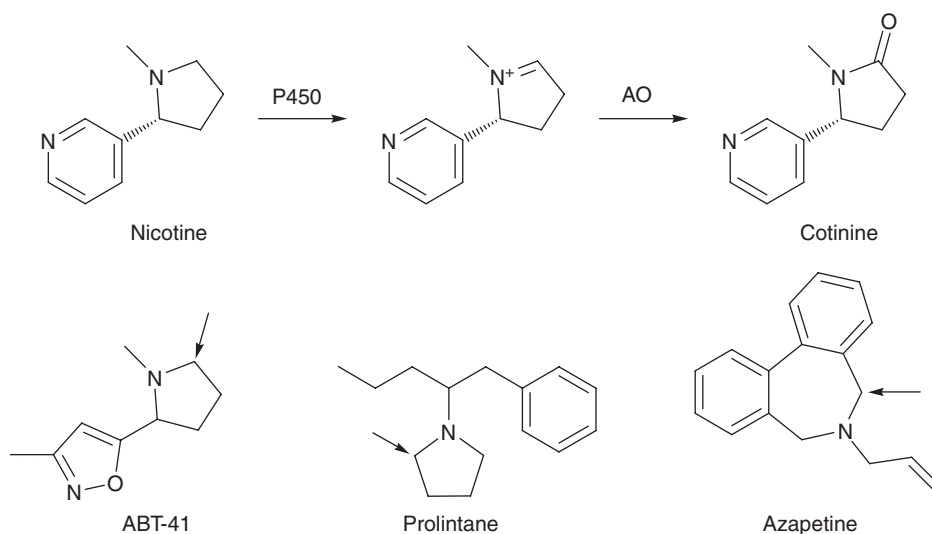


Figure 11.19 Examples of lactam metabolite formation via AO oxidation of iminium (arrow indicates site of oxidation).

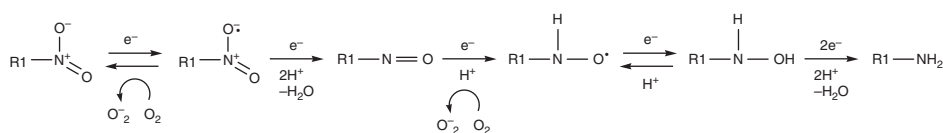


Figure 11.20 Proposed mechanism of AO/XOR reduction of nitro-containing compounds.

vasorelaxation NO via reduction of organic nitrates and nitrites such as glyceryl trinitrate, isosorbide dinitrate, isoamyl nitrite, and isobutyl nitrite (Fig. 11.22) [138–140].

11.4.7 N-Oxide and Sulfoxide Reduction

AO acts as reductase for *N*-oxide and sulfoxide under anaerobic conditions (Fig. 11.23). Nicotinamide *N*-oxide, *S*-(–)nicotine-1'-*N*-oxide, imipramine *N*-oxide, and cyclobenzaprine *N*-oxide are reduced to their corresponding parent amines when incubated in liver cytosols from rabbits, hogs, guinea pigs, hamsters, rats, and mice [141,142]. XOR also reduces *S*-(–)nicotine-1'-*N*-oxide to nicotine *in vitro* [143]. Sulfoxides (sulindac, sulfinpyrazone, phenothiazine sulfoxide, fenthion sulfoxide) are reduced by AO to their corresponding sulfides under anaerobic conditions [144–149]. Stereoselective reduction of sulfoxide has been reported for flosequinan, a racemic mixture of *R*- and *S*-sulfoxide, both rat liver and kidney cytosol reduced *R*-sulfoxide much faster than *S*-sulfoxide to the sulfide metabolite under nitrogen in the presence of electron donor 2-hydroxypyrimidine (Fig. 11.24) [150]. Both reduced amine and sulfide metabolites can be oxidized back to *N*-oxide and sulfoxide, which may prolong the residence time of these species via futile cycle of oxidation–reduction.

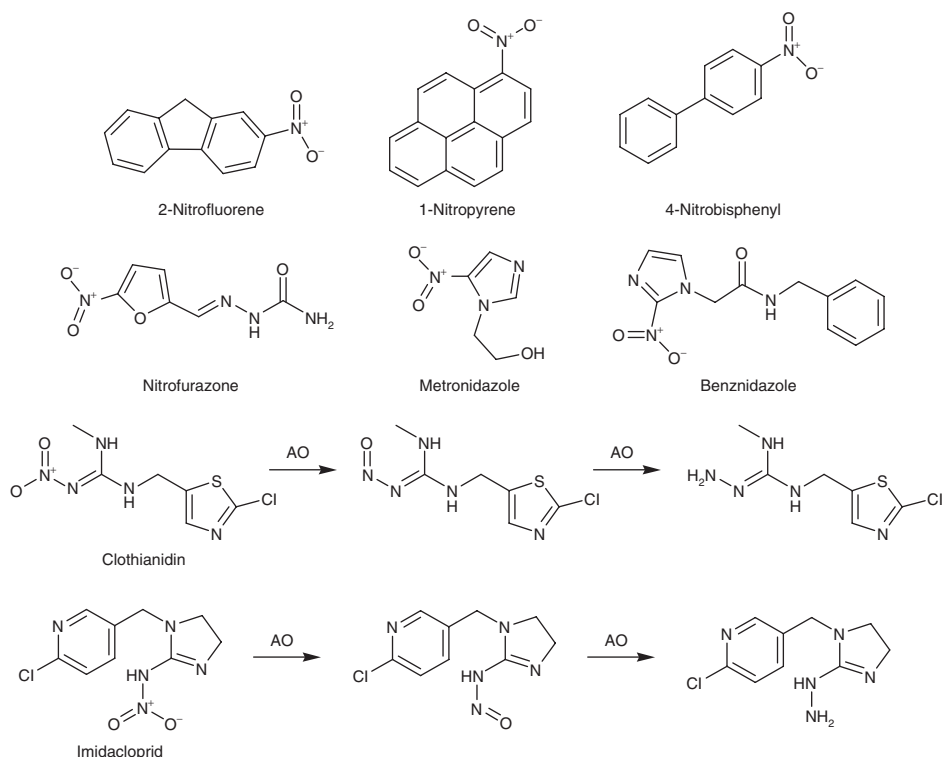


Figure 11.21 Examples of nitro-containing substrates of AO/XOR.

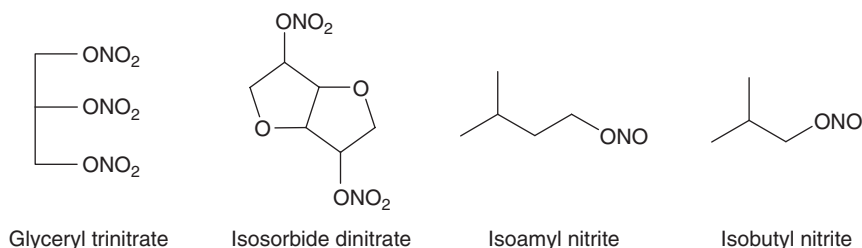


Figure 11.22 Organic nitrates and nitrites that are reduced by XOR to generate NO.

11.4.8 Reduction of *N*-Hydroxy Compounds

The reduction of *N*-hydroxy compounds such as hydroxamic acids, oximes, and *N*-hydroxyguanidines by AO/XO have been described in previous reviews [126,151]. Hydroxamic acids such as benzohydroxamic acid, nicotino hydroxamic acid, salicylhydroxamic acids, and *N*-hydroxy-2-acetylaminofluorene are reduced to their corresponding amide by AO [152,153] (Fig. 11.25). Guanoxabenz, an *N*-hydroxyguanidine metabolite of antihypertensive drug guanabenz, is reduced back to guanabenz by XO [154]. Acetophenone oxime and salicylaldoxime are reduced by AO under anaerobic

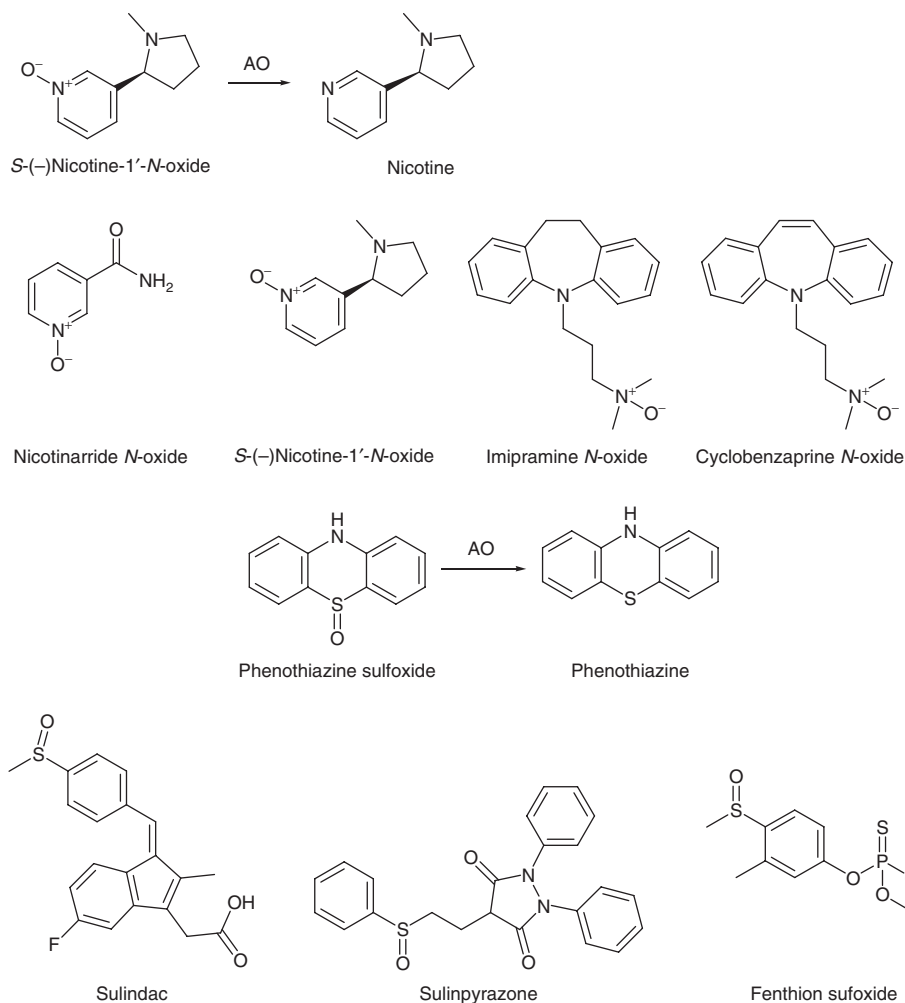


Figure 11.23 AO/XOR reduction of *N*-oxide and *S*-oxide compounds.

conditions to their corresponding ketimines, which are hydrolyzed to the oxo metabolites, whereas benzamidoxime is reduced to a ketimine [155]. Reduction of *N*-hydroxy groups is not limited to AO/XO. Recently, a new molybdenum-containing enzyme system named *mitochondrial amidoxime reducing component* (mARC) was reported to be involved in microsomal and mitochondrial reductions of hydroxylamines and amidoximes [156–158].

11.4.9 Heterocycle Reduction

AO has been suggested to be involved in the reduction of 1,2-benzisoxazole and 1,2-benzisothiazole in some drugs (Fig. 11.26). The 1,2-benzisoxazole ring of zonisamide was reductively cleaved to the ketimine intermediate, which is hydrolyzed to a keto metabolite in rat and rabbit liver cytosol [143]. It should be noted that the reductive

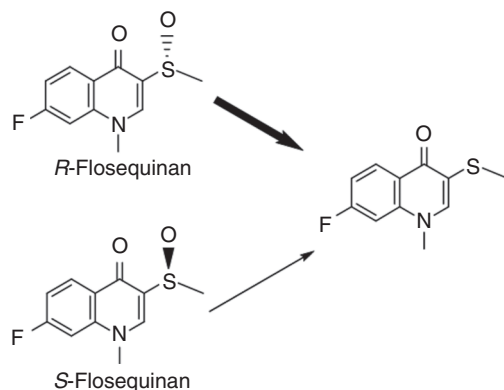


Figure 11.24 Stereoselective reduction of sulfoxide in flosequinan.

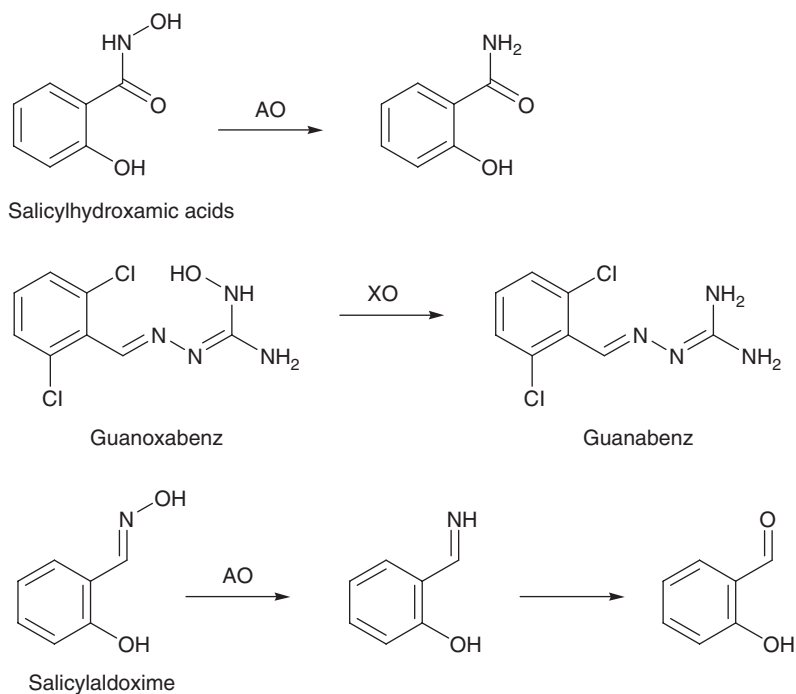


Figure 11.25 Reduction of *N*-hydroxy compounds by AO/XOR.

metabolism of zonisamide is also catalyzed by liver microsomal cytochrome P450, especially CYP3A4 [159], as well as mammalian intestinal bacteria [160]. Similarly the 1,2-benzisothiazole of ziprasidone underwent reductive cleavage of N–S bond to form a thiol intermediate. Subsequent S-methylation of the produced thiol moiety yielded a major metabolite *in vivo* [161]. *In vitro* studies suggested that AO is the major enzyme responsible for the reductive metabolism of ziprasidone [162,163].

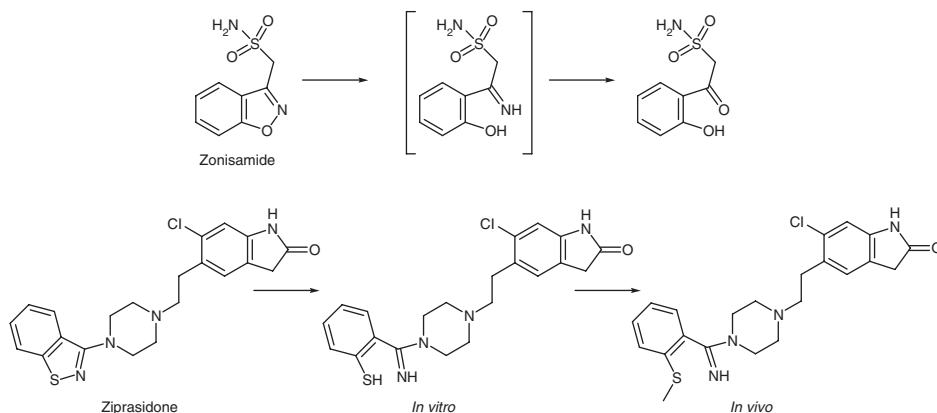


Figure 11.26 Heterocycle reduction catalyzed by aldehyde oxidase.

11.5 INHIBITORS OF MOLYBDENUM-CONTAINING HYDROXYLASES

11.5.1 Inhibitors of AO

11.5.1.1 In Vitro. Many inhibitors of the molybdenum-containing hydroxylases have been characterized and reported in the literature. Menadione is perhaps one of the most well-characterized inhibitors of AO, particularly *in vitro*, that does not potently inhibit XOR [164]. Thus, it is often used to distinguish between AO- and XOR-mediated metabolism in cytosolic or S-9 fractions [23,78,84,165]. Meanwhile, the use of menadione as an inhibitor of AO *in vivo* is unlikely to be effective since it is rapidly reduced by DT-diaphorase [166]. Menadione has been observed to be roughly equally potent against AO in multiple species [84,167], whereas other inhibitors of AO appear to show profound species differences for inhibitor potency. For example, estradiol was found to inhibit vanillic acid formation from vanillin in liver cytosol from human, monkey, rat, and mouse at potencies ranging from IC_{50} of 0.3 μM (human) to 12 μM (monkey) [167]. In addition, methadone and SKF-525A inhibit rat AO, with little inhibition of human AO [84,168]. These findings suggest that it is unlikely that inhibition of AO in a particular species can be directly extrapolated to human AO.

In perhaps the most comprehensive attempt to screen for inhibitors of AO, Obach *et al.* [169] tested 239 frequently used drugs for inhibition of AO utilizing phthalazine oxidation to phthalazinone as a probe reaction. One of the more interesting findings from these studies was that among the inhibitors tested, raloxifene was found to be exquisitely potent ($IC_{50} = 2.9$ nM). Other drugs from a similar therapeutic class were also identified as inhibitors, including tamoxifen, ethinyl estradiol, and estradiol, and overall, 16 drugs were found to inhibit AO with an $IC_{50} < 1$ μM . Overall, there was no one particular physical property that distinguished the most potent inhibitors from the weakest ones, although most inhibitors were weakly basic in nature. From a structural perspective, a tricyclic moiety was present in many potent inhibitors, including several tricyclic antidepressants, antipsychotic agents, and phenothiazines. Of these, perphenazine was found to be the most potent ($IC_{50} = 33$ nM). Other inhibitors of AO discovered in these studies worth mentioning (Fig. 11.27) are the potent CYP3A4

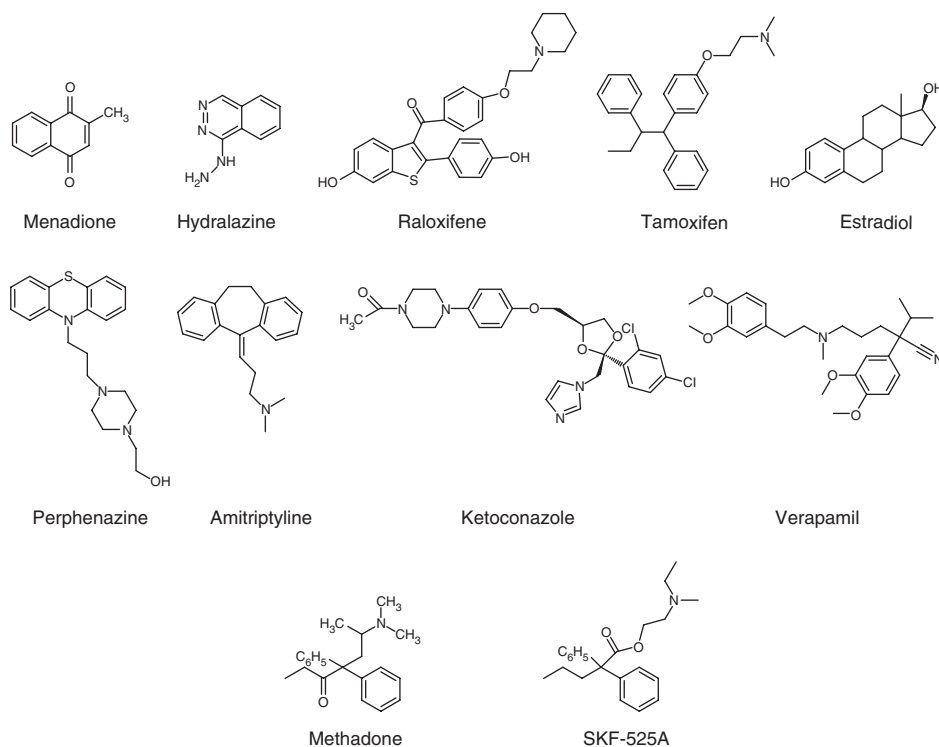


Figure 11.27 Inhibitors of aldehyde oxidase.

inhibitor ketoconazole ($IC_{50} = 3.5 \mu M$) and the CYP3A time-dependent inhibitor erythromycin ($IC_{50} = 15 \mu M$) and verapamil ($IC_{50} = 3.5 \mu M$). Most recently, it has been highlighted that flavenoids, one of the largest groups of naturally occurring compounds that have been shown to modulate the activity of several drug-metabolizing enzymes, may also be effective inhibitors of AO [170,171].

As previously discussed, distinguishing AO from XOR-catalyzed reactions is relatively straightforward, as menadione selectively inhibits AO and not XOR. Raloxifene has also been found to selectively inhibit AO [172], and has been used to confirm the role of AO in the oxidation of zoniporide and zebularine [77,88]. However, when attempting to differentiate AO from cytochrome P450-mediated oxidations in more enzymatically complete systems such as hepatocytes, raloxifene likely does not possess the desired selectivity, as it is known to be potent time-dependent inhibitor of CYP3A4 [173]. In addition, menadione appears to be fairly nonselective for inhibiting AO, as it also crosses over to inhibit multiple P450 isoforms (unpublished observations, R.S. Obach). In contrast, hydralazine is reported to be a potent inhibitor of AO, both *in vitro*, and *in vivo* in rabbit [174] and guinea pig, where it was used to demonstrate the role of AO in the extensive first-pass metabolism of carbazeran [175]. Hydralazine apparently does not inhibit the cytochrome P450 enzymes (unpublished observations, R.S. Obach) [176], which makes it a potentially useful *in vitro* tool for the selective inhibition of AO in effort to understand the fraction of metabolism mediated by AO

compared to the cytochrome P450 enzymes for drugs that may have both pathways contributing to their total metabolic clearance.

11.5.1.2 In Vivo Drug–Drug Interactions. From a clinical standpoint, only one drug interaction has been attributed to inhibition of AO [177]. In a study in human volunteers, following an 800-mg oral dose of cimetidine, the oral clearance of zaleplon was significantly decreased (to 56% of control), resulting in an increase in maximum plasma concentrations ($C_{\max}$) and area under the concentration–time curve (AUC) of zaleplon [178]. In addition, it was discovered that the 5-oxo metabolite (M2) of zaleplon was formed at a reduced rate after cimetidine administration, implicating inhibition of AO as the culprit. Follow-up *in vitro* studies by Renwick [177] demonstrated that cimetidine does in fact inhibit AO-mediated formation of M2 in a competitive fashion, albeit weakly, in both human liver cytosol ($K_i = 155 \mu\text{M}$), and liver slices ($K_i = 506 \mu\text{M}$). Considering this lack of potency for inhibiting AO, it is unclear whether this is the true mechanism of the observed *in vivo* interaction between cimetidine and zaleplon. However, despite the limited impact demonstrated in humans to date, it is conceivable that clinical drug–drug interactions mediated by inhibition of AO may become more prevalent. For example, there appears to be a shift in the chemical matter toward more heterocyclic-containing drug candidates in effort to reduce lipophilicity and cytochrome P450-mediated metabolism, which may result in increased metabolic clearance by non-cytochrome P450 drug-metabolizing enzymes such as AO. This observation, coupled with a number of clinically used drugs being identified as inhibitors of AO, suggests that this particular mechanism of drug–drug interaction may become more relevant in the future. The parameter that will ultimately drive the magnitude of any clinical drug–drug interaction will be fraction metabolized by AO ($f_{\text{m,AO}}$). If the $f_{\text{m,AO}}$ for any drug is >0.5 , then the likelihood exists for some measurable (e.g., greater than equal to twofold) DDI in the clinic [179].

11.5.2 Inhibitors of XOR

11.5.2.1 In Vitro. Historically, there has been much less structural diversity among the known inhibitors of XOR, as the most effective inhibitors appear to be purine-based analogs. In addition, inhibition of XOR has been viewed as therapeutically beneficial, as it is involved in the biosynthesis of uric acid (Fig. 11.28). Allopurinol is the gold standard inhibitor of XOR, and is used therapeutically in the treatment of gout [180], a condition resulting from overproduction or underexcretion of uric acid and subsequent deposition of urate crystals in joints. Allopurinol has been characterized as a mechanism-based inhibitor of XO, whereby the product formed following hydroxylation at the 6-position (oxipurinol) complexes with the partially reduced MoCo, which prevents further electron transfer [181,182]. Xanthine oxidase enzymes may also be inactivated by cyanide, which works to remove the required sulfur atom from the molybdenum, making the enzyme catalytically inactive [183]. Arsenite also appears to be a general inhibitor of XOR and acts in a similar manner, which is an interaction with the critical sulfur atom at the molybdenum center [184]. Research into the mechanism of non-purine inhibitors has continued, with the recent report of a crystal structure of arsenite-inhibited XOR [185].

While allopurinol has been the treatment option of choice for patients suffering from gout for >30 years, a small percentage of the population suffers from adverse

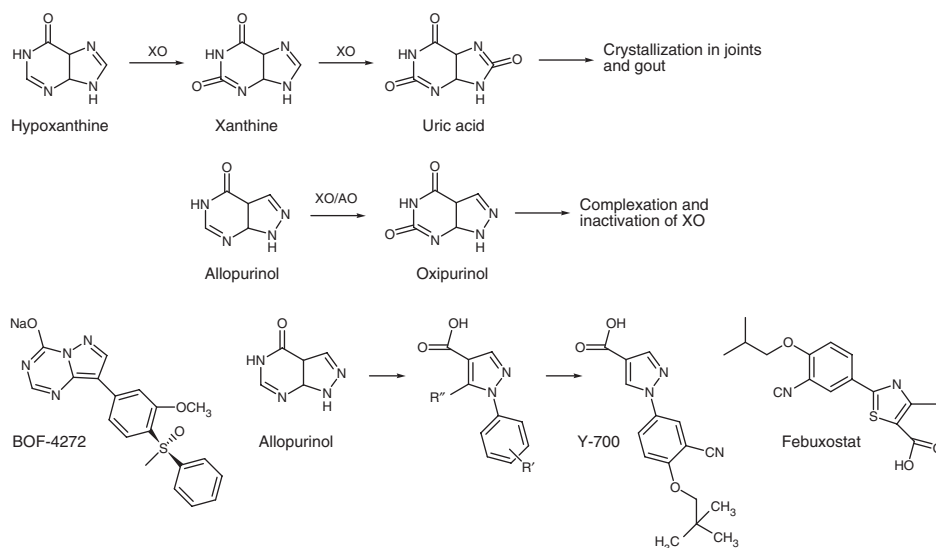


Figure 11.28 Inhibitors of xanthine oxidase.

events, including hypersensitivity reactions, toxic epidermal necrolysis, liver dysfunction, and acute nephritis [186,187]. These clinical observations have triggered numerous efforts to design potent inhibitors of XO that may be used therapeutically without the deleterious side effects. For example, BOF-4272, a pyrazolo-triazene derivative (Fig. 11.28), was found to bind tightly to both the oxidized and reduced forms of XOR, likely in the xanthine-binding site [188], and thus, was expected to inhibit the enzyme for a longer duration than allopurinol *in vivo*. This longer duration of action was in fact demonstrated *in vivo* in rats [189]. Structure–activity relationship (SAR) studies of a series of 1-phenylpyrazoles was reported in 2001 [190], with a number of compounds displaying enhanced inhibitory potency on XOR compared to allopurinol. The strategy employed here was to introduce a phenyl group at the N-1 position of allopurinol to effectively block conversion to unnatural nucleotides, as well as replacement of the 4-hydroxypyrimidine moiety with a carboxylic acid to enhance coordination with molybdenum (Fig. 11.28). From this effort, Y-700 (1-(3-cyano-4-neopentyloxyphenyl)pyrazole-4-carboxylic acid) was discovered as a potent inhibitor of XOR ($IC_{50} = 5.8$ nM) that displayed long-lasting hypouricemic effect in a rat pharmacology model, with an ED_{20} of 0.55 mg/kg (0–6 h) and 1.4 mg/kg (0–24 h), both superior to allopurinol. Y-700 was subsequently dosed in humans where a single-oral dose (5, 20, or 80 mg) led to a dose-dependent reduction of serum uric acid levels [191]. Febuxostat, a structurally related non-purine selective inhibitor of XOR, was also found to be more potent ($K_i < 1$ nM) than allopurinol, selectively inhibiting both the oxidized and reduced form of XOR. In addition, febuxostat was more effective than allopurinol at lowering serum urate levels in patients [192], and was subsequently approved in February 2009 by the US Food and Drug Administration for the management of hyperuricemia in patients with gout (Uloric®). These few examples make it clear that a purine core is not obligatory for potent inhibition of XOR. The work to identify additional diverse inhibitors of XOR is extensive and extends beyond what we

are able to review here. For a more comprehensive summary of current XOR inhibitors, the reader is directed to a recent patent survey [193].

11.5.2.2 In Vivo Drug–Drug Interactions. Drug–drug interaction due to inhibition of XOR by allopurinol has been demonstrated *in vivo* with the anticancer agent 6-mercaptopurine (6-MP), which is metabolized by XOR to 8-hydroxy-6-MP, and then to thiouric acid [194]. When rhesus monkeys and humans were pretreated with allopurinol, peak plasma concentrations ($C_{\max}$) of 6-MP following oral dose were markedly increased, in the order of 400% in monkeys and a 500% in man [102]. Allopurinol pretreatment also led to a 300% increase in plasma AUC in monkeys after oral 6-MP and a 500% increase in AUC in man. However, allopurinol pretreatment had minimal effect on the disposition of intravenously dosed 6-MP. This observed difference was concluded to be the result of inhibition of first-pass metabolism of orally dosed 6-MP by allopurinol, consistent with the known tissue distribution of XOR in the liver and intestine [195].

11.6 DISTRIBUTION OF MOLYBDENUM-CONTAINING HYDROXYLASES IN HUMANS

Subcellular distribution of molybdenum-containing hydroxylases is thought to be mainly centralized to the cytosol of the cell [116,196,197]. Therefore, these classes of enzymes are aqueous soluble proteins, and when prepared from tissue, these enzymes are primarily found in the 100,000 *g* supernatant fraction. There have been claims that both AO and XOR have been found in the mitochondrial fraction of liver homogenates from two species, rat and guinea pig [198,199], although there are claims that the activity observed could not be repeated in subsequent experiments [126]. Interestingly, Critchley *et al.* [198] provided evidence that in guinea pig liver XOR has 52% higher level of activity ($\mu\text{mol}/\text{min}/\text{mg}$) in the mitochondria than that of the cytosol. It was also found that AO in the cytosol versus the mitochondrial fraction have different substrate specificities, thus indicating at least two different forms of AO specific for a particular subcellular site. In a XOR specific study using electron microscopy by Frederiks and Vreeling-Sindelarova identified XOR in the peroxisomes of hepatocytes, and in multiple organelles of Kupffer and sinusoidal cells [196], including the rough endoplasmic reticulum, lysosomes, and endocytic vesicles. Since there is still some controversy as to the subcellular localization of both AO and XOR, more work to affirm such claims may be necessary.

The expression levels of mammalian molybdenum-containing hydroxylases have been studied with many different techniques. The techniques include immunohistochemistry, cytochemistry, Western blot, Northern blot, RT-PCR, and *in situ* hybridization of mRNA. Each one of these techniques has its strengths and weaknesses as pointed out by Garattini *et al.* [1], and Beedham [200]. The main issues with these techniques for molybdenum-containing hydroxylases are (i) the structural similarity of all the enzymes and the specificity of the antibodies used to assess the specific protein, (ii) the measurement of mRNA does not necessarily translate into active protein, and this is especially true with molybdenum-containing hydroxylases because of the complex assembly of the enzymes. However, when all the data is compiled for XOR and then AO, the results are telling. Utilizing all of these techniques, the basis of what we

have learned about the tissue distribution of XOR first came from experimental data in nonhuman species [201–203]. When we compare species, the mammary glands and the intestine seem to have higher levels of XOR than the liver. Human and cat are the exceptions to the rule, where these two species have approximately equal amounts in the intestine and liver. In most species, the proximal portion of the small intestine has the highest XOR enzymatic activity, and when quantified, the epithelial lining of the duodenum and the jejunum is rich in both mRNA and XOR protein [201,202]. A similar such abundance is supported in humans because of high observed activity in both epithelial and goblet cells throughout the villi of the intestine [204], which seems to peak midway through the villi. It has also been reported that activity, protein and mRNA indicative of XOR gene expression, is high in the human liver [201,203,205], but in human lung, expression is barely detectable [206]. Additionally, there have been observations that XOR is present in plasma [207,208]. In lactating mammary gland, acinar cells show high XOR activity compared to nonlactating mammary gland [204]. It has also been reported in humans that a large proportion (>80%) of XOR enzyme in milk is not active [209]. There are conflicting reports of XOR expression in the heart and brain [24,210,211]. Interestingly, XOR activity is increased in tumor tissue in the brain [212] and decreased or absent in aggressive breast cancer [213] hepatomas (rats) [214], and colon tumors [215]. A summary of relative amount of human XOR in tissues can be found in Table 11.2.

AO in humans is abundant in the liver [216] but has also been found in the kidney, pancreas, prostate, testis, ovary, and especially the respiratory system [25,216,217]. AO has also been observed via immunostaining in the adrenal gland, cortex, and the zona reticularis. It has been reported that AO in both the liver and adrenal glands has similar abundance and is the richest source of AO mRNA transcript [218]. AO has also been found in the glial cells in the spinal cord but not in neurons [24]. A summary of relative amount of human AO in tissues can be found in Table 11.3. An interesting comparison analysis by Garattini and Terao [55] of AO mRNA expression of human tumor and the corresponding normal tissues indicated that normal tissues produce large amounts of AO mRNA, where the corresponding neoplastic tissue produce very little. This finding is most relevant and potentially most useful for targeted drug delivery in the case of both liver and lung cancers.

11.6.1 Distribution of Molybdenum-Containing Hydroxylases in Other Species

In rodents, the distribution of AOX1 and AOX3 are overlapping. The most abundant source of these two enzymes in adult rodents is the liver. It was found that the stage of development of the mouse influences when one or the other is expressed. AOX3 is expressed in newborn mice, where AOX1 does not appear in measurable quantities until the mouse is fully grown [29]. Localization of AO in the liver of rats has been observed via histological staining. The staining of the hepatic tissue provides evidence that the distribution of the activity is not homogeneous, and seen mostly in the pericentral and not the periportal area [219]. The second richest tissue containing AOX1 and AOX3 in mouse is the lung; this observation has also been indicated in rat [220]. According to Garattini and Terao [55], mouse AOX3 mRNA is only expressed in the testis, where in mouse and rhesus monkey AOX3L1 is only expressed in the nasal mucosa. AOX1 mRNA has been detected in mouse testis, heart, and the epithelial layer of the esophagus [221], although, no translated protein can be found in either the heart

TABLE 11.2 Tissue Distribution of Xanthine Oxidoreductase (Number of “+” Denotes Relative Amount Observed, “–” Denotes None Observed)

Tissue	Human	Rat	Rabbit	Mouse	Guinea Pig	Bovine
Liver	+	++	—	++	+	+
Pericentral ^a	+	++	—	—	—	+
Periportal ^a	++	+	—	—	—	—
Kupffer cells ^a	+	—	—	—	—	+
Endothelial cells ^a	—	—	—	—	—	+
Kidney	+	+	—	+	+	—
Endothelial cells ^a	+	—	—	—	—	—
Glomerulus tubules ^a	—	+	—	—	—	—
Heart	–/+	+	+	+	—	+
Adrenals	+	+	—	—	—	—
Spleen	+	+	—	—	+	—
Skeletal muscle	+	+	+	—	—	+
Tongue	—	++	—	—	—	—
Esophagus	—	+	—	—	—	—
Stomach	—	+	—	—	—	—
Small intestine	++	+++	—	++	+	—
Large intestine	—	+	—	+	+	—
Lung	—	++	—	+	+	—
Bronchioles ^a	—	++	—	—	—	—
Alveolus ^a	—	++	—	—	—	—
Brain	–/+	—	+	—	—	—
Mammary gland	++	—	—	+	—	+
Milk	++	—	—	—	—	++

^aSubtissue.

Source: Data adapted from the work of Christine Beedham [200].

or esophagus [221]. Interestingly, Kurosaki *et al.* [221] found that brain AO activity was much higher than what the levels of mRNA would have dictated, suggesting that the synthesis of the mouse AO is under translational and posttranslational control. Unique to only a few species, including rodent, but not human and chimpanzee, AOX4 protein is highly expressed at the Harderian gland, a specialized structure in the orbital cavity of the eye region [222]. These types of unique sources of AO may be of consequence when trying to extrapolate human pharmacokinetics from other species and using multiple AO substrates. A summary of AO species differences in tissues can be found in Table 11.3.

Moderate XOR activity has been found in mouse intestine and liver, although XOR mRNA was found at low levels in all tissues studied [203]. Only when subjected to interferon and induction of XOR was XOR mRNA found in mouse liver, intestine, kidney, heart, and lung.

Krenitsky *et al.* [223] when comparing liver and intestinal XOR activity utilizing xanthine as a substrate, found that rabbit XOR oxidase activity was lower than that of mouse, rat, or guinea pig, although, xanthine dehydrogenase activity in rabbit has been shown in heart, brain, and skeletal muscle [224]. When studying guinea pig crude extracts for AO and XOR activity, Beedham *et al.* [225] measured activity in the liver, lung, kidney, intestine, spleen, and heart. Two of the three substrates were specific for

TABLE 11.3 Tissue Distribution of Aldehyde Oxidase (Number of “+” Denotes Relative Amount Observed, “–” Denotes None Observed)

Tissue	Human	Rat	Rabbit	Mouse	Guinea Pig	Bovine
Liver	+	++	+++	+	++	++
Pericentral ^a	+	++	—	—	++	—
Periportal ^a	—	+	—	—	—	—
Kidney	+	—	++	—	+	+
Glomerulus tubules ^a	—	+	—	—	—	—
Heart	—	+	+	+	—	—
Spleen	—	—	—	+	+	+
Skeletal muscle	—	—	—	—	—	—
Tongue	—	+	—	—	—	—
Esophagus	—	++	—	+	—	+
Stomach	—	+	1/4+	—	—	—
Small intestine	—	+	—	—	+	—
Large intestine	—	+	—	+	+	—
Lung	+	++	++	+	+	+
Bronchioles ^a	—	++	—	—	—	—
Alveolus ^a	—	+	—	—	—	—
Brain	++	—	+	++	—	—

^aSubtissue.

Source: Data adapted from the work of Christine Beedham [200].

AO (phenanthridine and phthalazine) and one was specific for XOR (xanthine). AO activity in guinea pig was observed highest in the liver, and for the kidney, the AO activity was ~40% of that observed in the liver. XOR activity was found highest in the small intestine, the jejunum, followed by the duodenum, ileum, liver, spleen, kidney, and lung. A summary of XOR species differences in tissues can be found in Table 11.2.

Unfortunately, studies on both dog and cat tissue-specific and cell-specific distributions are few, although, Terao *et al.* [56] have provided a substantial amount of AO and XOR distribution data for the dog in a single publication. It is reported that dogs are devoid of liver AO activity. Dogs, however, do express homologs of AO in very specific regions of the body. The expression of the AOX4 protein in dogs is found in the lacrimal gland, which is a functional substitute for the rodent Harderian gland. The other actively expressed AO protein, AOX3L1, is expressed similarly in the mouse and rhesus monkey, only in the nasal mucosa. On the other hand, dog XOR mRNA is ubiquitously expressed at low levels in multiple tissues [56].

11.7 XOR AND AO ACTIVITY VARIATION

Humans have a wide range of XOR activity, which spans >3.6-fold variation in enzyme activity [226]. Although the variation is substantial in humans when compared to other mammals, humans have low enzyme activity [227]. There are conflicting reports on the influence of gender on XOR activity [226,228]. Guercioli reports that males have higher XOR activity than females, while Relling reports the opposite. These studies were conducted quite differently, and may be influenced by study specific variables. Guercioli's study utilized cytosolic liver fractions from Caucasian patients, while

Relling's study measured caffeine metabolites in urine of both Caucasian and African-descent healthy volunteers. Unfortunately, Relling's reported results do not stratify by ethnicity and gender together to understand the impact of both at the same time. This stratification could potentially help explaining the different outcome on gender from the two studies.

Variation in active XOR has also been observed when comparing milk of different species. Abadeh *et al.* [209] found that although the expression levels were similar in both bovine and human milk, the human XOR activity for xanthine metabolism was 1–6% of that in bovine. It has been observed that a majority of this disparity can be attributed to two inactivated forms of XOR, either demolybdo-XOR or desulpho-XOR [209,229].

Early works by Krenitsky *et al.* [98,223] provided a unique insight in to the type of mammals that express high levels of AO and those which do not. In these works, it is noted that herbivores have extremely high levels of AO, where carnivores have low levels. It has been alluded that the high levels of AO in herbivores is because of certain dietary plants that might be rich in sources of AO substrates. Perhaps uniquely because of the omnivore status, humans and primates [230] possess some AOX1 activity in the liver.

Several works have displayed marked differences in AO activity within a single animal species, as well as between species [100,231], including some marked differences with famciclovir [104]. In an *in vitro* study conducted by Al-Samy *et al.*, variation of hepatic AO was observed utilizing two AO substrates, DACA and benzaldehyde. Differences in both the K_M and V_{max} was observed from 13 human individual cytosolic fractions from both males and females [232]. Clearance differences between donors ranged from 16.6-fold with DACA and 2.75-fold for benzaldehyde, which is similar to other reported human AO variation for benzaldehyde [233]. Another work has reported up to 50-fold variation for benzaldehyde human AO activity [234]. Additionally, work by Yerino *et al.* [235] presented at the 2007 International Symposium of the Study of Xenobiotic (ISSX) provides a look at 23 donor cytosolic fractions with two AO substrates, which is more than any other study in the literature [232–234]. When reanalyzing the data of phthalazine or *p*-vanillin in a histogram and applying the D'Agostino and Pearson omnibus normality test, the activities of the 23 donors suggests a Gaussian distribution (Fig. 11.29).

A larger cytosolic sample set would be a more appropriate measure of population distribution, but none is presently available. The granularity of a larger study would help the hypothesis that the source of this intraspecies variability is because of multiple polymorphic enzymes distributed throughout the population [59,236]. Recently, the development of a noninvasive method for determining AOX1 activity *in vivo* [237] has been employed in the understanding of developmental AO activity in rats and Japanese children [238,239]. In Japanese children and the adults that they were compared to, a considerable interindividual variation was observed with a fourfold range in activity in individuals greater than one year of age. This method could also be employed to understand the frequency of activity in large populations and distribution modality of polymorphic AO. One of the most revealing examples of the existence of a polymorphic AO enzyme, in a species which expresses AO, was a series of works by Itoh *et al.* [31,32,240] conducted in rats. This series of experiments provided some insight as to why intra- and interstrain differences in AO activity are observed, with little or no observable change in mRNA transcription. In the earliest of the works [31], multiple

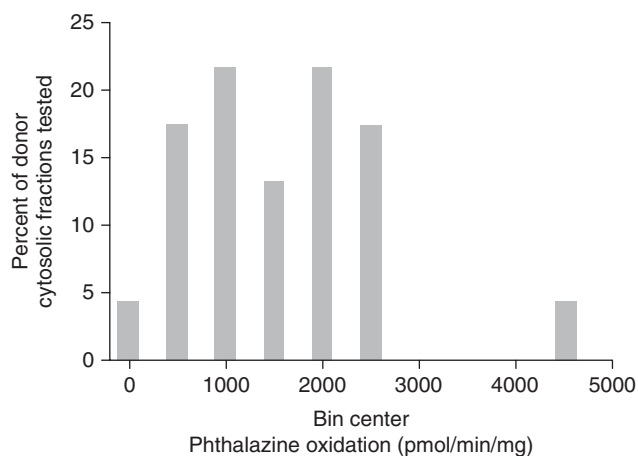


Figure 11.29 Histogram of phthalazine oxidation from 23 donors. *Source:* The data was acquired from the work of Yerino *et al.* presented at the 2007 ISSX meeting [235].

point mutations were observed, which divided the Donryu rats into ultra metabolizers (UM), extensive metabolizers (EM), and poor metabolizers (PM). As previously described in Section 11.2.1, two mutations were identified as being important, G377A and C2604T, thus translating to amino acid changes of 110Gly-Ser and 852Ala-Val. The UMs and PMs were homozygous at all four positions, where the EMs were heterozygous. Therefore, UMs translate to the amino acid at location 110 being a glycine and an alanine at amino acid 852, which differs in PMs translating to a serine and a valine, respectively. The observed specific enzyme activity in the UMs versus the EMs was greater than twofold $V_{\max}$, and a 20- to 40-fold difference in clearance ($K_M/V_{\max}$) of the S-enantiomer of RS-8359 was observable in the EMs versus the PMs. This effect was thought to be due to a change at amino acid position 110, which is conserved among rat species. Since this nucleic acid is near the Fe-S center, and the change shifts the isoelectric point from pH 5 to 6.2 from EMs to PMs. It was thought that this change of isoelectric point may cause a conformational change of the AO. In a later study investigating interstrain difference in activity, Itoh *et al.* [240] observed that a lack of homodimerization of the enzyme was responsible for the reduced activity in-between strains of rat. Further work implicated that the same point mutations (G377A and C2604T) drive the dimerization process and effectively drives the activity [32].

Species differences are most definitely due, in part, to the expression and the differing distribution of species-specific isoforms. There are many examples of variability in AO-metabolized drugs in different species. For example, Zaleplon [108] had been reported to have marked species differences between monkey and rat. SGX523 shows acute renal failure in humans because of obstructive nephropathy from a particular poor soluble AO urinary metabolite, not seen in preclinical toxicology species. Diamond *et al.* conducted studies to understand the production of this poorly soluble metabolite in multiple species. The specific AO metabolite was formed in humans and monkeys and to a much lesser extent in rat and none in dog. Interestingly, several prodrugs of an antiviral agent, 9-(3-hydroxypropoxy)guanine, are even activated by different molybdenum-containing hydroxylases. In rats, XOR was the responsible

enzyme, where as AO catalyzed the reaction in humans [241]. Carbazeran, a successful inotropic agent in dogs, is another interesting example of AO species differences. It has been observed that carbazeran is extensively metabolized utilizing baboon and human liver cytosol *in vitro* but stable in dogs [91,92]. The phenomenon in dog is not specific just to carbazeran. Dog AO activity has been measured with a variety of substrates and found to be very low or absent [100,223,242]. Also of interest is the data suggesting that carbazeran metabolism is species specific. Both rabbit [243] and mouse (unpublished work, Fig. 11.30) show little or no AO-specific metabolism of carbazeran. In humans, baboons, marmosets, and guinea pig, affinity in the single digit

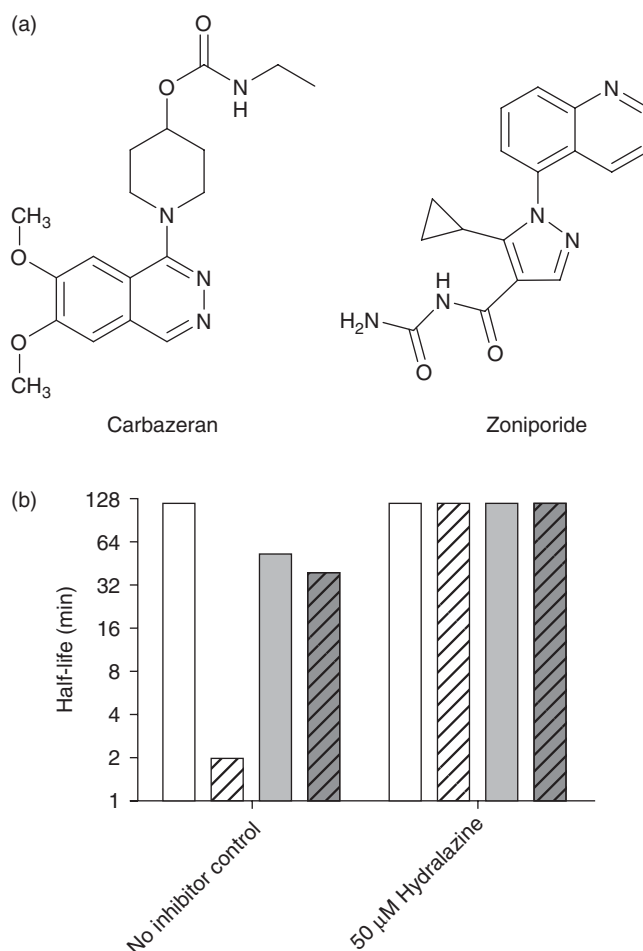


Figure 11.30 (a) Structures of carbazeran and zoniporide. (b) Unpublished results of a substrate depletion time course assessment of 1 μ M carbazeran (white) and zoniporide (gray), utilizing 2 mg/mL of mouse (no pattern) and human (diagonal lines) S-9 liver fraction in the presence and absence of 50 μ M hydralazine (a potent and specific AO inhibitor) [169,175] in 100 mM potassium phosphate buffer pH 7.4. Zoniporide [88] was assayed as a parent compound depletion positive control of both mouse and human AO. Maximum extrapolated half-life was 120 min. Y-axis displayed on the natural log scale for better visualization.

to 100 μM range with a moderate to very high V_{max} (20–150 mol/min/mg) is observed. The V_{max} for rat is much lower for carbazeran, which is similar to what is observed in the mouse (Fig. 11.30). This outcome may suggest that the rodent family may share the lack of activity with the rabbit.

11.7.1 Regulators and Inducers of Molybdenum-Containing Hydroxylases

XOR has a variety of mechanisms that govern its protein expression and level of enzymatic activity. One of the most studied is modulation by cytokines. Of the cytokines, interferon has been characterized by a number of laboratories [244–247]. The impact was shown via a mouse study using either an artificial increase in type I interferon or an interferon inducer such as bacterial lipopolysaccharide. A substantial induction of XOR was observed in multiple tissues in the mouse model [203]. In a complementary study using epithelial cells *in vitro*, a similar response was reproduced [244]. These studies suggest that this induction of XOR is a feedback mechanism to help mediate certain features of interferon activity. Studies inhibiting XOR activity do not support the involvement of XOR in influencing the therapeutic or detrimental effect of interferon [248].

In other examples of cytokines influencing XOR transcription, the proinflammatory cytokines, tumor necrosis factor α (TNF- α), interleukin-1, and interleukin-6 levels also increased XOR mRNA levels in human kidney epithelial cells [244,245]. Anti-inflammatory corticosteroids have also been found to induce XOR expression. This phenomenon is surprising, since both anti-inflammatory and proinflammatory cytokines induce XOR. Regardless, the induction triggered by both of these proinflammatory and anti-inflammatory cytokines suggests a potential XOR function in inflammation.

Other non-cytokine and exogenous stimuli are capable of inducing XOR as well. Linder [249] reports that oxygen tension has a significant effect on XOR expression, where hypoxia increases XOR, while hyperoxia tends to decrease the observed activity. It was noted that there are observations of both hypoxia and hyperoxia effecting XOR both on the transcriptional and posttranscriptional level, depending on the cell line studied and the species line was derived from. Both phorbol-12-myristate-13-acetate (PMA) and tetrachlorodibenzo-*p*-dioxin (TCDD) are inducers of XOR gene expression in many tissue types and animal species [250–256]. PMA has a questionable mechanism; it is unclear whether it directly induces or influences the production of inflammatory cytokine. Alternatively, TCDD is an environmental pollutant that induces XOR activity directly via activation of the TCDD receptor, or otherwise known as the *Ah receptor*. XOR induction derived from the TCDD receptor in the liver is suspected to be the reason for TCDD-induced liver damage. TCDD is thought to induce liver damage by the increased XOR expression and the coinciding increased production of superoxides.

Regulation of AOX has been proposed to be a consequence of multiple factors, both genetic and endogenous. Interindividual variability in AOX1 expression in humans has been suggested to be responsible for both methotrexate pharmacokinetic variations in the clinic [100]. Garattini and Steinberg [59,236] have posed that the variations seen in the methotrexate study maybe a consequence of allelic variants or polymorphic enzymes, which express a catalytically defective enzyme. Endogenous factors such as sexual steroid hormones have been shown to regulate gender-specific difference in AO levels in the liver of mice [221,257,258]. The mRNA of mouse AOX1 seems to

increase with levels of androgens, where as estrogen decreases the levels of AOX1 mRNA. This influence of androgens could be responsible for the observed difference in the non-preclinical species, the camel. A twofold increase in AO-specific activity in males opposed to female camels was observed using phenanthridine as a specific probe [259]. Another example is zebularine; when studied in cancer prone Apc(Min/+) mice, showed gender-specific expression which influenced its chemopreventive activity for intestinal polyposis [260,261]. In humans, *in vitro* and *in vivo* comparisons have taken place. The data suggests little if any gender-influenced AO activity differences. *In vitro* studies by Al-Salmy *et al.* [238] did not detect a relationship between gender and AO activity, which correlates with the aforementioned clinical study in Japanese children and adults. Additionally, with limited clinical data, penciclovir also shows little to no gender difference [262].

Exogenous compounds, such as the previously mentioned TCDD [263], as well as methanesulfonate [264], nitrosoureas [264], nitroguanidines [264], curcumin [265], and phenethyl isothiocyanate [266] have been demonstrated to induce AOX1.

11.7.2 Ontogenic Expression

No correlation has been associated with age and XOR enzyme activity, regardless of the method of analysis [226,228,267]. This also includes a study of allopurinol metabolism, which according to Turnheim *et al.* is a function of XOR activity. They observed that in elderly subjects compared to young controls, the clearance of allopurinol is unchanged. Others have reported that allopurinol is metabolized primarily by AO. If taken together, one could conclude that neither AO nor XOR vary in elderly subjects compared to younger controls. An AO-specific example is famciclovir, in which elderly subjects produced similar amount of the active metabolite penciclovir in urine as patients. This result is somewhat clouded because of the reduced renal clearance of penciclovir in the elderly [268,269]. There have been multiple studies looking at very early development stages in multiple species of both AO and XOR. In a rabbit study by Nichols *et al.*, a lack of AO variation across age groups and life events was observed. Their data indicated that hepatic tissue from nonpregnant, pregnant, fetal, and newborn rabbits did not differ significantly in its ability to reduce 2,6-dichloroindophenol in the presence of acetaldehyde substrate. In another study utilizing mice at various stages of development, Holmes [270] observed that up to three weeks from birth, no XOR was present, whereas AOX-1 could be detected in a 20-day-old fetus. There has been a single study in humans conducted comparing the relative amounts of nicotinamide and its metabolites in urine of young Japanese children and adults [238]. The study population consisted of 101 children, of both sexes ranging in age from shortly after birth to 10 years of age, and 26 adults. When relative urinary product values of the children were compared to the adults, it was found that AO activity rapidly increased in a linear fashion with age to adult levels within first 365 days of life. Also of interest was that AO activity of neonates was found to be ~10% of those observed in adults. No data were available for fetal AO activity, but the expectation is that the activity would not surpass those of neonates. It was suggested that since AO activity is immature in human neonates and infants, perhaps there is a need to make dose adjustments with drugs such as methotrexate and cyclophosphamide based on AO activity to avoid increase risk of side effects including leucopenia, thrombocytopenia, liver failure, renal

failure, and nephropathy [238]. Also, when all the species data is placed together, the data indicates that there is species-specific triggering of AO expression at certain stages of early life, although this phenomenon might be homolog influenced, thus also species specific.

11.7.3 Ethnic Differences

The activity of XOR in the human population appears to follow a normal Gaussian distribution [226,271,272], although marked variation in expression and activity has been observed in some ethnic populations. As previously mentioned in Section 11.7, Relling *et al.* measured caffeine metabolites as indicators of specific drug-metabolizing enzyme activities. Utilizing ratios of the metabolite (1-methyluric acid) to substrate (1-methylxanthine), a comparison of xanthine oxidase activity was made between Caucasians, subjects from African descent, males, and females. It was found that subjects from African descent and males, in general, have lower XOR activity. Interestingly, Relling identified that the lowest xanthine oxidase activities were observed in males from African descent, and that the highest activities were observed in the females of both ethnic groups, but stated that the distributions within each gender according to ethnic group are not bimodal. However, the author later stated, in a personal communication that compared the potential impact of environmental and iatrogenic influences, such as methotrexate, exercise, interferons, smoking, and other variables on xanthine oxidase activity, the impact of gender and ethnicity is unlikely to be substantial. As far as the authors are aware, this type of ethnic analysis has not been conducted with an AO-specific substrate.

11.8 *IN VITRO*–*IN VIVO* CORRELATION (IVIVC)

XOR has a narrow substrate pool compared to AO, and thus as far as the authors are aware, there has not been an attempt to perform an *in vitro*–*in vivo* correlation. In 2010, Zientek *et al.* [273] attempted an IVIVC for human AO. Eleven test compounds were assayed using two *in vitro* systems (pooled human liver cytosol and liver S-9 fractions) to calculate scaled unbound intrinsic clearance, and were then compared to calculated *in vivo* unbound intrinsic clearance, to examine if any correlation existed. The correlation offered a means by which newly synthesized compounds that are shown to be metabolized by AO can have a prediction made for whether it will have a high, medium, or low CL'_{int} (Fig. 11.31) in the clinic.

The *in vitro* system chosen, be it pooled human liver cytosol or S-9 fraction, can be calibrated using high, medium, and low CL'_{int} selected from the 11 described in Fig. 11.31. Then a CL'_{int} can be obtained for a new compound and measured in relation to the chosen controls. The authors suggest that compounds with AO-mediated CL'_{int} less than that of zaleplon would be predicted to be low CL'_{int} *in vivo* and compounds with CL'_{int} equal to or greater than 6-deoxypenciclovir, zonisporide, or *O*⁶-benzylguanine would be predicted to be high CL'_{int} . Thus, a relative scale has been provided that can be used for *in vitro*–*in vivo* correlation of AO clearance and can aid in directing drug discovery programs regarding when structural modifications are needed because of unacceptably high aldehyde-oxidase-mediated metabolism.

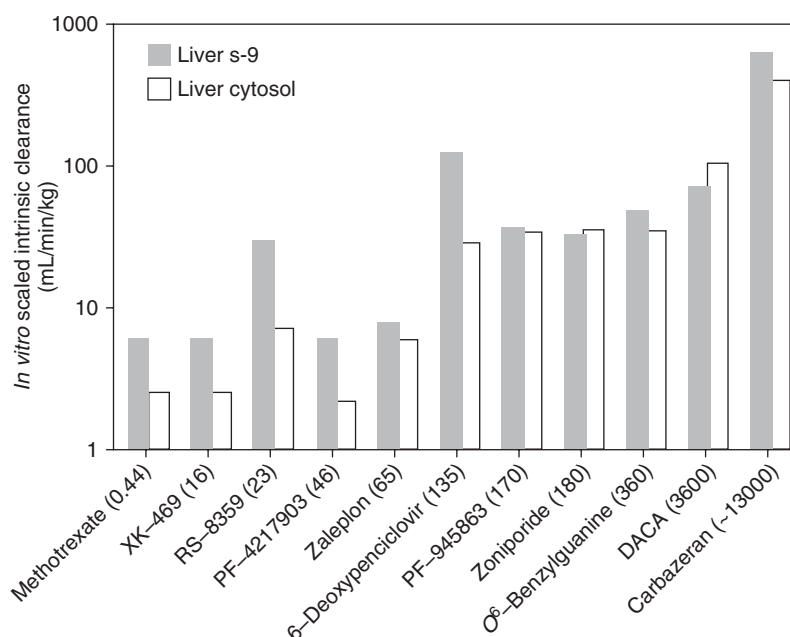


Figure 11.31 Bar graph calibrating *in vitro* scaled intrinsic clearance with *in vivo* free intrinsic clearance for 11 compounds metabolized by aldehyde oxidase in humans. Numbers in parentheses refer to estimated *in vivo* free intrinsic clearance. Bars are placed in the order of *in vivo* free intrinsic clearance (mL/min/kg). Source: Bar graph adapted from Zientek *et al.* [265].

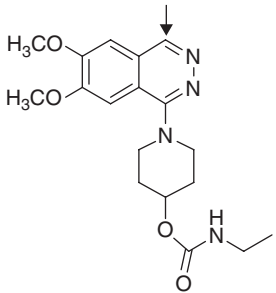
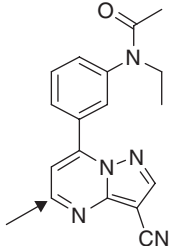
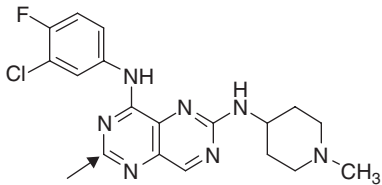
11.9 CLINICAL IMPLICATIONS

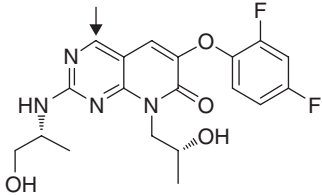
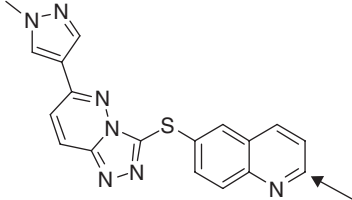
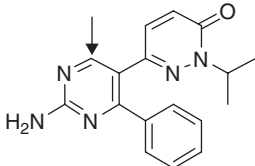
From a drug attrition standpoint, AO appears to have substantially more impact on clinical programs than XOR. Interest in AO by the pharmaceutical industry has increased dramatically of late [2,55]. Several recent literature reports have demonstrated that extensive metabolism by AO has led to the ultimate termination of clinical candidates, either due to toxicological outcomes or due to higher-than-predicted clearance in humans, yielding unacceptable pharmacokinetic properties. In each of these cases, unexpected results were encountered in the clinic, likely because metabolism of these candidates was only evaluated in liver microsomes, which are devoid of AO activity, or predictions of human clearance were extrapolated from preclinical species (e.g., rat, dog) that possessed significantly lower AO activity relative to humans. Metabolically, despite both oxidative and reductive mechanisms catalyzed by AO, oxidation of heterocyclic ring systems appears to be the most relevant pathway for drugs that have met their demise in clinical studies (Table 11.4).

11.9.1 Carbazeran

Carbazeran was an inotropic drug developed in the early 1980s that was perhaps one of the first examples of a clinical candidate that fell victim to extensive AO-mediated metabolism [92]. In early pharmacological investigations in dog, it was observed that carbazeran demonstrated good oral bioavailability (62–74%), which lead to a favorable

TABLE 11.4 Summary of Drug Candidates that have Either been Discontinued Because of Poor Oral Exposure in Early Clinical Studies, had Human Clearance Values Underpredicted, or Demonstrated Toxicity Attributed to Aldehyde Oxidase Metabolism

Drug	Structures	Preclinical Data Package Used for Human PK Predictions	Clinical Outcome	References
Carbazeran		Dog PK	<5% F-Termination	91
Zaleplon		Liver microsomes, mouse, rat, and dog PK	Clearance underpredicted (1 h half-life)	274
BIBX1382		Liver microsomes, mouse, rat, and dog PK	5% F-Termination	275

RO1		Rat, dog, and monkey PK	0.7 h half-life—termination	276
SGX523		Liver microsomes, rat and dog PK	Obstructive nephropathy—Termination	23
FK3453		Liver microsomes, rat and dog PK	M4 metabolite exposure >> FK3453—Termination	78

The arrow indicates the major site of metabolism by aldehyde oxidase.

pharmacodynamic profile in that species. However, following oral dosing to humans up to 350 mg, plasma levels of carbazeran were essentially undetectable, with oral bioavailability <5%. The observed profound differences in pharmacokinetic profile in dog and human has been attributed to the lack of AO activity in dog, with the predominant metabolite in both baboon and human liver cytosol being the AO-derived 4-phthalazinone metabolite [91], which illustrates the extreme importance of understanding species differences in metabolism and identifying a species with AO activity that is representative of human.

11.9.2 Zaleplon

Zaleplon is a sedative hypnotic used mainly in the treatment of insomnia. Clinically, the clearance of zaleplon was underpredicted roughly sixfold, which resulted in a short half-life in humans of 1 h [274]. It was later published that one of the predominant metabolites of zaleplon was 5-oxo zaleplon, confirmed to be AO derived by both chemical inhibition studies, and incubations conducted in the presence of $H_2^{18}O$ [165]. As it turns out, development of this drug was actually not discontinued since the short half-life actually provides advantages for a sleep aid.

11.9.3 BIBX1382

BIBX1382 was a drug candidate being evaluated clinically as an inhibitor of epidermal growth factor receptor (EGFR) for the treatment of cancer. In human liver microsomes, it was observed that BIBX1382 was mainly metabolized by CYP2D6, and in preclinical pharmacokinetic evaluations in rat and mice, oral bioavailability ranged between 50% and 100%. Following oral dosing to human patients, plasma levels were far below that expected to be efficacious (~5% oral bioavailability), and a previously uncharacterized metabolite was observed in the urine of one patient, that was also circulating in human plasma at high concentrations [275]. Retrospective experiments by Dittrich *et al.* also confirmed that BIBX1382 was metabolized by hepatic AO, and that the rhesus monkey appeared to be a suitable surrogate for human AO metabolism.

11.9.4 RO1

The pharmacokinetics of a novel p38 MAP kinase inhibitor was evaluated in six healthy male subjects following a 50-mg oral dose. Surprisingly, the observed terminal mean half-life was only 0.7 h, even though a half-life of 5.9 h had been predicted using common allometric scaling approaches based on pharmacokinetic data from rats, dogs, and monkeys [276]. The major drug metabolite of RO1 (M1) represented 220% of the AUC of parent drug, and was shown to be generated by AO.

11.9.5 SGX523

Extensive metabolism by AO may not only result in poor pharmacokinetic properties but also toxicity. SGX523 was a clinical candidate for an oncology target (c-MET, a receptor tyrosine kinase) that progressed because of favorable potency and selectivity profile for the target, as well as a comparable metabolite profile in liver microsomes from rat, dog, monkey, and humans. Thus, conventional drug safety studies were

conducted in rats and dogs to enable first-in-human (FIH) studies. However, following doses administered to human subjects of >80 mg, acute renal failure was observed [23]. Interestingly, metabolite profiling of human plasma indicated a major metabolite that was not observed *in vitro* in liver microsomes. Retrospective analysis by Diamond *et al.* demonstrated a major NADPH-independent metabolite (M11, 2-quinolinone-SGX523) when SGX523 was incubated with liver S-9 fraction, which was subsequently shown to be generated by AO. In addition, male cynomolgus monkey was shown to generate the M11 AO metabolite in substantial quantities *in vitro*, and safety studies were thus conducted in this species, where the adverse renal effects were reproduced. The ultimate cause of the renal toxicity was deemed to be the M11 metabolite that crystallized in the kidney because of poor solubility. This represents a great example of not just considering pharmacokinetic outcomes, but also toxicity outcomes, highlighting the critical importance of identifying an appropriate toxicology species with a comparable metabolite profile to humans.

11.9.6 FK3453

The most recent example of poor oral exposure in humans that was not predicted is FK3453, a novel adenosine A1/2 dual inhibitor being evaluated in Japan for the treatment of Parkinson's disease. While FK3453 underwent minimal cytochrome P450-mediated metabolism *in vitro* and demonstrated favorable preclinical pharmacokinetic properties in rat and dog, the development of this compound was discontinued because of extremely low oral exposure in humans [78]. It was again found that the poor oral exposure was the result of extensive metabolism by AO.

In summary, when one considers the first example of carbazeran failing in the clinic because of extensive AO-mediated metabolism, which is a time spanning over 25 years, it suggests that first principles in biotransformation are not being followed consistently. Understanding the complete metabolism of drug candidates, and not just the metabolism in human liver microsomes, is crucial. As medicinal chemists have successfully optimized small molecules via chemical modification to have less cytochrome P450-mediated metabolism, other drug-metabolizing enzymes such as AO may begin to play a larger role in drug biotransformation. Thus, a renewed focus by drug metabolism scientists on these types of non-P450 metabolic pathways should become standard practice. These standard practices, as suggested by Pryde *et al.*, should include evaluating metabolism in cofactor-supplemented liver S-9 fraction or isolated hepatocytes [2].

11.10 FUTURE PERSPECTIVES

Since molybdenum-containing hydroxylases are a complex enzyme family, and what is known about these enzymes is in its infancy compared to that of the cytochrome P450s and perhaps uridine 5'-diphospho-glucuronosyltransferase, much work is needed to put in perspective the implications of metabolism for this class of enzyme. As the science of molybdenum-containing hydroxylases in humans and preclinical species matures by building on the foundation of the current knowledge, it will become easier to relate these enzymes in drug research. There are active efforts underway to solidify chemical structure–activity relationships, and once better understood will become a

larger part of drug design. These structural advancements will provide scientists with the understanding as to the diversity of metabolic reactions and mitigation or minimization of molybdenum-containing hydroxylase metabolism. Tools such as specific inhibitors will provide ways to elucidate the contribution of such enzymes, once universally accepted enzyme abundance values has been achieved. From the literature, there have been efforts to understand the enzyme variability in population and the reasons why. This is an area where it is believed will benefit from more intensive research. Taken together, structural understanding, contribution to metabolism, and population variability will place this enzyme family in perspective compared to, and in addition to other drug-metabolizing enzymes.

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12 Amine Oxidases and Reductases

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12.1 Summary	1
12.2 Introduction	2
12.3 Amine oxidases	2
12.4 Azo reductases	11
12.5 Nitro reductases	16
12.6 Other enzyme-catalyzed reduction reactions	17
12.7 Conclusions/further perspectives	20
Acknowledgments	21
References	21

12.1 SUMMARY

In the drug and foreign compound metabolism literature, amine oxidation and carbon oxidation reactions are well documented for the reactions of the FAD-containing monooxygenase [1] and the cytochrome P450s [2]. However, there are many other enzyme systems that are capable of catalyzing these reactions. This review provides an overview of the various amine oxidases, such as the copper-containing amine oxidases (CAOs) and flavin-containing amine oxidases. The copper-containing enzymes also have a prosthetic group formed from a tyrosyl residue in a self-catalyzed oxidation reaction with copper. There are four different prosthetic groups that can be formed from tyrosine or tryptophan and that are critical for the various catalytically important reactions of these related proteins. The flavin-containing amine oxidases play important roles in the metabolism of neurogenic amines, D- and L-amino acids and polyamines. However, they metabolize exogenous compounds as well. A second set of enzymes are involved in the reduction of azo and nitro compounds, mainly involving bacterial and mammalian flavoproteins and the cytochrome P450s. These enzyme systems also reduce quinones, halogenated compounds, hydroperoxides, and α , β -unsaturated aldehydes. This chapter describes the biochemical and molecular details of these genes

and their gene products, with an eye to identifying tools that would be valuable in establishing which might be involved in oxidative and reductive metabolism of new molecular entities synthesized for drug therapy.

12.2 INTRODUCTION

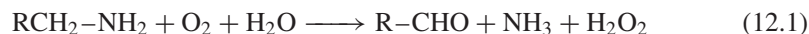
Among the many reactions involved in the metabolism of drugs and foreign compounds, the amine oxidation and reduction reactions are mechanistically the least well documented in the literature [2]. This is due in part to the common use of a single isolated cell types or tissue fractions (microsomes, nuclei, mitochondria, or cytosol) to study foreign compound metabolism in academe and industry. Amine oxidation and reduction often are missed unless intact cells or tissue are used and a careful tissue fractionation study is performed with any new compound of interest [3]. Often, this is due to two different enzyme systems contributing to the activity observed in the same tissue fractions/cell or the second enzyme system existing in an organelle other than the microsomal fraction. Thus, such studies result in an underestimation of the role of these interesting enzymes in the metabolism of nitrogen-containing organic compounds. For example, in our early studies on hydrazine metabolism, we discovered an enzyme activity in the mitochondrial fraction of rat liver that oxidized procarbazine, a 1, 2-disubstituted hydrazine, to its azo derivative [4]. We performed careful subcellular fractionation of rat liver to demonstrate the organelle the enzyme activity was associated with (i.e., microsomes or mitochondria) and further characterized the biochemical nature of the mitochondrial activity. After the use of monoamine oxidase (MAO) inhibitors and partial purification following kynuramine deamination activity, we determined that the enzyme responsible for this activity was mitochondrial MAO. We also demonstrated that procarbazine was a competitive substrate with kynuramine. While cytochrome P450 can oxidize nitrogenous compounds such as primary and secondary amines and hydrazines [5–7], other enzyme systems also oxidize these compounds to *N*-hydroxy or azo compounds. Many of these are the MAOs and diamine oxidases (DAOs) that are discussed in this chapter.

The reduction reactions of azo and nitro compounds are reactions that also have been shown to be catalyzed by cytochrome P450s and other electron-transfer proteins, such as NADPH:cytochrome P450 reductase and NAD(P)H:quinone oxidoreductase. These reactions are also underappreciated and can account for reduction of azo, nitro, and α , β -unsaturated alkene compounds, such as neoprotosil, nitrofur, and 4-hydroxynonenal, respectively. The work of Levine and Lu [8] demonstrated that specific cytochrome P450s, such as CYP4A and CYP3A4, account for the major catalysts for azo reduction in microsomal fractions from liver and intestine, in addition to flavoproteins, such as NADPH:cytochrome P450 oxidoreductase (POR).

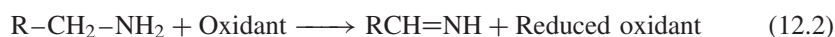
12.3 AMINE OXIDASES

There are two classes of amine oxidases, those that use covalently bound quinones and copper as cofactors and those that use a prosthetic flavin group for catalysis. In general, they catalyze the same chemical reaction, namely, oxidative deamination leading to formation of an aldehyde, ammonia, and hydrogen peroxide (Eq. 12.1). All

three products formed are chemically reactive and specific enzymes are required to dispose of the reactive products formed.



Both classes display bi-bi ping-pong kinetics, which implies that two half reactions are involved in amine oxidation (Eqs. 12.2–12.4). The oxidant is an enzyme-bound prosthetic group that undergoes cyclic reduction/oxidation reactions and varies with each class of amine oxidase. An aldimine intermediate is formed in the oxidation step, but it is chemically unstable and decomposes to ammonia and the aldehyde product (Eq. 12.2). The following section describes the characteristics of these two classes of amine oxidases and the prosthetic groups involved in amine oxidation.



12.3.1 Copper-Containing Amine Oxidases (CAOs)

The CAOs were the first quinoproteins discovered and their characteristics were described by Hauge [9] and Antony and Zatman [10,11] in the early 1960s for the bacterial enzymes glucose dehydrogenase and methanol dehydrogenase. However, the identity of the cofactor involved was not defined for some years and Anthony and Zatman [10] suggested it was a pteridine derivative. These proteins contain a covalently bound quinone formed from a tyrosine or tryptophan residue, which serves as an electrophilic group to covalently bind amines or alcohols, before their oxidation by a metal redox center, yielding hydrogen peroxide as a product. Through the study of bacteria and lower eukaryotes, a role for CAOs in the degradation of amines as a source of energy and basic nutrients was deduced. The presence of CAOs in mammalian tissues suggests that there may be other important roles for these enzymes. There are four known mammalian genes (*AOC1–4*) that are described in detail below. The cofactor for the bacterial enzymes was shown to be a pyrroloquinolone quinone (PPQ) by Salisbury *et al.* [12] and Knowles *et al.* [13], whose studies were based on the earlier work of Duine and coworkers [14]. No examples were known for such novel quinone cofactors in mammalian enzymes until the 1990s, when Hartmann and Klinman [15] suggested a quinone cofactor for bovine plasma copper-amine oxidase, first assumed to be PPQ. Since that time, a number of mammalian enzyme systems involved in amine oxidation have been described and a variety of quinone cofactors have been elucidated. These CAO quinoproteins are divided into four basic forms containing different posttranslationally modified tyrosine or tryptophan residues capable of enhancing the catalysis of oxidative metabolism of primary amines [16]. The four types of side chain-derived quinone cofactor groups that are formed

in eukaryotic organisms use posttranslational, self-driven oxidation of a tyrosine within a highly conserved sequence [Ser/Thr-X aa-X aa-Asn/Tyr (topoquinone (TPQ))-Asp/Glu-Tyr/Asn] to yield a peptide-bound 2,4,5-trihydroxy-phenyl-alanine quinone (TPQ) residue that is the critical catalytic prosthetic group on the enzyme for amine oxidation [17–19]. A second enzyme class consists of proteins containing a lysyl tyrosylquinone (LTQ) prosthetic group [20]. These oxidases have prosthetic groups that involve two critical amino acid residues, lysine and tyrosine, resulting in the formal adduction of the lysine residue to a TPQ intermediate similar to that deduced for bovine serum amine oxidase. The other oxidases have been shown to contain tryptophan-derived quinone cofactor quinoproteins [21–23], where cysteine tryptophanquinone (CTQ) and tryptophan tryptophylquinone (TTQ) residues are covalently linked to form a complex structure (Fig. 12.1).

The formation of the known quino-cofactors requires several steps, starting with copper-catalyzed hydroxylation of either tyrosine or tryptophan residues within a relatively conserved amino acid sequence to yield an *ortho*-quinone ring. The mechanism for the formation of these quinones has been studied with the purified *Hansenula polymorpha* amine oxidase by Klinman and coworkers [17,19]. In the case of TPQ in plasma amine oxidase, no further modification of the tyrosyl quinone occurs for productive catalysis. The unconjugated quinone carbonyl group at the para position allows for Schiff base formation with nucleophilic monoamine or adduction by other

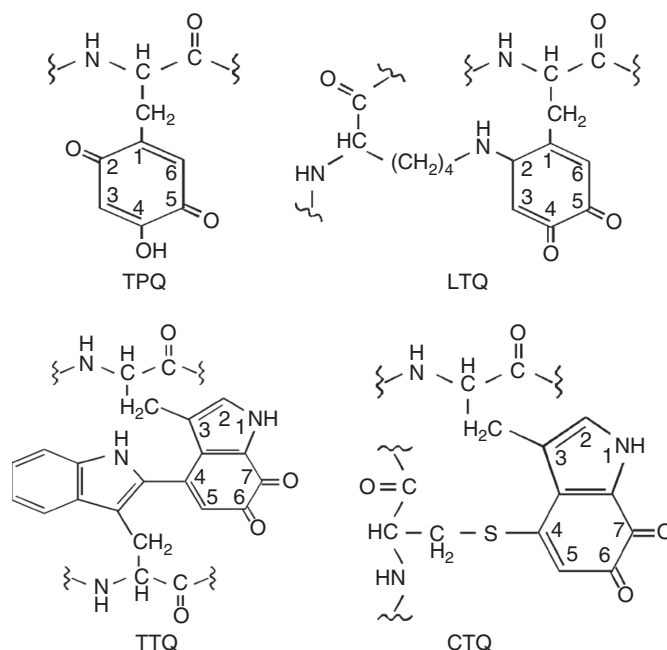


Figure 12.1 Quinone cofactors of various copper-containing amine oxidases as described by DuBois and Klinman [16]. TPQ, 2,4,5-trihydroxy-phenyl-alanine quinone; LTQ, lysine tyrosylquinone; CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone. *Source:* Modified from DuBois JL, Klinman JP. Arch Biochem Biophys 2005;433:255–265., with permission from Elsevier Inc.

inhibitory nitrogenous compounds, such as phenylhydrazine, semicarbazide, clonidine, and phenylethylhydrazine. The other quinoproteins have products of oxidized tyrosyl or tryptophyl rings cross-linked with other nucleophilic amino acid side chain residue, such as lysine, cysteine, or tryptophan. For CTQ, TTQ, and LTQ, the end products are oxidized tyrosyl or tryptophyl residues that are further cross-linked with another nucleophilic amino acid side chain residue, namely, tryptophan, lysine, or cysteine. This cross-linking process may provide a unique function for the proteins, forming a relatively stable cross-link [lysyl oxidase (LOX)], modifying the redox potential of the cofactor to enhance reactivity, protecting the reactive cofactor from side reactions, and/or reducing movement of the cofactor at the active site [24]. Further examples of such quinoproteins and the study of their reactions are needed to define the unique properties of these complex proteins. With that description of quinoproteins, we describe several classes of enzymes whose function might affect the study of drug metabolism, leading to new metabolic products not expected from the cytochrome P450s or FAD-containing monooxygenase.

12.3.1.1 Diamine Oxidase (DAO). The DAOs are a group of enzymes that display substrate specificity for oxidation of diamines, such as histamine [25]. DAO is expressed in the placenta, kidney, gut, lung, and brain. In the intestine and kidney epithelial cells, DAO is released from basolateral vesicles at the plasma membrane in response to heparin and is subsequently cleared from the plasma. The IUB nomenclature designation for DAO is EC 1.4.3.22 classifying it as an amine:oxygen oxidoreductase (deaminating) (copper) enzyme, and it is encoded by the *AOC1* gene found in nearly all mammalian species. The human enzyme appears to have a subunit molecular weight of 70–105 kDa and contains one atom each of copper and magnesium [26,27]. Histamine and 1-methyl-histamine are excellent substrates. A human intolerance syndrome in pregnancy has linked high circulating levels of histidine to decreased levels of plasma DAO [25]. Other substrates described for the human enzyme are spermine, spermidine, acylated putrescine, cadaverine, and related compounds, for example, [8-arginine]vasopressin, [8-lysine]vasopressin, collagen, and tropocollagen. Although DAO has been shown to have some activity toward monoamines, it displays little or no activity toward higher substituted amines. The pH optima of the human enzymes are in the range of 9–10, but other forms have been shown to have other pH optima.

Like the amine oxidases described below, DAOs contain copper and 2,4,5-trihydroxy-phenyl-alanine quinone. In a copper-deficient state in mammals, this enzyme activity may be diminished like the amine oxidases because it easily loses or gains copper depending on the concentration of free copper in the cell [25]. Because DAO contains TPQ and therefore a reactive carbonyl group, they are inhibited by carbonyl-specific reagents such as semicarbazide or phenylhydrazine. The enzyme also has been shown to be inhibited by drugs such as isoniazide (tuberculostatic agent), cimetidine (histamine H₂ receptor agonist), clonidine (antihypertension drug), and several antiprotozoal agents (pentamidine and berenil). 2-Hydrazinopyridine and benzylamine are inhibitors, as they are for the other CAO enzymes, because of the presence of the active carbonyl functional group of TPQ cofactor utilized in the deamination reaction.

12.3.1.2 Retina-Specific Amine Oxidase (RAO or Lysyl Oxidase, LOX). There are four LOX genes in humans (LOXL1, LOXL2, LOXL3, and LOXL4), and their full

comparison has not been fully documented to date. They are believed to be involved in the development of many tissues and also in invasive processes during cancer metastasis [28,29]. A retina-specific amine oxidase (RAO), described by Imamura *et al.* [30] as LOX, is composed of 729 amino acids giving a molecular weight of 80.6 kDa. It is encoded by the *AOC2* gene. Its IUB nomenclature designation is EC 1.4.3.13, and its substrates are collagen and elastin in the extracellular matrix (ECM) of ocular tissues. It is synthesized as a glycosylated proenzyme with molecular weight 50 kDa per subunit and is converted to its active form (molecular weight, 32 kDa) by procollagen C proteinase (bone morphogenic protein-1) before secretion to the ECM of the eye. The disparity in molecular weight may be due to the fact that it is a secreted protein and that it is heavily N- and O-glycosylated, leading to aberrant migration on SDS polyacrylamide gel electrophoresis [31]. Reports of multiple LOX isoforms can be found in the literature [31]. It is ~62.2% related in sequence to the bovine amine oxidase described by Janes *et al.* [18], and the active site is highly conserved among the amine oxidases. Imamura *et al.* [30] also demonstrated that this gene is expressed only in the eye, suggesting an important function of RAO in this tissue. Subsequent studies by Coral *et al.* [32] characterized a LOX enzyme in ocular tissues and suggested that proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RRD) result from ECM remodeling due possibly to improper collagen cross-linking. They demonstrated that LOX enzyme activity is significantly lower in the vitreous of PDR or RRD patients, relative to control subjects. This observation supports the report of Hewitt *et al.* [33] who provided a strong correlation between expression of a LOX variant in the ECM of individuals and the development of pseudoexfoliation syndrome, a condition in which the ECM appears to be aberrant and there is accumulation of abnormal fibrillar material in the anterior segment of the eye. They suggested that this syndrome may predispose humans to a glaucomatous optic neuropathy.

The roles of LOX in development and disease are numerous, as shown by the interest in its role in cancer metastasis, as well as normal tissue development [34]. It is known that strong nucleophiles (lathyrogenic agents), including nitriles, hydrazides, hydrazines, and ureides, cause defects in formation of connective tissues in developing animals. Agents such as β -aminopropionitrile, 2-chloroethylamine, and 2-bromoethylamine are also potent inhibitors of cancer cell metastasis, demonstrating the chemical nature of the quinoprotein. The critical function of LOX in processing collagen and elastin to complete the posttranslational processing of these molecules and its association with disease and defective connective tissue development provide a potentially unique future therapeutic target for future investigation.

12.3.1.3 Semicarbazide-Sensitive Amine Oxidase (SSAO). Semicarbazide-sensitive amine oxidase [SSAO; EC 1.4.3.6; (amine:oxygen oxidoreductase (deaminating) (copper-containing))] is a glycosylated dimeric copper-containing enzyme mostly bound to outer membranes of smooth muscle cells, endothelial cells, and adipocytes; it has been described as a vascular ectoenzyme [35–37] since it appears to be a secreted protein with full enzyme activity. This protein is encoded by the *AOC3* gene. It was initially discovered as a benzylamine oxidase activity found in cultured porcine aortic smooth muscle cells and smooth muscle cell homogenates [38–40]. The enzyme's activity is also present in other tissues, such as uterus, ureter, and vas deferens. Research characterizing the toxicity of allylamine on myocardium and other vascular tissues suggested that SSAO is responsible for the oxidative metabolism to

acrolein, the toxic intermediate responsible for tissue damage. The toxicity toward the myocardium was prevented by administration of hydralazine, a hydrazine-containing drug [41–44], which now can be explained by its adduction to the reactive carbonyl of the quinone cofactor. The unique toxicity of allylamine reported in the literature led Boor and coworkers to characterize the protein by following its semicarbazine-sensitive benzylamine oxidizing enzyme activity. Boor and coworkers [45,46] demonstrated that swine aortic smooth muscle cell homogenates or cultured smooth muscle cells could oxidize allylamine to acrolein and lead to the view that acrolein most likely is the toxic agent causing the deleterious effects of allylamine on myocardial and vascular dysfunction. The purified protein was shown to be a homodimer with each monomer displaying a molecular weight of 90–100 kDa when isolated from either bovine lung or swine aortic smooth muscle cells [42,45,47]. From the primary sequence of the bovine lung enzyme, it contains 762 amino acids to provide a molecular weight of 84.8 kDa. The first 16 amino acids of its N-terminus possibly represent a signal peptide involved in synthesis and secretion. The extracellular portion of SSAO is apparently cleaved to generate a soluble enzyme that circulates in the plasma [42,44], explaining why Boor and coworkers [45,46] could isolate the enzyme from the media taken from cultures of swine aortic smooth muscle cells. Although the protein was identified as a secreted extracellular protein, cell fractionation showed that the majority of intracellular SSAO activity is membrane bound with the principal intracellular site of localization in the microsomal fraction that also contain the golgi apparatus that participates in the secretion process. Careful fractionation of plasma membranes also provided evidence that SSAO is predominantly localized in the plasma membrane of rat and aorta smooth muscle cells, likely facing away from the cell membrane where it can encounter its substrates [35,42]. Since it can be found in the media during culture of swine aorta smooth muscle cells [46,48], it appears that it can be processed to a soluble form as discovered by Boor's group.

In addition to benzylamine and allylamine, circulating amines such as methylamine, 2-phenylethylamine, aminoacetone, histamine, tyramine, tryptamine, and protein-bound amines appear to be substrates for SSAO [35]. The substrate specificity varies depending on the species studied. As predicted by the reaction it catalyzes (Eqs. 12.2 and 12.3), it displays a ping-pong catalytic mechanism. A unique feature of the enzyme is that it binds copper tightly relative to the DAO and plasma amine oxidase (PAO) [35,46]. As its name implies, it is inhibited by strong nucleophiles, including semicarbazine, amiloride, hydralazine, phenelzine, and phenylhydrazine.

SSAO has been shown to be identical to vascular adhesion protein-1 (VAP-1) that is induced during inflammation and mediates interaction between lymphocytes and endothelium [49]. The tissue distribution of VAP-1 is similar to SSAO and is responsible for inducing lymphocytic cell adhesion and granulocyte extravasation [50]. Ongoing research in vascular biology suggests that baseline VAP-1/SSAO activity can be used as a predictor of hemorrhage after tissue plasminogen activator (tPA) treatment. Since semicarbazide and other compounds, such as hydralazine, ameliorate VAP-1/SSAO activity, it is proposed that anti-VAP-1/SSAO drugs given with tPA may prevent neurological disorders following with ischemic stroke [51].

12.3.1.4 Plasma Amine Oxidase. The plasma amine oxidases are the best studied mammalian CAO, as exemplified by the extensive research done by a number of groups and the seminal work of Klinman and her collaborators in identifying the cofactor [16].

Among the early publications on the plasma amine oxidases was a report by Mason and coworkers [52] demonstrating that this enzyme contains copper in its active state; the enzyme also has been suggested to contain zinc, which is essential for its activity. Yet a number of studies using electron paramagnetic resonance could not detect a redox change in the Cu(II) center during catalysis [53]. Blaschko and coworkers [54] also demonstrated the essential role of copper by administering a diet deficient in copper and measuring the resulting enzyme activity of the plasma. As a copper enzyme, its bluish color can be observed during the later steps of the purification process. In addition, Blaschko *et al.* [54] suggested the enzyme contained pyridoxyl phosphate because of its absorbance at around 440 nm, a common characteristic of the TPQ that can be followed spectrophotometrically during the course of self-catalysis of the native protein to the active posttranslational modified enzyme. Subsequently, several groups [13,54] documented that the purified enzyme did not contain pyridoxal phosphate, but on the basis of NMR spectra suggested that it contained PPQ. At the time Klinman began her studies on plasma amine oxidase, she referred to PPQ as the cofactor in her 1987 publication using reductive trapping of substrate/inhibitor to the bovine plasma amine oxidase [15]. Subsequently, she deduced that the cofactor was not PPQ, but a new quinone cofactor, TPQ. Her group also established the oxidative mechanism by which the tyrosyl residue was converted to the TPQ cofactor [17–19, 54].

Plasma amine oxidases are listed with the IUB nomenclature EC 1.4.3.21 as being amine:oxygen oxidoreductase (deaminating) (copper-containing). It is encoded by the CAO4 gene (*AOC4*) in humans [44]. These enzymes have been purified and characterized from several species (bovine, humans, swine, and pea seedlings); they share many properties in common. The PAOs display native molecular weights of 180 kDa and are composed of two subunits (70–90 kDa) accounting for the presence of two copper (II) molecules and two active carbonyl groups per mole of enzyme, the latter being susceptible to adduction with amines and hydrazines. Three critical histidine residues are proposed to be available for the binding of copper, and early work using zinc to displace copper demonstrated that this protein can easily be made replete of copper. This observation also provides an explanation for reduced levels of PAO in plasma during dietary depletion of copper [44,52,55]. Although benzylamine and methylamine have been the major substrates used in studies of this enzyme, there are reports that the natural substrates for this class of enzymes are physiologically active amines, such as spermidine and spermine, catecholamines, and tryptamine derivatives. Other amines such as tyramine have been shown to be oxidized but with low affinity, and tryptamine, epinephrine, serotonin, or agmatine do not appear to be substrates [52,55]. The pH optima for the reactions varies depending on the substrate in use; for example, for spermine, the pH optimum is 6.2, while for spermidine it is 7.2 [56].

AOC4 is known to be expressed in mammalian liver, with the encoded protein being a secreted protein, leading to the high levels seen in bovine and porcine blood. In humans and rodents, there are much lower levels of this activity in plasma, although these species all have an *AOC4*-related gene. In these species, *AOC4* exhibits a single base change that converts the codon 255 (tryptophan) to a stop codon, yielding a nonfunctional, truncated protein [44]. In humans, the mRNA can be detected in the liver, and splice variants of the gene have been reported [57]. In the mouse and rat, only fragments of the *AOC4* gene exist.

Since the enzyme is a quinoprotein, strong nucleophiles, such as aryl- and alkylhydrazines, and other nitrogenous compounds inhibit oxidation by forming hydrazide or

semicarbazide forms of the cofactor. Clonidine, an antihypertensive that binds to α_2 receptors, is an inhibitor that changes the orientation of the TPQ relative to copper, yielding an inactive cofactor–metal complex [58]. Adduction by these compounds to TPQ prevents the nucleophilic attack by the amine substrate as the first catalytic step, as shown by Klinman and DuBois [16] for phenylhydrazine.

12.3.1.5 Summary of CAOs. Since these CAOs have K_{cat} values equal to or greater than those of the cytochrome P450s, drugs and foreign compounds that contain a primary amine or that can be converted to a primary amine can be oxidatively deaminated to aldehyde metabolites. These aldehydes, such as acrolein in the case of allylamine, can cause toxicity, particularly if they are generated near sensitive target organs and have been proposed to result in aberrant cell signaling [59]. In addition, if primary amines contain an amino group that is a good nucleophile, but are not CAO substrates, they can serve as competitive inhibitors altering the disposition of other substrates. These properties allow the CAOs to be part of a drug–drug interaction mechanism that should be considered where warranted during drug development.

The CAOs are affected by dietary depletion of body stores of copper, and therefore, copper-deficient patients might experience difficulties with certain drugs or compounds that are CAO substrates, as has been seen with histamine-sensitivity syndrome seen during pregnancy. To date, relatively few studies exist related to the study of CAO regulation or limits on the self-driven posttranslational modification to form an active enzyme. Therefore, when amine compounds are a focus of study, one should be aware that CAOs are clearly involved in amine metabolism and are also critical for defined steps in collagen processing and connective tissue formation.

12.3.2 Flavin-Containing Amine Oxidases

A number of flavin-containing amine oxidases exist that rely solely on a flavin prosthetic group as the oxidizing cofactor in their catalytic mechanism (Eq. 12.2). These enzymes are distinctly different from the microsomal FAD-containing monooxygenases first described by Ziegler and Poulsen [1]. Owing to the role of flavin-containing amine oxidases in the metabolism of monoamines and D- and L-amino acids, they have been extensively studied and are defined as being amine:oxygen oxidoreductase (deaminating) enzymes with an IUB EC 1.4.3 designation. The enzyme family consists of several subclasses depending on their subcellular localization and amine substrate preferences. They are distinguished as MAOs, DAO, and polyamine oxidases. Because there is considerable sequence identity among these oxidase enzymes, it is presumed that they have similar molecular structures. In addition, there are multiple enzyme forms within each subfamily as seen below. The catalyzed reactions are a subset of the reactions as shown in Equations 12.1–12.4, except a flavin, such as FAD or flavin mononucleotide (FMN), is used as the prosthetic group. The first products formed in their reaction is reduced flavin and an aldimine intermediate (Eq. 12.5) that subsequently hydrolyzes to form an aldehyde and ammonium ion (Eq. 12.6). Molecular oxygen is the terminal oxidant for most reduced flavoproteins of this group (Eq. 12.7).



12.3.2.1 Monoamine Oxidase A (MAO A and MAO B). The MAOs are flavin-dependent oxygenases catalyzing the same basic reaction as the copper-containing quinoprotein class of enzymes (Eq. 12.1). A review article by Shih *et al.* [60] provides an overview of the MAOs and has an extensive reference list addressing the characteristics of the two enzyme forms (A and B) that are classified as EC 1.4.3.4 enzymes [61]. Their molecular weights deduced from their amino acid sequences are 59.7 and 58.0 kDa [62], respectively, and they have 70% amino acid sequence identity. MAO A and B are encoded by separate genes located on the X chromosome. They are membrane-bound enzymes found in the outer mitochondrial membrane in the brain and many other tissues, including liver and kidney [63]. Their cofactors are covalently linked FAD molecules, with a cysteine thiol linkage to the 8a position of the isoalloxazine ring [64]. The best studied substrates include dopamine, noradrenaline, 5-hydroxytryptamine, and tyramine (MAO A substrates), while benzylamine and 2-phenylethylhydrazine are preferred substrates for MAO B [60,65]. Excessive intake of tyramine, an MAO A substrate has been associated with the “cheese” reaction, in which processing of tyramine is inhibited by MAO inhibitors, leading to hypertensive crises. Clearance of some foodstuffs high in tyramine or other vasoactive amines is now known to be dependent on the MAOs, and their routine clearance is affected by the MAO inhibitors [39,66]. MAO B also oxidizes 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to its toxic metabolite. Production of this metabolite apparently is involved in the selective destruction of nigrostriatal neurons, resulting in a Parkinson-like disorder [67]. There are also form-specific inhibitors that allow differential inhibition in experimental studies, namely, clorgyline (MAO A specific) and deprenyl and pargyline (MAO B specific). Some species-specific differences in substrate specificity and inhibitor sensitivity are seen between the two enzymes.

In addition to amines, Coomes and Prough [4] demonstrated that MAO can oxidize monoalkyl- and 1,2-disubstituted hydrazines to form diazene and azo metabolites, respectively [68]. In the case of the monosubstituted hydrazines, the diazene intermediates are potent electrophiles capable of protein or nucleic acid binding, while the azo metabolites of 1,2-dimethylhydrazine and procarbazine (a 1-methyl-2-benzylhydrazine derivative) are stable but can be further oxidized to azoxy metabolites that are mutagenic [68]. Erikson and Prough [69] demonstrated that MAO catalyzed the oxidative metabolism of procarbazine, 1,2-dimethylhydrazine, *n*-propylhydrazine, and iproniazid. In the case of procarbazine, both clorgyline and propargyline were inhibitors of MAO A- and MAO B-catalyzed oxidation to azoprocabazine. Although endogenous amine metabolism by MAOs has been extensively studied because of their role in clearance of neuroamines, their metabolism of exogenous amines has been less well characterized.

12.3.2.2 D-Amino Acid Oxidases (DAAO). The pig kidney D-amino acid oxidase (DAAO) was one of the earliest flavoprotein oxidases discovered. This enzyme [EC 1.4.3.3, L-amino-acid:oxygen oxidoreductase (deaminating)] is a dimeric protein, with a subunit molecular weight of 38 kDa and containing a molecule of FAD per subunit [70]. DAAO principally is localized in the peroxisomal compartment of the pig kidney and is closely related to D-aspartic acid oxidases, comprising a small family of proteins with similar function. DAAO metabolizes most D-amino acids containing a single carboxylic acid group, from D-alanine to D-tryptophan, while D-aspartic acid oxidases metabolize dicarboxylic amino acids. The enzymes require substrates with both an amino and carboxylic acid group to achieve high levels of oxidative metabolism, and

the keto acids of the amino acids formed during oxidative metabolism by this enzyme are product inhibitors of the reaction. Since D-amino acids cannot be used for protein synthesis, the existence of DAAO in kidney lysosomes is believed to be critical for the disposition of D-amino acids.

12.3.2.3 L-Amino Acid Oxidases (LAAO). The substrate specificity for L-amino acid oxidase (LAAO), L-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2, includes the L-amino acids, namely, alanine, aspartate, methionine, valine, leucine, isoleucine, tyrosine, phenylalanine, and tryptophan. Similar to DAAO, the keto acid metabolites of these amino acids are also inhibitors of this enzyme system. The enzyme purified from rat kidney was shown to have a subunit molecular weight of 49.3 kDa determined by flavin content, and on the basis of this molecular weight and the amount of flavin associated with the protein, is consistent with two FAD molecules per molecule of enzyme [71]. It has been assumed that this enzyme allows the clearance of L-amino acids that accumulate in the kidney because of excessive protein catabolism and release from various tissues before excretion. In murine lymphocytes, this enzyme was shown to have a molecular weight of ~90 kDa, suggesting its existence as a dimer in this cell type [72]. The enzyme's function in lymphocytes has been suggested to be antigen processing in lysosomes. During the development of drugs, namely, levodopa and carbodopa, that protect against excessive dopamine metabolism in brain, researchers were concerned that LAAO might be involved in the metabolism of dopamine and therefore may be a drug target for inhibition or inactivation. Other LAAOs in nature have been purified from venoms of various vipers, that is, *Calloselasma rhodostoma*, *Naja naja kaouthia*, and *Ophiophagus hannah*. These enzymes have similar properties to those characterized in mammalian species.

12.3.2.4 Polyamine Oxidases. Another member of the flavin amine oxidase family are enzymes that oxidize polyamines (EC 1.5.3.11). These enzymes are highly conserved among humans, mice, and rats and act on the acetylated products of spermine and spermidine, namely, *N*¹-acetylspermine and *N*¹-acetylspermidine, to produce spermidine and putrescine as products. *N*¹, *N*¹²-diacetylspermine, formed by acetylation of spermine via spermidine/spermine *N*¹-acetyltransferase, is also a substrate for the enzyme. The molecular weight of the polyamine oxidases is 61.8 kDa, and they contain FAD as the prosthetic group [73,74]. These enzymes are believed to be critical in maintaining polyamine levels in cells, although in at least some cells, the coupling of the polyamine transporter, SLC3A2, to the spermidine/spermine *N*¹-acetyltransferase suggests an equally important mechanism to control intracellular polyamine levels [75].

12.4 AZO REDUCTASES

Another set of reactions that have not been as well characterized as the dealkylation or ring hydroxylation reactions of cytochrome P450 are the azo, nitro, and other reduction reactions [2]. The azo reduction reactions have been of interest to pharmacologists and toxicologists for some time because of their effect on the azo dye industry, which produces azo dyes that are largely used for cloth coloration or as pharmaceutical agents for preventing bacterial contamination. In his 1991 review, Levine [76] described the role of azo dyes in carcinogenesis and mutagenesis and also addressed reductive

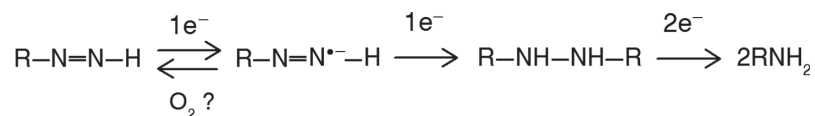


Figure 12.2 Steps of azo compound reduction to hydrazine and primary amines, including the putative reoxidation of radical intermediates by molecular oxygen. Hydrazine compounds have also been shown to be oxidized by cytochrome P450s, copper-containing amine oxidases, and monoamine oxidases [4–7].

metabolism in bacterial and mammalian cells. The oxidative metabolism of azo dyes includes N-dealkylation, aromatic ring hydroxylation, and N-hydroxylation to form azoxy derivatives, which have been extensively studied [68,77,78]. All these reactions tend to be catalyzed by the cytochrome P450s, and in the case of procarbazine, the oxidation of this 1,2-dialkylhydrazines to the azoxy derivatives leads to formation of a reactive methyldiazonium ion that probably accounts for the alkylation potential of this anticancer drug [7,79]. Azo functional group reduction occurs in the absence of molecular oxygen, and depending on the azo compound in question, reduction can occur in the presence of molecular oxygen. Since cytochrome P450 requires molecular oxygen for oxidation of C- and N-centers, only the reduction reaction can occur in the absence of oxygen. The reaction steps for azo reduction are shown in Fig. 12.2.

This review focuses on the unique azo reduction reactions catalyzed by mammalian liver microsomes and some bacterial systems. Interest in azo reduction was increased after the unique work of Bovet and others [80,81], who elucidated the need for reductive bioactivation of the antibacterial azo dye protosil, which on reduction yields sulfanilamide in rabbits. This is a classic prodrug reductive activation process. Other azo compounds were subsequently developed as effective antibacterial agents, but often, their azo linkage was not reduced, leading to excretion through a glucuronidation pathway. Miller and Miller [82] who studied the metabolic activation of butter yellow (*N,N*-dimethylaminoazobenzene) also demonstrated that NADPH in the presence of liver microsomes and oxygen could not only sustain the demethylation of this azo dye but also reduce it to *N,N*-dimethyl-*p*-phenylene-diamine [77,78]. The chemical properties of various azo dyes appear to dictate how easily they are reduced; these properties are addressed in the following sections as well. In addition to the cytochrome P450s, other enzymes play a role in azo dye reduction, principally flavoproteins.

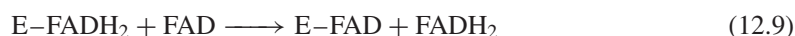
12.4.1 Flavoproteins

As mentioned by Levine in his 1991 review [76], many azo dyes used in agricultural or clinical applications are taken orally, and only a small fraction of the parent compound is excreted in the urine with the azo linkage intact, suggesting that reductive metabolism is a major route of disposition. If administered by routes other than per os, substantial biliary excretion of the intact dye can be observed in experimental animals. The lack of reduced azo compound in bile suggests that if azo reduction occurs, the metabolites are rapidly oxidized back to the azo compound in liver or that the gut microflora may be significantly involved in disposition of azo dyes. Their uptake is dependent on solubility because those with charged groups, such as sulfonates, are apparently not well absorbed. More lipid-soluble dyes, such as dimethylaminoazobenzene, were shown

by the Millers [77,78] to be extensively metabolized by both oxidative and reductive metabolism when administered by intraperitoneal injection. Their work demonstrated step-wise N-demethylation and ring hydroxylation reactions leading to products that are excreted into the bile as glucuronides, while reduction to stable products such as amine compounds leads to urinary elimination.

Walker published a review on azo compound metabolism in 1970 [83] and summarized for the first time the understanding of azo dye metabolism. Several important points were made in this review, including the fact that several groups had shown that water-soluble azo dyes were mostly reduced in the gut, based on the pronounced effects of antibiotics. Ryan and Wright [84] used the dye tartrazine, which was not reduced by liver extracts in the presence of NADPH. When added to cell-free extracts of *Proteus vulgaris*, several azo dyes were shown to be rapidly reduced, supporting the view that bacterial cells can reduce azo dyes. In subsequent work, Ryan and coworkers [85] showed that a number of bacterial isolates of differing origin can reduce azo dyes, and thereby, they established that gut bacteria are an important source of reduction in rodents and probably humans. At this time, others were purifying the bacterial NAD(P)H-dependent reductases and several enzymes were shown to reduce a number of dyes [86,87]. The bacterial flavoproteins normally do not bind FAD or FMN as tightly as the mammalian flavoproteins do, so the addition of FAD or FMN was required to optimize the azo reduction reaction.

Hernandez *et al.* [88,89] noted that purified lipase-solubilized NADPH:POR catalyzed the anaerobic reduction of the azo compound neoprotosil to sulfanilamide. Using inhibitors of the oxidoreductase, the microsomal azo reductase activity was concordantly inhibited with the activity of the purified enzyme. Studies at the NIH by Gillette's group also addressed nitro reduction reactions, and the group found that these reactions were stimulated by the addition of FAD. Similar experiments with exogenous FAD were performed by Walker [83], where he noted that if FAD was added to liver microsomal fractions, the azo reductase activity increased. The apparent azo substrate specificity approximates first-order kinetics, because of the slow step of reduction being the reaction of reduced flavin with the dye. Many bacterial flavoproteins have since been isolated and nearly all reduce azo and nitro compounds, suggesting that this reaction is a nonphysiological reaction of nearly all flavoproteins [86,87,90]. These reactions most likely account for the metabolism of azo dyes in the intestine (Eqs. 12.8–12.10), although flavin-independent reactions can occur with bacterial enzyme systems by direct reduction with reduced pyridine nucleotides [91]. However, many of these flavoprotein-catalyzed reductions are oxygen sensitive and the reduction reactions are normally only seen during anaerobic metabolism, depending on the dye substrate's chemical properties (electrochemical potential, etc.).



12.4.2 Cytochrome P450

The studies of Mueller and Miller [77,78] were among the first to address the possibility of cytochrome P450 serving as the reducing enzyme for azo reduction. Masters

et al. [92,93] had shown that steapsin, a pancreatic lipase preparation from pigs, could release a cytochrome *c* reductase enzyme activity from liver microsomes. Masters' group subsequently purified the membrane-bound form of the flavoprotein [94], which retains its azo reductase activity (Masters BSS, Prough RA, unpublished research). Their subsequent work demonstrated that the enzyme released by lipase treatment was a proteolytic form of the NADPH:POR. Hernandez *et al.* [88,89] used the method of Masters *et al.* [92] to purify the oxidoreductase to homogeneity and demonstrate that the pure enzyme could reduce neoprontosil under anaerobic conditions. When Hernandez *et al.* [88,89] utilized steapsin to release the oxidoreductase from the microsomal membrane, they noted that azo reductase activity was decreased to 25% of control, but NADPH:cytochrome *c* reductase activity actually increased 20%; neotetrazolium dye reduction to formazin also increased slightly. They also noted that on induction of cytochrome P450 in liver microsomes by 3-methylcholanthrene, the microsomal azo reductase activity increased without a concordant increase in reductase activity, suggesting that the polycyclic aromatic hydrocarbon inducible cytochrome P450s (the CYP1 family) were azo reductases. When they used phenobarbital as an inducing agent, both cytochrome P450 and oxidoreductase levels were induced. Subsequently, they used carbon monoxide as an inhibitor of cytochrome P450-dependent reactions and noted that CO decreased microsomal azo reductase activity by 30–40% with liver microsomes from control, 3-methylcholanthrene- or phenobarbital-treated rats. The azo reductase activity of purified oxidoreductase was unaffected by replacing the air atmosphere with CO. Hernandez *et al.* [88,89] proposed that there were two enzyme systems that resulted in anaerobic reduction of neoprontosil, the first was the NADPH:POR itself and the other system was cytochrome P450, which itself requires two-electron transfer from the oxidoreductase to maintain its catalytic process (Fig. 12.2).

The subsequent work of Levine *et al.* [76,95,96] addressed several points about azo dye reduction; first, which cytochrome P450s catalyze azo reduction and second, why some azo dyes required anaerobiosis for reduction by these systems and others do not. In their studies, they identified two types of azo dye substrates related to 4-dimethylaminoazobenzene, those substrates whose reduction was sensitive to O₂ and CO and those that were insensitive [95,97]. A common chemical feature of the two classes was that the insensitive class tended to have electron-donating ring substituents such as -OH, -NH₂, -NH-CH₃, or -N(CH₃)₂ group. The sensitive class of substrates contained electron-withdrawing groups, such as -SO₃H, -COOH, -COOCH₃, and -AsO₃H₂. They also noted that cyanide ion potentially inhibited the reduction of the O₂-insensitive azo dye substrates, suggesting that the insensitivity/sensitivity may be a property of the ferrous cytochrome P450 [98]. Azide did not differentially affect the azo reduction of their set of compounds. One explanation for the differences in O₂/CO sensitivity was that the hydrazine intermediates (Fig. 12.2) may have differential sensitivity to reoxidation by molecular oxygen to the azo intermediate or alternatively be reduced to the primary amine metabolite. On the basis of the work from our groups, we know that hydrazines are easily oxidized by cytochrome P450s and are also sensitive to metal-catalyzed oxidation [68,99]. The mechanism accounting for the oxygen sensitivity or insensitivity remains unsolved.

Pursuing the approach of Hernandez *et al.* [88,89], Levine demonstrated that induction of liver microsomal enzymes from animals treated with various chemicals greatly affected azo dye reduction and each condition displayed a different substrate specificity for azo dyes [100]. Induction by clofibrate increased azo dye reductase activity

for nearly all dyes, suggesting a role for the CYP4 family of cytochrome P450s in azo dye reduction, while phenobarbital, pregnenolone-16 α -carbonitrile, and isosafrole were all inducers of azo dye reduction for *O*-methyl red [8,100]. Their results demonstrated that members of the CYP1, CYP2, CYP3, and CYP4 families all reduce azo dyes under anaerobic conditions and depending on the structure of the azo dye, possibly under aerobic conditions. Mallett *et al.* [101,102] utilized purified NADPH:POR and CYP2B1 to reconstitute the anaerobic reduction of the azo dye amaranth, an oxygen-sensitive reaction. In addition, they added FMN and FAD to yield higher rates of reduction of the dye. In the absence of FMN, amaranth reduction was dependent on oxidoreductase, CYP2B1, and dilauroylphosphatidylcholine; in the presence of FMN, the oxidoreductase appeared to be the source of reduction. They proposed two mechanisms of reduction for amaranth: direct reduction by oxidoreductase that was stimulated by addition of FAD and cytochrome P450-mediated reduction in the absence of FAD. Clearly, CO-insensitive azo reduction reactions are apparently unaffected by flavin deficiency, while the flavoprotein-dependent azo reduction reactions are heavily affected by depletion of flavin *in vivo* or supplementation of flavin *in vitro* [103].

Milton *et al.* [104] demonstrated that clofibrate was a potent inducer of CYP4A1 in rat liver and defined several inhibitors of the enzyme, including saturated fatty acids and clofibrate. Raza and Levine [105,106] demonstrated that azo dyes were most effectively reduced in liver microsomes from ciprofibrate-treated rats. In addition, they demonstrated that addition of *N,N*-dimethyl-4-aminoazobenzene to reaction mixtures containing liver microsomes from clofibrate-treated rats inhibited lauric acid hydroxylation and that lauric acid inhibited *N,N*-dimethyl-4-aminoazobenzene reduction with these same microsomal fractions. Their work also demonstrated that a CYP4A-specific inhibitor, 10-undecynoic acid, inactivates laurate hydroxylation, but not azo reductase activity, suggesting that the active site for oxidative metabolism is affected by the inhibitor for laurate oxidation, but not that for azo dye reduction. In addition, they reconstituted NADPH:POR with purified CYP4A1 to demonstrate that *N,N*-dimethyl-4-aminoazobenzene azoreduction is catalyzed by this enzyme in the absence or presence of oxygen, but in the presence of oxidoreductase alone, the flavoprotein could only reduce it under anaerobic conditions. Finally, they noted that in the reconstituted system, inclusion of FAD greatly enhanced azo reduction; the oxidoreductase alone was nearly as effective in reducing azo dyes in the presence of FAD than the reconstituted system. Lu and Levine [8] performed a more detailed study that demonstrated that azo reduction by cytochrome P450 is a two-electron process yielding the stable hydrazine (two-electron) or amine (four-electron) reduction product, while the oxidoreductase apparently only catalyzes one-electron reduction to an intermediate that is rapidly oxidized to the azo derivative.

Walker [83] and Levine [76] discussed the one- and two-electron reduction processes involved in azo dye reduction, arguing that those that are oxygen-insensitive must form stable two-electron reduced products like the hydrazine derivative, while oxygen-sensitive processes may catalyze one-electron reduction to a radical. It is known that molybdopterin-containing enzymes [76], such as aldehyde and xanthine oxidase, also reduce azo dyes but are sensitive to cytochrome P450 inhibitors, such as SKF-525A and metyrapone. These complex redox centers have common properties to the cytochrome P450s that make defining specific inhibitors difficult. However, one should consider whether any reduction reactions are noted to be oxygen/CO sensitive when studying

new molecules that are reduced, as well as the relative sensitivity to cyanide and azide ions to characterize various reactions.

12.5 NITRO REDUCTASES

Similar studies have been performed with nitro compounds, and among the early works, Gillette's group at the NIH documented that anaerobic nitro reduction of *p*-nitrobenzoate was catalyzed by liver microsomal protein in the presence of NADPH [107,108]. Carbon monoxide atmosphere caused an 80–90% inhibition of nitro reduction. In addition, they studied the formation of aniline from nitrobenzene, nitrosobenzene, and phenylhydroxylamine, noting that nitrosobenzene and phenylhydroxylamine were rapidly converted to aniline in a CO-sensitive reaction. The oxygen sensitivity of aniline production from any of the proposed metabolites reported in the literature suggests that anaerobiosis is required for net reduction due to the ease of oxidation of the substrate and intermediates by oxygen. On the basis of their studies, they described a reaction that required four 1-electron reduction steps (Fig. 12.3).

Other groups studied the role of NADPH:POR in these processes. For example, Delaforge *et al.* [109] demonstrated that trinitroglycerin is also reduced to eventually allow liberation of nitric oxide. Aristolochic acid is a nutraceutical found in evergreen and deciduous woody vines and herbaceous perennials of *Aristolochia* and *Asarum* of the Aristolochiaceae plant subfamily and is a naturally occurring nephrotoxic nitro compound. On reductive bioactivation, it forms DNA adducts in rodent tissues *in vivo* [110]. Bioreductive activation of the 6-nitro group of aristolochic acid by members of the CYP1A family has been proposed by Stiborova *et al.* [110] as the bioreductive activation mechanism of this chemical. Reduction of the nitro group leads to formation of a putative nitrenium intermediate and its subsequent reaction with nucleic acid bases results in formation of DNA and protein adducts.

Harada and Omura [111] provided mechanistic details about nitro reduction by demonstrating that inhibitory antibodies to NADPH:POR and several cytochrome P450s inhibited anaerobic reduction of *p*-nitrobenzene, but not antibodies against NADH:cytochrome *b*₅ oxidoreductase or cytochrome *b*₅. They also reconstituted the purified oxidoreductase and cytochrome P450s to document that the latter were required for the final step of reduction of phenylhydroxylamine to aniline, while the oxidoreductase could catalyze reduction of nitrobenzene and nitrosobenzene alone in the presence of NADPH. Moreno *et al.* [112] utilized electron spin resonance (ESR) to study trapped radical species formed during *p*-nitrobenzene reduction. They observed that under aerobic conditions, the major radical trapped was superoxide

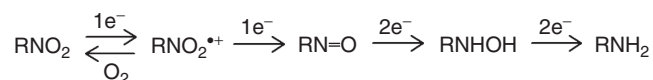


Figure 12.3 Steps of nitro reduction to nitrosoamine, hydroxylamine, and primary amine derivatives, including the putative reoxidation of radical intermediates by molecular oxygen. Hydroxylamine compounds have been shown to be oxidized to nitroso derivatives by cytochrome P450s [6].

anion radical. At low oxygen concentrations, the putative radical species undergo intramolecular electron transfer and rearrange to form carbon-centered nitrobenzyl radicals that could also be measured by ESR. Radical trapping was not inhibited by CO or the nitrogenous cytochrome P450 inhibitor metyrapone. Radical formation for *m*-nitrobenzyl anion radical did not achieve a steady-state concentration, unless metyrapone or CO was included in the reaction mixture. This observation suggests that the POR is a major source of reduction [113,114], and this oxidoreductase-dependent reaction is increased by inclusion of cytochrome P450 inhibitors.

The study of other nitro compounds, 4-nitroquinoline *N*-oxide and 4-hydroxylaminoquinoline *N*-oxide, strongly supports the role proposed for the oxidoreductase in their reduction [115]. Furthermore, the studies suggest that this reaction is due largely to one-electron reducing flavoproteins such as POR since two-electron-donating flavoproteins, such as NAD(P)H:quinone oxidoreductase, do not generate superoxide anion radical. However, POR can reduce the one-electron reduced nitro compounds further, unless molecular oxygen is present to oxidize the radical species [114]. Many bacterial flavoproteins in the gut also catalyze these reactions and may protect mammals from nitro compounds taken in the diet. Bacterial and mammalian flavoproteins appear to be particularly effective in catalyzing reduction of nitro compounds, and the role of cytochrome P450s is perhaps less important for these compounds.

12.6 OTHER ENZYME-CATALYZED REDUCTION REACTIONS

A number of other reports of substrates being reduced by flavoproteins and possibly by cytochrome P450s have been reported. These have not been studied in depth, and anecdotes of such reports are included in the discussion below.

12.6.1 Quinones

Quinones have long been known to be reduced by most flavoproteins. The studies of Masters *et al.* [92] show that POR reduces cytochrome *c*, menadione, and 2,6-dichlorophenol indophenol. Our laboratories [116–118] also have studied the reduction of *t*-butylquinone by both POR and NAD(P)H:quinone oxidoreductase, suggesting that benzocyclic, naphthocyclic, and complex polycyclic aromatic hydrocarbon quinones are good electron acceptors for the flavoproteins. POR serves as a one-electron reducing flavoprotein, while NAD(P)H:quinone oxidoreductase serves as a two-electron reducing flavoprotein. In the case of one-electron reduced semiquinone radicals, they rapidly react with molecular oxygen to generate superoxide anion radical, but two-electron reduced hydroquinones react more slowly with molecular oxygen in the presence of metal catalysts [116,119]. A consequence for this difference is that one attains higher levels of quinone reduction by two-electron reduction because less radical quinone intermediate is available for reoxidation by molecular oxygen (Fig. 12.4). For the purpose of this review, we address only information known about reactions catalyzed by cytochrome P450s.

Capdevila *et al.* [120] used spectroscopic methods to study quinone reduction and reoxidation of benzo[*a*]pyrene-3,6-quinone as a model quinone. Reduction and reoxidation could be monitored at 480 nm (wavelength maxima for B[*a*]P-3,6-quinone). The quinone was observed to be rapidly reduced by liver microsomal fractions. The

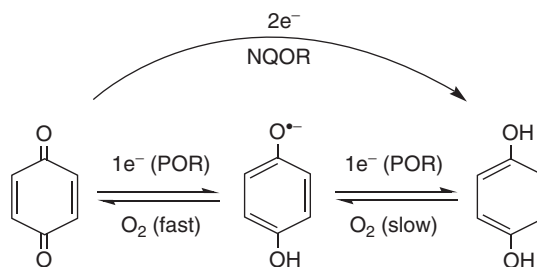


Figure 12.4 Reduction of quinones by the flavoproteins, NADPH:cytochrome P450 oxidoreductase (POR), or NAD(P)H:quinone oxidoreductase. Semiquinone radicals reoxidize rapidly in the presence of molecular oxygen to yield the parent quinone and superoxide anion radical, while hydroquinones react less well with molecular oxygen [119].

reoxidation process was coupled to molecular oxygen reduction and hydrogen peroxide formation. The initial rates of oxidation were inhibited by anti-POR globulin and the nitrogenous ligand, metyrapone. Of interest, anaerobiosis or CO had no effect on the initial rates of reduction, only reoxidation was prevented. During steady state of the reaction, the quinone exists largely as the hydroquinone, but the level of reduction increases under anaerobic conditions, suggesting a redox cycling phenomenon similar to that seen with azo dyes and nitro compounds under aerobic conditions. This reaction was partially supported by purified POR but was not inhibited by metyrapone or CO [120]. The reoxidation reaction indicated that insufficient reduction occurred to attain full steady-state reduction of the quinone as seen with NAD(P)H:quinone oxidoreductase, known to catalyze two-electron reduction of menadione and *t*-butylquinone. Alternatively, the B[a]P quinone may be further metabolized by oxidative metabolism forming more polar metabolites. With benzyl- and naphthylquinones, total reduction of menadione and *t*-butylquinone can be achieved with purified NAD(P)H:quinone oxidoreductase, but not with purified POR [119,120]. These studies suggested one-electron reduction processes are involved in reduction involving both POR and cytochrome P450s, but not NAD(P)H:quinone oxidoreductase (Fig. 12.4). Complete reduction was never achieved in the presence of oxygen with either flavoprotein because of the slow comproportionation equilibrium of the quinone/hydroquinone couple (Eq. 12.11).



Vermeulen and coworkers [121,122] also noted that 2,3,5,6-tetramethylbenzoquinone (TMQ) was reduced to a one-electron reduced quinone in the presence of CYP2B. This reaction was apparently inhibited by SKF-525A, another nitrogenous ligand inhibitor of cytochrome P450. In a reconstituted enzyme system of POR and cytochrome P450, they followed the reduction of TMQ by ESR and noted that the semiquinone radical was produced at a higher rate when cytochrome P450 was reconstituted with POR than POR did itself. Cytochrome P450 inhibitors and inhibitory antibodies also were effective in inhibiting TMQ reduction. They concluded that reconstitution of TMQ reduction by POR with increasing concentrations of cytochrome P450 was correlated with the level of the hemoprotein in the reactions, thereby proving the role of cytochrome P450 as a reductive catalyst. Subsequent work

from this group on adriamycin and mitomycin C documented roles for P450 2B1 in one-electron reduction of these quinone compounds and their bioactivation by this process [122–124].

12.6.2 Reductive Dechlorination

Guengerich [2,125,126] has provided a number of examples from the literature for reductive dechlorination reactions. Other reviews have also summarized these reduction reactions [127,128]. The best studied dechlorination reactions are those monitoring metabolism of carbon tetrachloride [129] and halothane under anaerobic conditions [130–132]. These reduction reactions principally form reduced products in which the halide group is replaced by hydrogen or by the formation of olefins (Fig. 12.5). In each case, cytochrome P450 provides an electron to the parent halide compound from its ferrous iron species, liberating a halide ion and a carbon-centered radical. The chemistry of the radical appears to determine the product formed. For example, the products can react with molecular oxygen to form an oxidized product, as seen with the trichloromethane radical produced from CCl_4 radical. Interaction with molecular oxygen allows production of phosgene. Alternatively, loss of a second halide ion from the radical allows formation of an olefin, as seen during the reduction of halothane. Both reactions led to the formation of reactive intermediates capable of forming adducts with nucleophiles such as protein and nucleic acid bases [130–132]. As pointed out by Guengerich [2], Castro and Castro [133] proposed a consistent mechanism for reductive dehalogenation reactions (Fig. 12.5).

12.6.3 Reduction of Hydroperoxides

Hydroperoxides are also reduced by cytochrome P450s to yield alcohol and water (Eq. 12.12), as discussed by Guengerich [2]. The mechanism of this reaction is not fully appreciated since organic hydroperoxides apparently function as alternate donors of an atom of oxygen to ferric cytochrome P450 to form a perferryl species, a reaction similar to the key step in the P450 catalytic cycle involved in heterolytic cleavage of molecular oxygen [134]. Many examples of these hydroperoxide reduction reactions exist in the literature for the prostaglandin synthetases, lipoxygenases, and cytochrome P450s, in which a lipid hydroperoxide is reduced to a lipid alcohol [135]. Cytochrome P450s can not only produce lipid peroxides during the course of catalysis but can

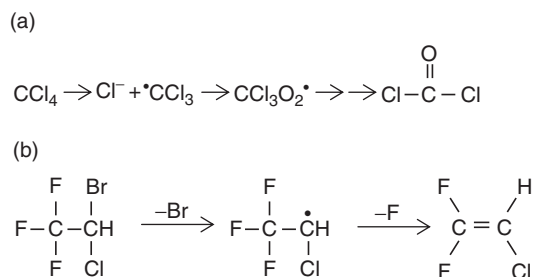
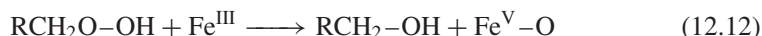


Figure 12.5 Steps of reductive dehalogenation reactions: (a) molecular oxygen reacts with a radical (halide) to lose halogen [133] and (b) reductive dehalogenation forms olefins [129].

also convert such intermediates to the hydroxyeicosenoic acid as part of their catalytic mechanism.



12.6.4 α,β -Unsaturated Aldehydes

Recently, we have studied the metabolism of α,β -unsaturated aldehydes derived from lipid peroxidation, such as 4-hydroxy-2-nonenal and acrolein, using primary hepatocytes and expressed cytochrome P450s. We have shown that many cytochrome P450s catalyze the oxidative metabolism in an NADPH- and O_2 -dependent manner [136]. Specific cytochrome P450s of the CYP3A, 2B, and 2C families were effective in oxidizing aldehydes to their carboxylic acid. In recent studies [137], we also have detected a reaction in which the 4-hydroxy-2-nonenal is reduced to 4-hydroxy-2-nonenol in the presence or absence of oxygen. The reaction was not affected by replacing the normal air atmosphere with carbon monoxide or addition of inhibitors, such as metyrapone, to block the reaction. The α,β -unsaturated bond in aldehydes may allow reduction of these aldehydes to their alcohols, much like the oxygen-insensitive azo reduction reactions. This precedent suggests that there may be other reduction reactions of olefinic compounds by cytochrome P450s, and the tools for the study described by Levine and others may be important approaches to characterize these reactions.

12.6.5 Summary of Cytochrome P450 Reduction Reactions

Except for our report of 4-hydroxynonenal reduction, no cytochrome P450-dependent reduction reactions for endogenous compounds are known. As pointed out by Guengerich in his 2001 review [2], it is peculiar that this heme protein catalyzes reduction reactions at all because of the high reactivity of the hemoprotein in its ferrous form with molecular oxygen. The scientific community assumes that all ferric cytochrome P450s bind oxygen and subsequently the molecular oxygen is rapidly reduced. In tissues with low oxygen tension, reduction reactions might be favored [138]. Because the oxidation–reduction potential for ferric cytochrome P450s is around -300 mV , in the absence or presence of substrate, this species of cytochrome P450 should be an excellent one-electron reductant. Further study of the chemical mechanism of the cytochrome P450s is needed to address this anomaly. However, discovery of a reduced metabolite might direct one to assume either a flavoprotein or cytochrome P450 may be involved in these reactions.

12.7 CONCLUSIONS/FURTHER PERSPECTIVES

In the past, considerable attention has been directed toward the role of cytochrome P450 and FAD-containing oxygenase in amine oxidation reactions. However, an increasing number of studies in the literature require researchers to rediscover the role of the quinone- and flavin-dependent amine oxidases. This chapter attempts to describe the enzyme systems that can catalyze these reactions and provides examples of the reactions with exogenous compounds that have been shown to involve these enzymes. In addition, this chapter addresses the rare reduction reactions of flavoproteins and the

cytochrome P450s as a guide to the reader for considering rare reactions that occur in drug metabolism. The study of metabolic enzymes has been extensive, but it is clear there is much to be learned about these proteins on a single gene/protein level. Because bioreductive reactions for foreign compounds are rare, the authors hope that this review has provided a road map to assist in identification of such systems.

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13 Epoxide Hydrolases

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13.1	Summary	1
13.2	Introduction to epoxide hydrolases	2
13.3	Microsomal epoxide hydrolase (EPHX1)	3
13.4	Soluble epoxide hydrolase (EPHX2)	15
13.5	Conclusions	20
	Acknowledgments	20
	References	20

13.1 SUMMARY

Epoxides are three-membered carbon–oxygen compounds that typically arise from the metabolism of endogenous and xenobiotic compounds via chemical and enzymatic oxidation processes. Many different oxygenase enzymes are capable of forming epoxide derivatives, from either arene or alkene substrate; however, the cytochrome P450 monooxygenases are principal contributors to their generation. Certain epoxides are highly electrophilic and chemically reactive, and they have been implicated as initiators of various cellular toxicities, including the formation of DNA mutations and ultimately, cancers. Therefore, it is of vital importance for the organism to regulate levels of these reactive species. A single mammalian enzyme, microsomal epoxide hydrolase (EPHX1, mEH), functions as a predominant pathway responsible for detoxification of xenobiotic epoxides, as well as bioactivation pathway for certain xenobiotic epoxides. In contrast, another important epoxide hydrolase, soluble epoxide hydrolase (EPHX2, sEH), has more recently been established as a mediator of epoxide hydrolysis of several endogenously derived epoxyeicosatrienoic acids (EETs), intermediates resulting from arachidonic acid metabolism. In this respect, EPHX2 is now characterized as an important regulator of physiological processes, such as blood pressure and inflammation, and has emerged as a promising therapeutic target for pharmacological intervention. This chapter reviews the biochemistry, biological regulation, genetics, and pharmacological relevance of these important enzyme systems.

13.2 INTRODUCTION TO EPOXIDE HYDROLASES

A wide variety of endogenously and exogenously derived lipophilic aromatic and aliphatic substances are metabolized initially through phase I biotransformation pathways, perhaps most notably by the cytochrome P450 monooxygenase enzymes (CYPs) [1]. The resulting oxidative metabolites include epoxide-containing intermediates that are subject to further metabolism by both phase I and phase II biotransformation pathways. Cells have developed the capacity to metabolize chemical epoxides through several pathways. Prominent among these are the epoxide hydrolases, including EPHX1 (EC 3.3.2.9) and EPHX2 (EC 3.3.2.10). The epoxide hydrolases belong to a subcategory of a broad group of hydrolytic enzymes that include esterases, proteases, dehalogenases, and lipases [2,3]. Epoxide hydrolases catalyze the hydrolysis of epoxides to their corresponding dihydrodiol (glycol) products. The hydration reactions often lead to changes in the biological functionality and/or the chemical reactivity of the resulting metabolite. Simple epoxides are hydrated to their corresponding vicinal dihydrodiols and arene oxides to *trans*-dihydrodiols. Although certain epoxides are relatively stable, for example, the pharmaceutical metabolite carbamazepine (CBZ) epoxide, other chemical epoxides are highly electrophilic and may react with cellular nucleophiles, such as DNA, RNA, and proteins [4]. With respect to xenobiotics, epoxide hydrolase metabolism results principally in detoxification. Importantly however, in certain instances, bioactivated toxic intermediates of xenobiotic substances may also be generated. Frequently, reactive and unstable epoxide metabolites, such as the bay-region and fjord-region diol epoxide intermediates arising from polyaromatic hydrocarbon (PAH) metabolism, have been identified as ultimate cytotoxic and carcinogenic reaction products [5,6]. The overall balance of xenobiotic metabolism between bioactivation and detoxification pathways catalyzed by EPHX1 determines the pharmacokinetic disposition and the ultimate fate of reactive intermediates within target cells. In contrast to EPHX1, the substrate specificity of EPHX2 is directed toward endogenously derived and generated epoxides, in particular, those resulting from the arachidonic acid metabolism cascade [3]. These intermediates function to regulate several important physiological processes, such as vascular reactivity and inflammatory responses.

13.2.1 Types and General Reactions of the Epoxide Hydrolases

Epoxide hydrolases are members of the α/β -hydrolase fold superfamily. The α/β -hydrolase fold domain structure α/β domain (ABD) is common to a number of hydrolytic enzymes of widely differing phylogenetic origins and catalytic functions. The α/β -hydrolase fold is characterized by a β -sheet core of five to eight strands linked by α -helices to form a $\alpha/\beta/\alpha$ sandwich structure [7]. The enzymatic activities contributed by members of this superfamily include esterase, lipase, and hydrolase functionalities [7,8]. Members of other gene families may also catalyze hydrolase activities, described briefly below.

Several types of mammalian epoxide-hydrolyzing enzymes have been characterized. These include (i) a microsomal enzyme specific for cholesterol 5,6-epoxide hydrolysis [9] that is little studied but distinct from the ABD-containing hydrolases otherwise discussed in this chapter; (ii) a membrane-associated hepoxilin A3 hydrolase, also likely distinct from the ABD domain structure, participates in arachidonic acid metabolism in the central nervous system and vascular cells [10]; (iii) a cytosolic leukotriene

A4 (LTA4) hydrolase that is quite distinct from the ABD protein family, as it possesses a zinc-binding domain and aminopeptidase activity, which mediates leukotriene metabolism and is consequently implicated in inflammatory and allergic responses [11,12]; (iv) an mEH (EPHX1) that is active in the metabolism of certain pharmaceuticals as well as a broad range of xenobiotic compounds, such as the mutagenic PAH-derived epoxides [2]; (v) an sEH (EPHX2) that is active in the metabolism of arachidonic-acid-derived epoxides and other endogenous epoxides [13]; and, the two more recently identified epoxide hydrolases, (vi) EPHX3 and (vii) EPHX4. EPHX3, also termed *ABHD9*, is a predicted α/β -hydrolase fold superfamily member that was identified in a genomics screen for DNA methylation markers associated with prostate cancer prognosis [14]. EPXH4 was identified by genomic-sequencing-based coding queries as a putative α/β -hydrolase-domain-containing protein, potentially associated, along with many other polymorphic markers, with altered response to hepatitis B vaccination [15]. Little other functional data are available describing EPHX3 or EPHX4, although apparent homologs of these hydrolases have been identified in *Caenorhabditis elegans*. EPHX3 and EPHX4 appear structurally much more closely related to EPHX2 than to EPXH1 and therefore may have a role in generating lipid-signaling intermediates [16].

The EPHX1 and EPHX2 members of the α/β -hydrolase fold family of proteins are two of the most well-investigated mammalian epoxide hydrolases. EPHX1 and EPHX2 are distinguished on their subcellular distributions, typically associated with microsomal and cytosolic distributions, respectively, but perhaps, more importantly, they possess highly distinctive substrate specificities and physiological roles. It is likely that two independent forces, namely, cytoprotection and cellular signaling, drove the evolution of EPHX1 and EPHX2, respectively [13]. Of the various epoxide hydrolases, that is, EPHX1, is *the only* hydrolase known to possess enzymatic activity toward xenobiotic-derived epoxides [13], and therefore, it is a principal focus in this chapter. However, EPHX2 is rapidly generating intense interest as a pharmaceutical target, with potential application in drug therapies aimed at treatment of inflammatory, cardiovascular, and other diseases. Consequently, this chapter reviews both these hydrolases and includes discussion of their respective functional, regulatory, and genetic features.

13.3 MICROSOMAL EPOXIDE HYDROLASE (EPHX1)

13.3.1 Introduction

As the earliest known epoxide hydrolase, EPHX1 plays a critical role in the metabolism of many xenobiotic compounds. The name of this epoxide hydrolase is derived from its primary cellular localization in the endoplasmic reticulum, that is, the microsomal environment. mEH appears unique among all the epoxide hydrolases for its exclusive role in the metabolism of xenobiotic-derived epoxides. EPHX1 is capable of hydrolyzing epoxides derived from xenobiotics of highly diverse structures, and the enzyme is especially capable of accepting substrates with bulky configurations, such as arene oxides [2,17]. The diol products of EPHX1 hydrolysis typically are less reactive chemical species relative to the parent compound, and the enzyme is therefore considered to generally foster detoxification. However, in the case of arene oxide metabolism, subsequent CYP-mediated ring oxidation of the hydrolyzed metabolite can lead to the

formation of vicinal diol epoxide species that may possess extremely high chemical reactivity [6,18].

The catalytic mechanism of EPHX1 involves two steps and is similar to that described for EPHX2 [19,20]. In this mechanism, first there is attack of a nucleophilic aspartic acid on the oxirane ring to yield an alkyl enzyme intermediate, and then subsequent hydrolysis of the intermediate by water. On the basis of sequence alignment analysis, the catalytic triad of EPHX1 was identified as consisting of Asp226, His431, and Glu404 [20,21]. Subsequently, site-directed mutagenesis experiments in a rodent model confirmed these catalytic amino acids as critical for EPHX1 enzymatic activity [3]. The human EPHX1 enzyme requires no cofactors for activity.

The human EPHX1 gene exists as a single copy and is located on chromosome 1, specifically at position 1q42.1 [22,23]. The coding region of the EPHX1 gene locus spans ~20 kb. The EPHX1 gene product exists as a monomeric protein of ~49 kDa and is composed of 455 amino acids [23]. Human EPHX1 is expressed in all tissues thus far examined, with highest levels in the liver, lower yet comparable levels in kidney and ovary, and lower levels in testis, lung, adrenal glands, and lymphocytes [13,24,25]. Developmentally, EPHX1 expression in human fetal tissues is relatively low during early gestation but increases as gestation progresses [26]. Research conducted with experimental rodent models indicates that the EPHX1 gene is responsive to chemical treatments [27–29]; however, evidence obtained from primary hepatocytes indicates that EPHX1 is only modestly transcriptional responsive in humans [30]. Early studies, cited above, drew attention to associations between EPHX1 and cancer. In humans, EPHX1 protein has been consistently identified in tumors from a variety of organs [31–35], fueling speculation that expression of this enzyme may play a molecular role in malignancy. Importantly, experiments employing null mice have demonstrated that EPHX1 expression is a critical determinant in PAH-induced carcinogenesis [36].

13.3.2 Biological Function of EPHX1

Over the last few decades, EPHX1 has been recognized primarily for its role in xenobiotic metabolism. However, several intriguing observations also suggest that EPHX1 may play a role in modulating physiological functions. For example, several lines of evidence indicate that EPHX1 may play a role in steroid synthesis and/or metabolism [13]. For example, estroxiol and androstene oxide are good EPHX1 substrates [37,38]. Consistent with this theme, an early study identified EPHX1 as a subunit of the anti-estrogen binding site [39]. Furthermore, EPHX1 is relatively highly expressed in ovaries [40,41], especially in follicle cells [42]. One hypothesis is that EPHX1 may be important for cellular protection against reactive metabolites of endogenous compounds, such as epoxy steroids. Human EPHX1 is expressed and developmentally regulated in fetal tissues [26], suggesting that it may be important for protection from toxic epoxide intermediates during embryonic and fetal development. In this light, a recent report indicated that oviductal EPHX1 expression is upregulated during the process of mouse embryogenesis and that increased oviductal EPHX1 may help reduce levels of reactive oxygen species [43]. These functions may represent protective effects of EPHX1, potentially enhancing mouse embryo developmental processes.

In addition to a potential role in steroid homeostasis, another study suggested that EPHX1 is a functional component of the vitamin K₁–oxide reductase complex in rat liver microsomes [44]. Also, a role for EPHX1 in bile acid homeostasis has been

suggested, as EPHX1 was identified by one group of investigators as part of a multiprotein transport system that is responsible for sodium-dependent bile acid uptake in liver [45–48]. To further this view, several reports have indicated that EPHX1 is expressed at the hepatocyte plasma membrane and in the endoplasmic reticulum where it can exist in two topological orientations and that bile acid uptake appears dependent on EPHX1 expression at the plasma membrane [48]. However, the specific role and relative importance of EPHX1 in the physiologic function of bile acid transport is controversial, in that another study was unable to reproduce these purported activities of EPHX1 [49]. Possibly, development and use of potent and selective EPHX1 inhibitors may help to address the still open questions as to a potential role of EPHX1 in bile acid transport and/or vitamin K reductase participation.

As EPHX1 is expressed ubiquitously in many species, including mammalian, plant, and single cell organisms [50], and several intriguing lines of evidence for its endogenous functionality have been advanced, it seems logical to postulate an important physiological role for its activity. However, it should be noted that an EPHX1 null mouse model has been established [36]. These null mice appear healthy and reproduce with normal efficiencies. Thus, lack of EPHX1 expression in the mouse does not appear to be physiologically detrimental, presenting somewhat of a puzzling dilemma regarding the potential endogenous role of the enzyme in mammalian systems.

13.3.3 Substrates and Inhibitors

EPHX1 exhibits a large range of substrate specificity, and most epoxide intermediates are generated *in situ* by phase I oxidation reactions [51]. The substrates of EPHX1 broadly range from aliphatic epoxides to polyaromatic oxides. The subsections below review a number of chemically derived, environmentally relevant, and pharmaceutical substrates for the enzyme, together with the biological implications of these reactions.

13.3.3.1 Environmental/Xenobiotic. EPHX1 has been recognized as an important contributor to the metabolism of xenobiotic epoxides, including numerous environmental contaminants. Historically, several different substrate probes for EPHX1 have been employed for routine assessments of enzymatic activity in biochemical reactions. These include benzo[*a*]pyrene (B[*a*]P)-4,5-oxide [52,53], *cis*-stilbene oxide [54], styrene oxide [55], CBZ-10,11-epoxide [56,57], and naphthalene-1,2-oxide [57,58]. Fluorescent substrates for EPHX1 have more recently been developed. The highest activity for both rat and human EPHX1 was obtained with the fluorescent substrate cyano(6-methoxy-naphthalen-2-yl)methyl glycidyl carbonate [59]; however, this compound cannot be used with crude enzymatic preparations due to interfering reactions.

Common environmental toxins that generate epoxide intermediates metabolized by EPHX1 include 1,3-butadiene [60,61], styrene [62], and benzene [63]. Illustrations of the structural features of these substrates and associated reactions are shown in Fig. 13.1. In addition, several other xenobiotic compounds are metabolized to epoxide intermediates, such as aflatoxin B₁ [64], the nitropyrenes [65,66], and the PAHs, including chrysene, naphthalene, anthracene, and B[*a*]P [67,68].

As a case in point, PAHs are products of incomplete combustion of organic matter and are widespread environmental contaminants found in automobile exhaust, cigarette smoke, air, water, food, and soil. PAHs are considered procarcinogens because they

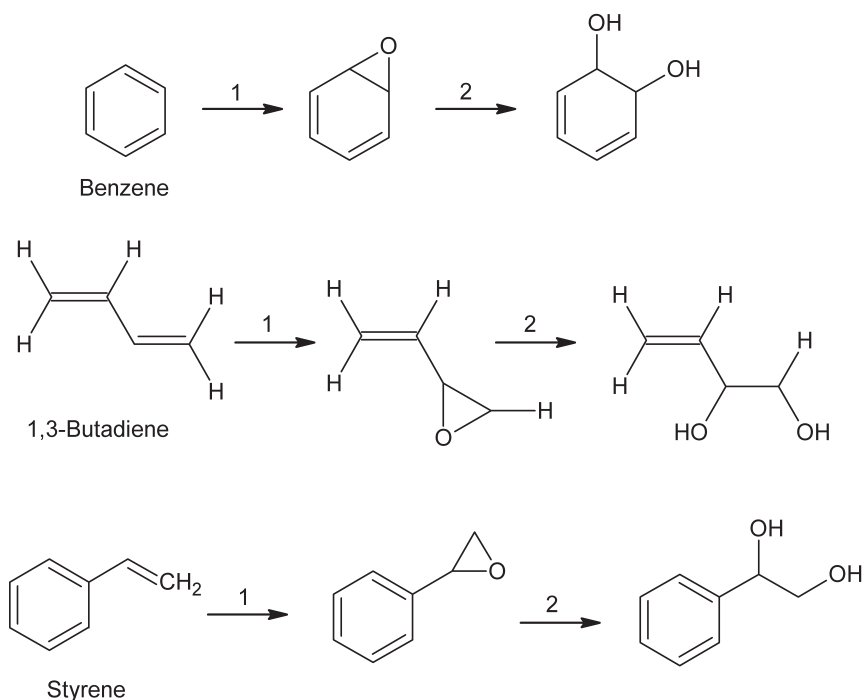


Figure 13.1 Common xenobiotic substrates of EPHX1 and their general reaction schemes. Step one is commonly mediated via cytochrome P450, while step 2 is mediated via EPHX1.

require metabolic activation to electrophilic reactive metabolites to exert their mutagenic and tumorigenic activities. Three principal pathways of metabolic activation have been proposed for the activation of the PAHs. These involve (i) the formation of diol epoxides by several CYPs, where CYP1A1, CYP1B1, and others can play a major role [69]; (ii) the formation of radical cations catalyzed by peroxidases [70]; and (iii) the formation of reactive intermediates catalyzed by dehydrogenases and other reductases [71,72]. However, the biological effects of a number of PAHs, including their mutagenicity and carcinogenicity, are largely dependent on enzymatic activation to dihydrodiol epoxide intermediates [73], which are formed by a three-step process: initial epoxidation by the CYPs, subsequent EPHX1-mediated hydrolysis to the *trans*-dihydrodiol, followed by a second epoxidation at the adjacent double bond [74]. The critical nature of EPHX1 bioactivation in PAH-induced carcinogenesis is further demonstrated in EPHX1 null mice, which are completely resistant to the tumorigenic effects of the PAH dimethylbenz[*a*]anthracene in a complete carcinogenesis assay [36].

The mutagenic and carcinogenic potency of the PAHs appears largely dependent on their structural features. As indicated previously, K-region PAH oxides, such as the B[*a*]P-4,5-oxide, are often used as model substrates for EPHX1 activity. Although mutagenic, these derivatives are not the principal determinants of PAH-induced carcinogenesis. Rather, it is the terminal ring PAH oxides, away from the respective bay or fjord region and when hydrolyzed by epoxide hydrolase, that lead to production of the proximate PAH carcinogen, the *trans*-dihydrodiol, which in turn is metabolized to the ultimate carcinogen, the diol epoxide. The strong steric interactions of the fjord-region

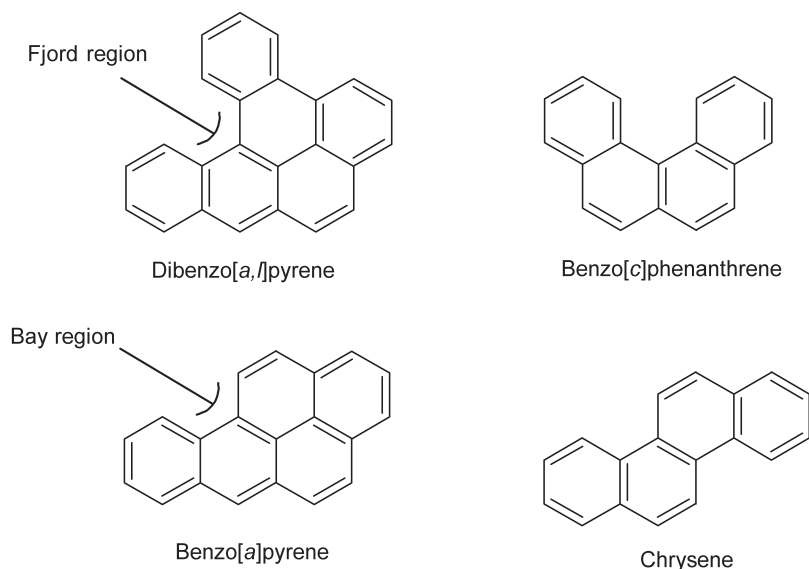


Figure 13.2 Examples of polycyclic aromatic hydrocarbons illustrating both bay- and fjord-region centers that are subject to epoxidation reactions by cytochrome P450s and subsequent hydrolysis by EPHX1.

force the molecule to distort from planarity. EPHX1 contributes both to the formation of highly reactive and mutagenic bay-region (planar PAHs) and fjord-region (nonplanar PAHs) diol epoxide intermediates [6,75,76], with the corresponding mutagenic and tumorigenic potencies of these substances largely dependent on their structural features. In particular, diol epoxides derived from PAHs that possess a fjord region exhibit substantially more potent carcinogenic activities than those derived from bay-region PAHs [6]. Examples of both bay-region and fjord-region PAHs are shown in Fig. 13.2. The diol epoxides thus generated react readily with mutational hot spots in DNA to form stable adducts both *in vitro* and *in vivo* [77,78]. If these adducts are not repaired, they result in misreplication and mutagenesis [77,78]. The fjord-region, nonplanar diol epoxides, such as dibenzo[a,h]pyrene, react more favorably with deoxyadenosine (dA) than deoxyguanosine (dG) in DNA and fjord-region-modified DNA adducts are more difficult to repair than a bay-region adduct [79–82]. Given this backdrop, it is of interest that several epidemiologic investigations have now implicated human EPHX1 gene-coding-region polymorphisms as a risk modifier for lung cancer incidence [83].

13.3.3.2 Pharmacological. Although many pharmaceuticals undergo oxidative metabolism, principally in the liver but also in other organs, and the CYPs play prominent roles in these initial enzymatic reactions, it is perhaps surprising that not many known pharmaceutical agents undergo sequential enzymatic processing by EPHX1. In part, this is likely due to the extensive preclinical testing that developmental drug candidates are subjected to, and it is logical to assume that compounds exhibiting an avid potential to be metabolized into reactive epoxides are typically eliminated from further development in early toxicity screens. However,

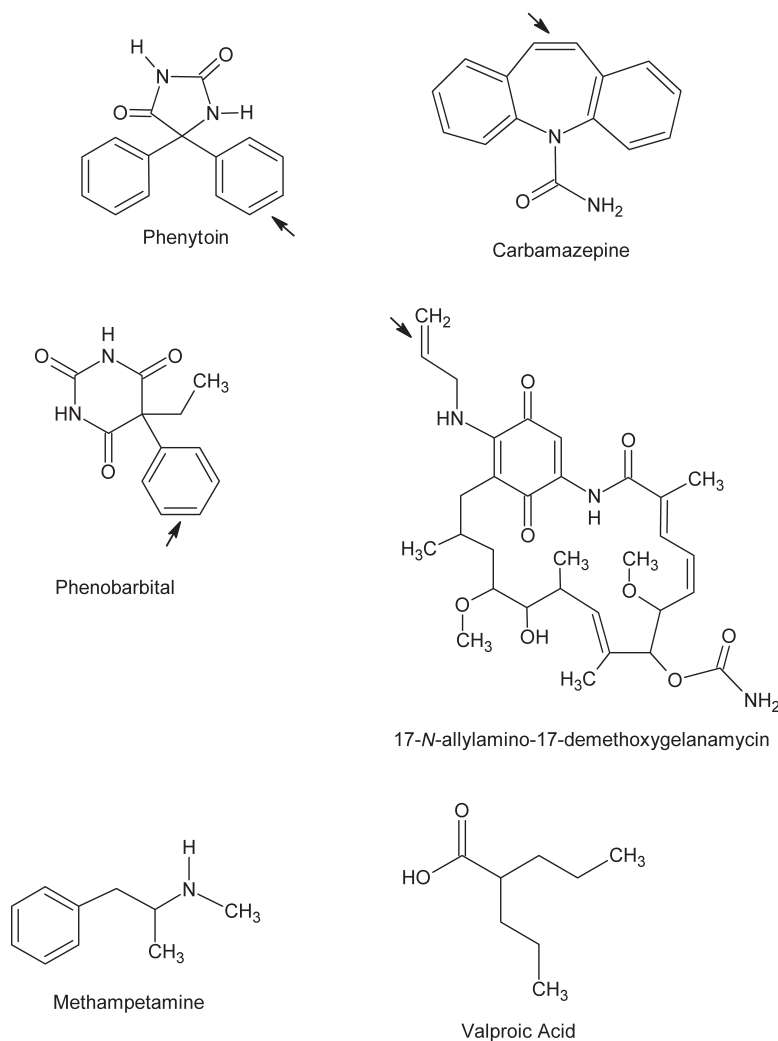


Figure 13.3 Examples of anticonvulsant medications and other pharmacological agents that are implicated in EPHX1 metabolism schemes. Arrows represent epoxidation reaction sites.

EPHX1 has been implicated in the metabolism of several categories of drugs, including commonly used anticonvulsant, psychostimulant, and anticancer drugs.

Historically, aromatic anticonvulsant drugs, such as phenobarbital (PB), diphenylhydantoin (phenytoin), and CBZ, have been widely employed in the treatment of seizure disorders and are the most readily recognized as having interactions with epoxide hydrolase [84]. Figure 13.3 illustrates chemical structures of selected anticonvulsant agents that are metabolized either to epoxide intermediates, which are in turn substrates for EPHX1, or pharmacological structures, which are otherwise known to affect the metabolism of the epoxide hydrolase pathway. Biotransformation pathways for these drugs typically involve initial phase I metabolism by various CYPs that can then result in the generation of arene oxides, which are substrates for EPHX1. Idiosyncratic

adverse drug reactions (ADRs) can occur following treatment with certain anticonvulsants, and in several early studies, polymorphism in EPHX1 expression was implicated as a risk factor for this response [85,86].

For example, phenytoin is an older anticonvulsant medication that has a widespread usage. It is likely metabolized, in part, through an arene oxide intermediate [87]. Toxicity concerns surrounding its use have been frequently raised, and certain evidence implicates EPHX1 in this respect. For example, a case study published in 1988 suggested the protective role for EPHX1 in anticonvulsant hypersensitive syndrome, indicating that patients susceptible to anticonvulsant toxicity had decreased epoxide metabolism activity [88]. In one instance of ADRs, strikingly reduced levels of mEH RNA were demonstrated in an affected patient [89]. The teratogenic risk of fetal hydantoin syndrome, related to exposure to phenytoin, was also reported as increased in a study in subjects with low EPHX1 enzymatic activity [90]. However, subsequent studies that specifically examined the association of EPHX1 amino acid polymorphisms with susceptibility to ADRs found no apparent association [91,92]. It should be noted that the population sizes evaluated in these latter studies were small and additional EPHX1 polymorphisms, for example, those residing in the respective promoter regions of the gene have now been identified [93,94] but have not yet been assessed as potential risk contributors in these respects.

Given the long-standing use of PB for the effective treatment of convulsant disorders, concerns with its use have also arisen. The metabolism of this agent is also projected to proceed, in part, through an arene oxide intermediate [95]. Although PB is a liver-tumor-promoting agent in rodents, epidemiological studies in PB-exposed populations have not uncovered enhanced risk to liver cancer in humans, even following its long-term use [96,97]. Therefore, the larger concern with the use of PB is likely due to its induction effects on various biotransformation pathways [98] that may in turn lead to ADRs or drug–drug interactions. However, the question of whether PB may induce epoxide hydrolase activities in humans is open. In studies conducted using primary hepatocyte cultures from human donors, this agent appears to only modestly effect EPHX1 levels [30], but in general, the issue of interindividual variability in response to chemical inducers certainly adds complexity to these issues [99].

CBZ is another older-generation therapeutic for the treatment of epileptic disorders and has historically been one of the most widely prescribed frontline medications for this syndrome [100]. The principal bioactive metabolite of this compound is the CBZ-10,11-epoxide derivative, an epoxide that is relatively stable and readily detected in serum [100,101]. CBZ-10,11-epoxide is a substrate for EPHX1. Using genetic haplotype-mapping approaches, Japanese investigators reported apparent correlation with EPHX1 coding region genotype status and intrinsic clearance measures of CBZ-10,11-epoxide [101]. More recently, results of independent studies also suggested that the genetic status of EPHX1 may be a useful predictor of CBZ maintenance doses in prescribed populations [102]. Furthermore, it has been reported that valproic acid, another anticonvulsant medication, potentially inhibits EPHX1 activity, thereby altering steady-state levels of CBZ-11,12-oxide in cotreated patients [103]. However, results of other investigations indicate that the glutathione transferase system is likely involved as well in determining the disposition of CBZ-11,12-oxide serum levels [104].

Methamphetamine hydrochloride (METH) is a highly addictive and popular illicit drug that is neurotoxic and presents an addiction that is very difficult to treat. The biotransformation of METH has been investigated. On smoking, pyrolysis products are

derived that include *trans*-phenylpropylene, a product that is likely to undergo oxidation metabolism by the CYPs, leading to a potentially reactive and neurotoxic epoxide intermediate [105]. Interestingly, experimental evidence indicates that this neurotoxic epoxide intermediate is indeed a substrate of EPHX1 [105]. Using an EPHX1 knockout mouse model, a recent report identified that EPHX1 expression plays an important role in detoxifying the reactive epoxide intermediates of METH and in attenuating METH drug addiction [106]. This finding offers a novel therapeutic approach for potential prevention or attenuation of the long-term consequences of METH use disorders in humans.

17-(Allylamino)-17-demethoxygeldanamycin (17AAG) is a potent antiproliferative drug that has been in clinical trials for treatment of chronic lymphocytic leukemia as well as kidney tumors, especially in young patients [107]. Its mechanism of action is through targeting of Hsp90-associated activities and disruption of protein-tethering interactions. 17AAG is extensively metabolized; a diol metabolite is among the major products detected. EPHX1 is likely involved in the production of the diol metabolite, subsequent to hydrolysis of an epoxide intermediate formed on the allyl side chain of 17AAG [108]

13.3.3.3 Inhibitors. In early studies, several epoxide-containing compounds were discovered as EPHX1 inhibitors. For example, 1,1,1-trichloropropene-2,3-oxide [109,110] and cyclohexene oxide [111,112] have been widely used as EPHX1 inhibitors. In addition, other investigators have described primary ureas, amides, and amines [113,114], as well as heavy metals such as divalent mercury and zinc [115], as EPHX1 inhibitors. More recently, Hammock and colleagues screened a series of amides and identified 2-nonylsulfanyl-propionamide as a competitive inhibitor of EPHX1 enzymatic activity, exhibiting a K_i of 72 nM, thus establishing this agent as the most potent inhibitor identified to date. These agents represent useful *in vitro* tools for assessing the involvement of EPHX1 activity in the characterization of chemical and pharmaceutical biotransformations.

13.3.4 Genetic Polymorphisms

Human pharmaceutical and xenobiotic metabolisms are subject to large interindividual differences [116,117]. A variety of parameters are known to contribute to this phenomenon, and genetic constitution is likely of major importance [118]. In addition to the inherited differences in chemical metabolism's capacity and specificity, additional contributors to interindividual differences in risk of toxicity resulting from xenobiotic exposure include age, gender, disease states, tissue differences, and environmental influences, such as diet, smoking habits, occupational exposure to chemicals, and concurrent exposure to other drugs [117,119]. Despite the present era of genomics, achieving a detailed comprehension of the interplay of such variables with man's inherent genetics remains a formidable challenge for the foreseeable future. The ability to predict potential hazards resulting from human exposure to pharmaceuticals as well as other foreign chemicals requires that we elucidate the genetic and regulatory factors that contribute importantly to the risk scenarios underlying human variability in biotransformation and xenobiotic disposition.

Large interindividual variations in EPHX1 activities have been described in human tissues. For example, EPHX1 activity was reported to vary from 8-fold to up to 63-fold

in panels of human liver samples [53,120,121]. After the full-length human EPHX1 DNA sequence was cloned and compared, two nonsynonymous amino acid changes were identified in the EPHX1 coding region. The polymorphism in exon 3 corresponds to amino acid position 113 and results in Tyr (Y) to His (H) substitution (rs1051740). An exon 4 polymorphism at position 139 (rs2234922) encodes a His (H) to Arg (R) substitution. For the polymorphism at position 113, 7.6% individuals were observed with homozygous His, and the frequency of 139 A/A is 4.6%. The distribution of the two alleles follows Hardy–Weinberg equilibrium [2,122]. There are other nonsynonymous polymorphisms reported or listed in the NCBI dbSNP database, but to the authors' knowledge, these polymorphisms either have not been validated or have only been identified at a very low allelic frequency. Consequently, the 113Y/H and 139H/R polymorphisms are recognized currently as the most common EPHX1 amino acid variants in the human population. As many commonly encountered environmental chemicals undergo epoxide metabolism, there is an interest in assessing genetic variation in EPHX1 as a potential risk modulator for other chemicals.

Since the initial identification of these two primary EPHX1 coding polymorphisms, a surprisingly large number of epidemiologic investigations have examined the potential association of disease incidence with EPHX1 genotype. An early review of selected molecular epidemiological investigations examining associations of EPHX1 genotype with cancer susceptibility has been published [123]. Perhaps, the most consistent associations implicate EPHX1 coding region polymorphisms as a risk factor for lung cancer [124–130]. In 2002, Lee *et al.* [128] conducted a meta-analysis of seven previously published studies and reported a significant decrease in lung cancer risk (OR = 0.70, 95% CI = 0.51–0.96) associated with the “low activity” exon 3 H113 homozygous EPHX1 genotype, after adjustment for age, sex, smoking, and center. In 2006, a human genome epidemiology (HuGE) review and meta-analysis of 13 case-control studies was published [83], similarly concluding that the low activity variant H113 genotype of EPHX1 was associated with decreased risk of lung cancer (OR = 0.65; 95% CI = 0.44–0.96). In contrast, other reports have indicated an increased risk of chronic obstructive pulmonary disease (COPD) [131,132] and emphysema [132] with the H113 genotype, despite both diseases being linked to smoking behaviors. Another report has indicated a protective association of the Y113H genotype with COPD [133]. The paradoxical results may perhaps be explained if the various EPHX1 allelic proteins exhibited differential activities on specific substrates involved in diverse disease etiologies. Disease associations with EPHX1 polymorphism at the exon 4/Y139R position have also been reported [134–136]. The mechanistic basis for these associations in relation to the enzymatic function of the xenobiotic hydrolase remains unknown.

Genetic differences in EPHX1 also have been reported as a potential risk modulator of toxicity caused by styrene [137] and butadiene [138,139] exposure in human worker populations, although other studies have not confirmed such associations [120,140]. Interestingly, bioactivation of aflatoxin B₁ by cytochrome P450 enzymes generates a highly reactive epoxide metabolite, and incubation with the EPHX1 enzyme is reported to reduce the aflatoxin's carcinogenic effect [64]. In these respects, an initial study indicated a risk association with hepatocellular carcinoma and EPHX1 genotype [141]; however, subsequent analyses were also not able to confirm this association [142].

It should be noted that many of the published EPHX1 molecular epidemiologic reports frequently refer to quantitative functional descriptors for the respective EPHX1

genotypes, indicating, for example, “the low activity allele” or “the high activity genotype.” However, the relative enzymatic contribution of the commonly studied EPHX1 Y113H and H139R polymorphisms has been examined functionally to only a limited extent, and only modest support for any enzymatic/catalytic difference among the EPHX1 variants exists. For example, using the K-region, B[a]P-4,5-epoxide as substrate, the four potential allelic variants of EPHX1 were evaluated for relative functional activities using sources of EPHX1 protein variously derived from transfected COS-1 cells, baculovirus-infected Sf9 cells, and genotyped samples of human liver microsomes [122,143]. Following normalization to immunoreactive levels of EPHX1 protein, only minimal differences in enzymatic specific activities were apparent among the variant isoforms. Similar results were reported using *cis*-stilbene oxide, another model substrate for EPHX1 [143]. Other investigators also examined the enzymatic capacity of the respective EPHX1 variants and failed to discern a correlation between EPHX1 polymorphism and enzymatic activity [57]. Therefore, despite the many epidemiologic studies that have used descriptors such as “low activity allele” or “high activity allele” in an attempt to explain the functional basis of their results, the underlying basis for the EPHX1 activity claim is not yet well substantiated. Given the important role of EPHX1 in the biotransformation of reactive xenobiotic epoxides and the apparent wealth of epidemiological association of genetically encoded differences in EPHX1 protein structure with the incidence of various diseases, the exact functional impact of EPHX1 polymorphism requires more rigorous study. Moreover, the interaction of EPHX1 with other metabolizing enzymes, such as the glutathione *S*-transferases and *N*-acetyltransferases, may additionally contribute to modulate the association with lung cancer [144]. Since the EPHX1 and EPHX2 metabolic pathways are each intimately connected to cytochrome P450-mediated catalysis, it is very likely that a web of polymorphisms among these proteins will prove to be important pharmacogenetic considerations with respect to medical diagnosis and therapy.

Some studies of the EPHX1 protein variants suggested that these coding region polymorphisms may affect protein stability. For example, in a panel of 40 human livers, EPHX1 enzyme activities demonstrated strong correlation with the respective protein levels [53]. However, neither the EPHX1 protein nor its activity was associated with the EPHX1 mRNA levels, which suggested some posttranscriptional-mechanism-regulated EPHX1 protein expression. The translational efficiency, mRNA half-life, and protein half-life of mEH allelic variants have been assessed by *in vitro* transcription and translation using constructs encoding four EPHX1 alleles. The coding region polymorphisms do not appear to affect translational efficiency or mRNA decay rate. Although the calculated EPHX1 variant protein half-lives suggested that polymorphic amino acid substitution may result in altered protein stability [145], these differences in protein half-lives were small, and it is unclear whether they affect overall functional protein levels.

As previously discussed, EPHX1 is largely responsible for the formation of PAH reactive metabolites, and fjord-region diol epoxides exhibit substantially more potent carcinogenic activities than those derived from bay regions, and fjord-region-modified DNA adducts are more difficult to repair than the bay-region adducts [79–82]. The role of the EPHX1 polymorphic variants in the formation of these carcinogenic PAH metabolites has not been assessed. It is interesting to speculate that the EPHX1 protein variants may exhibit differential catalytic activities toward these substrates, which in turn could render some individuals more susceptible to PAH-induced cancers. Similarly,

as also presented earlier, EPHX1 genotype has been implicated as a determinant of steady-state serum levels of certain anticonvulsant medications, as well as incidence of ADRs, following administration of select anticonvulsant agents. These potential functional relationships will require additional detailed study in *in vitro* assays and in clinical populations in order to ascertain.

In addition to the polymorphisms encoding amino acid change, DNA sequence variation has been identified in the 5'-flanking of human mEH, proximal to the coding region of the gene [93]. Heterologous expression of DNA fragments containing the polymorphisms indicated variable levels of reporter gene activity, suggesting that variation in this portion of the human EPHX1 gene may also contribute to functional expression levels. When considering the association of genetic polymorphisms with the risk of human disease, structural region EPHX1 polymorphisms alone likely do not account for the complete spectrum of variation influencing EPHX1 activity; polymorphisms occurring within the 5'-regulatory regions of the gene may represent additional and, perhaps, important considerations.

13.3.5 Regulation of Expression

Alterations in the EPHX1 protein level and/or activity will likely affect an individual's xenobiotic metabolism capacity. Therefore, both the genetics and regulation of EPHX1 expression in human tissues may represent an important determinant of interindividual differences in chemical responsiveness, resulting toxicities, and, perhaps, carcinogenic outcomes resulting from select chemical exposures.

13.3.5.1 Transcriptional Regulation. In rodents, EPHX1 expression can be highly inducible by a variety of compounds, including PB, 3-methylcholanthrene, polychlorinated biphenyls, *trans*-stilbene oxide [146], certain peroxisome proliferators [13], radiation [147], heavy metals [2], and select steroids [38]. In contrast, studies of human EPHX1 induction capacity have not yielded comparable results. For example, analyses of the EPHX1 transcript levels *ex vivo* in primary cultures of human hepatocytes obtained from seven individual donors subjected to *in vitro* exposures to five prototypic inducing agents, specifically, PB, dexamethasone, Aroclor 1254, ciprofibrate, β -naphthoflavone, and *bis*-hydroxyanisole, revealed that expression levels were only modestly affected by these agents in primary human hepatocytes [30]. Therefore, in this regard, the EPHX1 induction results that have been obtained in rodents cannot be easily extrapolated to humans.

13.3.5.2 Regulation by Alternative Promoters. It is well established that RNA diversity in mammals is expanded markedly through the use of alternative promoters and differential RNA splicing mechanisms, and it has been shown that these mechanisms play a role in modulating the EPHX1 gene. Initially, Gaedigk *et al.* reported complex splicing processes at the exon 1/2 boundary, which apparently generated eight putative alternative exon 1 variant sequences in addition to the well-known exon 1, E1, previously defined from liver [148]. However, it was later determined that many of the previously characterized alternative exon 1 sequences were misidentified and rather shared identity with sequences derived from the signal recognition particle 9-kDa structural gene (SRP9, NM003133), a gene that exists upstream of the EPHX1 coding region [24]. The latter study utilized a more stringent 5'-rapid amplification of cDNA

ends (RACE) technique to identify two bona fide unique first exons from human-liver-derived mRNA samples, termed *E1* and *exon 1b* (*E1-b*). The *E1* transcript initiated from the initially characterized promoter and was positioned immediately proximal to the exon 2 of *EPHX1* coding region, while the *E1-b* variant exon 1 was localized to a genomic region ~18.5 kb upstream of exon 2. Northern blot RNA hybridizations demonstrated that the *E1-b* variant was expressed ubiquitously throughout panels of both human fetal and adult tissues. In contrast, the *E1* transcript was expressed almost exclusively in liver [24]. A more recent study confirmed and extended these observations and further substantiated that the far upstream *E1-b* promoter functions as the primary driver of *EPHX1* expression in human tissues, including liver [25]. Furthermore, genome browser sequence comparisons of the *EPHX1* *E1-b* promoter conducted among 28 vertebrate species revealed that the *E1-b* promoter region was highly conserved only among primate species [94], suggesting that the regulation of *EPHX1* expression in species such as mouse and rat may involve distinct promoter regulatory mechanisms. Currently, the nuclear factor interactions and associated molecular mechanisms regulating this critically important transcriptional domain of *EPHX1* are under intensive investigation.

With respect to the proximal and initially characterized *E1* promoter, the basis for its liver-specific functionality was examined by several investigators. A number of cis-regulatory elements were identified that appear to contribute toward its hepatic transcription character, including GATA and HNF3 motifs [24]. For example, mutation of the GATA site at position -110/-105 resulted in a marked (70%) decrease in basal transcription activities driven by the *E1* promoter in human hepatoma cells, and GATA-4 was identified as the principal GATA family member interacting with the respective motif. Interestingly, both HNF3 α and HNF3 β were found to interact with the HNF3 element at position -96/-88, but they acted to negatively regulate GATA-4 function in hepatic cells. GATA-4 has been described previously as a regulator of *EPHX1* tissue-specific expression, with assistance from other tissue-restricted transcription factors including the CCAAT/enhancer-binding protein α (C/EBP α) and nuclear factor Y (NF-Y). These factors appear to form a complex and bind directly to the CCAAT box within the *E1* promoter, activating hepatic *EPHX1* *E1* transcription [149].

13.3.5.3 Transposable Elements. Characterization of the entire human *EPHX1* gene locus has uncovered a number of remarkable features. The use of an alternative gene promoter, located ~18 kb upstream from the coding region of the gene, was described above, and it is the predominant driver of *EPHX1* expression in human tissues. Another feature of the *EPHX1* gene locus relates to the presence of repetitive DNA elements. Mammalian genomes are complex and dynamic, merely a small fraction is occupied by protein coding exons, while up to 50% is contributed by repetitive elements, with the remaining 48% comprising “unique DNA” [150]. Nearly half of the human genomic sequences are derived from ancient transposable elements (TEs), which encompass both transposons and retrotransposons, including short interspersed element (SINE) and long interspersed element (LINE), long terminal repeats (LTRs), and DNA transposons [151]. TEs are DNA sequences that are capable of integrating from one site in the genome to a new one via a “cut and paste” mechanism [152]. First described as “junk” DNA, the importance and function of TEs have been increasingly appreciated recently. These TEs or mobile elements participate in genome formation and benefit the evolution of genes by affecting their functions. TEs have the ability to promote

genetic diversification and regulatory variation by serving as recombination hot spots, altering protein coding content or by regulating gene transcription [153,154]. TEs have been shown to serve as alternative promoters for many genes, including aromatase P450 (CYP19; [155]), carbonic anhydrase 1 [156], and bile acid CoA: amino acid N-transferase [157], resulting in tissue-specific regulation of gene expression.

Alu elements are a particularly important type of TE and comprise the major components of SINEs that exist in the human genome; their 1.1 million copies occupy over 10% of the human genome and contribute to a significant portion of human genetic diseases [158]. It is therefore of interest and potentially of functional importance that the far upstream E1-b promoter region of human EPXH1 is replete with repetitive elements, including those of the Alu class, and that humans are genetically polymorphic for the inclusion of a specific double Alu repeat cluster [94,159]. Presence of the double repeat element appears to reduce transcriptional activity of the E1-b promoter in cell lines of different tissue lineage [94]. If these results also apply to the *in vivo* situation, differences in EPXH1 expression and corresponding capacities to conduct xenobiotic metabolism, may arise from these or other promoter region polymorphisms and may have an important bearing on interindividual risk factors associated with epoxide catalysis in target cells.

13.3.6 Summary/Pharmacological Implications

In summary, EPXH1 is an important xenobiotic enzyme, contributing critical activities toward both detoxication and bioactivation processes. The identification of human genetic polymorphisms within the EPXH1 gene locus may represent risk variables to various disease syndromes that include cancers [83], certain pulmonary diseases [132,160], preeclampsia [161], METH addiction [106], and adverse reactions to pharmacotherapies. For example, in the case of adverse drug reactions, anticonvulsant hypersensitivity syndrome has been postulated to potentially involve EPXH1 [88,89]. The transcriptional regulation of human EPXH1 appears to differ markedly from that in rodents, for example, involving the primary contribution of a far upstream gene promoter region [24,94]. Elucidating the molecular mechanisms of EPXH1 transcriptional control in human tissues and the potential genetic contribution of EPXH1 to the etiology of human disease remain important venues for future research.

13.4 SOLUBLE EPOXIDE HYDROLASE (EPHX2)

13.4.1 Introduction

Although its existence was controversial in early studies, sEH (EPHX2) was first identified as a separate enzymatic activity several years after the discovery and characterization of EPXH1. Initially, the respective enzymes were largely distinguished by their soluble fraction versus microsomal subcellular localization [55,162]. Both enzymes were originally thought to participate in xenobiotic metabolism, although EPHX2 was recognized at its inception for its role in the metabolism of a terpenoid juvenile hormone mimic in insects [55,162]. Relatively early, EPHX2 was recognized for its preference for *trans*- over *cis*-substituted epoxides of sterically hindered substrates, and hence, *trans*-stilbene oxide has often used to distinguish EPHX2 activity

from EPHX1 activity, as the latter enzyme exhibits strong preference for *cis*-stilbene oxide [54]. However, more recent experimental evidence has firmly established that the major role of EPHX2 is in the metabolism of endogenous epoxy fatty acids, with the EETs emerging as the best studied EPHX2 substrates [13]. Although the endogenous role of EPHX2's metabolism seemingly shifts its focus away from its function as a drug-metabolizing enzyme, the theme of this text, the enzyme has taken on new and important identities that position it squarely as an important target for pharmacological intervention. Therefore, these latter therapeutic dimensions of EPHX2 are the primary focus of the subsequent sections.

Human EPHX2 is localized on chromosome 8 at position 8p21-p12 [163], a locus comprising a single gene that consists of 19 exons, and encodes a 62.5-kDa protein (EC 3.3.2.3) [164]. Unlike the monomeric EPHX1 protein, the EPHX2 protein exists largely as homodimers of its monomeric subunit [165–168]. Each EPHX2 protein monomer is composed of two functional domains joined by a proline-rich linker. The C-terminal domain contains the α/β -hydrolase structure homologous to haloalkane dehalogenase and is responsible for the epoxide hydrolase activity [13,169]. The N-terminal domain is similar to haloacid dehalogenase, and functions as a lipid phosphatase [13,170,171]. EPHX2 is widely distributed in numerous tissues, with highest activity in the liver [172], followed by the kidney [173], where its distribution is concentrated within the renal cortex. The primary isolation of EPHX2 is from the cytosolic or soluble fraction, but in some cases, EPHX2 activity was reportedly localized in peroxisomes [174]. In rodents, drugs such as the peroxisome proliferator-activated receptor α (PPAR α) agonists are strong inducers of EPHX2 [175,176], although functional PPAR α response elements have not been identified in the upstream of human EPHX2 gene.

13.4.2 Biological Functions

Numerous investigations have now demonstrated the importance of EPHX2 in the regulation of high blood pressure [177,178] and inflammation [179,180]. Recently, with the development of EPHX2 inhibitors, the generation of EPHX2 null mice and the analysis of EPHX2 genetic polymorphisms, a more detailed understanding of the important biological role of mammalian EPHX2 has emerged.

As indicated earlier, it has become increasingly clear that fatty acid epoxides are the major endogenous substrates for EPHX2, with the cytochrome P450-derived epoxides of arachidonate acids (EETs) being the most well studied [13]. An overview of arachidonic metabolism is presented in Fig. 13.4. Numerous investigations have now demonstrated that EETs are chemical mediators that play significant roles in cardiovascular and renal regulation, modulating physiological parameters including hypertension, inflammation, angiogenesis, and mitogenic effects in the kidney [181]. Over the past several years, selective pharmacological EPHX2 inhibitors, such as 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, have been designed with the potential to treat a variety of disease situations [3,114].

Several biologically imported EETs are diagramed in Fig. 13.5. Generally, the hydrolysis catalyzed by EPHX2 eliminates the biological activity of the lipids. EPHX2 was therefore postulated to play a role in blood pressure regulation, as 14,15-EET is a potent vasodilator. In subsequent studies, EPHX2 null mice were found to exhibit decreased blood pressure [177]. Moreover, recent physiological and genetic studies in a hypertensive rat model have further delineated the important role of EPHX2 in

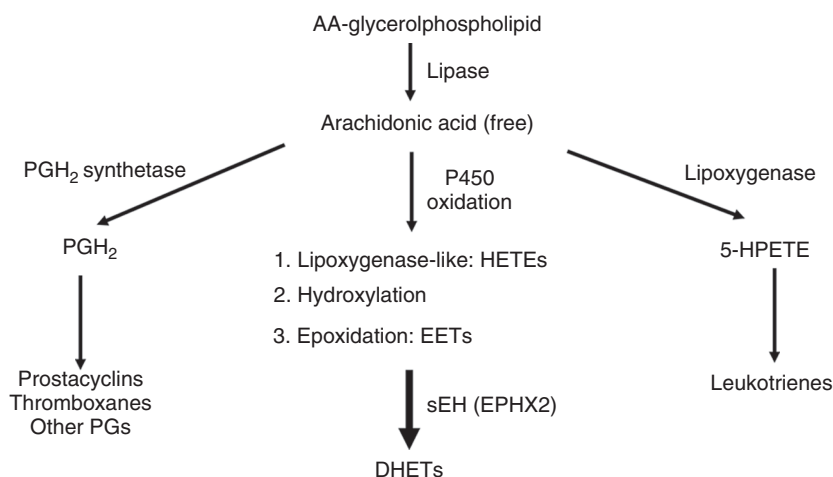


Figure 13.4 Overview of the arachidonic acid metabolism cascade. PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid.

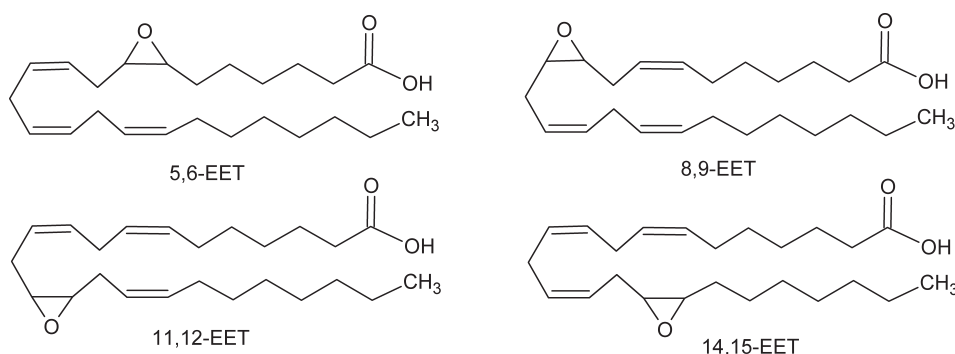


Figure 13.5 Illustration of several epoxyeicosotrienoic acid intermediates that display reactivity with EPX2.

blood pressure regulation and as a susceptibility factor for heart failure [182,183]. Therefore, inhibition of EPX2 is considered a new therapeutic approach for hypertension. In addition to vasodilatation, EETs also display anti-inflammatory role in vascular endothelial cells by inhibiting cytokine-induced NF- κ B transcription. It has been suggested that EPX2 may play a key role in the regulation of inflammation by metabolizing anti-inflammatory EETs and in bioactivation of the toxic and proinflammatory effects of leukotoxin. In cell culture, inhibiting EPX2 results in EET accumulation and enhances their anti-inflammation effect [3]. Also in these respects, investigations conducted in null mouse models have demonstrated that EPX2 deletion attenuates renal injury and inflammation in a high salt model [184].

Similarly, on the basis of studies conducted in cardiovascular models of ischemic injury and hypertrophy, EPX2 has emerged as a potentially important pharmacological target in cardioprotection. Indications are that inhibitors of enzyme may offer therapeutic potential for a broad range of cardiovascular diseases [185]. As endothelial dysfunction contributes to the development of coronary heart disease, contribution

of EPHX2 in the metabolism of the EETs in vasculature tissues is likely an important factor in the regulation of endothelial function [186]. Interestingly, EPHX2 was recently identified as a heart failure susceptibility gene. These studies reported that the metabolism of cardioprotective EETs are affected by EPHX2 allelic variants that result in altered transcript and protein levels [183].

13.4.3 Genetic Polymorphism

Approximately 755 single nucleotide polymorphisms (SNPs) for human EPHX2 are cataloged currently in the NCBI SNP database. However, the great majority of these SNPs reside in noncoding regions of the gene or code for synonymous nucleotide substitutions. Initial analyses of EPHX2 genetic polymorphism in humans revealed the existence of two nonsynonymous polymorphisms, resulting in altered human sEH/EPHX2 amino acid sequences with associated alteration in enzymatic function or stability [187]. One involved a polymorphism at residue 287 (Arg287Gln; rs751141), where the more common arginine is nonconservatively substituted with glutamine [187,188]. A second coding region variant (rs71868122) involved an unusual trinucleotide insertion encoding an additional arginine residue, following the normal serine in position 402 [187]. Additional nonsynonymous polymorphisms have since been identified, including a Lys55Arg (rs4150795) amino acid substitution. All these structural EPHX2 polymorphisms appear to affect enzymatic function. Results of *in vitro* enzymatic assays as well as comparative molecular modeling analysis based on the crystal structure of EPHX2 indicate that the Arg287Gln variant possesses decreased activity as well as decreased protein stability relative to the reference protein [187,189]. On the other hand, the Lys55Arg variant appears to encode a more highly active form of EPHX2 [186,189].

Results of molecular epidemiological studies in human populations assessing the potential association of EPHX2 genetic polymorphism in human disease have yielded interesting findings. For example, in a study of risk factors associated with familial hypercholesterolemia, plasma cholesterol levels were elevated in individuals carrying both the Arg287 allele and a mutation in the low density lipoprotein receptor (LDLR) [190]. Interactive effects of Arg287 with carriers of the LDLR polymorphism were also noted between increased risk of cardiovascular disease and incidence of familial hypercholesterolemia [190]. The R287Q polymorphism was further reported as associated with carotid artery calcified plaque disease in European Americans [191]. Furthermore, the Lys55Arg polymorphism has been reported as a risk factor in coronary heart disease [186]. In white Americans, Lys55Arg genotype was associated with vasodilator response to bradykinin, such that forearm blood flow was significantly lower ($P = 0.043$) and forearm vascular resistance was significantly higher ($P = 0.013$) in Arg55 variant allele carriers compared to wild-type individuals. Significant associations were also observed with methacholine and sodium nitroprusside, suggesting an important role for sEH in the regulation of vascular function in humans [192]. As these are complex disease phenotypes, likely dependent on the regulation of eicosanoid metabolism that involves both cytochrome P450 epoxidation to generate the EETs as well as EPHX2 hydrolysis in the degradation of EETs, the exact genetic relationships that dictate interindividual risk differences in these disease etiologies will require an additional detailed study.

13.4.4 Regulation of Expression

Although the biological function of human EPHX2 has been extensively investigated, much less is known about the molecular mechanisms of EPHX2 regulation. Several observations in rodent models have shown that EPHX2 can be regulated by endogenous chemical mediators and some xenobiotics. For example, both cigarette smoke and γ radiation appear to affect human EPHX2 expression levels [193,194]. It has also been noted that male mice exhibit higher EPHX2 activities in liver and kidney than female mice [13]. These observations suggest that sex hormones may be involved in EPHX2 regulation, at least in mice.

In rodents, EPHX2 expression is induced by the administration of PPAR α agonists. Peroxisome proliferators are compounds that induce the size and number of hepatic peroxisomes. PPAR α agonists include clinically used hypolipidemic drugs, endogenous compounds such as steroids, dietary fatty acids, and commercial plasticizers [195]. The response to these agonists suggests that EPHX2 may play role in peroxisome proliferator-induced liver toxicity in rodents. However, whether these responses are maintained in humans is an important consideration and one that has not been definitively evaluated. To this effect, the core promoter of the human EPHX2 gene was characterized recently by the Hammock group [196]. No PPAR α responsive motifs were identifiable in the human gene's promoter region. However, nuclear factor Sp1 regulatory elements were apparent that were localized in a GC-rich region of the promoter and appear to be important contributors to the basal levels of EPHX2 transcriptional regulation.

Another recent report indicated that angiotensin II (Ang II), a potent vessel constrictor that elevates blood pressure in animal models, directly upregulates EPHX2 transcription expression mediated by an AP-1 motif present in the human EPHX2 promoter [197]. These investigators further demonstrated that the transcriptional regulation mediating EPHX2 induction by Ang II involved c-Jun/c-Fos binding to the AP-1 site at the -446 position of the gene's promoter [197]. In addition, overexpression of a mutant c-Jun protein that was lacking its respective transactivation domain markedly attenuated the EPHX2 induction response otherwise facilitated by Ang II.

Alternative splicing of RNA transcripts may also play a role in the regulation of EPHX2 expression. Interestingly, in mouse ovary, a unique variant of EPHX2 was reported as generated by alternative splicing. This variant lacked the associated phosphatase activity normally encoded in the transcript [198]. This short isoform of EPHX2 possesses an altered N-terminal sequence spliced from the second intron. Examination of the alternative splicing databases further predicts a number of potential EPHX2 splice variants in humans; however, most of these variants would be predicted not to be functional as a consequence of the deletion of entire exons or due to the introduction of stop or out-of-frame codons. To our knowledge, no publications on splice variation in human EPHX2 have been reported. This area remains to be one requiring more detailed study.

13.4.5 Summary/Pharmacological Implications

Although originally thought of as a xenobiotic-metabolizing enzyme, this is no longer considered a functional role for EPHX2. Rather, this sEH has been identified as a major contributor to the metabolism of endogenous substances, in particular, epoxides derived

from the arachidonic acid cascade. In these respects, EPHX2 appears to contribute important biological functionality in processes that include control of blood pressure and inflammatory responses. Thus, the enzyme has been targeted by pharmacological modulators, facilitated by the development of highly selective and potent inhibitors of the enzyme that are currently in clinical trials. Historically, EPHX2 represents one of the few cases where an enzyme originally described as being involved in xenobiotic metabolism has evolved as an exciting clinical target.

13.5 CONCLUSIONS

Together, the epoxide hydrolases, in particular, the microsomal (EPXH1) and soluble (EPXH2) forms of the enzyme, have achieved prominent recognition for their respective biological roles in xenobiotic and endogenous metabolism. As outlined in this chapter, these enzymes also contribute to the spectra of drug metabolism, with EPXH1 involved in the metabolism of several drug substrates and EPXH2 as a pharmacological intervention target and mediator of physiological processes such as control of blood pressure and inflammation. Although the history of discovery and characterization of these respective enzyme systems is rich, a great deal of additional study is required to fully elucidate their extended functional roles, the details of their transcriptional control, and the influence of genetic polymorphism present in these systems as potential risk modifiers in pharmacotherapy and chemically induced disease.

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14 Carboxylesterases

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14.1	Summary	1
14.2	Functions of carboxylesterases	2
14.3	Classification and structural features	6
14.4	Catalytic mechanism, substrate specificity, activators, and inhibitors	10
14.5	Expression and regulation	16
14.6	Pharmacogenomics of carboxylesterases	18
14.7	Comparison between human and animal carboxylesterases	24
14.8	Conclusions/further perspectives	26
	Acknowledgment	27
	References	27

14.1 SUMMARY

Carboxylesterases (CESs, EC 3.1.1.1) constitute a large class of enzymes that hydrolyze chemicals containing a functional group such as a carboxylic acid ester, amide, and thioester. These enzymes are major pharmacokinetic determinants of ester/amide drugs and detoxify against organophosphorus and pyrethroid insecticides. In addition, these enzymes hydrolyze endogenous lipids and involve the assembling of lipoproteins. CESs exhibit overlapping substrate specificity; however, many drugs are hydrolyzed predominantly by a single CES. Although there are exceptions, the relative sizes between the alcohol and acyl (acid) moieties of an ester contribute significantly to the isoform-specific hydrolysis. CES activity is widely distributed in mammalian tissues, with the highest level in liver microsomes. High abundance of CES in the liver is linked to certain cellular roles, notably in directing protein trafficking. CESs belong to the superfamily of α/β -fold proteins and have similar crystal structure to other enzymes in this superfamily. CESs use a two-step mechanism for catalysis. Hydrolysis of carboxylic acid esters leads to the formation of an alcohol and a carboxylic acid. Compounds with these moieties are substrates for conjugation enzymes or transporters. Likewise, hydrolysis may create or eliminate a substrate of other phase I enzymes, particularly cytochrome P450s (CYPs). Like many other drug-metabolizing enzymes, the expression of CESs is regulated by many factors, including age, hormones, therapeutic agents,

TABLE 14.1 Primary Functions of Carboxylesterases

Function	Mechanism of Action	Example
Hydrolytic metabolism	Catalysis	Aspirin
Prodrug design	Catalysis	Oseltamivir
Mobilization of lipids	Catalysis	VDLD assembling
Detoxification	Catalysis/scavenging	Pyrethroids
Protein trafficking	Protein–protein interaction	C-reactive protein

and environmental chemicals. Mammalian species express multiple forms of CESs. However, there are notable differences in substrate specificity, tissue distribution, and regulated expression.

14.2 FUNCTIONS OF CARBOXYLESTERASES

The primary functions of CESs are to metabolize therapeutic agents [1–3], to mobilize lipids [4,5], to detoxify insecticides [6,7], and to target proteins to subcellular organelles [8,9]. While CESs act as catalytic proteins, not all the functions result directly from catalysis. As shown in Table 14.1, the catalytic action is responsible for drug metabolism and lipid mobilization but not for protein targeting. Retention of certain proteins in the endoplasmic reticulum (ER) is achieved by protein–protein interactions. CESs are known to protect against a large array of insecticides such as organophosphates and pyrethroids. The mechanisms for the detoxification vary depending on the type of insecticides. Pyrethroids, for example, are detoxified by hydrolysis. In contrast, organophosphates are detoxified by forming irreversible complexes, commonly referred to as the *scavenging mechanism*.

14.2.1 Hydrolytic Metabolism of Ester and Amide Drugs

It is estimated that 20% of drugs in the market undergo hydrolytic metabolism. These therapeutic agents contain functional groups such as carboxylic acid ester (e.g., esmolol), amide (e.g., procainamide), and thioester (e.g., spironolactone). Drugs metabolized by CESs usually have a single bond for hydrolysis, but there are exceptions such as lovastatin and spironolactone (Fig. 14.1, arrowed). In many cases, hydrolysis is a major determinant of the pharmacokinetic and pharmacodynamic behavior of ester drugs. For example, metoprolol and its ester analog esmolol are both β 1-selective adrenergic antagonists and widely used for cardiovascular disorders (e.g., cardiac arrhythmia). In contrast to metoprolol, esmolol is rapidly hydrolyzed by CESs and has a very short duration of action [10]. Therefore, esmolol is administered intravenously and used in critically ill patients who need a quick but short β 1-blockade therapy. In contrast, metoprolol is usually administered orally and used for chronic disorders.

Likewise, the hydrolytic rate of ester and amide analogs is a major determinant for therapeutic use. Although both carboxylic acid and amide bonds can be hydrolyzed by CESs, the hydrolysis of amide bonds usually proceeds slower than that of carboxylic acid esters. As a result, an ester drug and its amide counterpart are used for different

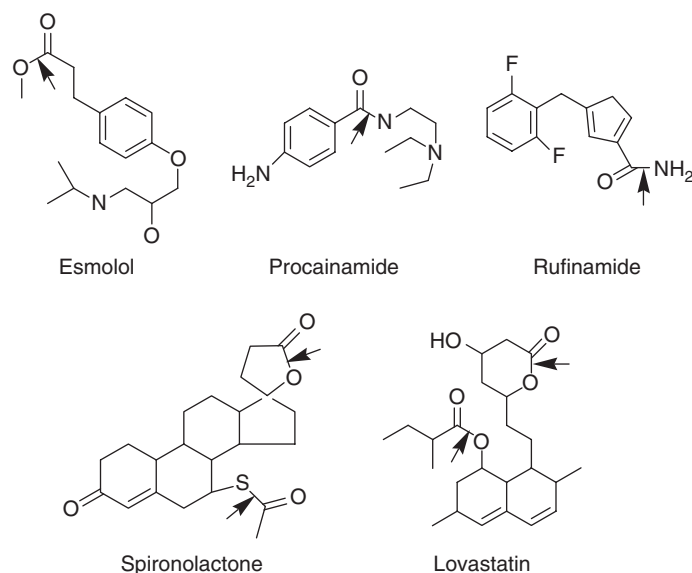


Figure 14.1 Chemical structure of representative drugs metabolized by carboxylesterases. The chemical bonds hydrolyzed by carboxylesterases are marked with an arrow.

indications. For example, procaine is hydrolyzed much faster than its amide counterpart procainamide [11]. Thus, procaine is used as a local anesthetic and has no systemic application. In contrast, procainamide reaches the systematic circulation and is used to treat cardiac arrhythmia. Consistent with the hydrolysis of amide bonds, CESs catalyze deamination. Rufinamide, a recently approved compound for Lennox–Gastaut syndrome (a special type of seizure), undergoes exclusively deamination [12].

In addition to hydrolysis, certain CESs are known to catalyze transesterification. For example, the antiplatelet agent clopidogrel, a methyl ester, normally undergoes rapid hydrolysis [13]. In the presence of ethyl alcohol, substantial amount of clopidogrel is converted to the corresponding ethyl ester, ethyl-clopidogrel (Fig. 14.2). The formation of ethyl-clopidogrel likely enhances the therapeutic activity of clopidogrel as hydrolysis of clopidogrel represents inactivation of this antiplatelet agent [14,15]. On the other hand, ethyl-clopidogrel is structurally similar to clopidogrel and presumably undergoes

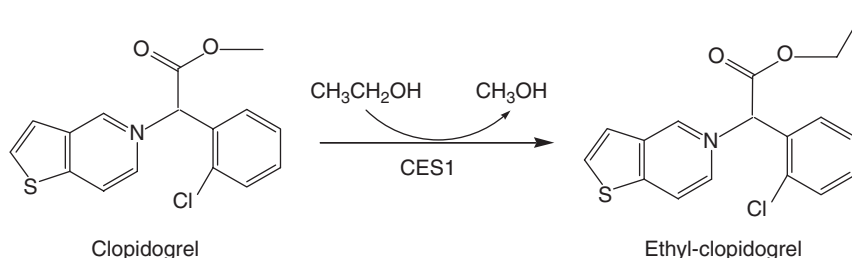


Figure 14.2 Transesterification of clopidogrel CES1 normally hydrolyzes clopidogrel. In the presence of ethyl alcohol, however, this enzyme catalyzes the formation of ethyl-clopidogrel.

similar oxidative activation [3]. Importantly, epidemiological analysis has shown that one-third of patients on clopidogrel drink alcohol [16], and these patients may have extended therapeutic effect. However, it remains to be determined to which extent the transesterification takes place in patients who are on clopidogrel and also drink alcohol.

14.2.2 Hydrolysis-Based Drug Design

CESs have a wide tissue distribution, and more importantly, these enzymes, compared with other enzymatic systems, have a high catalytic efficiency. Therefore, the CES system is widely incorporated into strategies for prodrug design [17,18]. One of the major advantages of ester prodrugs is to drastically increase the bioavailability [18]. For example, oseltamivir, an anti-influenza viral agent, has almost 80% bioavailability [19]. In contrast, its hydrolytic metabolite, although a potent inhibitor of the neuraminidase of the influenza virus, has an oral availability of 4% only [19]. In some other cases, ester prodrugs have much lower toxicity. For example, capecitabine, an ester prodrug of anticancer agent 5-fluorouracil, shows much lower intestinal toxicity than the therapeutically active compound [20]. To achieve tissue-specific activation, targeted expression of a prodrug CES has been tested through gene-delivery system [21].

14.2.3 Mobilization of Lipids

Many CESs are shown to hydrolyze lipid compounds such as choline esters, fatty acyl-CoAs, acylcarnitines, acylglycerols, phospholipids, retinyl esters, and cholesteryl esters [22–26]. Hydrolysis followed by re-esterification with different acyl moieties provides an important mechanism for generation of structurally diversified esters *in vivo*. CES-mediated action likely plays an essential role in the transportation and uptake of certain acyl esters or amides [24,25]. For example, very low density lipoprotein (VLDL) particles, a major delivery system of lipids from the liver to the periphery system, are assembled in and secreted by the liver. Assembling of these particles requires hydrolysis and re-esterification of triacylglycerol in the hepatic storage pool. Cells (e.g., RH7777 hepatoma line) lacking a CES called *triacylglycerol hydrolase* fail to assemble and secrete VLDL particles. Transfection of these cells with a cDNA encoding this CES causes a rapid depletion of intracellular triacylglycerol and a marked increase in the secretion of VLDL particles [26].

The importance of this CES in the mobilization of lipids has been recently confirmed by the knockout mouse model [4]. Consistent with a role of this CES in the assembling of VLDL, knockout mice show attenuated secretion of VLDL. Interestingly, the hepatic triacylglycerol level is not increased in the knockouts. Instead, the respiratory quotient and energy expenditure are increased. In addition, insulin sensitivity and glucose tolerance are improved in these knockouts. In humans, a role of CESs in the mobilization of lipids has also been implicated. CES1, a functionally related enzyme to the mouse CES, is abundantly expressed in human adipose tissues [27]. Inhibition of this CES in monocytes and macrophages increases the retention of intracellular lipids and enhances the development of the foamy phenotype [28]. Foamy macrophages are closely associated with the development of atherosclerosis, the major risk factor for cardiac and vascular diseases. Likewise, transgenic expression of CES1 leads to a more proatherogenic plasma lipid and apoB profile [29]. Apparently, further studies are required to determine the exact nature of CESs in the catabolism of lipids.

14.2.4 Detoxification of Insecticides

CESs, on the other hand, are also known to play important roles in detoxifying xenobiotics, particularly organophosphorus, carbamate, and pyrethroid insecticides [30–35]. These compounds constitute more than 80% total insecticides in the global use, including agriculture, residential setting, parasite eradication, and public facility. Animal studies demonstrate that inhibition of CES activity potentiates the toxicity of many organophosphates [34]. Intravenous administration of purified CESs affords considerable protection against the toxicity of soman, sarin, and paraoxon, providing direct evidence that CESs protect against chemical warfare agents and organophosphorus insecticides [36]. Similarly, CES activity is directly related to the toxicity of pyrethroid insecticides. For example, neonatal rats express only 10–15% of hepatic CESs of adult rats and exhibit a 16-fold greater sensitivity to pyrethroid deltamethrin [37]. More importantly, the relative insensitivity of adult rats is attenuated by pretreatment with tri-*o*-tolyl phosphate, a potent CES inhibitor.

The mechanisms of detoxification vary depending on the type of insecticides. CESs interact stoichiometrically and irreversibly with organophosphate [38]. Such an interaction is analogous to that between organophosphate and acetylcholinesterase, a vital enzyme that terminates neurotransmission of acetylcholine [38–40]. Interaction with CESs decreases the amount of organophosphate, which would otherwise inhibit acetylcholinesterase. Therefore, CESs detoxify organophosphates by acting as scavenging molecules. In contrast, CESs detoxify carbamates and pyrethroids by hydrolysis [41–47]. Another important difference is their insecticidal targets. As discussed above, organophosphorus insecticides (carbamate as well) target acetylcholinesterase. In contrast, pyrethroids target ion channels, particularly voltage-dependent sodium channels [48]. Such distinction provides a molecular basis for combined use of organophosphates and pyrethroids. Currently, there are hundreds even thousands of insecticide mixtures containing these two types of insecticides in the market [49]. The combined use is particularly effective against insecticide-resistant strains [50–52]. But at the same time, the toxicity to humans and other unintended species is presumably increased as well. While hydrolysis is generally considered to be detoxification, in some cases, a hydrolytic metabolite is more toxic than its parent compound. For example, hydrolysis of dehydropyrrolizidine alkaloids by CESs produces reactive dehydronecines, which form DNA/protein adducts and induce cell injury [53].

14.2.5 Protein Trafficking

High abundance of CESs in the liver is linked to certain cellular roles, notably in directing protein trafficking [8,9]. For example, two rabbit microsomal CESs have been shown to interact with and regulate the secretion of C-reactive protein [9], which is rapidly increased in the blood during the acute-phase response. Such a rapid release is likely from the C-reactive protein pool normally retained by interacting with microsomal CESs. The interaction is mediated by residues from 477 to 497 in the CESs. Interestingly, the interaction with C-reactive protein does not diminish the hydrolytic activity [9].

In contrast, β -glucuronidase interacts with CESs through their catalytically active site, thus leading to significant reduction or elimination in the CES activity [8,54]. Like C-reactive protein, the interaction of β -glucuronidase with microsomal CESs

results in the sequestration of β -glucuronidase in the ER [5,6], which is otherwise targeted to the lysosome. The microsomal β -glucuronidase has been shown to hydrolyze glucuronidated hormones (e.g., steroids), thus providing an effective mechanism that recycles physiologically important molecules [54]. Given the fact that CESs interact with β -glucuronidase through the active site, this interaction can be modulated by CES substrates and inhibitors [55]. It has been reported that administration of the anticancer agent irinotecan or exposure to organophosphates increases serum β -glucuronidase [56,57]. Irinotecan is a substrate of CESs, whereas organophosphates are potent inhibitors of these enzymes.

14.3 CLASSIFICATION AND STRUCTURAL FEATURES

Mammalian CESs are classified based on the sequence identity of amino acids [1,58,59]. CESs are all translated as secretory proteins; however, the majority of CESs are retained in the ER [60]. Secretory CESs are glycosylated to a much greater extent [61–63]. All CESs, even with less sequence identity, have the core segments arranged as alternate α -helix and β -sheets connected by loops with a varying length [40]. The crystal structure of CESs shows that these enzymes have three functional domains: the central catalytic domain surrounded by the $\alpha\beta$ -domain and regulatory domain [64,65]. Like acetylcholinesterase, the catalytic machinery is located at the bottom of the catalytic gorge [64–66].

14.3.1 Classification Methods

Several methods have been used to classify CESs. Early classification was based on the substrate specificity [67]. This approach soon became unsatisfactory because these enzymes have broad and overlapping substrate specificities. Classification was later made based on isoelectrophoretic points (IPs) [68]. This approach, although more definitive in terms of linking to individual CESs, was unsatisfactory because the same CES may have different IPs due to protein aggregation or differences in glycosylation. In addition, it is difficult to make a direct connection between the catalytic function (substrate specificity) and a CES based on its IPs. Currently, classification of CESs is made according to sequence identity of CES proteins. In this system, all mammalian CESs (e.g., human and rodents) are taken into consideration [2]. Members in the same family have a sequence identity of 60% or higher, otherwise, a different family is assigned. According to this method, six families are created: CES1, CES2, CES3, CES4, CES5, and CES6. The CES1 family contains the largest number of CESs and has eight subfamilies [2].

14.3.2 Salient Features of Carboxylesterases

Mammalian CESs have several salient structural features. These enzymes are synthesized as a large precursor with an N-terminal cleavable signal peptide [61–63]. Normally, this peptide directs newly translated proteins into the lumen of the ER for secretion. On translocation, this signal peptide is usually cleaved. However, many CESs are retained in the ER. These ER CESs have a C-terminal tetrapeptide HXEL. This consensus sequence is sufficient to keep a protein from being secreted [61–63]. In addition,

CESs are glycoproteins and secretory CESs are glycosylated to a much greater extent [62,63]. The extensive glycosylation of serum CESs suggests that glycosylation facilitates the secretory process and increases solubility. Another important posttranslational modification of CESs is the formation of disulfide bonds. All mammalian CESs contain at least four cysteines [2,69]; therefore, each CES has two or probably more disulfide bonds. Surprisingly, disruption of the disulfide bonds by reducing agents causes little changes in the catalytic activity toward 1-naphthylacetate, although reduced CESs are less compact than the native enzymes. On the basis of folding and refolding studies, it appears that the formation of the disulfide bonds plays critical roles in folding CESs into a catalytically active conformation [69].

14.3.3 Secondary and Crystal Structure

In addition to many salient structural features, CESs exhibit a unique assembly on the secondary structure. The core segments of CESs are arranged as alternate α -helix and β -sheets connected by loops with a varying length [40]. Such unique arrangements place CESs into the superfamily of proteins commonly referred to as the *α/β -hydrolase fold proteins*. Some members in this superfamily such as acetylcholinesterase are highly related to CESs in terms of the sequence identity and functions. On the other hand, some members such as neuroligin share little sequence identity with CESs and act as noncatalytic proteins [40].

Consistent with the similarity in the secondary structure, CESs share crystal structure with members of the α/β -hydrolase fold enzymes such as acetylcholinesterase and cholesterol esterase [64–66]. Overall, CESs have three functional domains: the central catalytic domain surrounded by the $\alpha\beta$ -domain and the regulatory domain. Like other members, CESs have the catalytic triad located at the base of a deep catalytic gorge. On the other hand, CESs show some important differences. For example, human CES1 has two substrate binding pockets: one is small and rigid and the other is large and flexible [65]. It has been proposed that the small/rigid pocket provides selectivity, whereas the large/flexible pocket is promiscuous. Interestingly, the sequence forming the small/rigid pocket is much more conserved than the sequence forming the large/flexible pocket. Even between CESs, notable differences exist. For example, an N-linked glycosylation chain is located at the product exit in a rabbit CES but not in human CES1 [64,65]. This carbohydrate chain presumably enhances catalysis by facilitating product exit. Furthermore, human CES1 exists as trimer or hexamer, whereas the rabbit enzyme exists as a monomer. The formation of the multimers of CES1 is likely due to the presence of a Z-shaped dimer interface [65].

14.3.4 Human Carboxylesterases

Like other mammalian species, humans express multiple forms of CESs. On the basis of molecular cloning and bioinformatics study, there are seven distinct CES genes in the human genome [1,58,59]. These genes are assigned to the CES1, CES2, CES3, CES5, and CES6 families, respectively. The CES1 family has three members, including CES1A1, CES1A2, and CES1A3. As described below, CES1A2 is an alternative form of CES1A3; thus, humans may express CES1A2 or CES1A3 but not both. All human CESs show a sequence identity of 39–44% [58,59]. The salient features of human CESs are shown in Fig. 14.3.

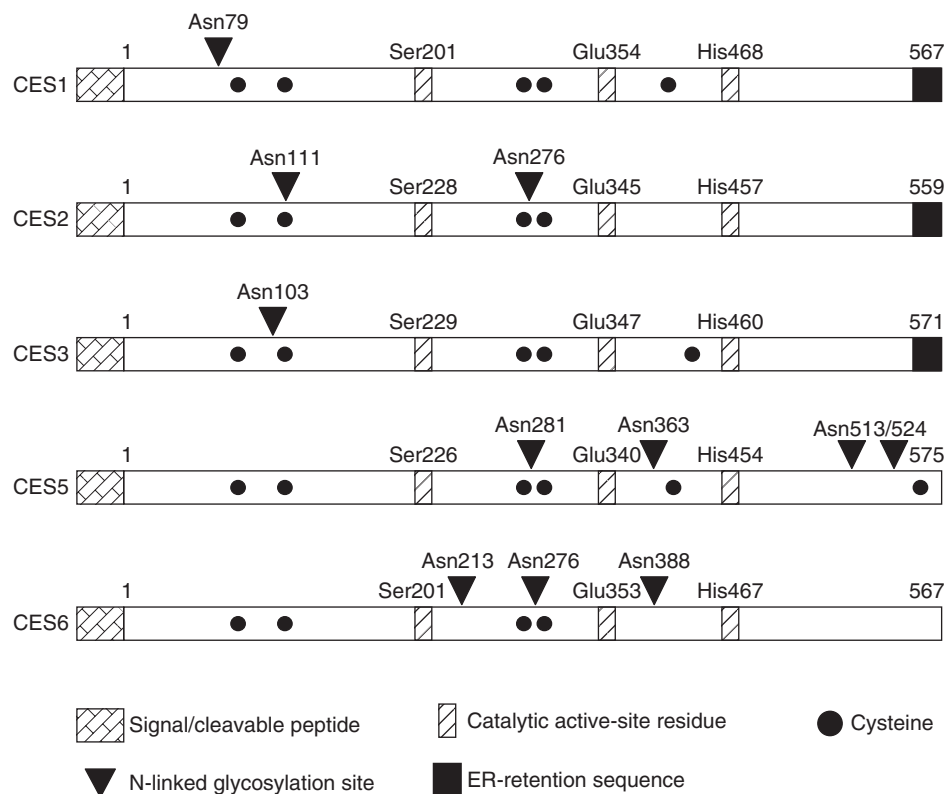


Figure 14.3 Salient features of human carboxylesterases. The protein sequences of carboxylesterases are derived from the corresponding cDNAs with the following accession numbers: NM_001025195 for CES1, NM_003869 for CES2, NM_024922 for CES3, NM_145024 for CES5, and FLJ37464 for CES6.

14.3.4.1 CES1 Family. Among the three CES1 members, only the *CES1A1* and *CES1A2* genes encode functional proteins [70,71]. The *CES1A3* gene, formerly termed CES1A4, has a premature stop codon thus is considered a pseudogene [72]. The *CES1A1* and *CES1A2* proteins differ by only four amino acids and these residues are located in the signal peptide. Therefore, these genes actually encode identical mature CESs. A recent study by analyzing a large number of individual samples has revealed that the *CES1A2* gene is a variant of the *CES1A3* gene [70]. Interestingly, the percentage of population carrying the *CES1A2* gene varies markedly depending on an ethnic group. African-Americans have the least percentage (5.1%), Japanese population has the highest percentage (31.3%), and Caucasians have 14%. In addition, a *CES1A1* variant exists and has the first exon converted from the corresponding exon of the *CES1A3* gene. Therefore, a total of four haplotypes of CESs can occur based on various CES1 genes: (i) *CES1A1* and *CES1A3*, (ii) *CES1A1* and *CES1A2*, (iii) *CES1A1* variant and *CES1A3*, and (iv) *CES1A1* variant and *CES1A2*. All CES1 genes are clustered at position 16q12.2, and the *CES1A1* gene has an orientation opposite to the *CES1A2* and the *CES1A3* gene. In addition, the *CES1A* genes produce alternatively splicing variants, leading to the deletion of Ala-18 and Gln-362 [73]. It remains to

be determined whether the splicing variants are derived from the *CES1A1* or *CES1A2* gene. Nevertheless, the deletion variants consistently have lower activity [1].

14.3.4.2 *CES2 Family.* In contrast to the CES1 family, humans have only a single member in this family [1]. This gene is located at 16q22.1, a second cluster of CES genes in the human genome. Like the *CES1A2* and *CES1A3* genes, the CES2 gene has the orientation opposite to that of the *CES1A1* gene. The CES2 gene uses three alternative promoters for transcriptions, and the relative activities of these promoters differ depending on a tissue [74]. Like the CES1 gene, the CES2 gene produces alternative splicing transcripts [1]. Several CES2 splicing variants share a 16 amino acid deletion, which occurs in exon 10. This deletion produces catalytically inactive protein. In addition, alternative use of the translation initiation codon leads to the addition of extra 64 amino acid to the N-terminus, although it remains to be determined whether such addition alters the hydrolytic activity and to which extent the alternative codon is used for translation initiation. Consistent with multiple alternative variants, multiple CES2 transcripts with marked differences in length have been identified [1].

14.3.4.3 *CES3 Family.* The human CES3 gene is clustered with the CES2 gene and has the same orientation in the genome as well [1,75]. Like the CES2 gene, multiple CES3 transcripts are identified with one being 2.1 kb and the other being 3.9 kb. These two transcripts differ in the 3'-untranslated region by 1750 bp. An alternative splicing variant in exon 12 was reported and this variant has a nine amino acid deletion (residues 481–483). Compared with CES1 and CES2, CES3 has a restricted tissue expression pattern. According to Northern blotting analysis, high level of CES3 mRNA is present in the liver, colon, and to a significantly less extent in the small intestine [75]. On the basis of a database of expressed sequence tags (ESTs) [1], the CES3 transcripts are also present in the trachea and placenta. However, it is not clear whether the EST transcripts are translated into protein. CES3 is much less active toward various commonly used substrates. On the basis of the catalytic efficiency on the hydrolysis of irinotecan, CES3 is 5 times less active than CES1 and 500 times less active than CES2 [75].

14.3.4.4 *CES5 Family.* Like the CES2 and CES3 families, the CES5 family has only a single member in humans [58]. The CES encoded by this gene is called *cauxin*, commonly referred to as *CES-like urinary excreted protein*. This protein is abundantly present in the urine of male animals such as cats, dogs, and sheep. It has been postulated that this CES is involved in the production of felinine, a precursor of the putative cat pheromone hormone. Nevertheless, the presence of this protein in the urine of humans remains to be established. However, an EST database shows that human CES5 is expressed in the brain, thymus, and testis. Like the *CES1A1* gene, the human CES5 gene is located at 16q22.1, the first cluster of CES genes in the human genome. Compared with CES1, CES2, and CES3, human CES5 has several unique features, notably in the ER retention consensus sequence, the potential N-linked glycosylation sites, and the charge clamp residues. CES5 lacks the C-terminal ER retention tetrapeptide, consistent with the fact that cauxin is a secretory protein. Additionally, CES5 has four putative N-linked glycosylation sites, the most among human CESs [58]. Coincidentally, several rodent serum CESs are highly glycosylated as well. And finally, CES5 does not have the conserved charge clamp residues. In CES1, these residues contribute to oligomerization [65].

14.3.4.5 CES6 Family. Human CES6 is a putative CES based on the sequence alignment analyses against the human genome. This putative enzyme has key residues involved in catalysis, formation of intramolecular disulfide bonds, oligomerization, and regulation of catalysis [59]. On the other hand, CES6 exhibits several major differences compared with CES1, CES2, and CES3. This CES lacks the ER retention tetrapeptide and has three potential N-linked glycosylation sites. These characteristics are consistent with the possibility of being a secretory CES. Like other CESs, CES6 variants exist due to alternative splicing. On the basis of reported ESTs, the CES6 gene is expressed in a broad range of tissues, including the skin, brain, lung, kidney, and placenta. The CES6 gene is clustered with the CES2 and CES3 genes with the same orientation in the genome.

14.4 CATALYTIC MECHANISM, SUBSTRATE SPECIFICITY, ACTIVATORS, AND INHIBITORS

As described above, CESs have a catalytic triad and use a two-step reaction mechanism for the catalysis [1–3]. In the past decade, tremendous progress has been made on the substrate specificity of CESs, particularly CES1 and CES2 [1–3,32,76]. It appears that both CESs, although with broad substrate specificity, show a strong structural preference for hydrolysis. CES1 preferably hydrolyzes esters with a relatively large acyl moiety, whereas the opposite is true with CES2. The catalytic activity of CESs can be enhanced by activators such as pinacolone [77] and inhibited by compounds such as benzil and trifluoromethyl ketone (TFK) derivatives [78–83].

14.4.1 Mechanism of Catalysis

CESs, like other esterases, use a two-step reaction mechanism for hydrolysis centered by the “catalytic triad.” This triad is made of the nucleophile (serine), the base (histidine), and the acid (glutamate) [2,64–67]. The first step of the reaction involves the attack of the nucleophile serine on the carbonyl carbon of a substrate, resulting in the release of the alcohol moiety from the substrate and simultaneously forming a covalent linkage between the remaining acid moiety of the substrate and the enzyme. Subsequently, the covalent linkage is cleaved through a water molecule activated by the base histidine, leading to the release of the acyl moiety of the substrate accompanied by the regeneration of the enzyme (Fig. 14.4).

The two-step hydrolysis mechanism, on the other hand, provides a molecular explanation to the toxicity of organophosphorus compounds. These insecticides, usually called *hemi-substrates*, interact similarly as true substrates. However, the resultant organophosphate–enzyme complex creates a steric exclusion, which prevents the second nucleophilic attack mediated by the activated water molecule [84]. Apparently, interaction with acetylcholinesterase represents toxicity, whereas interaction with CESs is considered detoxification against organophosphorus compounds.

14.4.2 Substrate Specificity of Human CES1 and CES2

CESs generally have high catalytic efficiency. This is particularly true with short-chain standard substrates such as *para*-nitrophenylacetate. Hydrolase A, a rat CES,

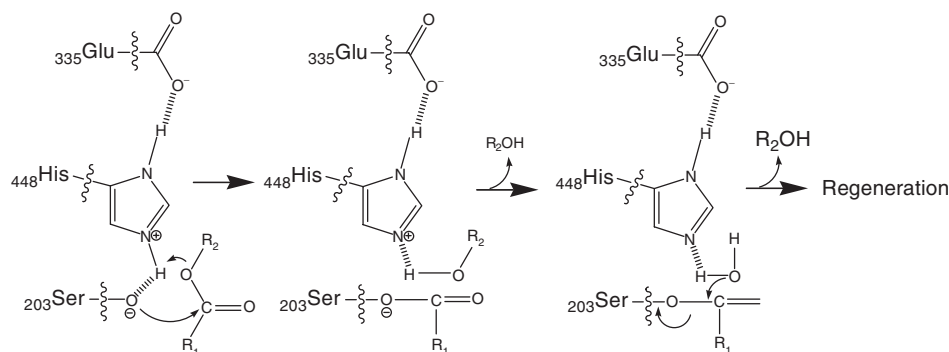


Figure 14.4 Catalytic cycle of carboxylesterases.

hydrolyzes this ester at a $K_{\text{cat}}/K_{\text{M}}$ ratio of $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$. This rate of efficiency is close to the diffusion rate of this ester, a measure of being a perfect enzyme. However, there are some exceptions for this general observation [60]. For example, human CES3 shows little hydrolytic activity toward this ester. As a matter of fact, human CES3 remains to be characterized for its preferable substrates.

Human CES1 and CES2, on the other hand, have been well characterized for their substrate specificity [1,2,13,85]. As described above, the hydrolytic preference of CES1 and CES2 is likely related to the sizes of the acyl and alcohol moieties of esters. On the basis of the molecular weights, the acyl moiety (clopidogrel carboxylate) of clopidogrel is 10 times as big as the alcohol moiety (methanol); therefore, this antiplatelet agent is hydrolyzed by CES1 but not CES2 (Fig. 14.5). Conversely, the acyl moiety of irinotecan is much smaller than the alcohol moiety; thus, this anticancer agent is predominately hydrolyzed by CES2. The alcohol/acyl size-based preference can apply to compounds with multiple ester bonds as well. Psychomotor stimulant cocaine, for example, contains two ester bonds and complete hydrolysis produces ecgonine, methanol, and benzoic acid. Among these products, ecgonine is the largest and acts as the acyl moiety relatively to methanol but the alcohol moiety relatively to benzoic acid. Consistent with the alcohol/acyl-based preference, CES1 hydrolyzes ecgonine-methyl ester, whereas CES2 benzoate-ecgoninyl ester [86,87]. Figure 14.5 shows several drugs that are selectively hydrolyzed by CES1 or CES2.

While the relative size of the acyl and alcohol moieties is a major determinant in the hydrolytic preference between CES1 and CES2, there are notable exceptions. For example, illicit heroine contains two ester bonds with both sharing the alcohol moiety [65] (Fig. 14.6). This alcohol moiety is much bigger than the acids in both cases. Interestingly, both ester bonds are preferably hydrolyzed by CES1 but not CES2. Although the crystal structure of CES2 remains to be elucidated, CES1 has a large/flexible substrate pocket, which accommodates bulky moiety of either alcohol or acid group. Such a unique structure likely contributes to the preferable hydrolysis of heroin by CES1 [6]. On the other hand, the *cis* and *trans* conformers of permethrin, even with the same sizes of acyl and alcohol moieties, are hydrolyzed differentially. For example, *trans*-permethrin is hydrolyzed comparably by both CES1 and CES2, but the *cis*-conformer is hydrolyzed by CES2 to a much greater extent than CES1 [6].

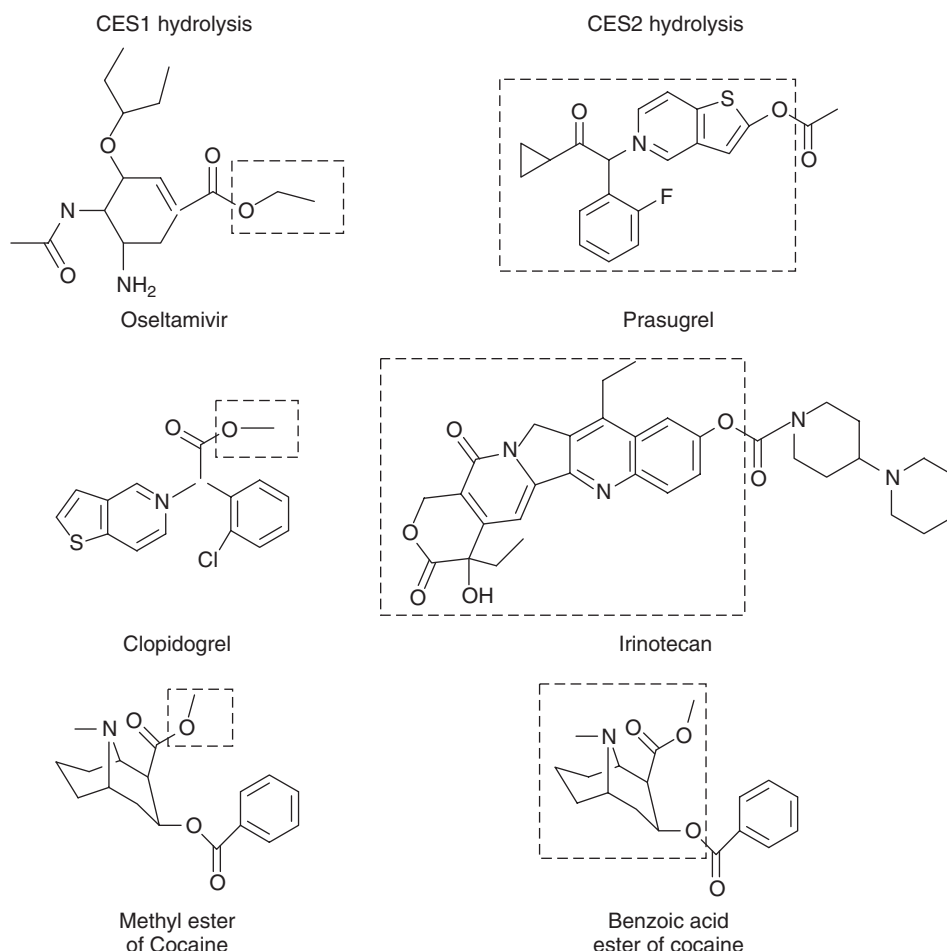


Figure 14.5 Substrate preference between human carboxylesterases CES1 and CES2. Drugs containing smaller alcohol moiety (boxed) are hydrolyzed by CES1 (left) and those containing larger alcohol moiety are hydrolyzed by CES2 (right).

14.4.3 Activators and Inhibitors

Like many other enzyme systems, the activity of CESs can be enhanced or inhibited. While the inhibition has been extensively studied, little information is known on the activation. Several chemicals such as pinacolone and pinacolyl alcohol have been shown to increase the hydrolytic activity of CESs. Enhancement has been observed *in vitro* and *in vivo* with pinacolone and pinacolyl alcohol [77]. These chemicals are metabolites of soman, a potent and irreversible inhibitor of serine enzymes, including CESs. The precise mechanism remains to be elucidated for the enhanced hydrolytic activity. Interestingly, commonly used solvent acetone also enhances hydrolytic activity of CESs [77], pointing to a possible mechanism of improving the accessibility of enzymes to a substrate.

In contrast to activation, several types of inhibitions are synthesized and well characterized. Ester drugs hydrolyzed by the same CESs may function as competitive

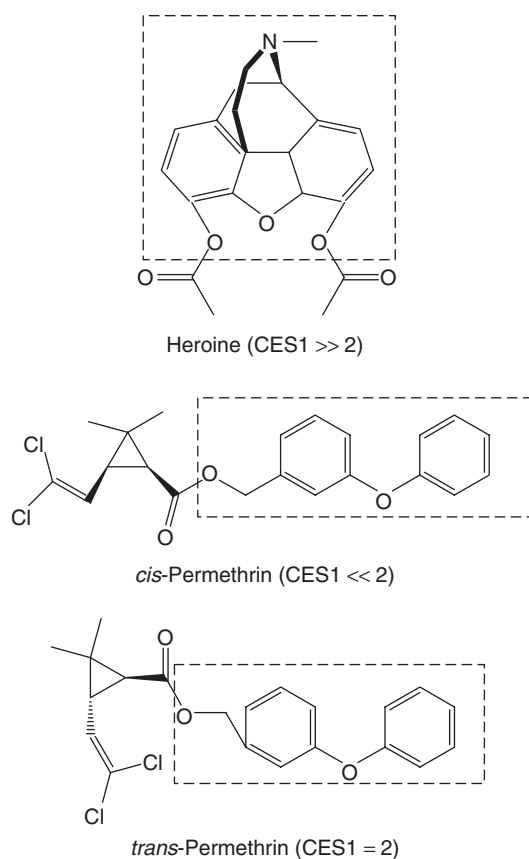


Figure 14.6 Examples of preferable hydrolysis independently of the relative sizes of the acyl/alcohol moieties. Boxed are the alcohol moieties of various esters. The two isomers of permethrin share the alcohol moiety, so do the two esters of heroine.

inhibitors toward each other. As described above, both clopidogrel and oseltamivir are substrates of CES1 with the former being kinetically favorable [76]. As a result, clopidogrel inhibits the hydrolysis of oseltamivir by as much as 90% when the same concentrations are used. The first-pass hydrolysis accounts for 90% of total oseltamivir activation [87–89], presumably due to the initial high concentration in the liver. Co-administration of clopidogrel inhibits the first-pass activation of oseltamivir and likely attenuates the activation of oseltamivir.

The second type of inhibition is achieved by chemicals that irreversibly modify the active-site serine residues, so-called serine enzyme inhibitors. In addition to organophosphorus insecticides, several irreversible inhibitors of serine enzymes are well characterized, including phenylmethylsulfonyl fluoride (PMSF) and inorganic salts such as sodium fluoride [60]. While these inhibitors generally act nonspecifically among serine enzymes, the IC_{50} values can vary markedly from one CES to another. For example, rat CES hydrolase A is ~ 1000 times more sensitive than hydrolase B based on the IC_{50} values (100 nM vs 100 μ M) [60]. Some chemicals such as malathion can function as inhibitors and substrates. This organophosphorothioate (OPT) contains a

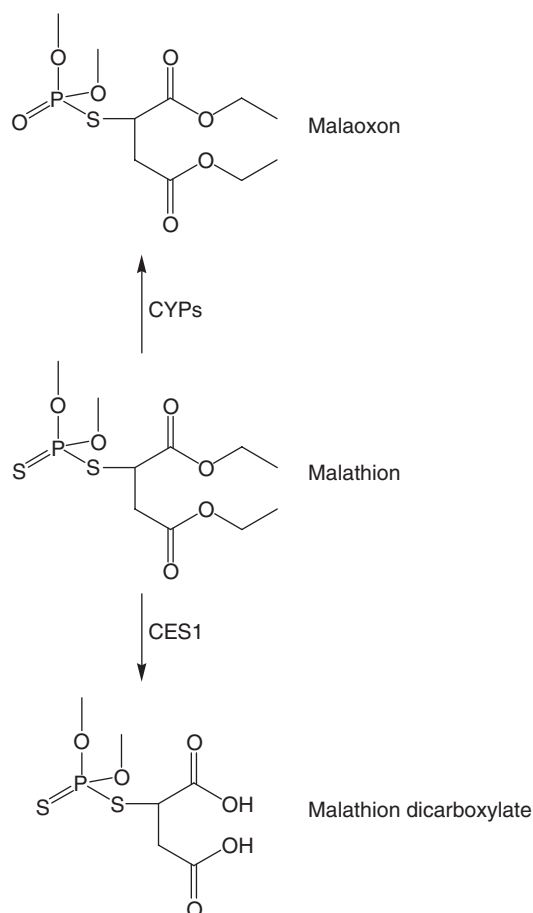


Figure 14.7 An example of substrate-inhibitor switch. Malathion is a substrate of carboxylesterases but can be a potent inhibitor on conversion to the corresponding oxon by multiple cytochrome P450s (CYP).

P=S bond and two carboxylic acid bonds (Fig. 14.7). Oxidative desulfuration of OPTs results in the formation of a P=O bond, the corresponding oxon. The oxon is an irreversible inhibitor of CESs. The carboxylic acid bonds, on the other hand, are subjected to hydrolysis by CESs. Hydrolytic metabolism of malathion represents detoxification (inactivation) and is correlated well with the abundance of CES1 in humans [90].

Two classes of compounds, with a potential of clinical use, are well characterized for the inhibition of CESs [78–83,91] (Fig. 14.8). Benzil derivatives belong to one of the classes and TFK-containing analogs belong to the other. Both benzil and TFK compounds inhibit catalysis of CESs by acting on the active-site residue serine. Compounds with this type of inhibition are called *transitional analog inhibitors*, and the inhibition is reversible. Among TFK compounds, thioether analogs are more potent than their sulfinyl or sulfonyl counterparts [82]. This is more evident with prolonged preincubation. The precise mechanism behind their different potency remains to be determined. It has been postulated that these substitution moieties may vary in supporting the

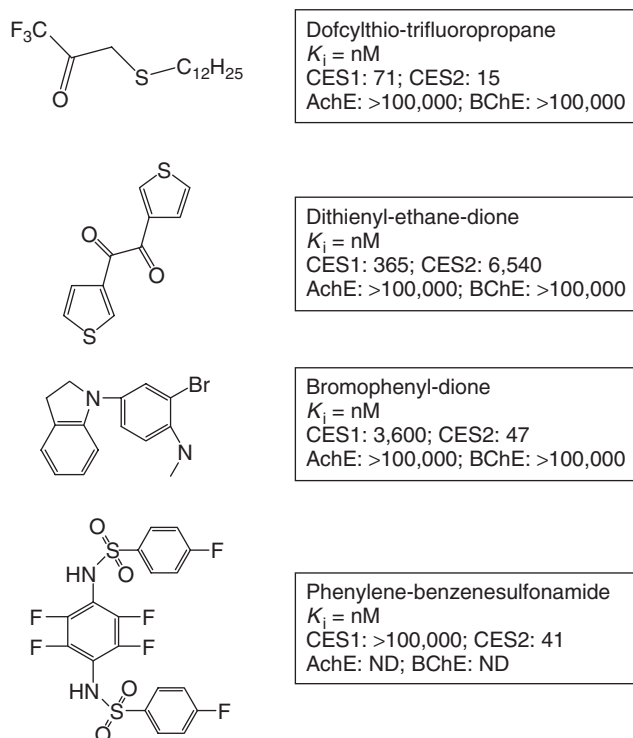


Figure 14.8 Selective inhibitors of CES1 and CES2. Data were compiled based on previous reports by Hicks *et al.* [79] on phenylene-benzene-sulfonamide, Hyatt *et al.* [81] on bromophenyl-dione and dithien-ylethane-dione [83], and Wadkins *et al.* on dofacylthio-trifluoropropane [62]. The K_i values (nM) for AchE (human acetylcholinesterase) and BChE (human butyrylcholinesterase) are shown for comparison. ND, not determined.

equilibrium between the ketone and *gem*-diol forms of a TKF compound. The ketone form is more inhibitory. Another interesting phenomenon with TFK compounds is their selectivity on acetylcholinesterase and butyrylcholinesterase. Many TFK compounds efficaciously inhibit CESs but surprisingly have little inhibition against cholinesterases (Fig. 14.8). This is interesting because CESs are highly related to cholinesterases in terms of the overall crystal structures and the sequence identity. On the other hand, TFK compounds show only some selectivity in inhibiting various CESs. For example, human CES1 and CES2 are comparably inhibited by a panel of TFK compounds [82]. Dofacylthio-trifluoropropane, the highest selectivity in this panel, exhibits only a five-fold difference in the inhibitory potency between CES1 and CES2 based on the K_i values (Fig. 14.8).

Benzil derivatives, in contrast, show much higher selectivity on the inhibition of various CESs [79,80]. These compounds, with a common feature of bulky structure, were initially synthesized for selective inhibition of CES2 to minimize the intestinal side effect (diarrhea) of irinotecan. Recently, structurally related compounds have been synthesized to contain an indole-dione or a sulfonamide [79,81] (Fig. 14.8). These compounds have shown improved selectivity with enhanced potency. For example,

dithienyl ethane-dione is ~20 times more potent in the inhibition of CES1 than CES2 (Fig. 14.8). Conversely, phenylene-chlorobenzenesulfonamide inhibits CES2 with a K_i value of 41 nM, three magnitudes lower than that on CES1 (Fig. 14.8). Actually, a panel of benzene sulfonamides tested shows little inhibition on CES1, although they all efficaciously inhibit CES2 [79]. The highly selective inhibitors are bulky in shape, and such bulky structure likely presents greater steric effect than TFK compounds. Apparently, combination of steric bulky structure with TFK compounds will likely produce more selective and potent inhibitors [80]. As described above, these compounds are transitional analog inhibitors; thus, they have hypothetical sizes of the alcohol and acyl moieties as seen in the substrates. Likewise, the hypothetical sizes are associated with the relative potency of inhibiting CES1 and CES2.

14.5 EXPRESSION AND REGULATION

In mammalian species, CES activity has a broad tissue distribution with the highest level in the liver. The expression of CESs is regulated by many factors, including age, hormones, pharmaceutical agents, environmental chemicals, and disease status [92–97]. Induction of CESs is generally minimal or moderate [93,94], whereas suppression can be profound [96]. On the basis of *in vitro* and clinical studies, both induction and suppression may significantly alter the metabolisms of ester/amide drugs and have severe clinical consequences.

14.5.1 Tissue Distribution of Human Carboxylesterases

CESs are generally present in a wide range of tissues and cells. However, the expression level varies markedly depending on a CES and also a tissue. Several experimental approaches have been used to determine the expression of various CESs, particularly CES1, CES2, and CES3 [75,92,94,96,98]. These methods include reverse transcription-quantitative PCR and Northern and Western blotting. Owing to intrinsic differences among these methods, the magnitude of differences in the expression levels may vary from one method to another method. Discrepancy can also rise from one laboratory to another. Nevertheless, there is a general agreement in all the reported data.

The expression level of CES1, CES2, and CES3 is summarized in Table 14.2. This represents composite data from various laboratories, therefore, plus or minus signs are used to reflect the relative levels. As expected, the liver expresses the highest level of all three CESs. Among them, CES3 has the most restricted tissue expression [1,75]. In addition to the liver, CES1 is also expressed at high levels in esophagus, larynx, and lung [1]. In contrast, CES2 is highly expressed in the intestine and kidney [1,98]. The intestine (colon) also contains high levels of CES3 [1,75]. The expression patterns suggest that CES2 has a dominant role in xenobiotic elimination, whereas CES1 is involved in the metabolism of both endo- and xenobiotics.

14.5.2 Ontogenic Expression

The hepatic expression of CES1 and CES2 shows an age-dependent expression. Overall, the adults express the highest level of CES1 and CES2 mRNA, pediatric donors express less CES1 and CES2 but fetal donors express the least level for both enzymes

TABLE 14.2 Levels of CES1, CES2, and CES3 in Various Tissues

Tissue	CES1	CES2	CES3
Adipose	++	++	—
Adrenal	+	++	—
Brain	+	—	—
Esophagus	++	+	—
Heart	+	+	—
Intestine	+	+++	+++
Kidney	+	++	—
Larynx	+++	+	+
Liver	+++	+++	+++
Lung	+++	+	—
Placenta	+	+	+
Spleen	+	+	—
Stomach	+	++	—
Testis	+	+	—
Trachea	++	++	++

Number of plus signs reflects the relative abundance of each carboxylesterase.

TABLE 14.3 Relative mRNA Levels of Carboxylesterases HCE1 and HCE2 in Fetuses, Children (0–10 Years Old), and Adults (≥18 Years)

Group	<i>n</i>	Minimum	Maximum	Variability	Mean	CV (%)
Fetus-HCE1	48	0.07	30.15	431 (fold)	3.32	172
Child-HCE1	34	12.18	2657.77	218	711.47	90
Adult-HCE1	22	225.50	2659.61	12	1059.59	61
Fetus-HCE2	48	0.20	4.28	21	1.63	91
Child-HCE2	34	7.00	148.78	21	65.82	53
Adult-HCE2	22	39.89	168.66	4	89.87	39

[92]. On the basis of the values of the means, the adult group has CES1 at levels 319-fold higher than the fetal group and ~50% higher than the child group (Table 14.3). Likewise, the adult group expresses CES2 at levels 55-fold higher than the fetal group and ~40% higher than the child group. In addition to the large difference among various age groups (intergroup), there is a large interindividual variability within a group. Interestingly, interindividual variability is inversely correlated with age. The fetal group, for example, has a 431-fold difference (ratio between the maximum and the minimum) in CES1 mRNA with a coefficient of variation (CV) of as high as 172% (Table 14.3). The pediatric and adult groups, on the other hand, vary less in CES1 mRNA with a 218- and 12-fold difference, respectively (Table 14.3). The inverse relationship of interindividual variability with age suggests that both developmental and xenobiotic regulations are involved in fetal and pediatric individuals. In contrast, the expression of CESs in adults is regulated by nondevelopmental factors only.

14.5.3 Induction of CES1 and CES2

CESs, like many other enzymes, can be induced by endogenous factors and xenobiotics. However, the induction is minimal or moderate at the most, presumably due

to high level of basal expression [93,94]. In hepatic cell lines, several antioxidants are shown to increase the expression of CES1A1 [99]. The induction is achieved by the transcription factor Nrf2. Interestingly, these antioxidants cause little induction of CES1A2, although the CES1A1 and CES1A2 genes are highly similar. In primary hepatocytes, several prototypical types of CYP inducers such as rifampicin, phenobarbital, and dexamethasone increase the expression of CES1 and CES2 by 20–50% at the protein level and ~80% at the level of mRNA [85,93,94]. The induction, although moderate, may have clinical implications. For example, coadministration of phenobarbital has been shown to markedly increase the hydrolysis of rufinamide, a recently approved antiepileptic by the US Food and Drug Administration. As a result, the steady-state plasma concentration of rufinamide decreases by as much as 46% [12]. The decrease is the most profound in children, pointing to the possibility that CESs are more inducible during the developmental stage. Phenytoin but not topiramate increases the hydrolysis of rufinamide as well [12]. Like phenobarbital, phenytoin is a potent activator of the constitutive androstane receptor [85,100]. These findings suggest that phenobarbital-type inducers may cause CES-based interaction, particularly in children.

14.5.4 Suppression of CES1 and CES2

It has been reported that hydrolytic biotransformation is decreased in patients with liver conditions such as hepatitis and cirrhosis [101–103]. In these conditions, the production of various proinflammatory cytokines (e.g., IL-6) is markedly increased [104–106]. In support of the role of cytokines in the reduced hydrolysis, IL-6 suppresses the expression of both CES1 and CES2 in primary hepatocytes [96]. The suppression is achieved primarily by transrepression as this cytokine decreases the activity of both CES1A2 and CES2 promoters. It appears that IL-6 represses the CES1A2 promoter through the upstream sequence located from -8251 to -7851.

The potential clinical significance of IL-6-suppressed expression of CESs is illustrated by inversely altering the cytotoxicity in response to ester drugs, including clopidogrel, irinotecan, and anti-influenza viral agent oseltamivir [13,76,96]. Hydrolysis of clopidogrel represents detoxification, whereas hydrolysis of irinotecan and oseltamivir increases cytotoxicity. As shown in Fig. 14.9, cells pretreated with IL-6 are spread and the projects are well extended when exposed to oseltamivir (right, top of Fig. 14.9). In contrast, cells without IL-6 pretreatment are rounded, isolated, and swollen with the presence of vesicles in the cytoplasm (left, top of Fig. 14.9). Conversely, when exposed to clopidogrel, cells pretreated with IL-6 exhibit profound morphological changes (e.g., aggregation), whereas cells without IL-6 pretreatment show normal appearance (middle of Fig. 14.9). As for irinotecan, cells without IL-6 pretreatment are isolated and shrank, whereas cells pretreated with IL-6 are morphologically normal (bottom of Fig. 14.9). The observed changes are opposite to the changes in cells in which CESs are overexpressed [13,76,107].

14.6 PHARMACOGENOMICS OF CARBOXYLESTERASES

Pharmacogenomics of CESs deals with several issues from altered hydrolysis in certain individuals to potential interactions with other drug elimination systems. Single nucleotide polymorphisms (SNPs) are increasingly identified in CES genes [1], and

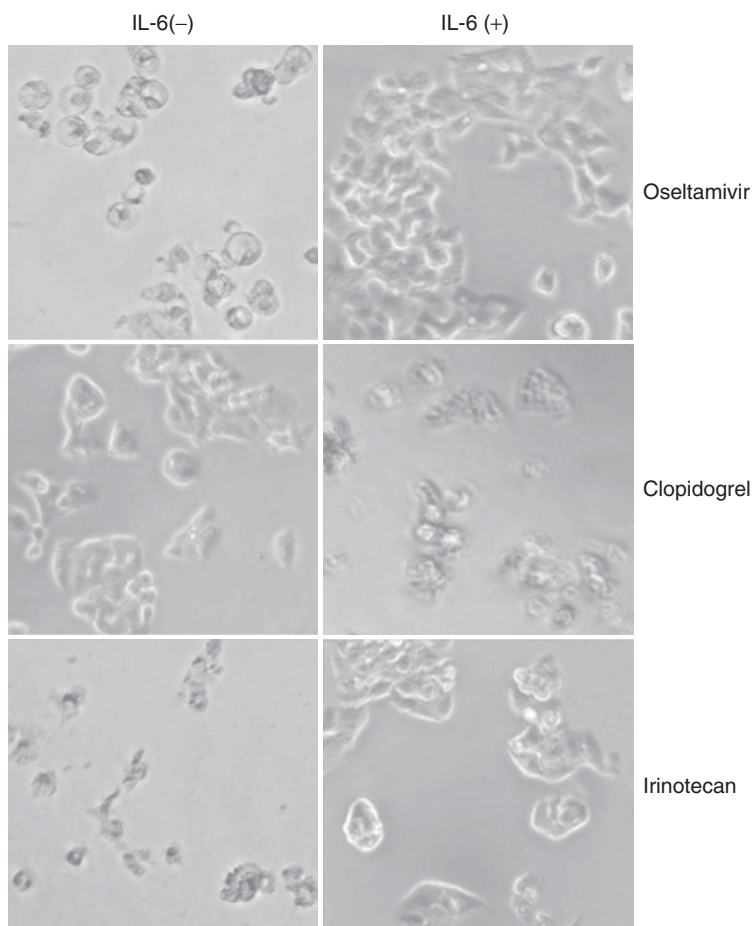


Figure 14.9 Morphological changes induced by interleukin-6 (IL-6) in response to oseltamivir, clopidogrel, and irinotecan HepG2 cells were seeded into 96-well plates at a density of 5000/well. After an overnight incubation, IL-6 (50 ng/mL) in 1% serum medium was added to half of the wells, and the treatment lasted for 12 h. Thereafter, the cells were washed with culture medium twice and treated with oseltamivir (100 μ M), clopidogrel (100 μ M), or irinotecan (3 μ M) in full-medium. The drug-medium was replaced with fresh drug-containing medium at 24 h. After an additional 24-h incubation, images were taken under bright field ($\times 250$).

some of them have been linked to poor clinical outcomes [108–114]. On the other hand, hydrolysis of carboxylic acid esters by CESs leads to the formation of products with an alcohol and a carboxylic acid. These functional groups can undergo further metabolism, notably conjugation reactions [115]. In addition, the carboxylic acid is negatively charged and elimination of the acid is likely achieved by transporters, presenting another possible interaction [116,117].

14.6.1 Polymorphisms

It has been shown that CES-based hydrolysis exhibits a large interindividual variability, particularly when population from various age is considered. As described above,

many factors such as hormones and xenobiotic exposure may alter the expression and contribute to individual variation in hydrolysis. On the other hand, genetic variation is increasingly recognized as an important contributor to the large interindividual variability. The NCBI SNP database lists a large number of SNPs in the *CES1*, *CES2*, and *CES3* genes. In addition, several investigators reported the existence of CES SNPs [108–114]. Generally, the *CES1* gene has the most and the *CES3* has the least reported SNPs [1]. SNPs can occur in the regulatory region, intron, and exon. Some exon SNPs result in the substitutions of amino acids.

Several SNPs have been linked directly to altered pharmacokinetics of ester drugs [108–114]. For example, a patient carrying a *CES1* Gly143Glu and Asp260 frame shift shows profound defect in the hydrolytic elimination of methylphenidate, a widely used psychostimulant [109]. Even for the Gly143Glu heterozygous genotype alone, dose reduction has been recommended. SNPs in the regulatory region may have therapeutic significance as well. For example, an A to C polymorphism at –816 in the 5′-flanking region of the *CES1A1* gene shows a 30% decrease in responding to the antihypertensive agent imidapril, which requires the activation by *CES1* [113]. A reporter containing this polymorphism has significantly lower promoter activity. Like *CES1*, certain SNPs in the *CES2* gene are linked to altered clinical outcomes. For example, patients carrying the R34W heterozygous genotype have a profound alteration in the pharmacokinetics of irinotecan with a decreased AUC ratio (hydrolytic species/parent drug) by as much as 40% [111].

14.6.2 The Cytochrome P450 Enzyme System

Many drugs metabolized by CESs are also metabolized by other enzyme systems, particularly the CYP system. Hydrolysis generally proceeds faster than oxidation, therefore, hydrolysis usually determines the fate of the parent drug [85,118–121]. In some cases, hydrolysis and oxidation compete for the metabolism of a drug and the relative activity has profound clinical consequences. For example, the widely used antiplatelet agent clopidogrel undergoes both hydrolysis and oxidation [13,118–123] (Fig. 14.10). Hydrolysis represents inactivation, whereas oxidation represents activation. The hydrolysis is catalyzed by *CES1* [13], whereas the oxidation is catalyzed by several CYPs such as CYP2C19 and CYP3A4 [120–123]. Normally, 90–95% of the dosed clopidogrel undergoes hydrolysis and only ~5% of the dose is oxidized [124,125]. While extensive study has been performed on the extent of the oxidative metabolite, no report has been made on the interplay between hydrolysis and oxidation. It is expected that a slight decrease in the hydrolysis likely favors the oxidation and enhances the antiplatelet activity.

14.6.3 Uridine Diphosphate (UDP)-Glucuronosyltransferases

In addition to phase I enzymes, in many cases, the action of CESs is directly associated with the action of phase II enzymes, notably uridine diphosphate (UDP)-glucuronosyltransferases [115,126,127]. These conjugation enzymes use the cofactor uridine diphosphate-glucuronic acid (UDPG) and add sugars to lipids and other nonpolar xenobiotics. This is commonly referred to as *glucuronidation* [115]. Conjugation with a sugar moiety drastically increases the hydrophilicity thus favoring elimination. While the site of glucuronidation in a compound usually occurs at an

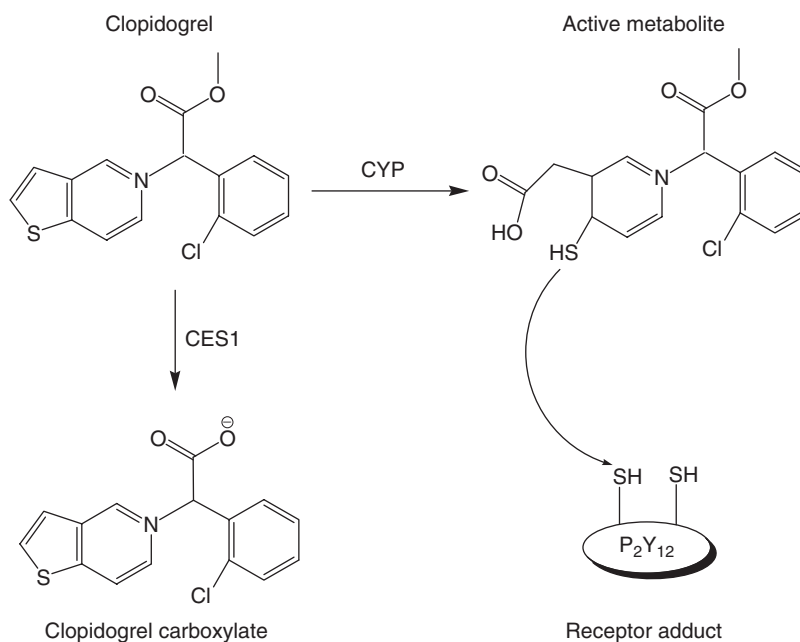


Figure 14.10 Hydrolysis and oxidation of clopidogrel. This antiplatelet agent undergoes both hydrolysis and oxidation. However, hydrolysis represents the major metabolic pathway (>90% of dosed clopidogrel). The P2Y₁₂ receptor belongs to a group of G protein-coupled purinergic receptors. Several CYPs have been found to catalyze the oxidation of clopidogrel, but the hydrolysis is exclusively catalyzed by CES1 [13].

electron-rich nucleophilic heteroatom such as O, N, and S, the majority of drugs for glucuronidation contain functional groups such as an alcohol/phenol or a carboxylic acid. These groups are produced by hydrolysis. In many cases, the relative activity between hydrolysis and glucuronidation has direct clinical consequences.

The hydrolysis–glucuronidation interaction is nicely illustrated by the anti-cancer prodrug irinotecan [126]. As shown in Fig. 14.11, this prodrug undergoes hydrolytic activation by CES2 to the cytotoxic metabolite SN-38, which is a potent inhibitor of DNA topoisomerase I. On the other hand, SN-38 is a substrate of UDP-glucuronosyltransferases, notably UGT1A1. Glucuronidated SN-38 no longer has therapeutic activity. As a result, an unbalanced hydrolysis over glucuronidation may increase systematic toxicity such as neutropenia. It has been demonstrated that increased SN-38 exposure is directly related to the severity of neutropenia [128]. Patients carrying the UGT1A1*28 polymorphism, particularly homozygous genotype, show a higher SN-38 AUC value and increased risk in the development of neutropenia [128].

14.6.4 Interactions with Drug Transporters

A third type of interaction as a result of the action of CESs occurs during the process of drug uptake and effluxing. Hydrolysis leads to the production of carboxylic acids, which are normally negatively charged. Charged molecules have poor membrane penetration

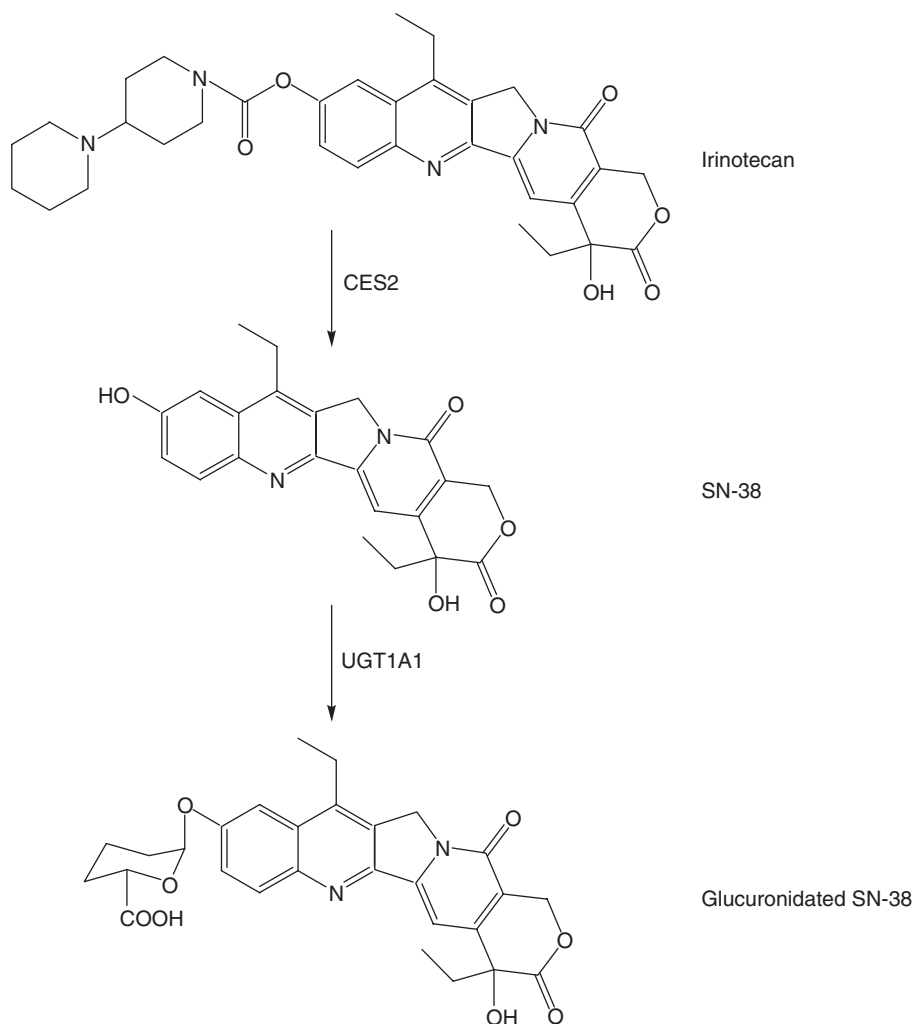


Figure 14.11 Hydrolysis-preceded conjugation reaction of irinotecan. This anticancer is hydrolyzed by CES2 to SN-38, which undergoes glucuronidation by uridine diphosphate-glucuronosyltransferase-1A1.

activity and are usually transported by drug transporters. Therefore, CES-coupled transporter activity is another major determinant of the efficacy and toxicity of ester drugs [129–131].

Oseltamivir, an anti-influenza viral agent, recently attracted a lot of attention from the public and scientific community because of the outbreaks of avian and swine flu. Oseltamivir, a substrate of CES1 [85], exerts potent antiviral activity through its hydrolytic metabolite. Several transporters have been shown to involve uptake or effluxing of this metabolite and parent drug as well. These transporters include multidrug resistance protein-4 (MRP4), peptide transporter-1, organic anion transporters, and P-glycoprotein [129–131]. MRP4 is localized to the basolateral membrane of

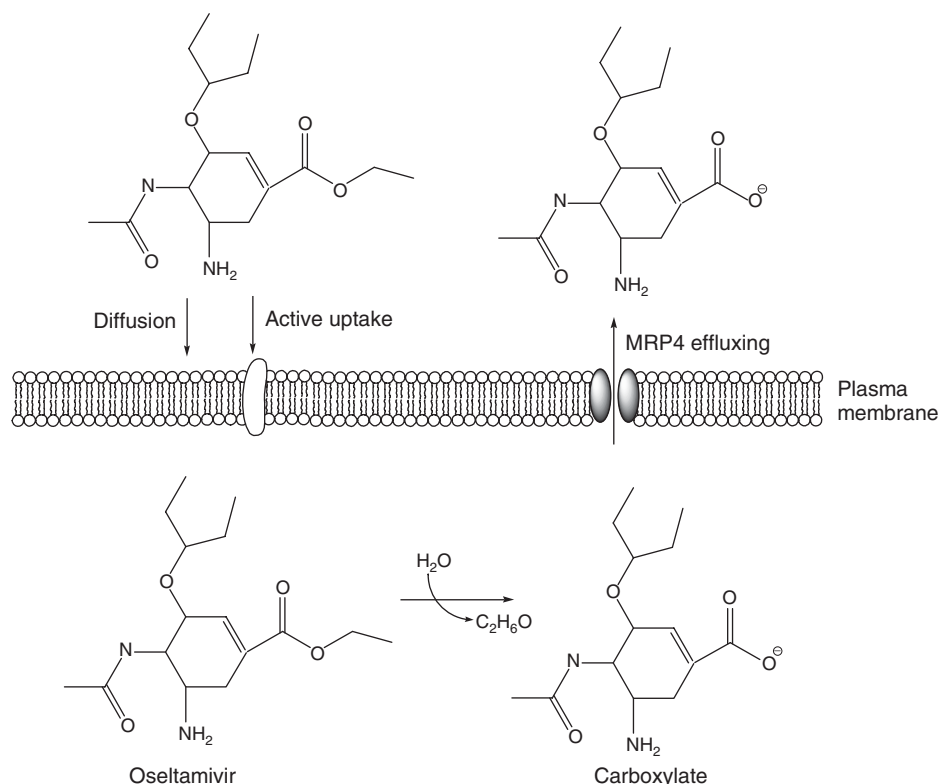


Figure 14.12 Hydrolysis-preceded transport of oseltamivir by multidrug resistance protein-4 (MRP4). The anti-influenza viral agent oseltamivir enters hepatocytes by passive diffusion and probably by active uptake as well. Subsequently, oseltamivir undergoes hydrolysis and the metabolite oseltamivir carboxylate is negatively charged and effluxed by MRP4.

hepatocytes and thus effluxes the metabolite into the blood (Fig. 14.12). On the other hand, overproduction of the hydrolytic metabolite is associated with cytotoxicity. Interestingly, oseltamivir is generally considered safe with only very few patients exhibiting liver toxicity. We recently tested whether increased expression of MRP4 protects against the cytotoxicity. Cells stably transfected with MRP4 or the corresponding vector were transfected again to overexpress CES1 and then exposed to oseltamivir. The cell proliferation rate was monitored for the toxicity. Vector cells showed marked decreases in cell proliferation when exposed to oseltamivir. In contrast, MRP4 cells showed little changes in the proliferation.

14.6.5 Drug–Insecticide Interactions

CESs are known to play critical roles in detoxification of several major types of organophosphorus, carbamate, and pyrethroid insecticides [30–35]. These hydrolytic enzymes detoxify organophosphates by acting as scavenging proteins. In contrast, CESs detoxify carbamates and pyrethroids by hydrolysis. Therefore, in all these cases, the metabolism of ester drugs should decrease. On the other hand, lower hydrolysis of

certain insecticides may alter the metabolism of drugs by other enzyme systems. For example, many pyrethroids are potent inducers of multiple nuclear receptors such as the pregnane X receptor (PXR). Activation of this receptor leads to the induction of many metabolizing enzymes such as CYP3A4. A recent study has demonstrated that the activation of PXR is significantly decreased in cells transfected with a CES [6]. It is therefore expected that coexposure to organophosphorus and pyrethroid insecticides likely increases the expression of genes regulated by PXR. It should be emphasized that the precise nature of the interactions between drug and insecticides remains largely unknown.

14.7 COMPARISON BETWEEN HUMAN AND ANIMAL CARBOXYLESTERASES

There are many similarities between human and animal CESs [2]. Like humans, all animal species studied express multiple forms of CESs with the highest CES activity being in the liver [60,65]. In many cases, compounds hydrolyzed by liver microsomes from animals are also hydrolyzed by human CESs [13]. On the other hand, there are notable differences, particularly in tissue distribution [60,62,63], regulation [85,94], and species-specific hydrolysis. Rodents but not humans, for example, have high levels of serum CESs [13,63]. The synthetic glucocorticoid dexamethasone exerts opposing effect on the regulation of several major rat and human CESs [85,94]. The local anesthetic procaine is hydrolyzed much faster by rat microsomes than their human counterparts [132]. These differences raise concerns regarding the relevance of animal models to human situation.

14.7.1 Tissue Distribution

CESs are expressed in all animal species studied. Without exception, the liver contains the highest overall CES activity in all mammalian species [65]. In addition, the gastrointestinal track, lung, and kidney have high levels of CES activity as well [1,13,63]. On the other hand, there are some notable differences. For example, rodents have high levels of CESs in the testis [62,98], in contrast, human testes contain less CES activity. Another major difference in the tissue distribution is the serum CES. Rodents but not humans contain abundant serum CESs. Actually, none of the well-characterized human CESs (i.e., CES1, CES2, and CES3) have been detected by Western blots in normal serum [13,63]. However, low level of CESs is expressed in leukocytes [133]. Certain liver diseases may increase the presence of liver CESs in the blood [134]. Nevertheless, the abundant presence of serum CESs in rodents may contribute to species difference in the metabolism of ester drugs. For example, rodents are relatively resistant to liver toxicity in response to oseltamivir [135]. One explanation is that rodents hydrolyze this antiviral agent in the blood, and only intracellular hydrolysis in hepatocytes has toxicity [76].

14.7.2 Species-Specific Hydrolysis

Like the tissue distribution, CES-based hydrolysis exhibits both similarities and differences among various species. Liver microsomes from human, monkey, dog, guinea

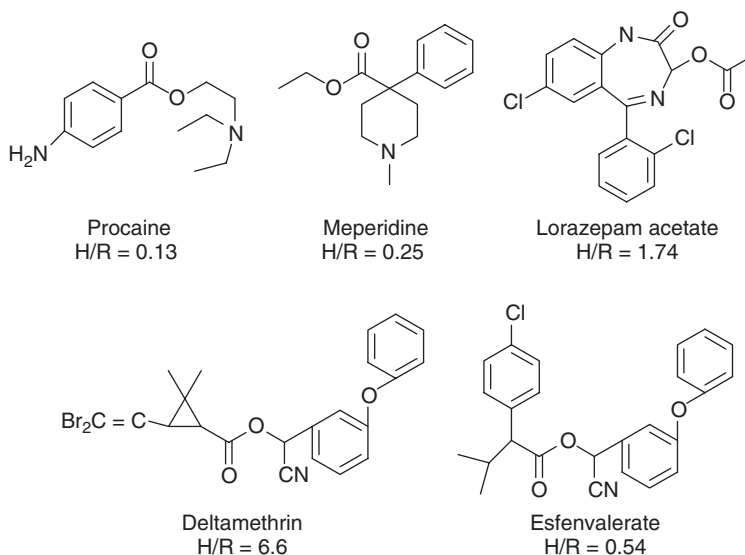


Figure 14.13 Examples of species-specific hydrolysis. The data were compared based on the specific activities of human and rat liver microsomes. The H/R ratios represent the specific activity on the hydrolysis of a compound by human microsomes over that by rat microsomes.

pig, hamster, mouse, and rat all contain two kinetically distinct hydrolytic activities toward *para*-nitrophenylacetate with the K_m values of 25–55 and 300–800 μM , respectively [60,93]. Significantly, the relative activity between low and high K_m CESs is comparable ranging from 40% to 60% with a single exception of guinea pig [60,93]. Microsomes from guinea pig contain predominately low K_m activity (80%). In both human and rat, the low K_m CES is responsible for the hydrolysis of clopidogrel, whereas the high K_m enzyme for the hydrolysis of aspirin [13].

Although there is remarkable similarity in the hydrolysis of certain esters among various species, these are equally remarkable in terms of species differences in hydrolyzing other esters. Figure 14.13 shows some esters that are differentially hydrolyzed by human and rat liver microsomes [44]. Deltamethrin and esfenvalerate are both pyrethroid type of insecticides. Based on the specific activity, human liver microsomes are six times as efficient as rat microsomes in hydrolyzing deltamethrin. In contrast, rat liver microsomes are twice as active as rat microsomes in hydrolyzing esfenvalerate. The local anesthetic procaine is hydrolyzed seven times faster by rat microsomes than their human counterparts [132], and likewise, meperidine is hydrolyzed much faster by rat liver microsomes than their human counterparts [136]. Conversely, lorazepam-3-acetate is a prodrug of lorazepam, a benzodiazepine type of anxiolytics, which is hydrolyzed by human microsomes approximately twice as fast as rat liver microsomes [137]. These findings demonstrate profound species differences in hydrolytic biotransformation of certain ester drugs.

14.7.3 Regulated Expression

Many factors have been shown to regulate the expression of CESs, including age, hormones, drugs, insecticides, and other chemicals [85,92–97]. Some of the factors

regulate the expression of these enzymes similarly across species. For example, the expression of CESs is very low in all species at neonatal and pediatric stage [92,97], and the anticonvulsant agent phenobarbital causes slight or moderate induction in rodents and humans [93,94]. On the other hand, some chemicals such as dexamethasone exert profound species-dependent regulatory effect. This synthetic glucocorticoid has been shown to slightly increase the expression of human CESs at micromolar concentrations [94]. In contrast, dexamethasone decreases the expression of multiple rat CESs, and significant decrease occurs even when nanomolar dexamethasone is used [85]. The decreases can be abolished by antiglucocorticoid RU486. Additionally, dexamethasone at nanomolar concentrations represses the promoters of CESs, and the repression is reduced by glucocorticoid receptor (GR)- β , a dominant negative regulator of the GR. In contrast, cotransfection of PXR increases the reporter activities, but the increase occurs only at micromolar concentrations of dexamethasone. These findings establish that both GR and PXR are involved in the regulated expression of rat CESs by dexamethasone, but their involvement depends on the concentrations [85].

In addition, structurally related compounds may have different effects on CES expression. For example, pregnenolone-16 α -carbonitrile (PCN) is structurally related to dexamethasone, and surprisingly, PCN consistently causes a moderate induction of rat hydrolase S, a secretory CES [63]. In contrast, dexamethasone suppresses the expression of this enzyme [85]. Furthermore, chemicals with the same or similar effects on the expression of CYP enzymes may differentially regulate the expression of CESs. For example, expression of rat hydrolase S is significantly suppressed by isoniazid but slightly increased by streptozotocin; both of them are CYP2E1 inducers [63,138]. The CYP1A enzyme inducers, β -naphthoflavone and 3-methylcholanthrene, have opposing effects; the former compound suppresses the expression of hydrolase S, whereas the latter compound slightly induces it [63,138]. The differential response of CES genes to the same type of CYP inducers suggests that these chemicals use multiple signaling pathways to exert their biological effects.

14.8 CONCLUSIONS/FURTHER PERSPECTIVES

CESs represent a large class of hydrolytic enzymes that are major determinants of ester/amide drugs and detoxify against organophosphorus and pyrethroid insecticides. In the past decade, tremendous progress has been made regarding substrate specificity and 3-D structure. Some progress has also been made on the regulated expression of these enzymes. Several important issues, on the other hand, remain to be established. First, increasing number of CES SNPs is identified, but it remains to be established how and to which extent these SNPs alter hydrolytic metabolism. Second, for many drugs, hydrolytic action is coupled with oxidation, conjugation, and active transportation; but the precise nature of the interactions among various systems remains to be determined. Third, it has long been postulated that CESs have a physiological role in lipid catabolism, but details on such a role are largely unknown. Finally, many drugs and disease status are shown to alter the expression of CESs, but the molecular mechanisms remain to be elucidated. In particular, how the expression of CESs is altered by the interplay between drugs and disease conditions.

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15 UDP-Glucuronosyltransferases: Pharmacogenetics, Functional Characterization, and Clinical Relevance

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15.1 Summary	1
15.2 Introduction	1
15.3 UGT pharmacogenetics	4
15.4 <i>In vitro</i> tools	5
15.5 Analysis of glucuronides	15
15.6 Individual UGT isoforms	16
15.7 UGTs in preclinical species	41
15.8 Conclusions	43
References	43

15.1 SUMMARY

Phase II conjugation by the UDP-glucuronosyltransferase (UGT) family of enzymes is an important metabolic pathway for both xenobiotics and endogenous compounds. This chapter attempts to catalog the vast depth of information surrounding UGTs, including pharmacogenetics, *in vitro* aspects, and clinical relevance. Individual sections on UGT isoforms cover expression, regulation, and polymorphisms; substrates; inhibitors; active site characteristics; and clinical relevance.

15.2 INTRODUCTION

Likely due to the observation that cytochrome P450 (CYP) was involved in the metabolism of the majority of drugs, most or all drug discovery organizations screen

for oxidative clearance in human liver microsomes (HLMs) [1]. The goal of this activity is to identify early the chemical space that results in high oxidative clearance, and thus attempt to “design out” this liability. Analysis of the reasons for drug development failures over the years suggests these activities have helped reduce drug attrition due to poor human pharmacokinetics [2]. This strategy, however, increased the frequency of involvement of non-CYP-mediated enzymes in clearance pathways for new chemical entities, and these are clearance mechanisms with which we have less experience [3,4]. It stands to reason that a better understanding of, and more experience with, these metabolic pathways (e.g., glucuronidation), the enzymes catalyzing them, and the *in vitro* systems in which we can model these processes will serve to keep drug attrition due to pharmacokinetics to a minimum.

Published in 2004, a systematic review of the top 200 drugs showed that glucuronidation was the second most common biotransformation pathway, being involved in the clearance of just <10% of drugs cleared by metabolism [5]. Glucuronidation, or glucuronide conjugation, is catalyzed by the UGTs. Historically, glucuronide conjugation catalyzed by the UGTs was classified by R. T. Williams as “phase II,” being a conjugation, or “synthesis,” resulting in detoxification; “phase I” was an oxidation, a reduction, a hydrolysis, or a transformation that could increase toxicity and also provide a functional handle for conjugation [6]. This terminology has been the conventional nomenclature for the better part of 40–45 years. There has since been recommendation that this terminology be phased out, and a gradual movement away from this convention has occurred [7]. It is recognized that most xenobiotics and endogenous compounds as well are functionalized with nucleophilic atoms to allow direct conjugation, in this case glucuronidation, to readily occur. Additionally, there is a growing pool of examples of metabolic products of direct conjugation reactions having become good substrates for oxidative enzymes [8,9].

Early in the twentieth century, G. J. Dutton performed some of the first *in vitro* glucuronidations and isolated the vital cofactor UDPGA (uridine diphosphate glucuronic acid) [10]. Since that early work, our understanding of this process has gradually advanced. We know the basic reaction where the substrate (aglycone) interacts with UDPGA (which is derived from UDP-glucose), resulting in the product containing glucuronic acid and the by-product UDP (Fig. 15.1). Glucuronidation can occur on any atom with sufficient nucleophilicity [11]: O-glucuronidation can occur on alcohols, phenols, or carboxylates; N-, S-, and even C-glucuronidation have also been reported [12]. The formed conjugate is usually much more water soluble than aglycone, resulting in greater elimination in excreta, both passively and through active secretion via efflux transporters such as the multidrug-related proteins (MRPs) [13]. Important to note is that unlike most oxidative biotransformations, glucuronidation is readily reversible via hydrolysis. Chemical hydrolysis can be acid or base catalyzed [14], whereas enzymatic hydrolysis may be catalyzed by the β -glucuronidase enzyme system [15]. This enzyme is discussed later.

The human UGTs are now recognized to be a superfamily of enzymes containing more than 15 members in humans. Since 1997 [16], the general isoform nomenclature has been standardized to a convention analogous to the CYP format: UGT family number (1, 2, 3, etc.), subfamily letter (A, B, C, etc.), and member number (1, 2, 3, etc.). The xenobiotic-metabolizing isoforms are thought to reside almost completely in the UGT1A and 2B subfamilies. These UGT isoforms show significant tissue-specific expression (Table 15.1), with most forms being expressed in the liver and/or intestine.

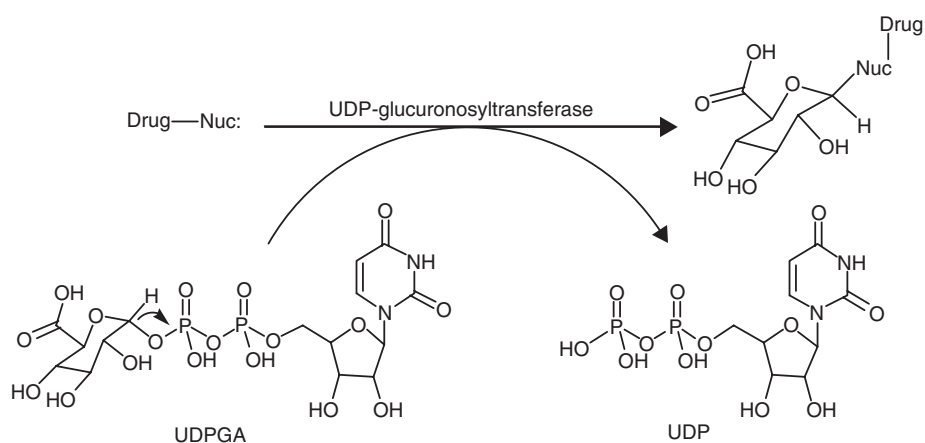


Figure 15.1 The UDP-glucuronosyltransferase reaction mechanism.

TABLE 15.1 Sites of Expression and Probe Substrates for Select UGT Isoforms

UGT Isoform	Major Sites of Expression	Probe Substrates	References
UGT1A1	Liver, intestine	Bilirubin	19
		Estradiol	19
		Ethinylestradiol	20
UGT1A3	Liver	F ₆ -1 α ,23S,25-trihydroxyvitamin-D ₃	21
		Lithocholic acid	22
		Fulvestrant	23
UGT1A4	Liver, stomach	Trifluoperazine	24
		Imipramine	25
UGT1A6	Liver, brain	Serotonin	26
UGT1A7	Esophagus, stomach	Benzo[α]pyrene metabolites	27
UGT1A8	Esophagus, intestine	Benzo[α]pyrene metabolites	27
		Dihydrotestosterone diglucuronide	28
UGT1A9	Kidneys, liver	Propofol	20
		Entacapone	29
UGT1A10	Kidneys, intestine, lungs	Dopamine	30
UGT2B4	Liver	Hyodeoxycholic acid (6 α -OH-gluc)	31
UGT2B7	Liver, intestine, kidneys	AZT	32
		Morphine	32
		Codeine	32
UGT2B10	Liver, prostate, breast	Nicotine	33
UGT2B15	Liver, prostate	(S)-Oxazepam	34
UGT2B17	Liver, prostate	Dihydrotestosterone	35

Interestingly, some forms show significant expression in extrahepatic tissues, such as UGT1A8 and UGT1A10 in the intestine and UGT2B17 in prostate [17,18]. Most isoforms are expressed in multiple tissues, as seen in Table 15.1, such as UGT1A9 in liver and kidney and UGT1A1 in liver and intestine.

The UGTs are membrane-bound enzymes, with the majority of the protein existing in the lumen of the endoplasmic reticulum, along with a membrane-spanning region and a short cytoplasmic tail [36,37]. The enzymes consist of two regions: an N-terminal and a C-terminal domain. The C-terminal domain is highly conserved across family members and has been demonstrated to be involved in cofactor binding [38]. The N-terminal region is much more variable and is involved in aglycone acceptor specificity. Recently, a crystal structure of the C-terminal portion of UGT2B7 was published [39], and this has yielded information about amino acids involved in cofactor binding and, along with site-directed mutagenesis experiments, residues likely involved in proton transfer and catalysis (histidines and aspartates) [38]. The mechanism of UGT-mediated glucuronidation has been the subject of a significant amount of research [40,41].

An advanced understanding of the UGTs has lagged behind the CYPs, and there are a few reasons for this: the more recent commercial availability of recombinant UGT isoforms, the lack of a complete and well-characterized battery of selective probe substrates and inhibitors, and the lack of a spectrally sensitive prosthetic group such as the heme in P450s. There are many aspects of the UGTs that are very similar to the CYPs, and parallels can be made between the two systems. However, there are many differences that have been and still are being elucidated regarding experimental approaches, biochemistry, enzymology, and *in vivo* predictions. We feel that the most important aspect to accelerating our understanding of the UGTs is to organize and catalog our knowledge to date in order to build on it. This chapter serves as one of the many steps that has and will occur toward this end. The rest of this work attempts to summarize our knowledge to date regarding UGT pharmacogenetics, *in vitro* phenomena, analytical tools, and finally, individual UGT isoforms. The section for each isoform is broken down, when appropriate, by expression, regulation, and polymorphisms; substrates; inhibitors; active site characterization; and clinical relevance.

15.3 UGT PHARMACOGENETICS

The effects of pharmacogenetic variability on drug profiles and disease states are well documented. While much of the initial work focused on the genetic polymorphisms of the CYP family of drug-metabolizing enzymes, the importance of UGT polymorphisms has gained increased attention in recent years. UGT polymorphisms have been shown to be directly related to pharmacological outcomes as well as adverse drug reactions and predisposition to cancer and other diseases [42,43]. Examples include irinotecan toxicity, Crigler–Najjar and Gilbert’s syndromes, and fluctuations in breast density (a potential biomarker of one’s potential of developing breast cancer) in pre- and postmenopausal women [42,44–47].

The UGT2B subfamily is made up of at least five human isoforms, and the UGT1A subfamily contains at least eight members. Gene transcription of each gene is controlled by unique regulatory elements, as described below for each isoform. Interestingly, the manner in which the genetic structure results in gene multiplicity is very different for the two subfamilies [48]. A distinct set of exons with a unique regulatory region exists

almost exclusively for each of the UGT2B members. The multiplicity of the UGT1A family arises via a very different mechanism: alternative splicing and exon sharing. The entire UGT1A subfamily contains a shared set of exons (exons 2–5) that make up the conserved C-terminal portion of the isoform. Distinct genes arise via alternative splicing of a unique exon 1 (exon 1a1, 1a3, 1a4, etc.) to the start of exon 2. Thus, each member contains a unique N-terminus and a common C-terminus. Recently, a new exon was identified in the common region of the UGT1A locus, between the coding exons 4 and 5 [49]. This new exon can either be a terminal exon or be spliced with the common exon 5. This newly discovered splicing event leads to the production of several new mRNAs and new human UGT proteins, resulting in the production of a complicated set of enzymes of widely varying activities (some inactive). While the implications of this discovery are still being evaluated, recent coexpression data from a variety of sources (see below) suggest that considerable protein–protein interactions occur, both as homo- and heterodimerization, and that these oligomers have significant implications on enzyme activity. Genetic polymorphisms have also been shown to influence the activity of each expressed isoform, and this is discussed later in detail in each isoform's section, as appropriate (Table 15.2).

The widely varied expression levels and catalytic functions of UGT enzymes across individuals and populations have been attributed to both coding region polymorphisms as well as polymorphisms in the regulatory elements of UGTs and their respective transcription factors [101]. Well-studied examples include coding region mutations in exons 1–5 of UGT1A1 leading to Crigler–Najjar syndrome (inactive UGT1A1), TATA-promoter element mutations resulting in Gilbert's syndrome (TA insert leading to a decreased production of active UGT1A1), and polymorphisms of hepatocyte nuclear factor 1 α (HNF-1 α) and caudal-related homeodomain protein (Cdx-2) that lead to altered expression of multiple UGT isoforms [47,101–103]. In addition, epigenetics, copy-number variations, and alternative splicing mechanisms have been implicated in leading to variations in UGT phenotype profiles [48]. More specific examples of the significance of pharmacogenetics for each UGT isoform can be found in the sections below.

Concurrent with the increased focus on UGT polymorphisms has been the development of tools to study their occurrence and impact [104]. Mammalian cell lines such as COS and human embryonic kidney (HEK) 293 cells are now routinely used to express variant UGT enzymes. Recombinantly expressed variant UGT enzyme systems are commercially available as well. Human tissue samples from both wild-type and disease models have also been utilized to study genotype–phenotype associations for various UGT polymorphisms [105,106]. More recent technologies to study UGT polymorphisms include the use of RNA interference and transgenic mouse models that encompass entire human UGT gene loci in a preclinical model [107,108].

The current UGT allele terminology used in this chapter has been obtained from the UDP-Glucuronosyltransferase Allele Nomenclature Page, accessed March 9, 2010 (http://www.pharmacogenomics.pha.ulaval.ca/sgc/ugt_alleles/).

15.4 IN VITRO TOOLS

There are multiple systems available with which to study glucuronidation [109]. Cellular systems, such as fresh or cryopreserved hepatocytes, have been used extensively

TABLE 15.2 UGT Polymorphisms

UGT Allele	Amino Acid Modification	Functional Change	References	UGT Allele	Amino Acid Modification	Functional Change	References
1A1*1	Wild type	—	50	1A1*45	Y74X	Unknown	47
*2	Frameshift	No activity	50	*46	Frameshift	Unknown	47
*3	S275F	No activity	51	*47	Frameshift	—	47
*4	Q357X	No activity	51	*48	V225G/f.s.	—	47
*5	Q331X	No activity	51	*49	Frameshift	—	47
*6	G71R	Reduced activity	52	*50	Frameshift	—	47
*7	Y486D	Reduced activity	52	*51	H376R/N	—	47
*8	R209W	Reduced activity	51	*52	G377V	—	47
*9	Q331R	Reduced activity	53	*53	W483X	—	47
*10	R341X	No activity	53	*54	W483X	—	47
*11	G308E	No activity	54	*55	L496X/N	—	47
*12	L175Q	Reduced activity	55	*56	Intron 1	—	56
*13	F170 deletion	No activity	50	*57	Intron 3	—	56
*14	G276R	No activity	55	*58	Q49X	—	56
*15	C177R	No activity	55	*59	Frameshift	—	57
*16	Q357R	No activity	54	*61	Intron 3	—	58
*17	S381R	No activity	54	*62	F83L	—	59
*18	A401P	No activity	54	*63	P364L	Reduced activity	57
*19	W355X	No activity	54	*64	Frameshift	Reduced activity	60
*20	A368T	No activity	54	*67	—	Reduced activity	61
*21	Frameshift	No activity	54	*69	I159T	No change	61
*22	A291V	No activity	54	*70	A321G	No change	61
*23	K426E	No activity	54	*71	I322V	No change	61
*24	K473X	No activity	54	*72	D359N	No change	61
*25	C280X	No activity	52	*73	P364L	No change	61

*26	Frameshift	No activity	55	*75	H533P	—	62
*27	P229Q	Reduced activity	52	*94	W461R	No activity	63
*28	—	Reduced activity	51	*95	R336Q	—	64
*29	R367G	Reduced activity	52	*96	R336L	—	64
*30	L15R	Reduced activity	55	*97	G395V	—	64
*31	P387R	No activity	65	*98	P387S	—	64
*32	R336W	No activity	65	*99	Frameshift	—	64
*33	I249T	Reduced activity	65	*100	Y192X	—	64
*34	M310V	Reduced activity	65	*101	W354R	—	64
*35	I431T	Reduced activity	65	*102	P34Q	—	64
*36	—	Increased activity	66	*103	R403C	—	64
*37	—	Reduced activity	66	*106	A478D	—	64
*38	N400D	Unknown	54	*107	W40R	—	67
*39	A401P/K437X	Unknown	54	*108	H132Q; 133/134 del	—	67
*40	A291V/K426E	Unknown	54	*109	Q185P	—	67
*41	Frameshift	Unknown	65	*111	Frameshift	No activity	68
*42	E463A	Unknown	69	*112	—	Reduced activity	70
*43	L233R	Unknown	71	*113	G493R	—	72
*44	H39D	Unknown	47		—	—	
1A3*1a	Wild type	—	73	1A3*3a	W11R/E27E/A159A	No change	73
*1b	D179D	—	73	*3b	W11R/E27E/A159A	—	73
*1c	A159A	—	73	*4a	R45W	Reduced activity	73
*1d	E27E	—	73	*5a	Q6R/W11R/E27E/A159A	Reduced activity	73
*1e	—	—	73	*6a	W11R/E27E/V47A/A159A/M270V	Reduced activity	73

(continued overleaf)

TABLE 15.2 *(continued)*

UGT Allele	Amino Acid Modification	Functional Change	References	UGT Allele	Amino Acid Modification	Functional Change	References
*1f	E27E	—	73	*7a	F110I	Reduced activity	73
*2a	W11R/E27E/ V47A/A159A	No change	73	*8a	A158V	Reduced activity	73
*2b	W11R/E27E/ V47A/A159A	Increased activity	73	*9a	W11R/E27E/ A159A/M208L	Reduced activity	73
*2c	W11R/E27E/V47A/ T78T/A159A	—	73	*10a	V47A	Reduced activity	73
*2d	W11R/E27E/ V47A/A159A	—	73	*10b	V47A	—	73
*2e	W11R/E27E/ V47A	—	73	*11a	W11R/E27E/V47A/ M114I/A159A	Reduced activity	73
1A4*1a	Wild type	—	74	1A4*3a	L48V	—	74
*1b	C157C	—	74	*3b	L48V	Reduced activity	74
*1c	L150L/P268P	—	74	*4	R11W	—	74
*1e	P10P	—	74	*5	43f.s. X22	—	74
*1f	N119N	—	74	*6	59f.s. X6	—	74
*2	P24T	Aglycone dependent	74	*7	L48V/R91C/ L150L/P268P	—	74
1A5*1	Wild type	—	42	1A5*5	A158G/6248I/ V249L/G259A	—	42
*2	A158G	—	42	*6	H225Y/6248I/ V249L/G259A	—	42
*3	H225Y	—	42	*7	A158G/H225Y/ L248I/V249L/ G259A	—	42
*4	L248I/V249L/G259A	—	42				

1A6*1a	Wild type	—	75	1A6*4a	S7A/L105L/R184S	—	76
*1e	D35D	—	76	*4b	S7A/L105L/R184S/ V209V	—	76
*2a	S7A/L105L/T181A/ R184S	Increased activity	76	*4c	S7A/L105L/R184S	—	75
*2c	S7A/L105L/ T181A/R184S	—	76	*5	T181A	—	75
*2 d	S7A/S93S/L105L/ T181A/R184S	—	76	*6	S7A/S103X/L105L/ T181A/R184S	—	76
*2e	S7A/L105L/ T181A/R184S	—	75	*7	S7A/R90H/L105L/ T181A/R184S	—	76
*3a	S7A	—	75	*8	T181A/R184S	Aglycone dependent	77
*3b	S7A/L105L	—	75	*9	R184S	—	77
1A7*1a	Wild type	—	78	1A7*8	N129K/R131K/ E139D/W208R	Reduced activity	79
*2	N129K/R131K	Increased activity	78	*9	G115S/N129K/ R131K	Reduced activity	79
*3	N129K/R131R/ W208R	Reduced activity	78	*10	N129R/R131K/ W208R	—	80
*4	W208R	Reduced activity	78	*11	R131Q	—	81
*5	G115S	Reduced activity	79	*12	W208R/R254X	Reduced activity	81
*6	E139D	Increased activity	79	*13	N276K	—	81
*7	N129K/R131K/ E139F	Increased activity	79	*14	C141S	—	82
1A8*1a	Wild type	—	83	1A8*2	A173G	No change	83
*1b	T255T	—	83	*3	C277Y	Reduced activity	83

(continued overleaf)

TABLE 15.2 (continued)

UGT Allele	Amino Acid Modification	Functional Change	References	UGT Allele	Amino Acid Modification	Functional Change	References
1A9*1a	Wild type	—	79	1A9*3b	M33T	—	84
*2	C3Y	—	79	*4	Y242X	Truncated protein	84
*3a	M33T	Aglycone dependent	79	*5	D256N	Reduced activity	85
1A10*1	Wild type	—	86	1A10*4a	L244I	—	86
*1b	Q42Q	—	86	*4b	A231A/L244I	—	86
*1c	H199H	—	86	*4c	H199H/A231A/ L244I	—	86
*1d	H199H/A231A	—	86	*5	M59I	No change	87
*2a	E139K	—	86	*6	T202I	Reduced activity	87
*3a	T240M	—	86	*7	I211T	Reduced activity	88
*3b	A231A/T240M	—	86				
2A1*1	Wild type	—	89	2A1*?	G308R	—	89
*?	D85Y	—	89	*?	V391I	—	89
2A3*1	Wild type	—	90	2A3*?	T497A	—	90
2B4*1a	Wild type	—	91	2B4*2b	D458E/R459R	—	91
*1d	A404A	—	91	*3	F109L/F369L	Reduced activity	91
*1n	A404A	—	91	*4	K455R	—	91
*1p	A404A	—	91	*5	A404A/C511R	—	91
*1q	A404A	—	91	*6	D458Q	—	91
*2a	D458E	No change	91				
2B7*1a	Wild-type	—	91	2B7*2c	P267P/H268Y	—	91
*1c	T245T/Y354Y	—	91	*2 d	P267P/H268Y/ V305V/L353L	—	91

*1d	R124R	—	91	*2e	H268Y	—	91
*1f	L353L	—	91	*2f	P267P/H268Y/ L353L	—	91
*1g	T245T/L353L	—	91	*2 g	H268Y	—	92
*1j	T245T/Y354Y	—	93	*3	A71S/R124R	—	91
*2a	H268Y	—	94	*4	T245T/Y354Y/ D398N	—	91
*2b	P267P/H268Y/ L353L	—	91				
2B10*1	Wild type	—	95	2B10*2	D67Y	Reduced activity	95
2B15*1	Wild type	—	96	2B15*5	D85Y/K523T	—	97
*2	D85Y	Aglycone dependent	96	*6	T352I	—	97
*3	L86S	—	98	*7	T352I/K523T	—	99
*4	K523T	—	97				
2B17*1	Wild type	—	100	2B17*2	Deletion	No activity	100

to generate glucuronides. It is important to note that without special treatment (e.g., inhibitors), all enzyme activities present (oxidative and hydrolytic as well as conjugative enzymes) in the cellular system will be able to act on the substrate being incubated, which can make it rather difficult to specifically study glucuronidation in this system for drugs possessing multiple metabolic pathways. The overall result, however, is likely more physiologically relevant (see below). Tissue preparations (e.g., liver microsomes or S9) are probably the most commonly used sources of UGT activity. However, special treatment, such the use of detergents or the pore-forming peptide alamethicin, is often used to unmask the maximum activity. An advantage to liver microsomes, however, is that oxidative or conjugative processes can be studied independently, simply by the use of the appropriate cofactor (NADPH alone, UDPGA alone, or a combination of both) [110]. Recombinant UGTs have become available over the last several years, and these can be a way to study the interaction of a single isoform with a single substrate. Nevertheless, the tools exist to begin attempts at reaction phenotyping: enzyme kinetics in these various systems and inhibition with inhibitors selective for a single isoform [111]. It bears mentioning that similar to the CYPs, another enzyme system with known promiscuity in substrate processing, the UGTs frequently show atypical or non-Michaelis-Menten kinetics [17,112]. This is just one phenomenon that can add complexity to reaction phenotyping.

Activation of UGT activity is an observation that has resulted in much research. It was noted that perturbation of the membrane caused an increase in enzyme activity by an unknown mechanism [113]. Agents such as detergents or *N*-acetylglucosamine have been shown to enhance enzyme activity. The exact mechanism of enzyme activation has been unclear for some time, although membrane disruption, activation of cofactor transporters, and generation of a conformational change in the enzyme to a more active conformation have been proposed. Indeed, the mechanism of “conformation” versus “compartmentalization” was debated as to the explanation for the latency of the native enzyme [114]. More recently, the pore-forming peptide alamethicin has gained widespread use as an activator of glucuronidation [3], lending support to the compartmentalization mechanism. Interestingly, it has also been shown that some UGT isoforms are very susceptible to detergent inhibition, making this mechanism of activation (detergent treatment) rather impractical for general use [115]. Membrane disruption to remove the barrier increases enzyme activity likely by allowing cofactors and by-products to more freely diffuse into and out of the lumen of the endoplasmic reticulum. This is especially important since the by-product, UDP, has been shown to be highly inhibitory to some isoforms. UGT1A1, in particular, appears to be exquisitely sensitive to inhibition by UDP generated during glucuronidation. This has been shown to be very important for UDP generated from other isoforms, as this could lead to some rather complex results (e.g., false positives/false negatives) [116].

15.4.1 Albumin Effect

Recent efforts to understand the tendency of HLMs to underpredict intrinsic rates of glucuronidation have identified the ability of albumin to attenuate inhibition of some UGT isoforms by long-chain fatty acids. As observed for CYP2C9, unsaturated fatty acids such as arachidonic, linoleic, and oleic acids are now known to be competitive inhibitors of various UGT isoforms, such as UGT2B7 and UGT1A9 [117,118]. As such, compounds such as lamotrigine and zidovudine that are glucuronidated by

UGT2B7 have shown a significant increase in intrinsic clearance rates when 2% bovine serum albumin was included in HLM incubations [119,120]. The increase in intrinsic clearance has been attributed to a decrease in the K_m values for each substrate. It is important to note that while bovine serum albumin and fatty-acid-free human serum albumin are able to bind unsaturated fatty acids and thus decrease their inhibition potential of UGT isoforms, crude human serum albumin cannot. This is most likely due to the fact that the fatty acid binding sites of crude human serum albumin are already saturated [117].

Regardless of the system used to study glucuronidation, the ultimate purpose is usually to predict *in vivo* glucuronidation. *In vivo* clearance can be scaled with a wide variety of success, depending on the system used. Several groups have noted a systematic underprediction, often 10-fold or greater magnitude of underprediction, when using liver microsomal preparations in order to scale-up to *in vivo* clearance [121–124]. Recent data has shown that the inclusion of albumin will greatly improve the predictability of *in vivo* clearance [117]. As previously mentioned, a mechanism for underprediction was shown to be the presence of natural but inhibitory fatty acids present in microsomal preparations and released during the incubations. These fatty acids are good ligands and are therefore inhibitory for some isoforms, and albumin acts to sequester these inhibitory factors away from the active site, which allows an increase in the affinity (K_m) and thus Cl_{int} of the process [118]. Similar effects on affinity and extrapolation accuracy have been observed when attempting to predict inhibitory drug–drug interactions [119].

Another possible factor possibly affecting extrapolation accuracy is the buffer type that affects the kinetics of glucuronidation. A change in enzyme kinetics was shown when buffer was changed from tris or phosphate to carbonate buffer, and this has been shown to greatly improve the *in vivo* prediction of clearance [122]. Interestingly, much better success has been obtained in general when scaling clearance from a hepatocyte system [124]. This now gives perspective to the affinity of ligands for the UGTs in general; the statement made in the past of these being just low affinity enzymes needs to be revisited [5] because clearly the use of a more physiologically relevant system (hepatocytes) or alteration of *in vitro* conditions (albumin) can greatly increase the observed affinity to likely improve the relevance of kinetic parameters such as K_m , IC_{50} , or clearance.

15.4.2 UGT Protein–Protein Interactions

As more examples of CYP–CYP protein–protein interactions have been observed to significantly alter their activities [125,126], it has become important to consider these phenomena with regard to the UGTs. Mounting evidence now suggests that UGTs may readily function as homo- and heterodimers with regard to glucuronide formation (Fig. 15.2). Indeed, coexpression studies, immunoprecipitation studies, and others performed to investigate oligomerizations involving UGTs and elucidate their effects have identified not only significant effects of UGT–UGT interactions but also significant UGT–CYP interactions [127]. For example, a soluble form of UGT1A9 was shown to form a hetero-oligomer with UGT1A4, and this interaction significantly altered the metabolite ratio for entacapone and scopoletin. In coexpression studies, CYP3A4 was shown to alter the kinetics and regioselectivity of UGT2B7-mediated morphine glucuronidation, while other CYP isoforms had little to no effect. Follow-up experiments

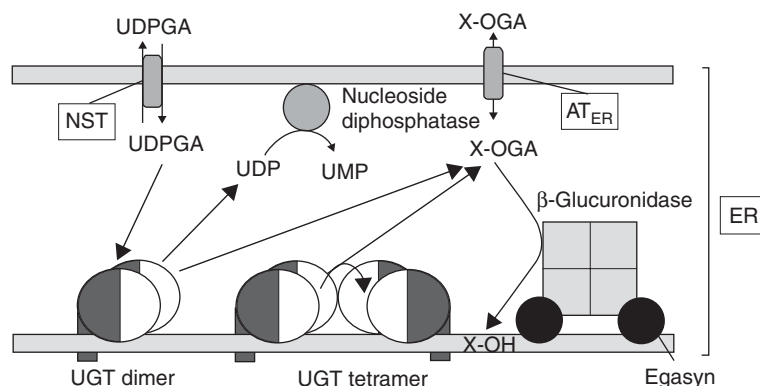


Figure 15.2 Functional characterization of UDP-glucuronosyltransferase protein–protein interactions within the endoplasmic reticulum. NST, nucleotide sugar transporter; AT_{ER} , organic anion transporters located within the endoplasmic reticulum; UDP, uridine diphosphate; UMP, uridine monophosphate; X-OH, aglycone; X-OGA, glucuronidated compound. *Source:* Reprinted from Bock, Köhle. *Biochem Pharmacol* 2009;77(9):1458–1465, with permission from Elsevier.

(coimmunoprecipitation studies, overlay assay, etc.) confirmed the specificity of this interaction. FRET (fluorescence resonance energy transfer) experiments using UGT1As tagged with either yellow or green fluorescent proteins showed that homo- and hetero-oligomers were very common occurrences between UGT proteins and that the extent of interaction varied widely among the isoforms [128]. For example, UGT1A7 appeared to homodimerize the most, and the UGT1A1/1A3 and UGT1A1/1A7 hetero-oligomer also showed a strong ability to FRET, among the isoforms tested. When assessed, coexpression had a significant effect on activity. Further work needs to be done to better understand this and classify it as a curiosity, a complexity, or a concern.

15.4.3 β -Glucuronidase

Human β -glucuronidase catalyzes the hydrolysis of glucuronide metabolites leading to what has often been referred to as *a futile cycle of glucuronidation and hydrolysis*. The enzyme has been identified in most mammalian tissues, with high levels of activity in the liver, kidney, spleen, and gastrointestinal tract [129–132]. It is normally located in lysosomal fractions within these tissues, with plasma levels of the enzyme being relatively low [133]. It should be noted that differences such as pH optimum and substrate selectivity do exist between human β -glucuronidase and *Escherichia coli* β -glucuronidase [134]. *In vitro*, the presence of β -glucuronidase in HLMs and recombinant UGT membranes has been shown to result in an underestimation of rates of glucuronidation [135–137]. As such, multiple inhibitors of β -glucuronidase have been evaluated, with the most widely used being D-saccharic acid 1,4-lactone [129,131]. Additional inhibitors include polymeric phosphates of benzesterol, diene-strol, diethystibesterol, diene-strol, and hexesterol, as well as heavy metals such as Ag^{2+} , Cu^{2+} , and Hg^{2+} [131]. The use of a β -glucuronidase inhibitor *in vitro* may be useful in obtaining kinetic parameters and intrinsic activities for UGT enzymes without introducing the competing hydrolysis pathway. However, in the case where one wishes

to extrapolate *in vitro* values to *in vivo* values, it may be most appropriate to measure the net glucuronide formation rates, taking into account the physiological relevant processes of both glucuronidation and hydrolysis [138].

15.5 ANALYSIS OF GLUCURONIDES

Special consideration must often be given to the analysis and quantitation of glucuronide metabolites. Factors such as temperature and pH stability, hydrolysis back to parent, acyl migration (for ester-linked acyl glucuronides), and varying degrees of lability toward analytical conditions such as mass spectrometer source environments should all be accounted for.

The glucuronidation of a carboxylic acid group results in the formation of an acyl glucuronide and can present a complicated scenario from both an analytical as well as a toxicological standpoint. Acyl glucuronides can undergo acyl migration, in which the acyl group relocates from the C-1 carbon to the C-2, C-3, or C-4 position of the glucuronic acid moiety [139]. Acyl migration occurs through a nucleophilic attack by an adjacent hydroxyl group on the acyl carbon, resulting in an intramolecular transesterification [140,141]. The result is β -glucuronidase-resistant glucuronides and compounds with the potential to covalently and irreversibly bind to proteins, a phenomenon commonly associated with drug toxicity (Fig. 15.3). Common drugs that form acyl glucuronides include zomepirac, diclofenac, mycophenolic acid, and ibuprofen [142–145]. Analytically, migration (to the C-2, C-3, and C-4 isomers) and hydrolysis (back to parent drug) can both result in the underestimation of the amount of glucuronide present, while the latter can also cause aglycone concentrations to be overestimated. General techniques for reducing both migration and hydrolysis include acidifying the samples (pH $\sim$ 2–4) with citric or phosphoric acid, cooling the samples on ice, and storing samples at -20°C until analysis [146,147]. Furthermore, although many mass spectrometry techniques are able to detect the presence of glucuronide metabolites, the choice of more mild conditions such as electrospray ionization (as compared to atmospheric pressure chemical ionization) can also reduce the amount of glucuronide converted back to parent [148].

The use of nuclear magnetic resonance (NMR) to study and characterize acyl glucuronides has advanced in recent years. Proton NMR and two-dimensional TOCSY (*total correlation spectroscopy*) techniques have been used to assign chemical shifts to each of the protons from the eight individual acyl migration species [149,150]. In addition, the unique coupling pattern, chemical shift, and coupling constant (doublet; $\delta \sim 4.9\text{--}6.0$ ppm; $J \sim 6\text{--}9$ Hz) of the C-1 anomeric proton have proven very useful in monitoring and estimating degradation rates for β -1-*O*-acyl glucuronides [151,152]. Though not a general rule, it has been proposed that the rate of degradation can be indicative of the inherent reactivity of the acyl glucuronide and as such, the potential to cause a toxicological response [153,154].

The formation of quaternary-ammonium-linked glucuronides is another scenario that may call for specialized sample preparation and analytical conditions. While the glucuronides of primary and secondary amines have been shown to readily undergo hydrolysis under acidic conditions, N^+ -glucuronides are relatively stable at low pH and degrade at higher pH levels (pH > 10) [155,156]. Mass spectrometric analysis of N^+ -glucuronides can be complicated by the thermolability of the compounds, and it

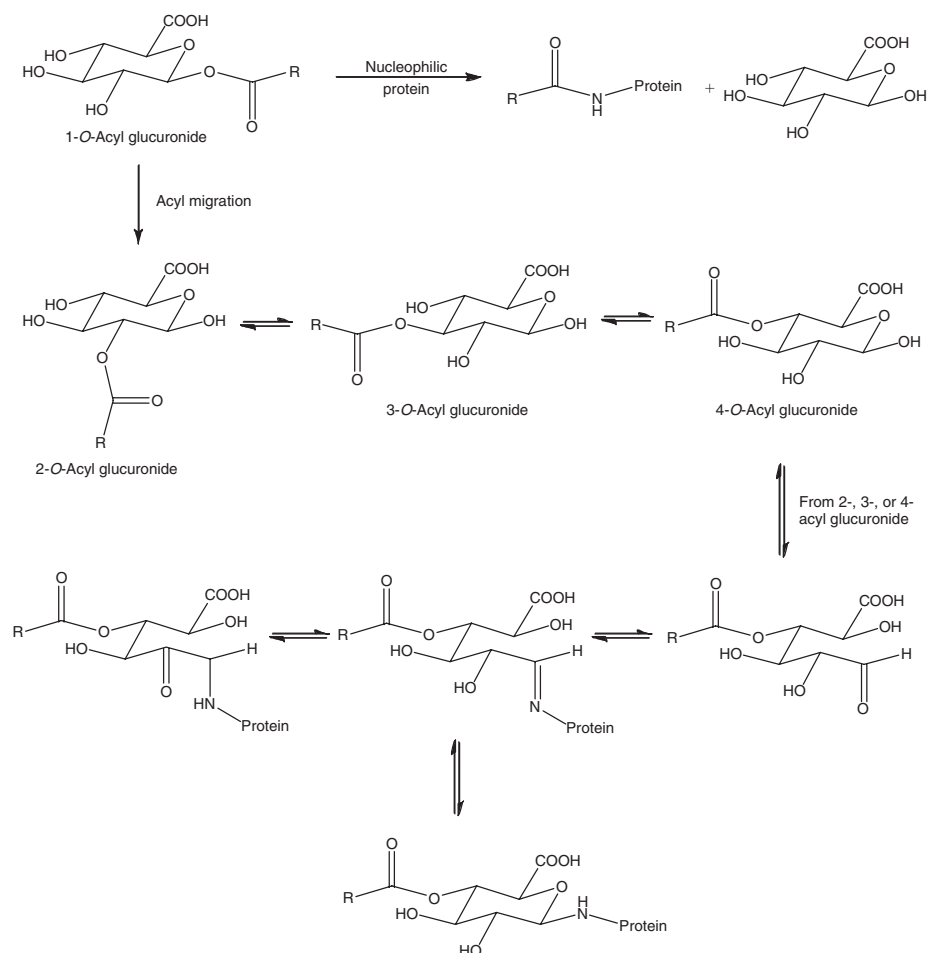


Figure 15.3 Reactivity and rearrangement of acyl glucuronides.

should be noted that owing to the permanent positive charge of the quaternary nitrogen, detection of these compounds occurs at a mass to charge ratio that corresponds to the M^+ ion and not the normally observed $M + H$ ion [14].

15.6 INDIVIDUAL UGT ISOFORMS

15.6.1 UGT1A1

15.6.1.1 Expression, Regulation, and Polymorphisms of UGT1A1. UGT1A1 has garnered a significant amount of attention in light of its role in the metabolism of bilirubin as well as the observed effects of its functionally relevant polymorphisms. The enzyme is primarily expressed in the liver and small intestine, though UGT1A1 mRNA has also been observed in the colon, bile ducts, bladder, and stomach [18,157].

There is evidence to support the fact that UGT1A1 exists and functions as a multimeric enzyme [158,159].

As with many of the UGT isoforms that are discussed in this chapter, UGT1A1 is inducible through the activation of various nuclear receptors. UGT1A1 expression is mediated by the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the aryl hydrocarbon receptor (AhR), with PXR effects potentially reaching clinical significance [160–162]. Both the PXR and CAR mechanisms of UGT1A1 induction may be synergistically influenced by activation of the glucocorticoid receptor [163]. Furthermore, oncostatin M (a member of the IL (interleukin)-6 cytokine family) may also increase UGT1A1 induction via the CAR, through cross talk between CAR and the oncostatin M receptor [164]. Finally, UGT1A1 expression has been shown to be upregulated by the HNF-1 α and upstream signaling factor 1/2 (USF1/2) while being downregulated by DNA methylation in colon cancer cells [165].

A major focus of UGT1A1 research has involved the pharmacogenetics of the enzyme and the clinically relevant outcomes attributed to its various polymorphisms. To date, over 100 polymorphic alleles of UGT1A1 have been reported in the literature [99]. Much of the interest surrounding UGT1A1 polymorphisms stems from the role of UGT1A1 polymorphisms in Crigler–Najjar (type I or II) and Gilbert’s syndromes, which result in hyperbilirubinemia, and in the observed toxicity profiles following irinotecan administration. The more common Gilbert’s syndrome is the result of a TA insert in the promoter region of the enzyme (UGT1A1*28, (TA)₇(TAA)) and variations in the exon 1 sequence of UGT1A1 (UGT1A1*6, G71R; UGT1A1*27, P229Q) [42,166,167]. Multiple other polymorphisms are also associated with the Gilbert’s syndrome phenotype, although it is believed that the UGT1A1*28 polymorphism is responsible for the majority of the observed variability in UGT1A1 activity [168]. Crigler–Najjar type II is associated with an additional TA insert in the promoter region (UGT1A1*37, (TA)₈(TAA)) and also results in a significant reduction in UGT1A1 expression levels [66]. The relatively rare Crigler–Najjar type I syndrome has been attributed to multiple UGT1A1 polymorphisms including UGT1A1*2 (frameshift), UGT1A1*3 (S375F), UGT1A1*4 (Q357X), and UGT1A1*5 (Q331X), and results in an absent or inactive form of the enzyme [58,169,170]. Patients with Crigler–Najjar type I syndrome generally require liver transplant [171]. A more comprehensive list of UGT1A1 polymorphisms can be found in Table 15.2.

15.6.1.2 UGT1A1 Substrates. UGT1A1 is primarily implicated in the glucuronidation of endogenous bilirubin as well as various catechols, estrogens, and flavanoids [19]. Exogenous probe substrates for the isoform include estradiol (3-OH-glucuronidation) and 17 α -ethynylestradiol [19,20]. A significant amount of research has also focused on the role of UGT1A1 in the metabolism of the topoisomerase inhibitors irinotecan, topotecan, and etoposide, as well as SN-38, the active metabolite of irinotecan [172–175]. The ability of UGT1A1 to catalyze the formation of *S*-glucuronides has been demonstrated in HLMs with the K_{ATP}-blocking agent HMR1098 [176]. Additional substrates include acetaminophen, anthraflavic acid, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), buprenorphine, morphine, and naltrexone [177–181]. A list of compounds that are glucuronidated by UGT1A1 can be found in Table 15.3.

TABLE 15.3 Substrates Glucuronidated by UGT1A1

Drug	Type of Glucuronide	References
Acetaminophen	O	177
Anthraflavic acid	O	180
Bilirubin	Acyl	19
Buprenorphine	O	19
Carvedilol	O	182
Desmethylnaproxen	O, acyl	183
Doxorubicin	O	184
Entacapone	O	29
Estradiol	O	19
Estrone	O	185
Etoposide	O	173
17 α -Ethinylestradiol	O	20
Ezetimibe	O	186
HMR1098	S	176
Hydroxytamoxifen	O	187
Morphine	O	179
Mycophenolic acid	Acyl	145
Nalorphine	O	178
Naltrexone	O	178
Naringenin	O	19
PhIP	N	188
Quercetin	O	19
Raloxifene	O	189
Retigabine	N	190
SN-38	O	71
Thiocoraline	O	191
Topotecan	O	175
Troglitazone	O	192

15.6.1.3 Inhibition of UGT1A1. A number of clinically relevant as well as endogenous compounds have been identified as inhibitors of UGT1A1. Sulfinpyrazone ($IC_{50} = 46 \mu M$), androsterone ($IC_{50} = 125 \mu M$), and phenylbutazone ($IC_{50} = 294 \mu M$) are all inhibitors of recombinant UGT1A1 [24]. The HIV-protease inhibitors atazanavir and indinavir are also reported to inhibit UGT1A1 activity through a linear-mixed inhibition mechanism in HLMs with K_i values of 1.9 and $47.9 \mu M$, respectively [193]. It has also been observed that ketoconazole, a potent inhibitor of CYP3A4, is also a competitive inhibitor of UGT1A1-catalyzed SN-38 glucuronidation with a K_i of $3.3 \mu M$ [194]. Finally, Williams *et al.* [181] report inhibition of UGT1A1 activity by the natural products flavone, chrysin, quercetin, nobiletin, tangeretin, and bilirubin.

15.6.1.4 Active Site Characterization. It has been suggested that the active site of UGT1A1 is capable of producing complex substrate and inhibition kinetics and may consist of multiple aglycone binding sites [181,185,195]. Indeed, multiple examples of homotropic and heterotropic activation in UGT1A1-catalyzed reactions have been reported. Within the active site of UGT1A1, a histidine residue at position 39 is believed

to be the conserved histidine acting as a catalytic base in the S_N2 glucuronidation reaction [196]. Interestingly, mutation of H39 to a proline residue retained UGT1A1 activity toward estrogen-like compounds while losing the ability to glucuronidate phenolic moieties. A conserved aspartic acid residue (D151) is believed to play an important role in UDPGA binding, and as such, any mutations to this amino acid result in an inactive enzyme. Site-directed mutagenesis studies also indicate the requirement for a phenylalanine residue at position 170, most likely due to the hydrophobicity and aromaticity of the residue [197]. Computer modeling studies aimed at elucidating the local protein structure in this region of UGT1A1 also revealed an important hydrophobic region from residues 159–177 (IVAQYLSLPTVFFLHALPC) as well as a buried helix with a center around amino acid positions 169–172 of the enzyme. In addition, the presence of multiple free thiol groups from six cysteine residues within the intracisternal domain of UGT1A1 appear to be essential for enzymatic activity, although most likely not through the formation of disulfide linkers [198]. The three cysteine residues that are located within the cytosolic tail of UGT1A1 (C509, C510, and C517) are thought to be involved in the internalization of UDPGA. Finally, recent studies indicate that D36, S38, and L41 are located within the van der Waals distance to UDPGA, while either Q84, D86, K115, and I116 or K115, I116, A174, V193, and D224 have significant interactions with bilirubin, depending on the binding orientation of the substrate [199].

15.6.1.5 Clinical Relevance. Much of the clinical relevance of UGT1A1 is centered on the functional relevance of the Crigler–Najjar and Gilbert’s syndrome phenotypes. As mentioned previously, patients diagnosed with hyperbilirubinemia due to Crigler–Najjar type I syndrome often require liver transplant, whereas those with type II or Gilbert’s syndrome can be treated with UGT1A1 inducers or may even be asymptomatic [171]. Changes in the pharmacokinetic and toxicokinetic profiles of clinically administered drugs have also been attributed to variations in UGT1A1 activity. Most notable is the glucuronidation of SN-38, the active metabolite of irinotecan, by UGT1A1. The hepatic glucuronidation rates of SN-38 have been shown to highly correlate with a patient’s UGT1A1 genotype [200–203]. As such, a decreased rate of SN-38 glucuronidation due to the UGT1A1 genotype is often associated with an increased risk of neutropenia and diarrhea in patients on irinotecan therapy. While the altered clearance of SN-38 is often attributed to UGT1A1*28, the risk of irinotecan toxicity has also been associated with UGT1A1*6 [204,205]. A number of excellent reviews on the clinical manifestations of UGT1A1 genotypes on irinotecan therapy have been published [200,201,206,207]. Finally, recent data suggests that UGT1A1 genotypes may also be involved in the risk of endometrial cancer and that the significance of UGT1A1 in determining this risk is also dependent on endogenous estrogen levels as well as soy intake [208].

15.6.2 UGT1A3

15.6.2.1 Expression, Regulation, and Polymorphisms of UGT1A3. UGT1A3 is primarily expressed in the liver, with additional sites of expression including intestinal, gastric, and biliary tissues. Small amounts of UGT1A3 mRNA have also been found in the bladder [157].

Multiple mechanisms appear to play a role in the regulation of UGT1A3. The requirement of an HNF-1 α binding element on the UGT1A3 gene for UGT1A3 activity

in vitro, coupled with an increase in enzyme activity when HEK293 kidney cells were coexpressed in the presence of HNF-1 α , implicates this pathway in the transcriptional regulation of UGT1A3 [209]. In addition to HNF-1 α , UGT1A3 is also transcriptionally regulated through a xenobiotic response element as well as AhR [210]. Induction studies in human hepatocytes have also identified the oxysterol-activated nuclear receptor liver X receptor α (LXR α) as a regulator of the UGT1A3 gene [211]. In addition, the peroxisome-proliferator-activated receptor (PPAR) and PXR are involved in the regulation of UGT1A3 [22,212]. Finally, a recent study by Erichsen *et al.* [213] demonstrates transcriptional regulation of UGT1A3 by chenodeoxycholic acid and the farnesoid X receptor (FXR), suggesting a potential feedback mechanism in cholestasis.

Over 20 UGT1A3 polymorphic alleles have been identified that may play a role in drug metabolism as well as estrogen-sensitive disease states. At least five of these alleles result in synonymous amino acid sequences [73]. UGT1A3*2 (W11R/V47A) results in nearly a fourfold increase in estrone clearance *in vitro* as compared to the wild-type enzyme [214]. UGT1A3*3 (W11R) exhibits activity similar to UGT1A3*1, while UGT1A3*4 (R45W), UGT1A3*6 (W11R/V47A/M270V), and UGT1A3*8 (A158V) displayed estrone glucuronidation capacities that were at least 10-fold less than the capacity of the wild-type enzyme [73]. UGT1A3*5 (Q6R/W11R), UGT1A3*7 (F110I), UGT1A3*9 (W11R/M208L), UGT1A3*10 (V47A), and UGT1A3*11 (W11R/V47A/M114I) all displayed clearance values that were moderately lower than the value of UGT1A3*1.

15.6.2.2 UGT1A3 Substrates. While UGT1A3 (together with UGT1A4) catalyzes the glucuronidation of many tertiary amines and aromatic heterocycles resulting in quaternary ammonium glucuronides, it can also glucuronidate flavanoids, nonsteroidal anti-inflammatory drugs (NSAIDs), statins, bile acids, estrogens, and structural analogs of vitamin D [21,213–216]. More specifically, substrates include estrone and 2-hydroxyestrone, benzo[α]pyrene metabolites, 4-aminobiphenyl, 2-aminofluorene, cyproheptadine, norbuprenorphine, 4-methylumbelliferone, scopoletin, naringenin, alizarin, and 4-nitrophenol [216,217]. Phenotyping studies have revealed that glucuronidation of fulvestrant and F $_6$ -1 α ,23S,25-trihydroxyvitamin-D $_3$ appear to be relatively selective for UGT1A3 [21,23]. A more complete list of compounds glucuronidated by expressed UGT1A3 can be found in the study by Green *et al.* [217].

15.6.2.3 Inhibition of UGT1A3. A number of compounds have been recently screened for inhibition of UGT1A3-catalyzed buprenorphine and norbuprenorphine glucuronidation [218]. Both substrates were able to inhibit the other's glucuronidation with similar IC $_{50}$ values (40–50 μ M), while norbuprenorphine was also inhibited in expressed UGT1A3 by amitriptyline and temazepam. Buprenorphine has also been reported to significantly inhibit bopiramine-O-glucuronidation by UGT1A3, while diclofenac appears to be a weak inhibitor of UGT1A3 [219,220].

15.6.2.4 Active Site Characterization. Using chimera-based approaches, Kubota *et al.* [221] have shown that isoleucine 36 and histidine 40 are crucial for conveying the substrate specificity of UGT1A3 toward tertiary amines and planar phenolic compounds. It can be assumed that H40 also plays an important role as a catalytic base in the S $_N$ 2 glucuronidation reaction by UGT1A3 [41].

15.6.2.5 Clinical Relevance. *In vivo* evidence exists that describes a potential role for UGT1A3 in the outcome of patients on statin therapy. UGT1A3*2 (W11R/V47A), an allele with a frequency of 0.125 in a Japanese population and an increased glucuronidation activity as compared to wild-type UGT1A3, has been shown to increase the lactonization of atorvastatin both *in vitro* and *in vivo* [222]. As the statin lactones are known to be cleared more rapidly than the statins themselves, an increased rate of lactonization could potentially result in failure of statin therapy as well as potential adverse events. UGT1A3 may also be involved in the promotion and regulation of various disease states. For example, breast density, a potential biomarker of breast cancer risk, has been shown to be slightly lower in women who have at least one copy of the UGT1A1(TA₇)-UGT1A3(R11/A47) haplotype [223].

15.6.3 UGT1A4

15.6.3.1 Expression, Regulation, and Polymorphisms of UGT1A4. The importance of UGT1A4 is often attributed to the isoform's ability to catalyze the formation of quaternary ammonium glucuronide metabolites. The cDNA of UGT1A4 has been cloned and characterized, and its major sites of expression include the liver and the stomach [50,224]. Additional sites of expression for UGT1A4 are the bile ducts, gastrointestinal tract, and colon, while Nakamura *et al.* report that UGT1A4 mRNA is detectable in all the aforementioned tissues as well as in lung, kidney, bladder, adrenal glands, ovaries, uterus, and testis [157,225,226]. A statistically significant decrease in UGT1A4 mRNA levels has been observed in patients in an exacerbated inflammatory state [227].

As with many of the UGT isoforms, the involvement of nuclear receptors has been reported for the regulation of UGT1A4 expression. UGT1A4 induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) demonstrates a role for AhR in the regulation of UGT1A4, although mutation of the promoter region of UGT1A4 containing two putative xenobiotic response elements resulted in an attenuation of the observed induction [228]. The results indicate a potential dynamic interplay between the xenobiotic response elements, AhR, and UGT1A4 promoter region polymorphisms in the regulation of UGT1A4. More recently, studies in transgenic mice have identified CAR, PXR, and PPAR- α as regulators of UGT1A4 expression [229]. Finally, involvement of the estrogen receptor and specificity protein-1 have been reported in the regulation of UGT1A4 during pregnancy [230].

UGT1A4*2 (P24T) and UGT1A4*3 (L48V) are two commonly occurring polymorphic alleles of UGT1A4. The two polymorphisms have been found in 8% and 9% of German Caucasians, respectively [231]. The occurrence of UGT1A4*3 in a Japanese population has been reported to be as high as 12.5% [74]. The effect of the UGT1A4*2 polymorphism appears to be substrate dependent, while the UGT1A4*3 protein generally leads to a decreased activity compared to the wild-type UGT1A4 [231,232]. Additional polymorphisms include UGT1A4*4 (R11W), UGT1A4*5 (43fsX22), UGT1A4*6 (59fsX6), UGT1A4*7 (L48V/R91C/L150L/P268P), and UGT1A4*8, which result in a splicing defect [74]. The combined frequency of UGT1A4*4 through UGT1A4*8 was ~2% in a Japanese population.

15.6.3.2 UGT1A4 Substrates. UGT1A4 is primarily regarded for its ability to glucuronidate primary, secondary, tertiary, and aromatic amines, although it also

conjugates androgens, steroids, and progestins [224,233,234]. Substrates include the anticonvulsant lamotrigine, the antipsychotic clozapine, and compounds such as imipramine, trifluoperazine, 4-hydroxytamoxifen, senecionine, 1-substituted imidazoles, nicotine, cotinine, and the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [232,233,235–239]. Trifluoperazine and imipramine are often considered to be selective probes for UGT1A4 activity *in vitro* [24,25].

15.6.3.3 Inhibition of UGT1A4. At relatively low concentrations, hecogenin has been characterized as a selective inhibitor of UGT1A4, with an IC_{50} of 1.5 μ M [24]. Valproic acid, lamotrigine, and androsterone have also been noted to be inhibitors of UGT1A4 activity [24,119,240].

15.6.3.4 Active Site Characterization. In contrast to most of the other UGT isoforms, UGT1A4 does not maintain the conserved histidine residue within the active site of the enzyme [221]. This may explain the preference of the isoform for glucuronidating tertiary amines, a reaction that does not require deprotonation of the aglycone by a basic amino acid residue. The mechanism of O-glucuronidation by UGT1A4 is currently unknown. The lack of the histidine residue (replaced by a proline at position 40) and the presence of a threonine residue at position 36 may convey substrate selectivity for UGT1A4 versus UGT1A3 [221]. QSAR (quantitative structure–activity relationship) modeling has identified the importance of hydrophobic regions and hydrogen bonding with higher affinity UGT1A4 substrates [241]. In addition, the active site of UGT1A4 also appears to be able to accommodate complex or atypical kinetic processes. Studies assessing the interactions of dihydrotestosterone, *trans*-androsterone, tamoxifen, and lamotrigine suggest the presence of multiple aglycone binding sites that may allow for homotropic and heterotropic atypical kinetics with UGT1A4 [242,243].

15.6.3.5 Clinical Relevance. Oral clearance values for the anticonvulsant lamotrigine are known to increase by as much as 50–90% in pregnant women [244–246]. The involvement of the estrogen receptor in the upregulation of UGT1A4 may indicate that the enzyme plays an important role in the clearance of lamotrigine *in vivo* [230]. In pediatric studies, UGT1A4 enzyme activity is believed to reach its maximum levels by 1.4 years of age [247]. Potential drug interactions involving UGT1A4 have been noted for lamotrigine and olanzapine, with the former causing a modest increase in mean plasma concentrations of olanzapine [248]. Conversely, combination treatment with atazanavir/ritonavir in healthy men resulted in a slight decrease in lamotrigine exposure, most likely caused by induction of UGT1A4 by ritonavir [249]. Effects of UGT1A4 polymorphisms in clinical trials have been varied. Interestingly, the presence of a 142T>G single nucleotide polymorphism (coding for UGT1A4*3) resulted in a decreased olanzapine exposure *in vivo* [250]. This would seemingly contradict *in vitro* studies that indicate UGT1A4*3 has a decreased glucuronidation capacity, although it is entirely possible that the effects are substrate dependent. Adding to this conclusion is the observation that intrinsic clearance values for clozapine in patients carrying the UGT1A4*3 allele were twice as high as those values observed in wild-type patients [251].

15.6.4 UGT1A5

UGT1A5, previously thought to be a nonfunctional UGT due to its lack of detectable expression, was identified in human hepatocytes, Caco-2, and HepG2 cells by Finel *et al.* in 2005 [252]. Expression of the isoform was also detected in hepatic and intestinal tissue samples, with a high degree of variability in the intestine. UGT1A5 mRNA has also been reported in the kidney, bladder, and uterus [157]. Finel *et al.* also demonstrated that UGT1A5 was inducible by rifampicin and 3-methylcholanthrene, implicating a role for PXR in the regulation of UGT1A5. Known substrates for UGT1A5 include 1-hydroxypyrene, zolarsartan, SN-38, 4-methylumbelliferone, and scopoletin; however, glucuronidation rates for the last three substrates are very low [252–254].

15.6.5 UGT1A6

15.6.5.1 Expression, Regulation, and Polymorphisms of UGT1A6. UGT1A6 is a polymorphically expressed enzyme that is found in the liver, stomach, gastrointestinal tract, kidneys, bile ducts, and brain [18]. UGT1A6 mRNA has also been detected in the bladder [157]. Interestingly, while most UGT isoforms are located in the endoplasmic reticulum, UGT1A6 (together with UGT2B7) is expressed in the nucleus of HEK293 cells, potentially implicating UGT1A6 in a protective or homeostatic role in the nucleus [255].

Regulation of UGT1A6 is complex and involves multiple pathways, which result in a high degree of interindividual variability in UGT1A6 expression and activity levels. UGT1A6 is regulated by nuclear receptors such as the AhR, CAR, PXR, nuclear factor E2-related factor 2 (Nrf2), and peroxisome PPAR- α [256–261]. HNF-1 α and 4 α are also thought to be involved in UGT1A6 regulation [262]. Additional factors that may play a role in upregulating UGT1A6 expression include oxidative stress levels, vitamin A, and phosphorylation by protein kinase C, while exposure to lipopolysaccharide has been shown to result in a significant downregulation of UGT1A6 mRNA, protein, and activity [258,263–265].

Expression of UGT1A6 is highly polymorphic with at least 20 haplotypes being identified, which code for 9 UGT1A6 variants. Polymorphisms include UGT1A6*2 (S7A/T181A/R184S), UGT1A6*3 (S7A), UGT1A6*4 (S7A/R184S), UGT1A6*5 (T181A), UGT1A6*6 (S7A/S103X/T181A/R184S), UGT1A6*7 (S7A/R90H/T181A/R184S), UGT1A6*8 (T181A/R184S), and UGT1A6*9 (R184S) [75–77, 266,267]. UGT1A6*2 has been reported to occur in ~20–30% of the Caucasian and Japanese populations, while the other alleles appear to be less common and occur with frequencies of <10% each [76,266]. Compared to wild-type UGT1A6, the *2 allele is generally thought to exhibit higher rates of glucuronidation, while the *8 allele may result in a substrate-dependent alteration of glucuronidation activity [77,266,267]. It appears that many of the UGT1A6 polymorphic alleles exist in linkage disequilibrium with each other and potentially with variants of other UGT isoforms such as UGT1A1*28 [268].

15.6.5.2 UGT1A6 Substrates. UGT1A6 is generally thought to catalyze the glucuronidation of planar phenolic compounds, although the enzyme has the ability to accommodate various other structural classes as well. Substrates

for UGT1A6 include acetaminophen, polycyclic aromatic hydrocarbons, carboxylic acids such as valproic acid, and the indole-containing compounds 5-hydroxytryptophol and serotonin, which appear to be highly selective for UGT1A6 [18,26,41,75,269,270]. UGT1A6 has also been reported to be responsible for the metabolism of the anesthetic drug propofol in the human brain [271]. Additional substrates include deferiprone, *cis*- and *trans*-resveratrol, 1-naphthol, 3,4-dihydroxybenzaldehyde, 4-methylumbelliferone, 4-nitrophenol, daphnetin, and 1-hydroxypyrene [272–278].

15.6.5.3 Inhibition of UGT1A6. A number of compounds have been characterized as inhibitors of UGT1A6, including those found in natural products and food additives. Rose bengal, phloxine, and erythrosine B, all components of chemical food dyes, were shown to inhibit UGT1A6 with IC_{50} values of 15, 40, and 50 μ M, respectively [279]. Bisphenol A, used in the manufacture of plastics and a known endocrine effector, also inhibits the glucuronidation of serotonin and 4-methylumbelliferone by UGT1A6 [280]. Finally, *in vitro* experiments have identified troglitazone as a mixed-type inhibitor of UGT1A6, with a K_i value of 20 μ M [281].

15.6.5.4 Active Site Characterization. As UGT1A6 is one of the more highly characterized UGT isoforms, a fair amount of information is available regarding the protein structure of the enzyme including assessments of the aglycone and cofactor binding sites. In regard to protein positioning and localization, D488 has been shown to be the terminal residue of the transmembrane domain of UGT1A6 (at the luminal interface) and is critical for UGT1A6 location and activity within the endoplasmic reticulum [196]. In addition, deletion of a 43-amino-acid stop-transfer sequence encompassing the C-terminal transmembrane domain and adjacent cytoplasmic tail resulted in a significantly decreased enzyme activity, most likely due to the role of the sequence in endoplasmic reticulum targeting and translocation [282]. An internal signal retention sequence at residues 140–240 is also involved in the translocation of UGT1A6 into the endoplasmic reticulum [283].

Catalytically, site-directed mutagenesis studies have implicated H38 and D150 in substrate specificity and catalysis, respectively [196]. Furthermore, H54, R52, and H485, while playing key roles in the structure and function of UGT1A6, are not essential for catalysis by UGT1A6 [284]. Conversely, H370 was found to be involved in UGT1A6 catalysis, while H361 is located in the UDPGA binding site. The structural integrity of the aglycone binding site in UGT1A6 is thought to be highly dependent on interactions of the thiol moiety of C129, with other amino acids in the active site [285]. Mutation of the cysteine residue to a valine residue resulted in complete loss of enzyme activity, although substitution with serine restored activity, indicating that the inferred amino acid interactions probably did not involve a disulfide linkage. Finally, a phenolic acid binding motif has been suggested for amino acids 76–81 (KIYPVP), which are located in the substrate-binding region of the enzyme and are similar to other motifs found in phenol-conjugating enzymes [286].

QSARs and pharmacophore modeling have provided insight into the molecular properties that confer favorable physicochemical characteristics for UGT1A6 catalysis. Analysis of para-substituted phenols revealed that smaller substituents at the para position resulted in higher K_m and V_{max} parameters [287]. A negative correlation between V_{max} and molecular volume was readily apparent. Pharmacophore modeling

suggested that the presence of an aromatic ring adjacent to the nucleophilic site of glucuronidation increased the frequency of catalysis by UGT1A6 [288].

15.6.5.5 Clinical Relevance. Genetic polymorphisms of UGT1A6 have been associated with altered pharmacokinetics and increased disease risk. A more rapid clearance of salicylic acid has been observed in female patients who were characterized as being homozygous for the UGT1A6*2 allele [289]. In addition, a more rapid rate of salicylic acid glucuronidation was suggested for healthy individuals who received 650 mg of aspirin [290]. In regard to disease treatment, the UGT1A6 genotype was associated with an altered effect of aspirin on colorectal adenoma, and functional variants of UGT1A6 have been associated with a decreased risk of colorectal adenoma recurrence, as compared to carriers with wild-type UGT1A6 [291,292]. Finally, linkage disequilibrium between UGT1A6 polymorphic alleles and UGT1A1*28 may result in simultaneous hyperbilirubinemia and hyperserotoninemia as well as a subpopulation of Gilbert's syndrome or Crigler–Najjar syndrome patients who are at greater risk for altered drug metabolism and developing malignant disease [293,294].

15.6.6 UGT1A7

15.6.6.1 Expression, Regulation, and Polymorphisms of UGT1A7. UGT1A7 is primarily an extrahepatic enzyme that plays an important role in detoxification processes and may be involved in the regulation of multiple disease states. The isoform is primarily expressed in the esophagus and gastric epithelium, though UGT1A7 mRNA has also been detected in the liver, kidneys, mouth, intestine, pancreas, ovary, spleen, and lungs [18,31,157,295,296].

UGT1A7 is regulated by HNF-1 α , though unlike other extrahepatic isoforms such as UGT1A8 and UGT1A10, there is no cooperative regulation from the caudal homeodomain transcription factor Cdx2 [295]. The enzyme has been shown to be inducible by compounds such as β -naphthoflavone, 3-methylcholanthrene, and oltipraz [297–301].

UGT1A7 has been reported to be a highly polymorphic enzyme with at least 14 alleles identified to date. The different alleles have varying activity levels, as compared to wild-type UGT1A7. UGT1A7*2 (N129K/R131K), UGT1A7*6 (E139D), and UGT1A7*7 (N129K/R131K/E139D) all retain relatively high glucuronidation activity relative to UGT1A7*1 [78,79]. In contrast, UGT1A7*3 (N129K/R131K/W208R), UGT1A7*4 (W208R), UGT1A7*5 (G115S), UGT1A7*8 (N129K/R131K/E139D/W208R), UGT1A7*9 (G115S/N129K/R131K), and UGT1A7*12 (W208R/R254X) have been shown to have a decreased capacity for glucuronidation as compared to the wild-type enzyme [78,79,81,302]. UGT1A7*10 (N129K/R131K/W208R), UGT1A7*11 (R131Q), UGT1A7*13 (N276K), and UGT1A7*14 (C141S) have not been fully characterized. UGT1A7*12 results from a functional TATA-box polymorphism and exists in linkage disequilibrium with UGT1A7*3 and UGT1A7*4. The polymorphic allele may also be associated with variants of UGT1A1 such as UGT1A1*28, resulting in a significantly decreased clearance of SN-38, an active metabolite of irinotecan [302].

15.6.6.2 UGT1A7 Substrates. The localization of UGT1A7 allows the enzyme to serve an important role in the detoxification of carcinogens, pollutants, and

xenobiotics. The enzyme exhibits a broad substrate specificity with the ability to accommodate mono- and polycyclic aromatic compounds, planar as well as nonplanar compounds and compounds with sterically bulky substituents [303]. Substrates for UGT1A7 include heterocyclic amines, benzo(α)pyrene metabolites, SN-38 (7-ethyl-10-hydroxycamptothecin; both lactone and carboxylate), bisphenol A, propofol, epicatechin, thyroxine and 1-hydroxypyrene, 17 β -estradiol [27,254,277,303–306].

15.6.6.3 Inhibition of UGT1A7. *In vitro* studies have characterized amitriptyline, canrenic acid, phenylbutazone, quinidine, and quinine as nonselective inhibitors of UGT1A7 [24]. Quinine and phenylbutazone both appeared to be noncompetitive inhibitors of UGT1A7 catalyzed 4-methylumbelliferone glucuronidation, with K_i values of 784 and 3.9 μ M, respectively.

15.6.6.4 Clinical Relevance. As UGT1A7 metabolizes many compounds with carcinogenic properties, links between the activity of UGT1A7 and various disease states have been proposed. UGT1A7 genotypes, in combination with UGT1A9, have been suggested to predict both response and toxicity in colorectal cancer patients on capecitabine and irinotecan combination therapy [307]. Similarly, low activity UGT1A7 alleles have been shown to have a reduced capacity for SN-38 glucuronidation and, as such, may play an important role in irinotecan-induced toxicities [79]. In addition, the use of both the UGT1A7 and UGT1A1 genotypes appears to be a better predictor of irinotecan toxicity than the occurrence of Gilbert's syndrome alone [308]. Other disease markers such as an increased risk of hepatocellular carcinoma in hepatitis B patients, the development and extent of liver cirrhosis and the susceptibility to polycyclic aromatic hydrocarbons induced toxicities have also been associated with UGT1A7 polymorphisms [309–312].

15.6.7 UGT1A8

15.6.7.1 Expression, Regulation, and Polymorphisms of UGT1A8. Human UGT1A8 is an extrahepatic glucuronosyltransferase that appears to be expressed strictly in the esophagus, jejunum, ileum, and colon [31,295,296,313]. A single report exists of UGT1A8 mRNA in the liver and kidney [314].

Regulation of UGT1A8 in the lower gastrointestinal tract occurs through the caudal homeodomain transcription factor, Cdx2, in tandem with HNF-1 α . Expression of UGT1A8 is upregulated in the presence of 3-methylcholanthrene and rifampicin [315].

A number of polymorphisms have been identified and characterized for UGT1A8, namely, UGT1A8*1b, UGT1A8*2, and UGT1A8*3 [83]. UGT1A8*1b encodes for a nucleotide change (765A>G) that does not result in any amino acid change (T255T). UGT1A8*2 (A173G) has similar activity to the wild-type enzyme, while UGT1A8*3 (C277Y) results in a significant reduction in the glucuronidation capacity of UGT1A8 [83,316]. The relative frequency of each allele is 0.551, 0.282, 0.145, and 0.022 for UGT1A8, UGT1A8*1b, UGT1A8*2, and UGT1A8*3, respectively. Additional polymorphic alleles that have been identified include UGT1A8*4 (A144V/A173G), UGT1A8*5 (A173G/T240A), UGT1A8*6 (S126G), UGT1A8*7 (A231T), UGT1A8*8 (S43L), and UGT1A8*9 (H53N/A173G). UGT1A8*4 exhibits an increase in the formation of mycophenolic acid relative to wild-type UGT1A8 (mainly due to a decrease

in the observed K_m value), while the remaining polymorphisms generally result in a decrease in activity [317].

15.6.7.2 UGT1A8 Substrates. UGT1A8 displays a relatively broad substrate specificity and has been shown to catalyze the glucuronidation of anthraquinones, catechol estrogens, coumarins, flavanoids, 17-hydroxyandrogens, opioids, and primary amines such as that found in 4-aminobiphenyl [313]. Similar to UGT1A7, UGT1A8 also shows significant activity toward metabolites of benzo[α]pyrene [303]. UGT1A8 is uniquely capable of catalyzing the formation of a diglucuronide metabolite of dihydrotestosterone [28]. It has also been reported that the intestinal glucuronidation of raloxifene and troglitazone may be due in part to UGT1A8 as well [189,192,318]. Additional substrates for UGT1A8 include emodin, valproic acid, tea catechins, mycophenolic acid, ciprofibrate, diflunisal, furosemide, 4-hydroxytamoxifen, and tolcapone [171,192,317,319,320].

15.6.7.3 Inhibition of UGT1A8. Few reports of UGT1A8 inhibition are available; however, the effects of emodin, propofol, and β -estradiol have been described [192,321]. Emodin inhibited the glucuronidation of troglitazone in human jejunal microsomes with an IC_{50} value of 15.6 μ M; however, the influence of UGT1A10 on this interaction must also be taken into consideration [192]. Experiments aimed at assessing the inhibitor potential of propofol and β -estradiol toward UGT1A8 resulted in weak inhibition ($\sim$ 50% at 500 μ M propofol) and enzyme activation, respectively [321].

15.6.7.4 Active Site Characterization. Examination of the amino acid residues that convey glucuronidation activity to UGT1A8 has been accomplished through site-directed mutagenesis studies and characterization of the multiple UGT1A8 variants. Substitution of a highly conserved cysteine (C277) or histidine (H53) residue results in a significant loss in UGT1A8 activity, demonstrating the importance of these two residues [317]. It has been suggested that C277 may be involved in disulfide formation that is required for UGT1A8 to achieve a certain conformation [83]. Interestingly, the fairly conservative change observed in UGT1A8*7 (A231T) also results in a large decrease in the ability of UGT1A8 to catalyze mycophenolic acid glucuronidation. From the substrate perspective, compounds that exhibit a high degree of planarity and have multiple aromatic ring systems or functional groups, which lie in the plane of the aromatic ring, appear to be efficiently metabolized by UGT1A8 [303].

15.6.8 UGT1A9

15.6.8.1 Expression, Regulation, and Polymorphisms of UGT1A9. UGT1A9 is primarily expressed in the kidneys, with significant amounts of the enzyme also found in the liver, colon, testis, and ovaries [226,322,323]. UGT1A9 is thought to be the most abundantly expressed UGT isoform in renal tissue and accounts for the extrahepatic metabolism of many xenobiotics [171].

Multiple response elements are believed to be responsible for the regulation of UGT1A9. The induction of UGT1A9 by PXR ligands such as rifampin, phenobarbital, and tetrachlorodibenzodioxin suggests a role for PXR in the increased expression of UGT1A9 [324,325]. In addition to PXR, both HNF-1 α and 4 α regulate the expression

of UGT1A9 [262,326,327]. Finally, treatment of human hepatocytes and macrophages with PPAR- α and γ activators such as rosiglitazone resulted in an increase in UGT1A9 expression, suggesting that UGT1A9 is also a target gene for these nuclear receptors [328].

A number of polymorphic alleles of UGT1A9 have been identified and characterized. UGT1A9*2 (C3Y), UGT1A9*3a and *3b (M33T), UGT1A9*4 (Y242X), and UGT1A9*5 (D256N) are fairly rare, with none of the individual alleles occurring in more than 4% of the population [79,85,231,329]. The UGT1A9*3 polymorphism has a functional effect that is substrate dependent, as demonstrated by a decreased capacity for SN-38 glucuronidation but no observed effect on flavopiridol glucuronidation [79]. UGT1A9*5 has only been identified in Asian populations and results in a decreased activity compared to the wild-type enzyme [85,329].

15.6.8.2 UGT1A9 Substrates. Typical substrates for UGT1A9 include sterically hindered phenols such as 2,6-diisopropylphenol (propofol), which is a commonly used probe substrate for the enzyme [20,330,331]. UGT1A9 also catalyzes the glucuronidation of flavanoids such as luteolin, quercetin, kaempferol, and isorhamnetin; the dihydroxycoumarin analog daphnetin; and the NSAID indomethacin [275,332,333]. A considerable amount of stereoselectivity has been observed for UGT1A9, as seen with higher glucuronidation rates for (*S*)-etodolac, (*S*)-propranolol, (*R*)-oxazepam, and (*S*)-5-(4'-hydroxyphenyl)-5-phenylhydantoin as compared to their respective enantiomers [34,334–336]. Interestingly, UGT1A9 also catalyzes the C-glucuronidation of sulfinpyrazone and phenylbutazone, relatively rare instances of glucuronidation occurring at a carbon atom [337,338]. Additional substrates for UGT1A9 include 1-hydroxypyrene, 4-hydroxytamoxifen, raloxifene, mycophenolic acid, valproic acid, and clofibric acid [171,277].

15.6.8.3 Inhibition of UGT1A9. A number of compounds have been characterized as inhibitors of UGT1A9 *in vitro*. Ketoconazole, an antifungal agent and common CYP inhibitor, has been shown to inhibit the glucuronidation of SN-38 in cDNA-expressed UGT1A9 [194]. The NSAIDs niflumic acid, diflunisal, diclofenac, and indomethacin are also potent inhibitors of UGT1A9 *in vitro*, with K_i values of 0.0275, 0.710, 53.3, and 69.9 μ M, respectively [339]. As propofol is a common substrate used for UGT1A9, it is not unexpected that structural analogs of propofol would have the potential to inhibit the enzyme, as seen with 2,5-diisopropylphenol and 2,5-dimethylphenol [340].

15.6.8.4 Active Site Characterization. Various studies have been undertaken to characterize the aglycone binding site and the overall protein structure of UGT1A9. Similar to other UGTs, UGT1A9 biological activity appears to be dependent on N-glycosylation during translation [341]. The isoform is unique within the UGT family in regard to its resistance to inactivation by detergents [115]. Truncation of UGT1A9 immediately upstream from the transmembrane helix resulted in an active and water-soluble protein and implicated the transmembrane helix in potential aglycone–protein interactions [342]. Site-directed mutagenesis studies have been used to further probe for the importance of specific amino acid residues in the UGT1A9 protein structure. While UGT1A8 and UGT1A9 share a very high amino acid similarity (93–95%), there have been observed differences in substrate selectivity and these differences may be due to Arg42 and Asn152 in UGT1A9 [343]. In addition, substitution of H37, a

conserved histidine found in most UGT isoforms, resulted in almost complete loss of O-glucuronidation activity by UGT1A9, while less of a reduction was seen for N-glucuronidation [40]. Conversely, replacing D143 with an alanine residue appeared to affect both routes of glucuronidation. The authors speculate that the conserved histidine is necessary to deprotonate an oxygen nucleophile before glucuronidation. The aspartic acid residues at D143 and D148 potentially serve to stabilize the positive charges found when nitrogen is the nucleophile or on H37 after deprotonation of an oxygen nucleophile. Interestingly, a recent study with chimeric UGTs has shown the amino acids in positions 69–132 of UGT1A9 play an important role in substrate selectivity and affinity of the enzyme for phenylbutazone and 7-hydroxy-4-(trifluoromethyl)coumarin [344]. Finally, a cysteine residue at position 183 has been shown to be necessary for UGT1A9 homodimerization, although lack of this residue was not enough to fully eliminate UGT1A9 activity [345].

15.6.8.5 Clinical Relevance. As with many other UGTs, the clinical significance of UGT1A9 may be primarily related to the functional variants of the isoform. For example, the intronic UGT1A9 polymorphism I399C > T (UGT1A9*1q) appears to result in an increased rate of glucuronidation for SN-38 [105,346]. A relationship may also exist between polymorphisms that lead to reduced UGT1A9 catalysis and the prevalence of catechol-*O*-methyltransferase inhibitor-induced asymptomatic hepatic dysfunction [347]. Finally, while clinical results vary, there are examples of the effects of UGT1A9 polymorphisms on the pharmacokinetics of mycophenolic acid as well as irinotecan-related toxicities [307,316,324,348,349].

15.6.9 UGT1A10

15.6.9.1 Expression, Regulation, and Polymorphisms of UGT1A10. Expression of UGT1A10 is mostly limited to the intestine, kidney, and lung [27,157,350–352]. Although the isoform is generally considered to be a strictly extrahepatic enzyme, reports do exist of its expression in primary human hepatocytes [315]. Unlike most UGTs, UGT1A10 has been shown to be localized in an extramicrosomal fraction rather than in the endoplasmic reticulum [353].

As with UGT1A8, UGT1A10 regulation is also a cooperative effort between the caudal homeodomain transcription factor, Cdx2, and HNF-1 α [354]. Experiments in Caco-2 cells support the hypothesis that the UGT1A10 promoter does not involve a TATA-box binding region but rather occurs through overlapping Sp1/initiator binding sites [355]. UGT1A10 appears to be inducible by both 3-methylcholanthrene and rifampicin [315]. Involvement of xenobiotic and antioxidant response elements has been reported for the regulation of UGT1A10, as observed by their induction through Nrf2 and AhR activation [356]. Finally, evidence exists for the control of UGT1A10 mRNA expression through the estrogen receptor, implying that UGT1A10 may represent a unique estrogen-regulated target gene in certain cell lines [357].

Twelve polymorphic alleles have been described for UGT1A10, and a number of the polymorphisms are the result of synonymous nucleotide substitutions and do not result in any changes in amino acid sequence of enzyme activity. UGT1A10*1b (Q42Q), UGT1A10*1c (H199H), and UGT1A10*1d (H199H/A231A) are all synonymous polymorphic substitutions [86]. Other UGT1A10 polymorphisms with either uncharacterized or unchanged enzyme activities include UGT1A10*2a (E139K),

UGT1A10*3a (T240M), UGT1A10*4a (L244I), UGT1A10*4b (A231A/L244I), UGT1A10*4c (H199H/A231A/L244I), and UGT1A10*5 (M59I) [86,87,358]. Two functionally relevant mutations, UGT1A10*6 (T202I) and UGT1A10*7 (I211T), result in a 50% decrease in enzyme activity and a nonfunctional enzyme, respectively [87,88,358].

15.6.9.2 UGT1A10 Substrates. UGT1A10, in conjunction with other extrahepatic isoforms, is known to glucuronidate anthraquinones, coumarins, various amines, estrogens flavanoids, and especially phenols with considerable steric bulk [18,88,351,359]. Dopamine has been characterized as a potentially useful *in vitro* probe of UGT1A10 activity [30]. The enzyme can exhibit a stereoselective preference in regard to aglycone conjugation, as is seen with the higher rate of (*R*)-propranolol glucuronidation than that of the (*S*)-enantiomer [360]. Additional substrates include mycophenolic acid, raloxifene, menediol, the hallucinogenic indole alkaloid psilocin, and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [361–365].

15.6.9.3 Inhibition of UGT1A10. Tacrolimus has been found to be an inhibitor of UGT1A10-catalyzed mycophenolic acid glucuronidation *in vitro* [365]. The reported K_i value for tacrolimus in kidney liver microsomes was 27.3 ng/mL. Both 6-hydroxywarfarin and 7-hydroxywarfarin were shown to be competitive inhibitors of UGT1A10; however, this was dependent on the UGT1A10 haplotype [366]. A series of bidentate inhibitors containing both a uridine and lipophilic moiety also demonstrated moderate inhibition of UGT1A10 [367].

15.6.9.4 Active Site Characterization. Site-directed mutagenesis studies, homology modeling, and assessment of the polymorphic UGT1A10 haplotypes have allowed for a thorough characterization of the amino acid structure necessary for UGT1A10 activity. Threonine 202 and isoleucine 211 have been determined to be essential for UGT1A10 to catalyze glucuronidation reactions, while the amino acids at positions 90–93 (phenylalanine-methionine-valine-phenylalanine) play an important role in substrate recognition [30,87,88,366,368]. It has been suggested that the neutral phenylalanine residues may orient substrate molecules through hydrophobic π -stacking interactions. However, overall UGT1A10 substrate selectivity is most likely conferred by interactions among multiple amino acid residues rather than one or two individual residues [369]. Regarding the binding of UDPGA by UGT1A10, an aspartic acid residue (D393) is thought to be critical in binding the cofactor [370]. Replacement of D393 with an alanine residue resulted in complete loss of UGT1A10 activity. Lysine residues at positions 314 and 404 of UGT1A10 are also required for UDPGA binding and have been shown to be conserved across most UGT isoforms [371].

15.6.9.5 Clinical Relevance. The UGT1A10*2a polymorphism (E139K) has been associated with a significant decrease in orolaryngeal carcinoma risk [86]. Elahi *et al.* suggest that the decrease in carcinoma risk may be related to an increase in the ability of the UGT1A10*2a polymorphism to glucuronidate and thus detoxify carcinogens such as benzo[α]pyrene-7,8-dihydrodiol; however, though it is possible that the polymorphism may be linked to other polymorphisms in the UGT1A locus. Alterations in UGT1A10 expression and activity may also be related to the risk of breast cancer [372].

15.6.10 UGT2A1, UGT2A2, and UGT2A3

UGT2A1 and UGT2A2 are primarily expressed in adult and fetal nasal mucosa, while UGT2A3 expression is highest in adult hepatic and intestinal tissues [90,373]. Relatively small amounts of UGT2A2 mRNA have been reported in the lung and small intestine. Broad and overlapping substrate specificities are observed with UGT2A1 and UGT2A2 [374,375]. Interestingly, UGT2A3 maintains a highly conserved substrate specificity [90]. Substrates for UGT2A1 and UGT2A2 include 4-methylumbelliferone; 4-nitrophenol; 2-, 3-, and 4-phenylphenol; scopoletin; 1-hydroxypyrene; and estriol [373]. UGT2A3 appears to specifically glucuronidate bile acids such as hyodeoxycholic acid and is inducible through the activation of PXR [90]. Observed polymorphisms include UGT2A1 (D85Y), UGT2A1 (G308R), UGT2A1 (V391I), and UGT2A3 (T497A) [89,90].

15.6.11 UGT2B4

15.6.11.1 Expression, Regulation, and Polymorphisms of UGT2B4. UGT2B4 is an ~52-kDa protein that is primarily involved in the glucuronidation of endogenous bile acids. Although mainly expressed in the liver, UGT2B4 mRNA expression has also been detected in adipose tissue, adrenal glands, intestine, kidney, lung, mammary glands, placenta, prostate, skin, and testis [225,376]. Localization of UGT2B4 also appears to correlate with tissues commonly targeted by endogenous steroids and, as such, may play an important role in the regulation of certain cancer types [377].

As with other members of the UGT2B family, expression of UGT2B4 is linked to nuclear receptor response elements. UGT2B4 has been shown to be inducible through activation of the PPAR- α as demonstrated by the increase in UGT2B4 activity and mRNA levels in the presence of fenofibric acid or Wy 14643, both synthetic PPAR- α agonists [378]. In a similar fashion, increases in UGT2B4 mRNA, protein, and activity have been observed on activation of FXR in hepatocytes or HepG2 cell lines [379]. Interestingly, induction of UGT2B4 may serve as a negative feedback signal in the control of bile acid toxicity by decreasing the susceptibility of FXR to activation.

Significant interindividual differences have been noted for the hepatic expression of UGT2B4 [227]. The polymorphisms that have been identified for the isoform include UGT2B4*2 (D458E) and UGT2B4*3 (F109L/F369L) [94,376,377,380]. UGT2B4*2 demonstrates a similar tissue expression profile to UGT2B4 and appears to have comparable specificity and activity characteristics as the wild-type enzyme. While UGT2B4*2 is a rare polymorphism in Asian populations, a frequency of ~0.25 has been reported in a Caucasian subgroup [377]. UGT2B4*3 differs from the wild-type UGT2B4 and UGT2B4*2 in its lack of hyodeoxycholic acid activity; however, the polymorphism appears to be relatively rare [94].

15.6.11.2 UGT2B4 Substrates. UGT2B4 predominantly catalyzes the glucuronidation of bile acids, catecholestrogens such as 17-epiestriol and 4-hydroxyestrone, reduced androgens, C19-steroids, and compounds containing phenol or monoterpenoid functional groups [224,376,381–383]. The glucuronidation of hyodeoxycholic acid at the 6 α -hydroxyl group is often used as a probe for UGT2B4 activity *in vitro* due in part to its relative selectivity for UGT2B4 [31,225]. Xenobiotic substrates for UGT2B4 include the β -adrenoceptor blocker carvedilol and the “profen”

NSAIDs such as ibuprofen, ketoprofen, and flurbiprofen [182,215]. The NSAIDs diclofenac and naproxen also demonstrate glucuronidation catalyzed by UGT2B4, while indene-containing NSAIDs such as sulindac do not appear to be as readily metabolized by the enzyme. Modeling studies suggest two potential pharmacophores that lend themselves to glucuronidation by UGT2B4, the first being three hydrophobic regions within 2–4 Å of the nucleophile and the second describing a symmetrical formation of a hydrophobic region and hydrogen bond acceptor with the nucleophile of the aglycone [288]. The hydrogen bond acceptor and nucleophile were separated by ~10 Å in the pharmacophore model, with each being within 2–3 Å of a hydrophobic region of the molecule.

15.6.11.3 Inhibition of UGT2B4. Relatively few examples of UGT2B4 inhibitors are discussed in the current literature, with most displaying overlapping inhibition with UGT2B7. For example, diclofenac is often used as an *in vitro* inhibitor for UGT2B4, although it is not selective for the isoform [384]. Tredaptive (laropiprant) is a rare example of a drug noted to potentially cause clinically relevant inhibition of UGT2B4 (prescribing information).

15.6.11.4 Active Site Characterization. Information regarding the active site characteristics of UGT2B4 is primarily derived from site-directed mutagenesis studies. The introduction of a leucine residue in place of a phenylalanine residue at position 33 of UGT2B4 resulted in a significant decrease in the glucuronidation capacity of the enzyme toward hyodeoxycholic acid, 4-hydroxyesterone, and 17-epiestriol, while substitution of a tyrosine residue in the same position showed little change from the wild-type enzyme [385]. The previous results suggest the importance of an aromatic residue at this position and the potential for π -stacking interactions in substrate recognition and binding by UGT2B4. In addition, it has been proposed that a cysteine residue at position 511 in the UGT2B4 cofactor-binding domain may play an important role in the activity of the enzyme, but the functional relevance of this residue remains to be determined [91].

15.6.11.5 Clinical Relevance. While few drugs are predominantly metabolized by UGT2B4, the clinical relevance of the enzyme may stem more from its role in steroid and bile acid homeostasis. For example, the glucuronidation of cholestatic bile acids by UGT2B4 may play an important role in their detoxification by blocking the formation of carcinogenic quinone estrogens [385]. In addition, UGT2B4 may also be involved in the regulation of intracellular bile acid levels by preventing the harmful accumulation of the acids [379].

15.6.12 UGT2B7

15.6.12.1 Expression, Regulation, and Polymorphisms of UGT2B7. It has been estimated that approximately one-third of the most commonly prescribed drugs in the United States have clearance mechanisms catalyzed by UGT2B7 [5]. The ~56-kDa enzyme is primarily expressed in the liver, intestine, kidneys, brain, prostate, lungs, colon, and testis [224,386]. It has also been reported to be expressed in human placental tissue [387]. While UGT2B7 gene transcripts have not been detected in human fetal liver samples, the protein appears to be fully expressed by six months of age [388].

Recent evidence supports the involvement of nuclear receptors in the expression of UGT2B7. Levels of UGT2B7 mRNA were suppressed in Caco-2 cells when co-incubated with lithocholic acid, with involvement of the human farnesoid X receptor (hFXR) being suspected [389]. UGT2B7 expression also appears to be associated with the expression of HNF-1 α [390]. Protein expression may also be linked to disease state, with UGT2B7 mRNA levels significantly lower in patients with severe inflammation [227].

Multiple polymorphisms have been identified for UGT2B7, including UGT2B7*2 (H268Y) and UGT2B7*3 (A71S) [91,391–393]. The UGT2B7*2 polymorphism has been reported to be present in more than 50% of Caucasians and has been associated with larger cortical bone size and higher serum testosterone levels in young men [94,377,394]. Although less prevalent, the UGT2B7*3 polymorphism has been shown to affect the pharmacokinetics of carvedilol in a Japanese population [395].

15.6.12.2 UGT2B7 Substrates. UGT2B7 catalyzes the glucuronidation of a wide range of clinically relevant drugs and endogenous substrates (Table 15.4). The formation of glucuronides from morphine, codeine, or 3'-azido-3'-deoxythymidine (AZT) are often used as probe reactions for UGT2B7 activity [32,396,397]. Glucuronidation of 6 α -hydroxyprogesterone and 21-hydroxyprogesterone, as well as glucuronidation at the benzylic hydroxyl group of the β_1 -adrenoceptor-selective partial agonist denopamine, have also been suggested as being potentially selective for UGT2B7 [398,399]. UGT2B7 has demonstrated both acyl and N-glucuronidation capability as well, as observed with the glucuronidation of the carboxylic acid of diclofenac, at the primary amine moiety of carbamazepine and at the amide moiety of Maxipost [400–402]. In addition to multiple xenobiotics, UGT2B7 also catalyzes the glucuronidation of endogenous retinoid, mineralocorticoid, and glucocorticoid substrates [403–405]. The enzyme can display stereoselective metabolism, as is observed with the increased rate of glucuronidation for (*S*)-carvedilol, (*R*)-oxazepam, or (*R*)-flurbiprofen as compared to their respective enantiomers [34,406,407].

15.6.12.3 Inhibition of UGT2B7. A number of inhibitors of UGT2B7 activity *in vitro* have been characterized. Diclofenac, flurbiprofen, and mefenamic acid have been identified as potent inhibitors of UGT2B7 [406,430,431]. In addition, the anticancer drug tamoxifen; the tricyclic antidepressants imipramine, clomipramine, amitriptyline, and desipramine; and the benzodiazepines diazepam, lorazepam, and oxazepam have all been shown to inhibit morphine 3- and 6-O-glucuronidation in HLMs [432]. Ketoconazole has been reported to inhibit UGT2B7 through linear mixed-type inhibition, with K_i values of 118 and 108 μ M for morphine-3-glucuronidation and morphine-6-glucuronidation, respectively [433]. Fluconazole has been identified as a selective inhibitor of UGT2B7 [431]. In light of recent reports of herb–drug interactions, it is also important to note that several herbal components of Kampo medicine, a traditional Japanese alternative therapy, have been shown to be relatively potent inhibitors of UGT2B7 [434].

15.6.12.4 Active Site Characterization. Similar to other UGT isoforms, the characterization of the UGT2B7 active site has been somewhat hampered by the inability to generate soluble, full-length human UGTs that are amenable to crystallization. The N-terminal substrate binding site is thought to be capable of accommodating multiple

TABLE 15.4 Substrates Glucuronidated by UGT2B7

Drug	Type of Glucuronide	References
Abacavir	O	408
Acetaminophen	O	3
Almokalant	O	409
Benoxaprofen	Acyl	410
Buprenorphine	O	397
Carbamazepine	N	401
Carvedilol	O, N	182
Chloramphenicol	O	411
Clofibric acid	Acyl	94
Clonixin	Acyl	412
Codeine	O	32
Cyclosporin	O	413
Diclofenac	Acyl	400
Diflunisal	Acyl	414
Epirubicin	O	415
Ezetimibe	O	186
Fenoprofen	Acyl	416
Gemfibrozil	Acyl	417
1'-Hydroxyestragole	O	418
3-Hydroxyrofecoxib	O	419
5-Hydroxyrofecoxib	O	419
Ibuprofen	Acyl	420
Indomethacin	Acyl	94
Ketoprofen	Acyl	412
Lorazepam	O	421
Maxipost	N	402
Menthol	O	422
4-Methylumbelliferone	O	423
Morphine	O	424
Nalorphine	O	397
Naltrexone	O	424
Naproxen	Acyl	94
1-Naphthol	O	423
4-Nitrophenol	O	425
Octylgallate	O	425
[<i>R</i>]-Oxazepam	O	34
[<i>S</i>]-Oxazepam	O	34
Pitavastatin	Acyl	426
Propranolol	O	427
Tacrolimus	O	413
Temazepam	O	171
Tiaprofenic acid	O	94
Valproic acid	Acyl	428
Zaltoprofen	Acyl	429
Zidovudine	O	32
Zomepirac	Acyl	139

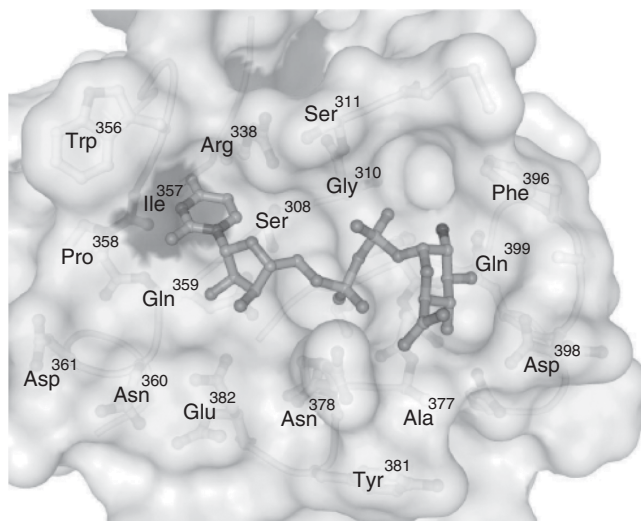


Figure 15.4 UGT2B7 cofactor-binding domain. *Source:* Reprinted from Miley *et al.* J Mol Biol 2007;369(2):498–511, with permission from Elsevier.

substrate molecules, and homology modeling with UGT2B7 has predicted that a histidine residue (H35) acts as a catalytic base to deprotonate the acceptor molecule in an acid–base type mechanism [39,179,423]. The resulting charged histidine residue is subsequently stabilized by a proximal aspartic acid residue (D151). Substituting H35 with a proline or alanine residue either abolished or greatly reduced the activity of UGT2B7, thus confirming the pivotal role of this amino acid in glucuronidation by UGT2B7 [39,435]. Finally, using 1D and NOESY (nuclear Overhauser effect spectroscopy) NMR experiments, Coffman *et al.* [436,437] have suggested that amino acid residues 84–118 of UGT2B7 comprise an opioid-binding pocket within the aglycone binding site.

More recently, the cofactor-binding domain of UGT2B7 has been crystallized, allowing for a more complete characterization of the interactions between UGTs and UDPGA [39]. For example, taking into account the human UGT sequence conservation and mapping it to the molecular surface of the C-terminal domain of UGT2B7 demonstrates that the UDPGA binding site is highly conserved across the various UGT isoforms (Fig. 15.4). For UGT2B7 specifically, W356, Q359, and E382 appear to have important interactions with the uracil base, while T373, H374, and N378 are predicted to interact with the diphosphate group of UDPGA through hydrogen bonding and charge stabilization. Finally, D398 and Q399 are thought to form hydrogen bonds with the glucuronic acid moiety of UDPGA and are essential for UGT2B7 activity [39].

15.6.12.5 Clinical Relevance. As mentioned previously, UGT2B7 plays at least a partial catalytic role in the metabolism of approximately one-third of prescribed medications in the United States. As such, a number of clinically relevant pharmacokinetic and pharmacodynamic phenomena involving UGT2B7 have been discussed in the literature. Most notably, the 6-*O*-glucuronide of morphine is pharmacologically active

and is ~30- to 50-fold more potent against the CNS μ -receptor than the parent compound [171,438–442]. A similar hypothesis has been put forth for the formation of codeine-6-glucuronide from codeine by UGT2B7, whereby the majority of the analgesic activity of codeine is attributed to the glucuronide metabolite and not the parent drug, though, this has not been shown clinically [443]. Interestingly, however, coadministration of diclofenac (an inhibitor of UGT2B7) to patients on codeine therapy resulted in no decrease in either the efficacy of the codeine treatment or the formation of codeine-6-glucuronide [444].

Multiple clinically relevant drug–drug interactions involving UGT2B7 have also been reported. For example, inhibition of UGT2B7 activity by probenecid or valproic acid resulted in a decreased clearance and increased AUC of lorazepam [171,445–447]. In addition, probenecid has been shown to decrease the *in vivo* clearance of clofibrac acid, diflunisal, and zomepirac [448–450], while therapeutically relevant concentrations of valproic acid are also known to affect the clearance of 3'-azido-3'-deoxythymidine (zidovudine) *in vivo* [428]. Finally, a decrease in the plasma AUC of zidovudine was observed in HIV patients who were coadministered rifampicin, possibly due to induction of UGT2B7.

15.6.13 UGT2B10

15.6.13.1 Expression, Regulation, and Polymorphisms of UGT2B10. UGT2B10 was originally isolated and characterized as an orphan protein with no known substrates or clinical relevance [451]. The enzyme is primarily expressed in the liver; however, there is evidence for UGT2B10 mRNA expression in the bladder as well [157,452]. Few reports exist describing the regulation of UGT2B10, though, increases in fibrosis and inflammation scores resulted in a downward (albeit statistically insignificant) trend in UGT2B10 mRNA expression [227].

The substitution of an aspartic acid residue for a tyrosine residue at codon 67 (UGT2B10*2; D67Y) is the sole polymorphism currently characterized for UGT2B10 [95]. UGT2B10*2 results in 5- and 16-fold lower levels of nicotine and cotinine glucuronidation, respectively. The polymorphism is present in ~9% of Caucasians and may also play a role in the decreased glucuronidation of nicotine by African-Americans, but this currently remains unclear [95,453].

15.6.13.2 UGT2B10 Substrates. The original substrates identified for UGT2B10 were endogenous metabolites of linoleic and arachidonic acid. Turgeon *et al.* [454] characterized the glucuronidation of 13-hydroxyoctadecadieneoic acid (13-HODE) as well as 12- and 15-hydroxyeicosatetraenoic acid (12-HETE, 15-HETE) by UGT2B10. Xenobiotic substrates include nicotine, cotinine, and several tobacco-specific nitrosamines such as 4-(methylbitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N'-nitrososnicotine, N'-nitrosoanabasine, and N'-nitrosoanatabine [33]. More recently, a number of tricyclic antidepressants (amitriptyline, imipramine, clomipramine, and trimipramine) have been shown to be glucuronidated by UGT2B10 *in vitro* [455].

15.6.13.3 Active Site Characterization. Though characterization of the active site of UGT2B10 is limited, site-directed mutagenesis studies as well as the observation of regio- and stereospecific metabolism by UGT2B10 have provided some insight into the active site architecture. As mentioned previously, a highly conserved histidine residue

(H39 based on UGT1A1 sequence) is found in most UGT isoforms. Interestingly, UGT2B10 is one of only two known UGT isoforms (with UGT1A4) that does not maintain this conserved histidine. Instead, UGT2B10 has a leucine in place of the conserved histidine [456]. As UGT1A4 and UGT2B10 are the primary UGT isoforms catalyzing N-glucuronidation, the authors speculate that the replacement of the histidine residue may confer some preference toward this catalytic pathway. It is also noted, however, that a different histidine substitution in UGT1A4 (proline) suggests that more complex phenomena may also be involved in the preference for N-glucuronidation by these two isoforms. Finally, UGT2B10 has also been shown to be both regio- and stereospecific in regard to N-glucuronidation, as observed for the glucuronidation of the medetomidine enantiomers, dexmedetomidine and levomedetomidine [457]. UGT2B10 catalyzed the glucuronidation of dexmedetomidine ((*S*)-medetomidine) at both the N-1 and N-3 positions of the imidazole ring. Conversely, only the N-3 glucuronide of levomedetomidine ((*R*)-medetomidine) was observed for UGT2B10, albeit at markedly higher rates of formation than that observed for either dexmedetomidine glucuronide.

15.6.13.4 Clinical Relevance. The major clinical relevance of UGT2B10 is currently based on the effect of UGT2B4 polymorphism and the detoxification of tobacco-specific by-products. It has been noted that the UGT2B10 haplotype may predict rates of nicotine glucuronidation *in vivo*, although it is probably not the sole determinant [453]. In addition, it has been proposed that the presence of the UGT2B10*2 allele may result in an increased susceptibility to smoking-related cancers [458].

15.6.14 UGT2B11

UGT2B11 is a human orphan UGT that has been isolated and characterized, but currently has no known function [451,459]. The 52-kDa protein has a 91% sequence homology with UGT2B10 and is expressed in the liver, kidney, mammary glands, prostate, skin, adipose, adrenal, and lungs [157,459]. While UGT2B11 is thought to have a very limited range of substrates, it does catalyze the glucuronidation of 13-HODE as well as 12-HETE and 15-HETE [454]. Finally, UGT2B11 mRNA levels have been shown to be highly inducible by R1881 (methyltrienolone) [460].

15.6.15 UGT2B15

15.6.15.1 Expression, Regulation, and Polymorphisms of UGT2B15. UGT2B15 is primarily expressed in the liver and prostate, with UGT2B15 mRNA also being detected in the gastrointestinal tract, bladder, kidney, placenta, adipose, esophagus, mammary glands, ovaries, uterus, and testis [18,96,157]. In addition to the tissues mentioned above, UGT2B15 activity has been noted in the colon and trachea [452].

UGT2B15 expression is downregulated in the presence of androgens such as R1881; however, this effect may be androgen specific, as no changes in UGT2B15 expression were seen in LNCaP cells cultured in the presence of dihydrotestosterone [460,461]. Regulation of UGT2B15 occurs through ligand binding to the androgen receptor, with the effect being indirect and requiring *de novo* protein synthesis [460]. Levels of UGT2B15 appear relatively insensitive to the epidermal growth factor or cytokines such as IL-1 α [461,462]. Finally, characterization of UGT2B15 levels in an estrogen-receptor-positive cell line has demonstrated that the isoform is upregulated in the

presence of estrogen and structurally similar compounds such as propylpyrazoletriol and genistein [463].

Multiple polymorphisms have been identified and characterized for UGT2B15. UGT2B15*2 (D85Y) occurs in ~55% of Caucasians and 72% of Asians and results in a significant decrease in (*S*)-oxazepam activity [97,377]. Interestingly, UGT2B15*2 has been reported to have an increased glucuronidation capacity toward androgens [171]. UGT2B15*4 (K523T) is found in ~35% of Caucasians and 64–79% of Asians, but with no apparent effect on (*S*)-oxazepam glucuronidation activity [97,464]. UGT2B15*6 (T352I) is a rare allele that may actually result in increased UGT2B15 activity. UGT2B15*3 (L86S), UGT2B15*5 D85Y/K523T), and UGT2B15*7 (T352I/K523T) have been identified but are not well characterized at present.

15.6.15.2 UGT2B15 Substrates. UGT2B15 catalyzes the glucuronidation of endogenous steroids such as dihydrotestosterone and androstane-3 α ,17 β -diol [465,466]. The enzyme also shows some activity toward other androgens such as 5 α -androstane-3 α ,11 β ,17 β -triol and estrogens such as 4-hydroxyestrone [467]. Xenobiotic ligands for UGT2B15 include eugenol, nandrolone, oxazepam, phenolphthalene, 4-hydroxyphenytoin, 4-hydroxytamoxifen, 5-hydroxyrofecoxib, 8-hydroxyquinoline, and bisphenol A [280,419,467–469]. UGT2B15 has also been shown to catalyze the glucuronidation of a secondary metabolite of 4-hydroxy-3-methoxymethamphetamine (MDMA; Ecstasy), indicating that it may play a role in the overall metabolism of MDMA [470]. (*S*)-Oxazepam has been proposed to be a selective probe substrate for UGT2B15 [34]. Other compounds metabolized to a glucuronide metabolite by UGT2B15 *in vitro* include flavanol derivatives such as naringin, 7-hydroxyflavone, galangin, chrysin, anthraquinone analogs such as emodin and anthraflavic acid, and the D₁-dopamine receptor antagonist SCH23390 [467].

15.6.15.3 Inhibition of UGT2B15. Multiple drugs have been reported to be inhibitors of UGT2B15 *in vitro*. For example, high concentrations of valproic acid, which is not a substrate for UGT2B15, have been shown to inhibit glucuronidation of dihydrotestosterone, 5 α -androsterone-3 α ,17 β -diol, and 8-hydroxyquinoline [428]. In addition, diclofenac, ibuprofen, and flunitrazepam have been reported to be inhibitors of UGT2B15 activity in HLMs, as well as in recombinant UGT2B15 membranes [186,375]. Phenobarbital and phenytoin are also inhibitors of UGT2B15-catalyzed acetaminophen glucuronidation, with IC₅₀ values of 303 and 607 μ M, respectively [471]. Finally, soy extract, a popular herbal medicine consisting of phytoestrogens and isoflavones, has been identified as an inhibitor of dihydrotestosterone glucuronidation in recombinant UGT2B15 [472].

15.6.15.4 Active Site Characterization. Consistent with most other UGT isoforms, UGT2B15 maintains a conserved histidine residue that most likely acts as a catalytic base to deprotonate nucleophiles before glucuronidation [456]. While the specific active site residues that confer substrate specificity to UGT2B15 are unknown at this time, recent studies have shown that the isoform has the ability to demonstrate stereoselectivity in the glucuronidation of multiple compounds. For example, while (*S*)-oxazepam has been proposed as a selective probe for UGT2B15, no UGT2B15

activity toward (*R*)-oxazepam has been observed [34]. Similarly, the rate of *cis*-4-hydroxytamoxifen O-glucuronidation by UGT2B15 is ~20-fold higher than that of *trans*-4-hydroxytamoxifen glucuronidation [237]. Finally, UGT2B15 has the ability to glucuronidate both β -estradiol and epiestradiol; there is a clear preference for the latter [473]. While many complex factors convey both substrate specificity and stereoselectivity for a given enzyme, it is tempting to consider such data as an indirect analysis of the active site topography of UGT2B15.

15.6.15.5 Clinical Relevance. Much of the data surrounding the clinical relevance of UGT2B15 involves the effects of polymorphisms on the pharmacokinetics or pharmacodynamics of UGT2B15 substrates. For example, the UGT2B15*2 polymorphism has been shown to be a major determinant of the observed interindividual variability of lorazepam in healthy subjects [474]. Similarly, the same polymorphism is associated with a decrease in the *in vivo* clearance of oxazepam [475]. Multiple groups have also attempted to link variations in UGT2B15 activity with an increased risk of prostate cancer, although no direct link has been established [476,477].

15.6.16 UGT2B17

15.6.16.1 Expression, Regulation, and Polymorphisms of UGT2B17. UGT2B17 shares a 95% sequence homology with UGT2B15 and is expressed in the liver and prostate, as well as the colon, small intestine, stomach, kidneys, testis, uterus, placenta, mammary glands, adrenal glands, brain, and skin [157,452,465]. *In vitro*, UGT2B17 appears to be one of the more labile UGT proteins, as demonstrated by cycloheximide treatment of stably transfected HK293 cells expressing various UGT isoforms [383].

UGT2B17 regulation is modulated by endogenous androgens, epidermal growth factor, and IL-1 α . Treatment of prostate cancer cells with the synthetic androgen R1881, epidermal growth factor, or IL-1 α all resulted in the downregulation of UGT2B17 [460–462]. Neither IL-4 nor IL-6 appeared to have much effect on UGT2B17. Conversely, inhibition of UGT2B17 expression by small interfering RNA (siRNA) resulted in an increase in dihydrotestosterone levels in LNCaP cells and indicated that UGT2B17 may play a role in androgen-dependent gene expression and cellular reproduction [478]. Finally, UGT2B17 expression has been shown to be dependent on HNF-1 α activation, and interactions with a Pbx2-Prep1 heterodimer indicate that this complex may further modulate HNF-1 α regulation of UGT2B17 [479,480].

A single polymorphism, resulting in the deletion of a 150-kb portion of the UGT2B17 gene, has been identified and characterized [100,481]. The UGT2B17*2 deletion polymorphism results in the absence of UGT2B17 DNA in human DNA samples. The frequency of the deletion allele in humans is 0.27, with a slight difference between African-Americans and Caucasians (0.21 vs 0.33, respectively) [100].

15.6.16.2 UGT2B17 Substrates. Although one of the primary substrates of UGT2B17 is the androgen dihydrotestosterone, the isoform has shown the ability to glucuronidate various other classes of compounds including coumarins, anthraquinones, and flavanoids [35]. Specific examples include alizarin, eugenol, scopoletin, galangin, chrysin, borneol, and the NSAID ibuprofen. Unlike UGT2B15, UGT2B17 is able to glucuronidate C19-steroids at both the 3 α - and 17 β -hydroxyl

moieties [465]. However, UGT2B17 appears to selectively glucuronidate both β -estradiol and epiestradiol at only the 17 β -hydroxyl position [473]. Recent reports also note that UGT2B17 may play an important role in the metabolism of suberoylanilide hydroxamic acid, a histone deacetylase inhibitor prescribed in the treatment of cutaneous T-cell lymphomas [482].

15.6.16.3 Inhibition of UGT2B17. In addition to being substrates for UGT2B17, the NSAIDs diclofenac and ibuprofen have also been shown to be relatively weak inhibitors of UGT2B17 testosterone glucuronidation *in vitro* [375]. The two drugs appear to differ in their mechanism of inhibition of UGT2B17, with diclofenac inhibiting the enzyme through competitive inhibition, while ibuprofen appears to go through mixed inhibition.

15.6.16.4 Active Site Characterization. As mentioned previously, UGT2B17 shares at least 95% sequence homology with UGT2B15 [465]. The enzymes differ at amino acid 121, with UGT2B17 having a tyrosine residue in place of the serine residue found in UGT2B15 [96,465,483]. UGT2B17 maintains the conserved histidine residue found in most UGT isoforms [456]. Through the use of structure–activity relationships with chiral secondary alcohols, it has been proposed that UGT2B17 has a relatively flexible active site that still maintains the ability to confer significant stereoselectivity [484]. While UGT2B17 preferentially glucuronidated the (R)-enantiomers over their respective (S)-enantiomers with eudismic ratios as high as 256, it was also shown that the stereoselectivity was probably due to spatial arrangement in the active site of UGT2B17 and not due to vastly different binding affinities between the two enantiomers.

15.6.16.5 Clinical Relevance. UGT2B17 genotypes have displayed an increasing clinical relevance in recent years, both with respect to glucuronide-mediated clearance and an increased propensity for certain disease states. With a worldwide increased focus on antidoping efforts, it is interesting to note that measurements of testosterone to epitestosterone in healthy male volunteers were highly dependent on the presence of the UGT2B17*2 polymorphism [485]. Another measurable effect of the UGT2B17*2 polymorphism is that the absence of UGT2B17 protein results in an altered immunogenicity for the enzyme and influences its role as a human minor histocompatibility antigen [481]. It has also been proposed that the UGT2B17 genotype may play a role in determining risk factors for both lung and prostate cancers, although results vary [486–488].

15.6.17 UGT2B28

UGT2B28 is a 52-kDa protein that is expressed primarily in the liver, mammary glands, and LNCaP cells [489]. Significant mRNA expression has also been detected in the bladder [157]. Two polymorphisms have been identified for UGT2B28, both resulting in truncated forms of the protein with no glucuronidation activity. Known substrates of UGT2B28 include androsterone, testosterone, androstane-3 α ,17 β -diol, estradiol, hyodeoxycholic acid, and lithocholic acid [489].

15.6.18 UGT3A1 and UGT3A2

Characterization of UGT3A1 has demonstrated the major sites of expression to be in the liver and kidney, with lesser amounts of UGT3A1 mRNA detected in the stomach, intestine, and testes. Significant levels of UGT3A2 mRNA have been found in the kidneys and testes [490,491]. UGT3A1 appears to be a unique UGT isoform, in that it primarily utilizes *N*-acetylglucosamine as the cosubstrate in conjugation reactions. UGT3A1 may play an important role in bile acid homeostasis, and as such, ursodeoxycholic acid has been shown to be a good substrate for UGT3A1 [491]. 17α -estradiol, ethynylestradiol, and estrone are also glucuronidated by UGT3A1. The substrate specificities of UGT3A2 are under investigation.

15.6.19 UGT8A1

A single member of the UGT8 family, UGT8A1, has been cloned and characterized [492,493]. The UGT isoform plays an important role in the synthesis of glycosphingolipids, cerebrosides, essential myelin membrane components, and sulfatides [89]. The main catalytic function of UGT8A1 is to transfer the galactose moiety of UDP-galactose to ceramide in the biosynthesis of galactocerebrosides [494].

15.7 UGTs IN PRECLINICAL SPECIES

Many UGT isoforms have also been characterized in preclinical species such as mouse, rat, guinea pig, rabbit, canine, and nonhuman primates (NHPs) (Table 15.5). Considerable knowledge of human UGT structure and function can be gained from the study of preclinical UGTs due to the significant homology across species, especially for the UGT1A family [495]. While an exhaustive review of preclinical UGTs is beyond the scope of this chapter, some mention of their characterization, expression, regulation, and substrate specificities is most certainly deserved.

A total of 26 UGT genes or pseudogenes have been identified in mice, with 20 UGT genes being reported in rats [495,496]. Similar to human UGT isoforms, rodent UGTs are primarily located in the liver but also demonstrate a wide tissue distribution. Rodent UGTs also exhibit a sexually dimorphic profile, as observed with UGT1a1, UGT1a2, UGT1a10, and UGT2b1 in mice (all are female predominant) and UGT2B1 and UGT2B3 in rats (female and male predominant, respectively) [314,496,497]. Regulation of UGT isoforms in mice has been shown to be dependent on inflammatory or infectious disease states. Treatment of mice with either lipopolysaccharide or *Citrobacter rodentium* resulted in a downregulation of hepatic UGT1a1, UGT1a9, and UGT2b5 [498]. Furthermore, rat UGT1A1 has been shown to be inducible by 3-methylcholanthrene administration [499]. Finally, the functional relevance of rodent UGT isoforms has also been assessed, mainly in rats. Rat UGT1A6 and UGT1A7 have been characterized as the major contributors to acetaminophen glucuronidation in rats, while rat UGT2B1 is the primary isoform involved in the detoxification of NNAL and bisphenol A [500,501]. Rat hepatocytes and microsomes are also known to efficiently catalyze the glucuronidation of zidovudine *in vitro* [502].

Additional mechanistic studies have been carried out on UGT isoforms from guinea pigs. As noted earlier, UGTs are known to form homo- and heterodimers that can

TABLE 15.5 UGT Isoforms Identified in Preclinical Species

Mouse	Rat	Guinea Pig	Rabbit	Canine	NHP
UGT1a1	UGT1A1	UGT1A07	UGT1A04	UGT1A6	UGT1A1
UGT1a2	UGT1A2	UGT2A3	UGT1A6	UGT2B31	UGT1A02
UGT1a3	UGT1A3	UGT2B21	UGT1A07		UGT1A03
UGT1a4	UGT1A ^a	UGT2B22	UGT2A2		UGT1A6
UGT1a5	UGT1A5	UGT2B32	UGT2B13		UGT1A08
UGT1a6	UGT1A6		UGT2B14		UGT1A09
UGT1a6a	UGT1A7		UGT2B16		UGT2B9
UGT1a7a ^a	UGT1A8				UGT2B18
UGT1a7b ^a	UGT1A9*				UGT2B19
UGT1a7c	UGT2A1				UGT2B20
UGT1a8	UGT2A2				UGT2B23
UGT1a9	UGT2A3				UGT2B30
UGT1a10	UGT2B1				UGT2B33
UGT1a ^a	UGT2B2				UGT8A1
UGT2a1	UGT2B3				
UGT2a2	UGT2B6				
UGT2a3	UGT2B8				
UGT2b1	UGT2B12				
UGT2b5	UGT2B34				
UGT2b34	UGT8A1				
UGT2b35					
UGT2b36					
UGT2b37					
UGT2b38					
UGT3a1					
UGT3a2					

^aPseudogene.

affect both their catalytic capacities and substrate specificities. Ishii *et al.* [503] demonstrated that while guinea pig UGT2B21 primarily glucuronidates morphine at the 3-hydroxyl moiety, UGT2B22 shows only minor activity toward morphine. Interestingly, when the two isoforms were coexpressed in COS-7 cells, a significant increase in morphine-6-glucuronide formation was observed, indicating an altered substrate specificity potentially caused by UGT protein–protein interactions.

Finally, the examination of UGT isoforms in NHPs has revealed an important role in the metabolism of steroids, mineralocorticoids, glucocorticoids, androgens, oestrogens, opioids, and xenobiotics containing either a carboxylic acid or hydroxyl moiety [504–506]. UGT2B9 glucuronidates xenobiotics such as morphine, codeine, hydromorphone, (*S*)- and (*R*)-ibuprofen, fenoprofen, naproxen, and the natural products borneol and menthol [506]. NHP UGT2B20 has been shown to catalyze the glucuronidation of C19-steroids such as testosterone, dihydrotestosterone, and 5 α -androstane-3 α ,17 β -diol, as well as exogenous compounds such as scopoletin, 4-methylumbelliferone, and *p*-nitrophenol [504]. Finally, NHP UGT2B30 has the capacity to glucuronidate C18-, C19-, and C21 steroids as well as compounds such as 1-naphthol and *p*-nitrophenol [505]. The extrahepatic localization of NHP UGTs has implicated the enzymes as playing an important role in estrogen and androgen metabolism as well as in various steroid signaling pathways [507,508].

The use of humanized mouse models to study human UGT enzymes is an area of research that deserves mention. As multiple caveats exist when using isolated human UGT isoforms *in vitro*, the ability to study them in a more complete *in vivo* setting is of the utmost importance. A recent example of such a model is the expression of human UGT1A1*28 in a humanized mouse model [509]. The mice are hyperbilirubinemic and display a decreased rate of SN-38 glucuronidation, a known substrate of UGT1A1 [202]. The authors suggest that the model may be viewed as an important tool to be used toward understanding the effects of human UGT1A1 polymorphisms on drug metabolism.

15.8 CONCLUSIONS

The aim of this chapter was to present a comprehensive overview of the current information available on the UGT family of phase II conjugating enzymes. As research continues on the pharmacogenetics, functional characteristics, and clinical relevance of UGTs, our understanding of the catalytic mechanism, potential for drug interactions, and ability to predict *in vivo* outcomes involving this family of enzymes should greatly increase. Future challenges will include understanding the impact of UGT protein–protein interactions on *in vitro* and *in vivo* data; developing better homology models and, perhaps, crystal structures to study the structural aspects of UGTs; and refining our ability to use *in silico* modeling and simulation software to predict UGT-mediated clearance and drug interactions.

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16 Sulfotransferases

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16.1 Introduction to human sulfotransferases	1
16.2 Sulfation reaction	8
16.3 Clinical perspectives	15
16.4 Conclusions and future prospects	20
References	20

16.1 INTRODUCTION TO HUMAN SULFOTRANSFERASES

In the nineteenth century, one of the difficulties associated with surgery was the high postoperative mortality rate that many patients suffered (as high as 50%) most often due to infection [1,2]. Carbolic acid (phenol) was an early antiseptic that was used to reduce postoperative infections and thus reduce mortality [3]. In the late 1870s, a German chemist named Eugen Baumann was studying the elimination of carbolic acid in urine. He was able to isolate and characterize phenol sulfate from the urine of a patient who was administered carbolic acid as an antiseptic [4]. Many years later (1957), the molecule which served as a sulfate donor in this process was characterized [5]. Robbins and Lipman demonstrated that when protein fractions from liver cytosol were separated, one fraction contained the sulfate-activating system and a second fraction contained the transferring enzyme or a “phenol sulfokinase.” The identity of the sulfate donor molecule was determined from the sulfate-activating fraction. These investigators were able to isolate and identify what is now known as *3'-phosphoadenosine 5'-phosphosulfate* (PAPS), which is the physiological donor molecule or cofactor in sulfotransferase (SULT)-mediated conjugation reactions [5,6]. Since these early discoveries, from a historical perspective, the SULT field has moved forward relatively rapidly.

Today, SULTs are recognized as an important family of enzymes. They are not only involved in the metabolism of xenobiotics such as carbolic acid but also involved in important processes such as supporting placental estrogen biosynthesis, modulating estrogenic stimulation of the endometrium, and influencing the levels of receptor-active

thyroid hormone [7–17]. Thus, the SULT field has grown beyond the conjugation of xenobiotics, and it is now recognized that SULTs play an important role in biological processes as well. It should be noted that many insightful and complete reviews of SULT enzymology have preceded this chapter [18–24]. Moreover, the reader is directed to the references in this chapter to expand into additional topics according to his or her level of interest. However, for the purposes of this book, the scope of the current review focuses most directly on the topic of drug metabolism and the role that SULTs play in this area. An emphasis is placed on the practicality of studying SULTs as they apply to drug discovery and drug development. While a great deal of information is available for cytochrome P450 *in vivo*–*in vitro* correlations, *in vitro* assay conditions, selective substrates and inhibitors, and so on, this information is not always readily available for the SULT family of enzymes. In many cases, selective probe substrates or inhibitors do not exist, and in other cases, the selectivity of certain inhibitors of substrates has not been well characterized. For the drug metabolism scientist determining, for example, the contribution of a specific SULT isoform(s) to the formation of a sulfate drug metabolite, it is a difficult task; many of the expectations learned from dealing primarily with cytochrome P450 enzymology do not translate to the world of SULTs. It is the authors' intent that the following chapter reviews some topics that may help applied drug metabolism investigators to point themselves in the right direction for solving practical issues in drug discovery/development.

16.1.1 Classification and Nomenclature

There are two classes of mammalian SULTs: SULTs that are membrane bound and those that are found in the cytosol [18]. Membrane-bound SULTs are located in the Golgi apparatus, and these enzymes are involved in the sulfation of endogenous substrates such as protein and carbohydrates. Membrane SULTs do not currently appear to be involved in the sulfation of xenobiotics [25,26]. As a result, focus is directed toward cytosolic SULTs, which are capable of conjugating a variety of small molecules including both exogenous and endogenous substrates. It is only relatively recent that the nomenclature system for cytosolic SULTs has been established [27]. The members of the cytosolic family of SULTs are all assigned to the superfamily "SULT." In addition, based on the genetic sequence identity, the members of this superfamily are categorized and subcategorized. Those SULTs that share at least 45% amino acid sequence identity belong to the same family. Subfamilies are determined by at least 60% amino acid sequence identity. Thus, SULT1A is a designation for the SULT1 family (family designation uses an Arabic numeral after the superfamily name) and SULT1A also specifies the "A" subfamily. SULT1A1 is the name for isoform "1" (Arabic numeral) within the SULT1A subfamily. Gene names have the same designation as the proteins, but the gene names are listed in italics. Alleles are identified with an asterisk and an Arabic numeral directly after the subfamily isoform number (e.g., *SULT1A1*1*). SULT2B1a and SULT2B1b refer to splicing variants encoded by the same gene but with different amino acid sequences [28]. However, new nomenclature has designated these enzymes as SULT2B1_v1 and SULT2B1_v2, respectively.

This last point serves to illustrate a very real and sometimes confounding issue when dealing with the SULT literature. Much of the SULT body of literature consists of papers written before standardized nomenclature. Readers who are unfamiliar with the subject will be confronted with several names designated for the same protein,

which can lead to potential misunderstanding. Table 16.1 has been constructed to aid in the process of reading “older” literature. For example, the following paper published as recently as 2002 was titled “Phenol Sulfotransferase, ST1A3, as the Main Enzyme Catalyzing Sulfation of Troglitazone in Human Liver” [29]. The term ST1A3 is similar to SULT1A3; however, consultation with Table 16.1 will clarify that this paper is actually referring to SULT1A1.

16.1.2 Protein Structure and Function

The first reported SULT crystal structure was a mouse estrogen sulfotransferase (mSult1e1) [30,31]. Since then, three-dimensional (3D) structures have been determined for human SULTs 1A1, 1A3, 1E1, 2A1, 2B1_v1, and 2B1_v2 [22,32]. SULTs are globular proteins with a single α/β domain containing a central five-stranded parallel β -sheet [20]. Although many structural features identified from mSult1e1 are also observed in human SULTs, the mouse enzyme exists as a monomer, whereas human SULTs are generally present as homodimers [20,33]. A conserved sequence consisting of 10 amino acid residues (KXXXTVXXE; also known as the *KTVE motif*) located near the C-terminus in most human SULTs has been identified as being involved in dimerization [20,33]. Mutation of the valine residue alone (Val270Glu in SULT1A1, Val266Glu in SULT1E1, and Val260Glu in SULT2A1) is sufficient to convert the dimeric enzymes into monomers [33,34]. The monomeric mSult1e1 has Pro269-Glu270 residues in place of Thr and Val and, as anticipated, a double mutation of Pro269Thr and Glu270Val leads to dimerization of the enzyme [34].

One of the conserved motifs identified in mSult1e1 and in various human SULTs is the structural element that contributes to the binding of cofactor PAPS. This motif consists of nine amino acids corresponding to residues 45-TYPKSGTTW-53 in mSult1e1, and is named the 5'-phosphosulfate binding loop (5'-PSB-loop) because of its interaction with the 5'-phosphate group of 3'-phosphoadenosine 5'-phosphate (PAP) or PAPS [31,32]. Several amino acid residues have been determined to constitute the 3'-phosphate binding (3'-PB) site for PAP or PAPS, including the highly conserved residues Arg130, Ser138, and 257-RKG-259 [30]. Site-directed mutagenesis of Arg257 to Ala or Glu in SULT1A1 results in inactive proteins, illustrating the importance of this residue for the catalytic activity of the enzyme [35]. In addition to two binding motifs, aromatic–aromatic interaction (π -stacking) has also been observed for the adenine ring of the PAP molecule with residues Trp53 and Phe229 [36].

The catalytic mechanism for SULTs has been suggested to involve an inline S_N2 -type nucleophilic attack on the PAPS sulfonate by a suitable substrate nucleophile. One early study that helped to shed light on this process was a solved crystal structure of mSult1e1 with the vanadate ion (VO_4^{3-}) [31]. Vanadate (oxidation state +5) can readily acquire a stable 5-coordinated trigonal bipyramidal geometry, which mimics the transition state of both phosphate and sulfate ions. Indeed, Kakuta *et al.* were able to show that the vanadate anion adopted this bipyramidal geometry in the active site, with the three bonded oxygen atoms forming the trigonal plane surrounding the vanadium atom and the apical positions occupied by water on one side and by the terminal oxygen of the 5'-phosphate of PAP on the other. In addition, the overall structure of the mSult1e1-PAP-vanadate complex was shown to be virtually the same as the mSult1e1-PAP- β -estradiol complex obtained by the same investigators [30,31]. Subsequent crystal

TABLE 16.1 Naming Conventions and Properties of Human Sulfotransferases

Human SULT	Other Names	Major Expression Sites	Known Drug Substrates	Known Endogenous Substrates
SULT1A1	P-PST, P-PST-1, TS-PST, TS-PST-1, H-PST, HAST-1, ST1A3	Adult: liver, intestine, kidney, lung, platelets, brain, placenta Fetus: liver	Minoxidil, troglitazone, acetaminophen, ethinyl estradiol	β -Estradiol, T ₂ , T ₃ , T ₄
SULT1A2	P-PST-2, TS-PST-2, ST1A2, HAST4	Adult: liver, intestine	Minoxidil	Not known
SULT1A3	M-PST, TL-PST, HAST3, ST1A5	Adult: intestine, kidney, lung, platelets, brain, placenta Fetus: liver	Salbutamol, terbutaline, minoxidil, ethinyl estradiol	Dopamine, 3-methyl dopamine, norepinephrine
SULT1B1	ST1B2	Adult: liver, intestine, kidney, lung	—	T ₂ , T ₃ , T ₄
SULT1C2	ST1C2, SULT1C1, HAST5	Adult: stomach Fetus: liver, kidney	—	Not known
SULT1C4	SULT1C2, hSULT1C	Fetus: liver, kidney, lung	—	Not known
SULT1E1	EST, hEST, ST1E4	Adult: liver, intestine, lung, endometrium Fetus: liver, kidney, lung	Ethinyl estradiol, troglitazone	β -Estradiol, estrone, 24OH-cholesterol, T ₂ , T ₃ , T ₄
SULT2A1	DHEA-ST, HST, ST2A3	Adult: liver, intestine, lung, adrenal Fetus: liver, adrenal	Ethinyl estradiol, minoxidil,	DHEA, 24OH-cholesterol, T ₂ , T ₃ , T ₄
SULT2B1_v1	SULT2B1a	Adult: placenta	—	DHEA, pregnenolone
SULT2B1_v2	SULT2B1b	Adult: placenta, prostate	—	DHEA, pregnenolone, cholesterol, 24OH-cholesterol
SULT4A1	BR-STL	Adult: brain	Not known	Not known

structures obtained from human SULT1E1–PAPS complex and the ternary hSULT1E1–PAP- β -estradiol complex provided additional evidence for the catalytic mechanism of SULT [37]. His107 (of hSULT1E1) has been hypothesized to be a catalytic base and to activate the hydroxyl group of the substrate for nucleophilic attack via proton abstraction. Mutation of this residue (His107Asn) leads to an enzymatically inactive protein [37]. Once activated, the oxygen anion attacks the sulfur atom of the PAPS molecule, resulting in the dissociation of the bridging oxygen between the 5'-phosphate and the sulfate leaving group. A conserved lysine residue (Lys47) has been suggested to act as an acid catalyst by interacting with the bridging oxygen and therefore facilitating the dissociation of the sulfate group from PAP. Interestingly, in the absence of substrate, the crystal structure of PAPS-bound human SULT1E1 showed that Lys47 actually interacted with Ser137 via a side chain interaction (hydrogen bonding) and that this interaction prevented the hydrolysis of PAPS. It was proposed that once the substrate was bound in the active site, and the reaction progressed, the bridging oxygen between the 5'-phosphate and the sulfate group then attained a partial negative charge after the attack of the substrate oxyanion (Fig. 16.1). At this point, the side chain of Lys47 switched from the Ser137 interaction to the partial negative charge of the bridging oxygen, stabilizing this intermediate and thus making the phosphate a better leaving group. Taken together, the results from crystallography and site-directed mutagenesis studies have suggested a detailed structural catalytic mechanism. Although there may be refinements to this proposed mechanism, the data indicate that the conserved serine, lysine, and histidine residues work in concert during the sulfonate transfer process [37].

16.1.3 Tissue Distribution

Sulfation has been shown to be a major biotransformation pathway for a number of xenobiotics (e.g., ethinyl estradiol, salbutamol, and troglitazone) [38–41] as well as an inactivation/regulation mechanism for endogenous chemicals such as dopamine [42]. Section 16.1 briefly touched on some of the important endogenous roles for SULTs. Not surprisingly, broad expressions of SULT enzymes have been identified in various tissues as would be expected for a family of enzymes with many different roles. Major expression sites for different SULT enzymes are summarized in Table 16.1. Using quantitative immunoblotting, Riches *et al.* [43] determined the expression levels of five major SULTs (SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) in adult liver, small intestine, kidney, and lung. The overall amount of SULT expressed in liver and intestine was greater than 10-fold of that observed in lung and kidney. In liver, SULT1A1 and SULT2A1 were determined to be the major SULT isoforms, with SULT1B1 and SULT1E1 expressing at relatively lower levels. There was no detectable level of SULT1A3 in liver. The expression pattern of various SULTs in intestine was markedly different from that in liver, with SULT1A3 and SULT1B1 more abundant than SULT1A1, SULT1E1, and SULT2A1. In a similar study conducted by Teubner *et al.* [44], high SULT expression levels were detected in ileum compared to the remaining intestinal sections (jejunum, cecum, colon, and rectum). Subsequent immunohistochemical analyses revealed that SULTs were primarily expressed in mature (differentiated) enterocytes (similar to cytochrome P450 3A), consistent with the anticipated role of xenobiotic detoxification. It is interesting to note that SULT1C2 was detected in stomach, while SULT1A2 was present at very low levels in liver and intestines. In addition, significant interindividual differences in SULT expression levels were observed in the tissues examined.

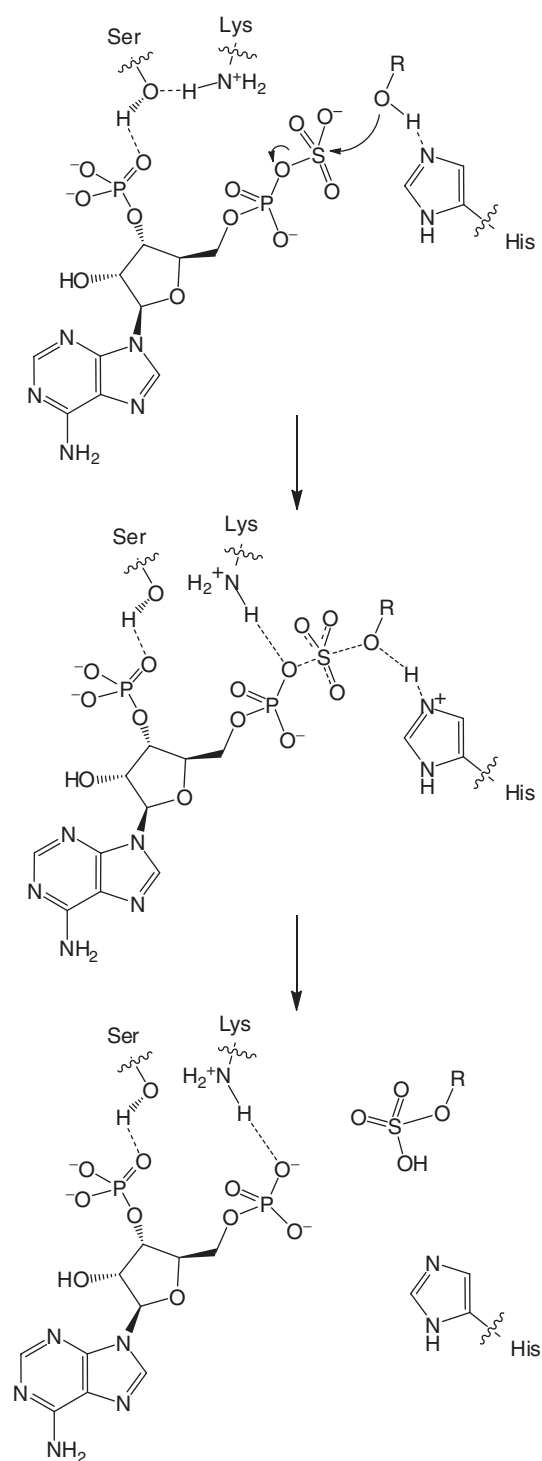


Figure 16.1 Proposed mechanism of sulfotransferase-catalyzed sulfonyl transfer.

Results obtained from reverse transcriptase-polymerase chain reaction (RT-PCR) analyses indicated that SULT1A1, SULT1A3, and SULT4A1 were readily expressed in human brain [45,46]. Immunoblot analyses showed that SULT1A1, SULT1A3, and SULT4A1 proteins were present in all regions of the brain examined; however, the expression patterns for these SULTs were vastly different [47,48]. SULT1A1 was preferentially expressed in cerebellum, occipital, and frontal lobes, whereas SULT1A3 was highly expressed in superior temporal gyrus, hippocampus, and temporal lobe. The expression level of SULT4A1 was relatively consistent among various regions tested. At the cellular level, immunoreactivities for SULTs 1A1 and 1A3 were found in neurons as well as glial cells, but localization of SULT4A1 was mainly associated with neurons. Currently, despite abundance, the role and function of SULT4A1 in human brain is undetermined.

16.1.4 Role in Fetal Development

Similar to adults, the developing fetus is potentially exposed to a number of environmental toxins; it has been hypothesized that SULTs play a protective role during development because a number of SULTs are expressed at high levels during this period [18]. However, it should be added that other xenobiotic-metabolizing enzymes are also present in the fetus, one notable example would be CYP3A7 and other P450 enzymes [49,50]. Several investigators have shown that SULT2A1 [dehydroepiandrosterone (DHEA) SULT] increases during fetal development, and the expression of SULT2A1 is very high in the adrenal from ~12 weeks of gestation onward, which lends credence to the idea that DHEA sulfate is needed during this period to support placental estrogen biosynthesis [51,52]. DHEA is sulfated primarily by SULT2A1 in the adrenal cortex and then desulfated in the placenta to release free DHEA [53]. The DHEA sulfate in placenta is from both maternal and fetal adrenal origin [54,55], and once free DHEA is released, it is then converted into androstenedione by the 3 β -hydroxysteroid dehydrogenase type 1 (3 β -HSD1), next aromatized to estrone by the cytochrome P450 aromatase and finally reduced by the 17 β -HSD type 1 to generate estradiol [56].

Thyroid hormones are critical for development of the fetal and neonatal brain, as well as for many other aspects of fetal growth (e.g., thyroxine). Hypothyroidism in either the mother or fetus often results in fetal disease; in humans, this can include a high incidence of mental retardation [57–59]. The fetal thyroid gland reaches maturity by week 11–12, close to the end of the first trimester, and begins to secrete thyroid hormones by about week 16 [60]. During this period, an adequate supply of maternal thyroid hormones must be sustained to ensure normal neurological development.

The mechanism by which thyroxine (T_4) is converted to the receptor active form 3,3',5-triiodothyronine (T_3) is a complex interplay of sulfation and deiodination between free T_4 and T_3 . When T_4 is secreted by the thyroid gland, it can be activated to form T_3 (outer ring deiodination) or inactivated to reverse T_3 (inner ring deiodination). However, when T_4 is sulfated, it is almost completely converted to receptor inactive reverse T_3 . Sulfation of T_3 also accelerates the deiodination of T_3 to T_2 (3,3'-diiodothyronine) [14,15,17,60].

It has been shown that iodothyronine sulfates are present at very high levels in fetal blood and postnatal life, and thus, it has been speculated that sulfation may play part of a protective mechanism that prevents the fetus from excessive T_3 exposure [61,62]. However, other mechanisms are also present and low fetal serum concentrations of

T₃ are due, in part, to the increased activity of the inactivating deiodinase (D₃) and a low expression of the activating deiodinase D₁ (the major activating deiodinase in adults) [63]. Although the major contributing mechanism may be subject to debate, it is interesting that the expression of deiodinase and high sulfate levels of thyroid hormones are all mechanisms that would reduce fetal exposure to T₃.

16.2 SULFATION REACTION

As discussed, since the early work of Eugen Baumann, examples of drug sulfation have grown substantially. Many review papers on this subject have wording such as “Sulfonation of the low molecular weight compounds catalyzed by members of the cytosolic SULT multigene family is an important determinant of the pharmacology and toxicology of a vast array of endogenous and foreign chemicals...” [21]. However, in the pharmaceutical industry as well as in the literature, the overall contribution of sulfation to drug metabolism is often overlooked. For example, in a recent review of cytochrome P450 and other drug-metabolizing enzymes, the author presents a pie schematic outlining the contribution of cytochrome P450s (CYPs or P450s), UDPGA-glucuronosyltransferases (UGTs), esterases, *N*-acetyltransferases (NATs), and monoamine oxidases (MAOs), but no slice of the pie was designated for the contribution from SULTs [64]. In addition, over the course of many collective years of industrial experience (and many hundreds of compounds), we have worked on relatively few drugs that undergo sulfation as the primary means of elimination. However, we have also made the observation that sulfation has grown in importance in the industrial setting in recent years. Several suggestions may be offered to rationalize this undocumented observation. First, in early drug discovery, much attention is focused on P450- and UGT-mediated metabolism. As a consequence, when discovery teams seek to minimize metabolism/clearance caused by these enzymes, the process (likely inadvertently) shifts the chemical space of compounds such that other pathways (e.g., active transport and sulfation) play an increasing role in compound elimination. Second, microsomes-based metabolic stability studies have been utilized as front-line assays for years. Given that drug-metabolizing SULTs are cytosolic, the microsomal system will not yield useful information regarding the potential sulfation of drug candidates. However, because the contemporary quality and availability of cryopreserved hepatocytes has improved, microsomal assays are increasingly complemented by *in vitro* assays using hepatocytes, which generate metabolism information from a whole cell system, and therefore include the contribution to metabolism from many enzymes including SULTs [65]. Regardless of the true reason for the increasing role of sulfation, given the potential for SULTs to conjugate a wide variety of xenobiotic compounds, it would be prudent for drug metabolism scientists (academic or industrial) to be familiar with the basics of SULT conjugation. Accordingly, this section focuses on the basic catalytic mechanism, *in vitro* assays, enzyme kinetics, practical considerations, and examples of conjugation leading to the formation of potential deleterious reactive intermediates.

16.2.1 Catalytic Mechanism

Sulfation (or sulfonation) is the transfer of a sulfonate group ($-\text{SO}_3^-$) to an acceptor molecule. A structural mechanism for this reaction was discussed in Section 16.1.2.

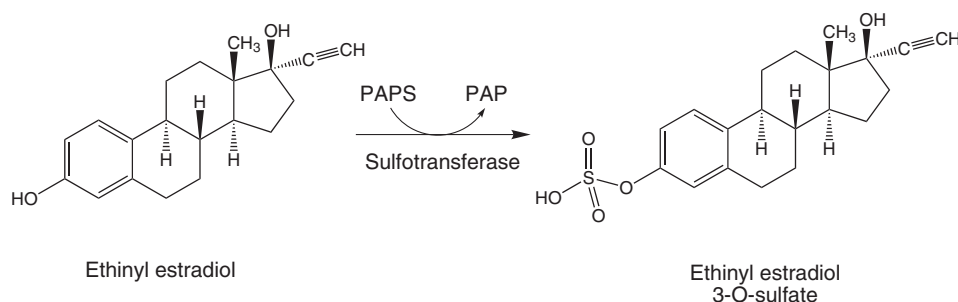


Figure 16.2 Example of a sulfonate conjugation reaction.

The acceptor in this reaction is commonly a hydroxyl group, in which case a sulfate is formed ($\text{RO-SO}_3\text{H}$; Fig. 16.2). It should be noted that some authors prefer to use the term “sulfonation” rather than “sulfation,” since technically it is the sulfonate group, not the sulfate group ($-\text{OSO}_3^-$), that is being transferred in the reaction. Regardless of terminology, a hydroxyl group can serve as the nucleophile/acceptor and it is also possible for nitrogen or sulfur to act as a nucleophile and form a sulfamate or thiosulfate metabolite, respectively. As discussed in Section 16.1.1, for small molecules, this reaction occurs primarily in the cytosol, catalyzed by cytosolic SULT enzymes. In general, the catalytic turnover of substrates catalyzed by SULT enzymes is relatively low. For example, the k_{cat} for sulfation of β -estradiol by SULT1E1 is $\sim 1.3/\text{s}$ [66]. However, the K_{m} values for many substrates are low (in the nanomolar range), resulting in high catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$).

Early crystallography studies were not able to determine a structure with both PAPS and an acceptor molecule bound to SULT simultaneously. Instead, structures of mouse Sult1e1 were solved as mSult1e1-PAP- β -estradiol complex and mSult1e1-PAP-vanadate complex (*vide supra*) [30,31]. Similarly, structures of human SULT1E1-PAPS and human SULT1E1-PAP- β -estradiol complexes were also obtained [37]. Insight into the mechanisms of sulfonate transfer was gained by superimposing the structures of the later two complexes, and the resulting superposition revealed a ternary complex that positioned PAPS with the acceptor substrate in a $\text{S}_{\text{N}}2$ -like inline displacement reaction [37]. In Section 16.1.2, a detailed description (hypothesis; Fig. 16.1) of this mechanism was presented. Recently, a group was able to provide a snap shot of the Michaelis–Menten complex with both PAPS and 4-nitrophenol bound to mouse Sult1d1 [67]. The data from this complex also strongly supported the $\text{S}_{\text{N}}2$ inline displacement mechanism as proposed by earlier studies.

16.2.2 Sulfation Assays

For many decades, the barium precipitation assay developed by Foldes and Meek [68] has been commonly adopted for determining SULT activities. This assay takes advantage of the highly effective precipitation process of PAPS in the presence of barium ions. With the use of $[^{35}\text{S}]$ -labeled PAPS as the cosubstrate, the unreacted $[^{35}\text{S}]$ PAPS can be easily removed from the incubation mixture by precipitation, leaving the soluble, $[^{35}\text{S}]$ -labeled sulfate conjugates readily available for quantification by scintillation counting.

However, this simple, quick, and widely applicable [^{35}S]PAPS assay does have some limitations. One major disadvantage is the inability to differentiate what exactly has been sulfated. It has been shown that contaminants of substrate and buffering agent, as well as small peptides present in tissue preparations, can undergo significant sulfation, and as a consequence, there is potential to confound experimental results [69]. The background signal caused by these undesired sulfation products are particularly problematic in determining product formation rates at low substrate concentrations. Many steroids, for example, have K_m values in the low nanomolar region. To overcome this hurdle, thin-layer chromatography (TLC) has been commonly utilized in recent years to replace barium precipitation when [^{35}S]PAPS is used [42,70,71]. The ability to separate various [^{35}S]-labeled sulfate conjugates also enables a more thorough characterization of metabolism and kinetic analysis. For compounds such as raloxifene and 24-hydroxycholesterol, TLC analyses reveal the formation of secondary metabolites (i.e., disulfates) in addition to monosulfates from *in vitro* incubations. In the case of dopamine, sulfation products of dopamine and its metabolite, 3-methyldopamine, have been identified in the incubation of dopamine with human neuroblastoma cells. The usefulness of TLC analysis, however, is limited by resolving power, as illustrated by the inability to separate isomeric monosulfate conjugates [42,70,71].

High performance liquid chromatography (HPLC)-based methods provide much needed resolution for various sulfate conjugates, including isomeric conjugates, leading to more precise measurements of SULT activities and kinetic analyses. Traditional reversed-phase column chromatography using a C18 column is broadly applicable for many endogenous substrates and xenobiotics [70–72]. For highly hydrophilic substrates such as dopamine and 3,4-dihydroxymethamphetamine, separation can now be readily achievable with a fluoro-substituted alkyl column or a hydrophilic interaction chromatography (HILIC) column [73,74]. Depending on the application, column effluent can be analyzed using one or more online detectors, including UV/VIS spectrophotometer, fluorescence spectrophotometer, radiochemical detector, and mass spectrometer. Liquid chromatography-mass spectrometry (LC/MS) appears to be the most versatile and convenient, albeit expensive, method among those listed above. Although the pK_a of the sulfate moiety is ~ 1 , a sulfate conjugate can be detected using a positive or negative ionization mode depending on the pH of the mobile phase used [73–75]. Constant neutral loss of 80 Da (sulfonate moiety) is a generic yet selective way for the detection of sulfate conjugates. It should be noted that the loss of sulfonate moiety can readily occur via in-source collision-induced dissociation (i.e., in-source fragmentation). As such, extra care should be taken to optimize the ionization condition for sulfate conjugates. The labile nature of the sulfonate moiety in mass spectrometric analysis makes the identification of the sulfation site very difficult. Additional nuclear magnetic resonance (NMR) analyses and/or preparation of synthetic standards are commonly performed in conjunction with LC/MS analyses [73,74].

16.2.3 Enzyme Kinetics and Some Practical Considerations

16.2.3.1 Kinetics. In Section 16.2.1, the catalytic mechanism of SULTs was presented. As discussed in this section, published data strongly support a ternary S_N2 inline displacement mechanism. One additional question that has been studied is the exact order of binding and the extent to which reaction products (e.g., PAP) are also

bound. In particular, understanding the binding order offers a mechanistic understanding for the phenomena of substrate inhibition (i.e., lower product formation as substrate concentration is increased). Atypical kinetics (e.g., substrate inhibition and autoactivation) displayed by drug-metabolizing enzymes such as P450s and UGTs can also be observed in SULTs [36,66,76–79]. For example, in the case of human, SULT1E1-catalyzed sulfation of β -estradiol, substrate inhibition was so prominent that it was observed even at concentrations below 100 nM [66].

Early studies by Duffel and Jacoby found that the kinetic binding mechanism of aryl SULT (purified from rat liver) was described by a random Bi Bi mechanism with two dead-end product–inhibitor complexes, which was also consistent with a study conducted by Zhang *et al.* using recombinant human SULT1E1 [66,80]. On the other hand, a sequential/ordered mechanism was proposed to describe kinetics determined in human SULT1A3 as well as SULTs obtained from rat and rhesus monkey liver [81–84]. Regardless of the exact order of binding, enzyme kinetic studies have postulated that substrate inhibition can result from noncatalytic dead-end complexes (e.g., enzyme bound to substrate and PAP).

However, formation of dead-end complexes is not the only hypothesis that can rationalize substrate inhibition kinetics. A second mechanism involves more than one substrate bound simultaneously to the active site. This kinetic mechanism was first proposed by Korzekwa *et al.* [76] to rationalize cytochrome P450 3A4 atypical kinetics. Active site occupation by multiple substrates results in a heterogeneous population of enzyme: Free enzyme (E), enzyme bound with a single substrate (ES), and enzyme bound with multiple substrates (ESS, etc.). The amount of each complex is dependent on the concentration of substrate and the relative substrate binding affinities for each complex. When the catalytic properties of ES differ from those of ESS (e.g., k_{cat} or rate of product release), atypical kinetics can be observed. In the SULT literature, evidence for the plausibility of this mechanism was found when a crystal structure of SULT1A1 was found to contain two molecules of 4-nitrophenol bound in the active site simultaneously [36]. These investigators postulated that overall rate of product formation from ESS was less than ES. Since ESS was formed at higher concentrations of substrate, the product formation curve displayed substrate inhibition as 4-nitrophenol concentration increased.

In addition, when SULT2A1 was cocrystallized with DHEA, two binding orientations were shown to exist for this substrate [85]. One binding orientation was consistent with a catalytic orientation (ES_1), while the other binding site penetrated further into the active site (ES_2); the authors postulated that this orientation might give rise to substrate inhibition [85]. Although different from multiple substrates within one active site, this mechanism would also result in two populations of bound enzyme (ES_1 and ES_2) with different product formation rates. Thus, as the concentration of substrate increases, it is expected that the ES_2 complex (in this case with reduced product formation) would be formed preferentially at higher concentrations of substrate, and consequently, at higher concentrations, substrate inhibition would characterize the product formation curve. On the basis of a kinetic argument, Gamage *et al.* [79] disputed this idea and proposed that the proportion of ES_1 and ES_2 complexes would most likely stay constant as substrate concentration increased and thus would not result in substrate inhibition. Finally, the stoichiometry of β -estradiol binding to human SULT1E1 was found to be 2:1 for each homodimer [66]. In this report, investigators noted that the binding of two molecules of estradiol to each

SULT1E1 subunit suggested that one of the binding sites was a catalytic site, whereas the other site could potentially be allosteric and regulate the turnover of the enzyme.

The allosteric binding site hypothesis was supported by studies with celecoxib, which in *in vitro* could modulate the stereoselectivity of ethinyl estradiol (EE) sulfation [86]. Celecoxib was first shown to cause product switching in SULT2A1-catalyzed sulfation of EE and then later with β -estradiol (E2) (from the 3-*O*-position to the 17 β -*O*-position) [86,87]. Detailed kinetics studies revealed that increasing concentrations of celecoxib decreased the $V_{\max}$ of EE 3-*O*-sulfation by three- to fourfold (without changing the K_m value significantly) and also increased the $V_{\max}$ and decreased the K_m for 17 β -*O*-sulfate formation by seven- and eightfold, respectively [86]. In addition, the inhibitory effect of celecoxib on EE 3-*O*-sulfation, as indicated by IC_{50} values, was independent of the EE concentrations. Increasing EE concentrations decreased the apparent kinetic constant for celecoxib (as indicated by EE 17 β -*O*-sulfation), suggesting that the formation of EE complex does not inhibit the binding of celecoxib. Collectively, these results lead to the conclusion that EE and celecoxib interacted with separate binding sites of SULT2A1 and the site bound by celecoxib could modulate the activity and regioselectivity of EE sulfation.

16.2.3.2 Practical Considerations. In a study conducted by Maiti *et al.* [88], it was shown that human SULT1E1 was inhibited in a time- and concentration-dependent manner by oxidized glutathione (GSSG) and that the redox status of Cys83 was implicated in affecting the enzyme activity. In this study, the presence of PAPS was found to have a protective effect against GSSG oxidative inactivation [88]. Moreover, these investigators also found that SULTs 1A1, 1A3, and 2A1 were not affected by redox conditions. Thus, it would appear that depending on the isoform, and perhaps the substrate, the redox state of key active site cysteine(s) may or may not influence SULT activity. Although the *in vivo* significance of this remains to be determined, the implications for *in vitro* experiments are clear. Investigators performing *in vitro* studies should be cautious to maintain reduced incubation/storage conditions, so as to more closely mimic the standard *in vivo* redox state.

Organic solvents have also been demonstrated to modulate SULT-mediated reactions [72]. In bench-top incubations, the presence of 0.4% (v/v) acetonitrile-activated SULT1E1-catalyzed 1-hydroxypyrene sulfation by ~ 2.6 -fold. When a two-site kinetic model was applied, studies demonstrated that acetonitrile affected $V_{\max 1}$, $V_{\max 2}$, and the K_i value of 1-hydroxypyrene sulfation, while there was no significant effect on the K_m value (the second binding site was postulated to be inhibitory and the binding constant was termed K_i). This suggests that solvent may potentially alter binding interactions of the second substrate molecule but not the first. Of course, there are other potential interpretations for this data; however, given the potential effects of organic solvents on SULT activities, it was recommended that ethanol should be used as the preferred solvent vehicle [72].

Divalent cations also have been shown to affect the activity of SULT enzymes. SULT enzymatic activity may be stimulated in the presence of both Mn^{2+} and Mg^{2+} . Using point-mutated SULT1A3 enzyme, Pai *et al.* [89] demonstrated that Mn^{2+} may exert a stimulatory effect by interacting with Glu86. Zhang *et al.* [66] found that Mg^{2+} stimulated SULT1E1 in a bell-shaped manner. Unfortunately, these findings were not supplemented with additional experimental work. In addition to SULTs, other drug-metabolizing enzymes are also affected by divalent cations. It has been recommended

that CYP3A4 incubations include Mg^{2+} , and in particular, since the free concentration of Mg^{2+} in liver is ~ 0.5 mM, it was suggested that incubations with CYP3A4 should include Mg^{2+} at a similar concentration [90]. By analogy, it may be necessary to include divalent cations in SULT incubations. However, the significance of cation inclusion, in terms of improving the ability to extrapolate *in vitro* data to *in vivo*, is not yet known.

Finally, careful attention to the amount of PAPS used in laboratory incubations is important. For example, the inhibition of 1-hydroxypyrene sulfation (using expressed SULT1E1) by mefenamic acid revealed that different inhibitory IC_{50} values could be obtained when different concentrations of PAPS were used in the incubation (data not published). In addition, when Kudlacek *et al.* [91,92] studied the sulfation of minoxidil, they reported a K_m value of 2.9 mM using a PAPS concentration of $0.4 \mu M$. In human platelets, Johnson and Baker [93] studied minoxidil sulfation and reported data using a concentration of $0.86 \mu M$ PAPS. The K_m value for PAPS has been reported to range from 0.16 to $0.66 \mu M$. However, the amount of PAPS in liver has been estimated to be ~ 17 – 77 nmol/g of tissue, or much higher than the concentrations used in the studies referenced above [33,37,66,94,95]. Saturation conditions for PAPS would be expected to be four to five times the K_m value or ~ 0.8 – $3.3 \mu M$. *In vivo*, it would appear that normal PAPS concentration is at a saturating level. Therefore, in laboratory incubations, it would be prudent to also use saturating PAPS concentrations to more closely estimate a relevant *in vivo* concentration and to avoid confounding kinetic results obtained when PAPS concentrations are no longer sufficiently above the K_m value. Moreover, it is important to ensure that sufficient amount of PAPS is used in kinetics experiments such that saturation of product formation observed at high substrate concentrations (e.g., 10 mM) is not an artifact caused by PAPS depletion.

16.2.4 Sulfation of Endogenous Substrates

In Section 16.1.4, the role of SULTs in fetal development was briefly discussed. In this section, other endogenous roles for SULT are explored. One such role is the sulfation of catecholamines. Catecholamines are mediators of physiological stress, and they prepare the body for physical activity. Some physiological responses include increased heart rate, blood pressure, and an increase in blood glucose levels [96]. Circulating levels of catecholamines, such as dopamine, are present primarily in the sulfated form and derive from the sulfation of dietary dopamine or sulfation of dopamine from the myenteric plexus [97]. In fact, the total circulating concentration of free dopamine was found to be less than 4% [98]. This observation is consistent with early experiments by Richter [99], who showed that the majority of orally administered epinephrine was excreted in urine as a sulfate conjugate. Also consistent is the observation that SULT1A3 is expressed at high levels in the intestinal tract (Section 16.1.3), which would contribute significantly to sulfated dopamine derived from the myenteric plexus.

The high level of SULT1A3 expressed in the intestinal tract has been suggested to serve as a “gut–blood” barrier [100]. This hypothesis is supported by the observation that fetal livers contain high levels of SULT1A3 levels, but that after birth (when exogenous dopamine levels will be obtained through the gastrointestinal (GI) tract) the expression of SULT1A3 is “turned off” in the liver [101]. Thus, while fetal livers contain significant SULT1A3 activity, adults do not. The capacity of SULT activity in

the GI tract appears to form a mechanism for detoxifying dietary biogenic amines in postnatal life, whereas the fetal liver takes on this role in prenatal life.

The two most abundant steroid sulfates present in plasma are DHEA and cholesterol. Blood concentrations of both can achieve low micromolar levels; however, unlike DHEA, cholesterol sulfate levels remain relatively constant throughout life [102]. Cholesterol sulfate is perhaps one of the more interesting sulfate conjugates because its physiological role and importance have been extensively studied. Initially, cholesterol sulfate was studied in the context of serving as a precursor for the synthesis of sulfonated steroids. The sulfate conjugate is a direct substrate for the synthesis of other adrenal steroids such as pregnenolone sulfate and DHEA sulfate, without removal of the sulfate moiety [103,104]. Moreover, cholesterol sulfate has also been shown to be a normal constituent of biological membranes (where it acts as a stabilizer) and a regulator of the activity of a number of enzymes (e.g., activating Factor XII, epidermal protein kinase C enzymes, etc.) [97,105,106]. Perhaps the most fascinating role that the sulfate conjugate serves is its involvement in keratinocyte differentiation and development of the epidermal barrier. Cholesterol sulfate conjugate is formed in the basal and spinous layer of the epidermis; these two layers are generally the lowest of the four to five skin layers that compose the epidermis [107,108]. Cholesterol reaches its highest concentration in the granular layer (third layer from the surface) and then concentrations decrease in the stratum corneum (first layer) because of increased sulfatase activity. Thus, sulfated cholesterol begins its journey through the epidermis in the lower layers, and as keratinocytes start to differentiate and migrate out to the surface, cholesterol sulfate is hydrolyzed by steroid sulfatase releasing free cholesterol, which is then available for conjugation in lower layers [107,108]. It has been hypothesized that this cycle plays an important role in normal desquamation (skin peeling or shedding) [109]. This idea is supported by the fact that patients with recessive X-linked ichthyosis, caused by steroid sulfatase deficiency, have difficulty (among other symptoms) with desquamation [110]. Finally, it is interesting that cholesterol is a substrate for SULT2B1_v2 (a splice variant of the *SULT2B1* gene) and that SULT2B1_v2 is significantly expressed in the granular layer, which, noted above, is the layer where cholesterol sulfate reaches the highest concentration [111].

16.2.5 Bioactivation

In the fields of drug metabolism and toxicology, bioactivation of compounds is a crucial topic that has been studied extensively [112,113]. The majority of the bioactivation processes are caused by cytochrome P450-mediated reactions that generate chemically reactive species, potentially leading to various types of toxicity. It has been shown that oxidative metabolites containing electron-deficient groups, such as epoxide and quinone/quinone imine/quinone methide, can form DNA adducts and eventually cause mutagenicity and genotoxicity [114–116]. Sulfation, as it turns out, is another bioactivation mechanism that can generate mutagenic products [117,118]. Sulfate conjugates of *N*-hydroxy aromatic amines (e.g., *N*-hydroxy-acetylaminofluorene) undergo N–O cleavage to form reactive nitrenium ions (Fig. 16.3), resulting in subsequent formation of DNA adducts and gene mutations in bacteria and mammalian cells [119,120]. Similarly, carbocations have been identified to be the reactive species generated from sulfation products of polycyclic benzylic alcohols (e.g., 1-hydroxymethyl pyrene) and allylic alcohols (e.g., 1'-hydroxyestradiol) [121,122]. Note that the positive charge of

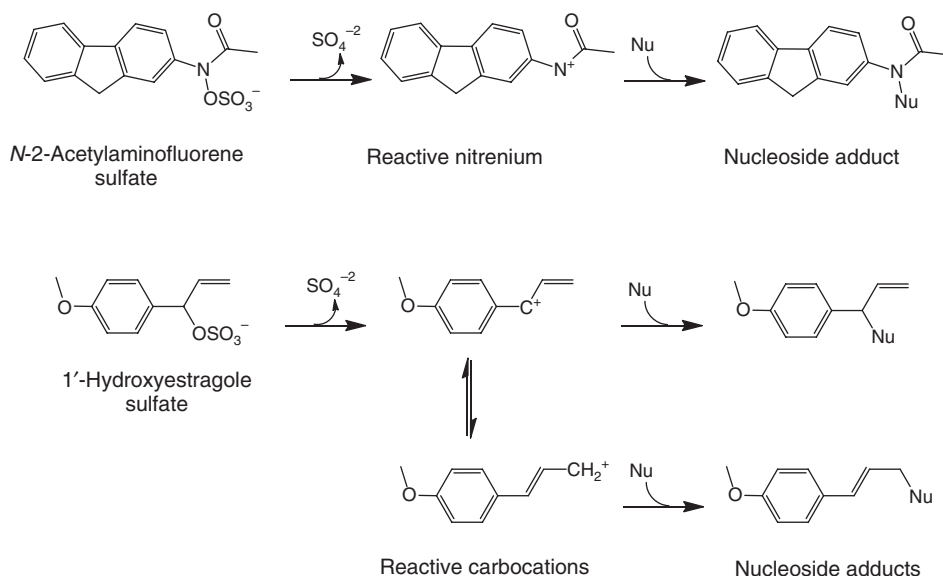


Figure 16.3 Bioactivation of sulfonate conjugates via the formation of reactive nitrenium and carbocation intermediates. Nu, nucleoside.

carbocation/nitrenium is stabilized by the conjugated system as evidenced by DNA adducts with alternative adduction sites arising from resonance structures. Mutagenicity observed from *in vitro* systems has been shown to be compound-dependent and SULT isoform-dependent [117,118]. It has been hypothesized that tissue expression and genetic polymorphism of a particular SULT isoform may influence the genotoxic effects of a given promutagen or procarcinogen. Further evaluations using humanized animal models have been proposed to gain insights on this matter.

16.3 CLINICAL PERSPECTIVES

16.3.1 Polymorphisms

Pioneer studies conducted by Eichelbaum *et al.* and Mahgoub *et al.* in the 1970s illustrated that the reduced metabolism of sparteine/debrisoquine was caused by deficiency of a single gene (now known as *CYP2D6 polymorphism*) [123]. Since then, genetic polymorphisms have been studied for CYPs [124] as well as other drug-metabolizing enzymes such as glutathione *S*-transferases [125], *N*-acetyltransferases [126], UDP-glucuronosyltransferases [127,128], and SULTs [18,129–134].

Polymorphism of SULT1A1 is the most extensively studied among various SULT enzymes. A number of single nucleotide polymorphisms (SNPs) have been identified for *SULT1A1* gene, however, only a few of these SNPs are located at the coding region and affect the amino acid sequence (nonsynonymous cSNPs). *SULT1A1**2 allele, the most common allelic variant other than *SULT1A1**1 allele (wild type), has a G→A mutation at nucleotide 638 in the coding region, resulting in an arginine to histidine substitution at amino acid 213 (Arg213His). By measuring SULT1A1 activity

in human platelet cells with 4-nitrophenol as a probe substrate, individuals homozygous for the *SULT1A1**2 allele were shown to have ~15% of the enzyme activity of *1A1**1/*1A1**2 heterozygotes or *1A1**1 homozygotes [132]. The lower enzyme activity of *SULT1A1**2 allozyme appears to result from, at least in part, preferential degradation via the proteasome pathway as compared to *SULT1A1**1 [133]. The second most common *SULT1A1* SNP is A667G, leading to amino acid substitution Met223Val. This variant is termed *SULT1A1**3. Kinetics studies showed that *SULT1A1**3 allozyme generally exhibits lower K_m and higher V_{max} values for various substrates when compared to the wild-type enzyme. Ethnic variation in allele frequencies for *SULT1A1**2 and *SULT1A1**3 has been reported [130,134]. For *SULT1A1**2, the frequency was higher in Caucasian (0.254–0.365), African (Nigerian and African-American; 0.269–0.294), and Turkish (0.238) than Japanese (0.168), Chinese (0.045–0.110), Korean (0.116), and Egyptian (0.135) populations. Interestingly, *SULT1A1**3 allele is highly prevalent in African-Americans (frequency 0.229). It is detected in low frequency in Caucasians (0.000–0.029) and is virtually absent in other ethnic groups.

Nonsynonymous SNPs have also been identified for *SULT1E1* and *SULT2A1* [18,129–131]. Three SNPs (G64T, C95T, and C758A) were observed in *SULT1E1*, resulting in amino acid changes Asp22Tyr, Ala32Val, and Pro253His, respectively. These variants appear to be very rare (allele frequency 0.008) in Caucasians and African-Americans combined. Although the Val32 (*SULT1E1**2) allozyme has similar affinities for β -estradiol and cofactor PAPS relative to the wild-type allozyme, the K_m values determined for Tyr22 and His253 allozymes are significantly higher. For *SULT2A1*, nonsynonymous polymorphisms (A63P, K227D, and A261T) have been identified in African-Americans but not in Caucasian Americans or Japanese. Allozymes expressed in COS cells generally showed decreased enzyme activities and lower immunoreactive protein levels. Surprisingly, the Tyr261 allozyme, resulting from a G→A mutation at nucleotide 781, appears to be a monomer rather than dimer. Despite that difference, the Tyr261 and the wild-type allozymes display comparable affinities for DHEA and PAPS. The allelic frequency for the G781A variant is 0.101–0.144 in different African-Americans populations.

It is important to point out that polymorphisms observed in SULTs have not been reported to significantly alter the elimination of drugs [135,136]. This is likely due to the fact that virtually all drugs undergo sulfation also having alternative elimination pathways such as direct excretion, oxidation, and glucuronidation.

16.3.2 Induction

Many xenobiotics act as perpetrators in pharmacokinetics drug–drug interactions by inducing drug-metabolizing enzymes, which results in an increased elimination of the victim compounds [137,138]. Elevation of drug-metabolizing enzymes usually begins at the transcriptional level when nuclear receptors are activated by the binding of chemical inducers. In the case of CYP enzymes, nuclear receptors such as aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and farnesoid X receptor (FXR) have been shown to regulate enzyme expression levels. *In vitro* studies conducted in primary human hepatocytes indicated that activation of AhR, PXR, CAR, LXR, and FXR did not significantly induce mRNA expression of various SULTs [139–141]. In fact, repression of gene expression was

observed for *SULT1E1* and *SULT2A1* in human hepatocytes treated with rifampicin [141,142], a known ligand for human PXR. Such repression was shown to be mediated by hepatocyte nuclear factor 4 α . Human hepatocytes treated with dexamethasone and ciprofibrate, prototypical ligands for GR and PPAR α , respectively, led to increases in *SULT2A1* mRNA and protein levels [140,143]. However, the clinical significance of *SULT2A1* induction remains to be determined.

16.3.3 Reaction Phenotyping

Identification of drug-metabolizing enzymes responsible for the biotransformation of a new drug (new chemical entity, NCE) is a necessary and pivotal study in the drug discovery and development process. The results of these studies, usually referred to as *reaction phenotyping studies*, are utilized to evaluate several issues: the potential of the NCE to be involved in a potential drug–drug interaction via inhibition of the enzyme(s) responsible for NCE metabolism; evaluation of the effect of induction on the NCE metabolism; and finally, the possible effects of genetic polymorphism of NCE metabolism. Although the historical focus of reaction phenotyping studies has focused on CYP enzymes, there is an increasing effort in recent years to characterize biotransformations catalyzed by non-CYP enzymes such as SULTs [29,70,75,144,145].

It has been well established for CYP enzymes that the reaction phenotyping studies generally consist of (i) correlation analyses utilizing a bank of individual tissue preparations characterized for enzyme activities; (ii) kinetic determinations with recombinant enzymes and tissue preparations; and (iii) inhibition experiments using isoform-selective chemical inhibitors and/or antibodies [146]. In the authors' experience, the last method is by far the most commonly used in industry. Attempts have been made to adopt these approaches for SULT enzymes; however, the success appears to be handicapped by limited knowledge and/or reagents available.

Correlation analysis involves the comparison of the interindividual variability of the sulfation rate of interest with the previously determined activity or protein level of a given SULT isoform. Hence, in order to determine SULT isoform enzymatic activity, the availability of SULT-isoform-selective probe substrates is vital to the correlation approach. Reasonably good correlation between protein expression and enzyme activity was observed for *SULT1A1*, *SULT1E1*, and *SULT2A1* (using 4-nitrophenol, β -estradiol, and DHEA as probe substrates, respectively) in human liver cytosol ($r \sim 0.64\text{--}0.86$) [43]. However, a selective probe substrate for *SULT1B1* has not been identified to-date. Apart from this, the fact that SULTs usually have overlapping substrate specificities [70,145,147] make correlation studies challenging and the results difficult to interpret. As such, the correlation approach has not been widely used for SULT phenotyping.

Screening of sulfate conjugation with recombinant SULT enzymes has been commonly employed as the initial approach, facilitated, at least in part, by the commercially available SULT enzymes (*SULT1A1*, *SULT1A3*, *SULT1E1*, and *SULT2A1*) representing the majority of isoforms detected in human liver and GI tract [43]. Kinetic studies are then conducted in recombinant enzymes and in tissue preparations to determine the affinity for the substrate (K_m) and the type of kinetics (e.g., hyperbolic vs substrate inhibition). Since the substrate affinity for a single enzyme should be similar regardless of the source/preparation (ideally), comparison of the K_m values determined in recombinant enzymes with that obtained in tissue preparations provide additional evidence

in identifying the SULT enzyme(s) responsible for the sulfation reaction observed in tissue.

Inhibition studies represent a primary methodology for reaction phenotyping. In particular, isoform-selective chemical inhibitors and/or immunoinhibitory antibodies allow direct quantitative assessment of the contribution of individual isoforms at a given drug concentration (often the concentration used is physiologically relevant), as exemplified in a countless number of CYP-mediated biotransformations. Unfortunately, isoform-selective inhibitory antibodies for SULTs are not yet commercially available. 2,6-Dichloro-4-nitrophenol (DCNP) is a potent inhibitor of SULT1A1 ($IC_{50} < 0.1 \mu M$) with selectivity of more than 2 orders of magnitude over SULT1A3, SULT1B1, SULT1E1, and SULT2A1 [144,148–150]. The compound is suitable to be utilized as a SULT1A1-selective inhibitor in human liver and GI preparations. Apart from DCNP, there has not been any other isoform-selective inhibitor reported for SULTs. It has been suggested that an ortho-hydroxy-polychlorinated biphenyl (6'-OH-PCB) could potentially serve as a specific inhibitor of SULT1B1 [151]. However, inhibition results in this study may have been confounded by substrate inhibition, poor inhibitor/substrate solubility, as well as the use of a substrate concentration (3-hydroxybenzo[a]pyrene) that was ~6-fold greater than the reported K_m value for SULT1A1*1, and ~20-fold lower than the reported K_m for SULT1B1 [152]. Because 6'-OH-PCB may serve as an isoform-selective inhibitor, further experiments to confirm the inhibition of SULT1B1 would be useful. In addition, compounds such as quercetin and estrone could also serve as probe inhibitors but further experiments are needed [75,144]. At $1 \mu M$, quercetin strongly inhibited sulfation activities catalyzed by recombinant SULT1A1 and SULT1E1 but had no effect on SULT1A3 and SULT2A1 activities. Similarly, estrone (at 40 nM) greatly inhibited (>75%) SULT1A3 and SULT1E1 activities but had minimal effect on SULT1A1 and SULT2A1 activities. Caution should be exercised when interpreting inhibition results obtained with quercetin and estrone as their inhibitory effects on SULT1B1 and other SULT enzymes have not been examined. Overall, reaction phenotyping for SULTs can be performed to some extent, and the quality will certainly improve when additional reagents become available in the future.

16.3.4 Drug–Drug Interactions

A large number of *in vitro* studies have been published, which suggest potential *in vivo* SULT drug–drug interactions. However, there are few confirmed *in vivo* drug interactions that are mediated by this class of enzymes. Moreover, as noted above, polymorphisms affecting SULT activity (which can mimic chemical inhibition) have not been clearly associated with significant changes in drug elimination [135,136].

The most commonly published example of a SULT-mediated drug–drug interaction involves an indirect mechanism. In humans, a single dose of acetaminophen (1.5 g) has been shown to cause partial depletion of inorganic sulfate [153]. This observation is relevant to SULT drug–drug interactions because the level of inorganic sulfate affects the level of PAPS [154,155]. The activity of all SULT reactions depend on the availability of PAPS as a cofactor, which in turn is dependent on the rate of PAPS synthesis and degradation [156]. Cellular uptake of inorganic sulfate depends on two uptake mechanisms: one is Na^+ independent and the other is Na^+ dependent [157–159]. Once uptake has occurred within the cell, PAPS is synthesized from inorganic sulfate by what is thought to be a bifunctional protein, associated with ATP-sulfurylase and adenosine

5'-phosphosulfate kinase activities [160]. Thus, indirect drug interactions can occur through a variety of mechanisms. To illustrate one mechanism, the concomitant administration of acetaminophen and a compound that undergoes significant sulfation can potentially lead to a competitive interaction in which a capacity-limited pool of PAPS is depleted by sulfation of one or both drugs [161–163]. In addition, other mechanisms may also include the inhibition of transporters involved in the cellular uptake of inorganic sulfate, or inhibition of PAPS synthase itself [156]. In the case of acetaminophen where doses are high (on a milligram per kilogram basis), competition for and depletion of the PAPS pool has been hypothesized as the mechanism behind interactions with fenoldopam and EE [164,165]. However, a study conducted in healthy male volunteers showed that codosing acetaminophen with troglitazone resulted in no statistically or clinically relevant differences in troglitazone exposure area under the plasma drug concentration-time curve ($AUC_{0-\infty}$) [166]. Troglitazone is metabolized to a sulfate conjugate, a glucuronide conjugate, and an oxidative quinone metabolite. Under steady state conditions, the AUC of the sulfated metabolite was 7–10 times greater than that of parent, whereas quinone exposure was similar to parent. The glucuronide was a minor metabolite (20–40% of parent) [167]. Thus, sulfation represented a major pathway for troglitazone metabolism, yet the coadministration of acetaminophen did not affect exposure. In fact, when troglitazone and acetaminophen were concurrently administered only a slight decrease in the troglitazone sulfate metabolite was observed; the decrease was not judged to be clinically relevant [166].

In addition to an indirect mechanism, SULT-mediated drug–drug interactions may result from a more traditional mechanism where a single SULT serves as the locus of the interaction. One example of this is the interaction of etoricoxib with EE. EE is a primary active component used in many oral contraceptive (OC) formulations; the metabolism of EE is complex, involving cytochrome P450, glucuronosyltransferases and SULT [168]. The absolute oral bioavailability of EE is variable and ranges from 20% to 65% [169]. In urine, the major metabolites excreted are glucuronides (up to 85%) followed by sulfate conjugates (up to 19%) and then low levels of monooxidation products [170]. Because more than one enzymatic pathway is involved in EE metabolism it would be expected that EE should be relatively resistant to drug–drug interactions. However, EE is subject to significant gut first-pass metabolism, and it has been shown that up to 38% of the EE dose forms conjugates in human jejunal mucosa and ~90% of the conjugates can be hydrolyzed by sulfatase [171]. These results imply that sulfation of EE is considerable in the GI tract and may be a major factor affecting bioavailability. As a consequence, it has been postulated that inhibition of SULTs in the GI can serve as a locus for drug–drug interactions, affecting the bioavailability of EE [172]. In support of this hypothesis, acetaminophen was orally administered 1 h before OC administration and subsequently, the pharmacokinetics of EE demonstrated that AUC_{0-24} was increased 22% [165]. It was hypothesized that the bioavailability of EE increased in response to depletion of the PAPS pool. Interestingly, although the investigators in this study did not report C_{max} values of EE, the C_{max} appears to have increased 60–70%. This increase in C_{max} was consistent with the hypothesis of lower intestinal SULT activity, which then resulted in increased absorption from the gastrointestinal lumen.

In an example of a direct drug–drug interaction caused by SULT inhibition, etoricoxib has been reported to inhibit EE sulfation activity catalyzed by SULT1E1, the major SULT isoform catalyzing the sulfation of EE [144]. It was further hypothesized

that after an oral dose, the high local intestinal exposures of etoricoxib could interfere directly with first-pass sulfation of EE via inhibition of SULT1E1. Evidence for this inhibition was published later by an *in vivo* drug–drug interaction study involving EE and etoricoxib. Data presented within this report show that coadministration of EE with etoricoxib was associated with a 50–60% increase in EE exposure, and a decrease of EE-sulfate exposure by ~40% [173].

16.4 CONCLUSIONS AND FUTURE PROSPECTS

SULTs are an important class of enzymes and are involved in many endogenous processes such as estrogen biosynthesis, influencing receptor-active thyroid hormone levels and sulfation of catecholamines and steroids (e.g., DHEA or cholesterol). For drug metabolism scientists, sulfation is of interest because it can potentially lead to electron-deficient species, and therefore, it is important to be aware of potential structural alerts that may increase the risk of SULT-mediated reactive intermediates. However, relative to other drug-metabolizing enzymes, SULT-mediated elimination of therapeutic compounds is rather limited; for this reason, and others, the contribution of SULTs to drug metabolism has been underappreciated, particularly in industry. With increased availability and use of high quality hepatocytes, the importance of SULTs to drug metabolism will become more evident in the future. As a result, drug metabolism scientists will be faced with many challenges. Foremost is the lack of a “chemical toolbox” to study SULT isoform involvement in drug elimination. While reaction phenotyping is readily accomplished with cytochrome P450s, there is no panel of well-characterized SULT-isoform-specific inhibitors. During the course of determining the mechanism for a clinical drug–drug interaction of etoricoxib with ethinyl estradiol, the authors of this chapter screened several hundred compounds in an effort to obtain specific inhibitors for a number of SULT isoforms (unpublished). This effort was largely unsuccessful. Thus, approaches other than screening (such as rational design from crystallography/computational chemistry) may have to be utilized to efficiently generate isoform-specific inhibitors.

The literature contains several examples of isoform-specific or isoform-selective SULT substrates, but the characterization of those substrates against all the relevant SULT isoforms is often lacking. As a consequence, the study of SULT inhibition and/or induction is hampered by the ambiguity surrounding the use of “probe” substrates. In the future, the discovery, development, and characterization of a panel of isoform-selective inhibitors and substrates will greatly increase the success of metabolism studies during drug discovery and development. Increased success in this area will also serve to further highlight the importance of this class of enzymes in drug metabolism [97,98].

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17 Functional Genomics of the Human Glutathione Transferases

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17.1 Summary	1
17.2 Introduction	2
17.3 The GST gene families	3
17.4 The cytosolic GSTs	3
17.5 Cytosolic GST genes	5
17.6 Genetic polymorphism of human GST genes	7
17.7 Structure and function of the cytosolic glutathione transferases	17
17.8 GST-deficient mice	21
References	21

17.1 SUMMARY

The glutathione transferase (GST) superfamily contains a number of enzymes that catalyze the conjugation of glutathione (GSH) to a wide range of compounds of both endogenous and exogenous origin. In addition to catalytic reactions, the GSTs also modulate several cell signaling kinases and ion channels such as Jun N-terminal kinase (JNK) and ryanodine receptors via protein/protein interactions. Pharmacogenetic studies have identified variations in drug response and susceptibility to cancer and other disorders associated with variant GST isoforms. In humans, the cytosolic GSTs have been subdivided into seven distinct classes termed *Alpha*, *Mu*, *Pi*, *Theta*, *Sigma*, *Zeta*, and *Omega*, and there are multiple genes in some classes. So far, 48 allelic variants that cause amino acid substitutions have been documented, and in many cases, the variant proteins have been functionally and structurally characterized. Common deletions of the *GSTM1* and *GSTT1* genes have been extensively investigated in relation to cancer susceptibility and drug response, and they have also been the subject of a vast number of disease association studies. Some polymorphisms in promoter regions have been shown to affect the level of GST expression *in vitro* and *in vivo* and this area requires further investigation.

The various members of the GST superfamily are characterized by striking differences in the reactions they catalyze and their substrate specificities. The structures of

representatives of all the human GST classes have been determined by X-ray crystallography and their differing reaction mechanisms have been investigated by mutagenesis and functional studies.

17.2 INTRODUCTION

The GST superfamily contains a number of enzymes that catalyze the conjugation of GSH to a wide range of compounds of both endogenous and exogenous origin. The GSTs have been intensively studied because of their capacity to protect cellular components from attack by electrophiles, and consequently, they have been implicated in the development of drug resistance and susceptibility to carcinogens. Pharmacogenetic studies have identified variations in drug response and susceptibility to cancer and other disorders associated with variant GST isoforms. A review by Hayes and Pulford [1] provides an excellent summary of the types of environmentally derived compounds that are conjugated or transformed by the action of GSTs. In addition, the Comparative Toxicogenomics Database provides an extensive compilation of publications reporting chemical/GST/disease interactions [2]. GSTs contribute to around 10% of the cytosolic protein in rat liver, and as they can be detected as discrete bands on coomassie blue stained SDS-PAGE gels of liver cytosol, it seems likely that GSTs are involved in additional physiological processes. An early study noted the identity of a rat GST with “ligandin,” a protein known for its capacity to bind a range of hydrophobic compounds, including carcinogenic azodyes, corticosteroids, and bilirubin [3,4]. In recent years, it has been shown that the physiological roles of GSTs extend well beyond the disposition of xenobiotics and include both enzymatic and nonenzymatic functions. Some GSTs catalyze cis–trans isomerase reactions that are important in steroid hormone synthesis and the catabolism of tyrosine [5–7], while others protect cells against the products of lipid peroxidation by catalyzing the reduction of lipid hydroperoxides [8] or the conjugation of reactive α,β -unsaturated carbonyl compounds such as 4-hydroxy nonenal [9–11]. In addition, GSTs have been identified, which catalyze thioltransferase reactions and the reduction of biologically significant molecules such as dehydroascorbate [12,13]. Members of the GST superfamily have been shown to engage in protein–protein interactions that can have a direct effect on several signaling pathways. The stress-activated kinase Jun N-terminal kinase, apoptosis-stimulating kinase 1 (ASK1), and tumor necrosis factor (TNF) receptor-associated factor 2 have all been shown to be modulated by GST binding [14–16] and there is evidence that Fanconia anemia group C protein (FANCC) can modulate the activity of GSTP1-1 during apoptosis [17]. A number of studies have shown that the chloride intracellular channel (CLIC) proteins are members of the GST superfamily and can enter membranes to form or modulate ion channels [18–21]. The human muscle-specific GST, GSTM2-2, has been shown to be a potent inhibitor of the cardiac ryanodine receptor Ca^{2+} channels and may play a key role in modulating excitation contraction coupling in the heart [22–24].

GSTs and GST-like proteins are almost ubiquitous in nature and there are close to 30,000 papers concerning GSTs listed in PubMed. Many aspects of this large field have been extensively reviewed [1,8,25–37]. This chapter focuses on the genetic variation in the human GSTs and its impact on their structure and function.

17.3 THE GST GENE FAMILIES

The evolutionary history of the GSTs is complex. Phylogenetic analysis has shown that at least four families of proteins with GST activity have emerged by convergent evolution [38]. In mammals, there are three well-recognized structurally and genetically distinct families of proteins that catalyze GST reactions. These families have been generally termed the *cytosolic*, *microsomal*, and *mitochondrial GSTs* [8,33,39–41]. The cytosolic GSTs are a large multiclass family of proteins that share a common structural fold and an ability to catalyze a variety of GSH-dependent reactions. Classically, they have been studied as drug and xenobiotic detoxication enzymes, conjugating GSH to small hydrophobic molecules in phase II drug metabolism reactions. However, as the number of members of this gene family has expanded with the genome era, so has the type of reactions and functions associated with these proteins. The membrane-bound microsomal GSTs have also been termed the *membrane-associated proteins in eicosanoid and glutathione metabolism* (MAPEG) [39]. There are at least six members of this family in humans, and although most have the capacity to conjugate GSH to 1-chloro-2,4-dinitrobenzene (CDNB), they appear to play prominent roles in the synthesis of leukotrienes and prostaglandins *in vivo* [39]. Although some cytosolic GSTs are also found within mitochondria, the Kappa class GST is found specifically within mitochondria and peroxisomes [42]. There is a single Kappa class gene in humans, rats, and mice and their amino acid sequences show little identity with the cytosolic or MAPEG GST families. Although the structure of the Kappa class GSTs contains a thioredoxin fold domain that is reminiscent of the cytosolic GSTs, it contains a polyhelical domain embedded in the linear sequence between the N-terminal region β -strands [40,41]. This contrasts with the cytosolic GSTs where the α -helical domain is encoded entirely within the C-terminal sequence (Fig. 17.1). This difference in structure suggests that the Kappa class GSTs also evolved via a distinct but convergent evolutionary path.

17.4 THE CYTOSOLIC GSTs

17.4.1 A Historical Perspective

This chapter focuses primarily on the cytosolic GSTs as they are considered to be of most significance in drug and xenobiotic metabolism. Originally, multiple cytosolic GSTs were identified by the reactions they catalyzed, their elution profile from ion

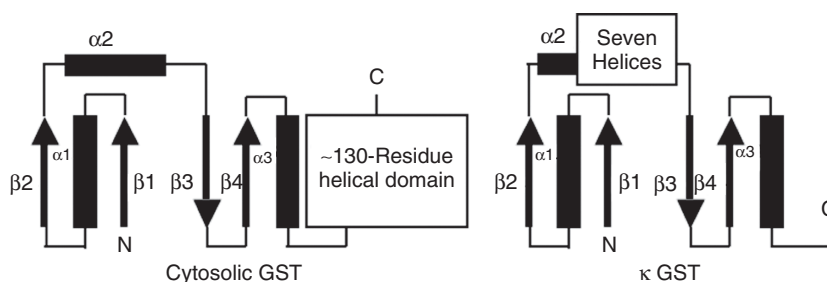


Figure 17.1 Topology diagrams comparing the secondary structural organization of cytosolic GSTs with the Kappa class GST [41,43]. β -Strands are shown as arrows and α -helices are shown as rectangles.

exchange chromatographic columns, their molecular weight, their isoelectric point, and their native electrophoretic mobility [44–47]. These and many other studies led to the development of a range of largely laboratory-specific nomenclature systems that are quite confusing to new readers of the older literature. Although there was early evidence that some GST isoenzymes had other substrate preferences [45,48,49], most GSTs were found to utilize CDNB as a substrate. The discovery of GSH affinity chromatography and the use of CDNB as a substrate allowed the biochemical characterization of many GSTs; however, this focus also delayed the discovery of GSTs that do not bind to GSH affinity matrices and do not use CDNB as a substrate [50–52]. The discovery and characterization of the Theta, Zeta, Omega, and CLIC proteins in mammals came from the utilization of different substrates and the use of bioinformatics to analyze sequence databases [12,18,53,54]. The discovery of novel GSTs by the use of bioinformatics subsequently presented an interesting challenge to identify their substrates.

17.4.2 Classification of the GSTs

The confusion in GST nomenclature was resolved in the early 1990s. The cytosolic GSTs were classified into a number of different classes that are designated by the Greek alphabet. The classes were originally defined in humans, rats, and mice on the basis of their amino-terminal sequence and their antigenic identity. An international nomenclature convention has been established that is theoretically extendable to other species [55,56]. Readers should refer to these reports for a definitive description of the accepted nomenclature system. Seven distinct classes have been well characterized in mammals and have been termed *Alpha*, *Mu*, *Pi*, *Theta*, *Sigma*, *Zeta*, and *Omega*. Proteins in each class are termed by the initial letter of the class name. For example, GSTA1-1 specifies the homodimeric product of the Alpha class *GSTA1* gene and GSTA1-2 specifies the heterodimeric product of the *GSTA1* and *GSTA2* genes. Other mammalian proteins that share the same structural fold but have no known enzymatic activity have been identified but not formally classified as GSTs. These include the CLIC family of proteins that have been shown to form chloride channels and to modulate the activity of ryanodine receptor Ca^{2+} channels in muscles [18]. In addition, molecular models of ganglioside-induced differentiation-associated protein 1 (GDAP1) and its paralog GDAP1-like 1 (GDAP1L1) suggest that they have a GST fold, but they appear to be inactive with common GST substrates [57,58]. Although the role of GDAP1 has not been clearly defined, mutations in GDAP1 have been shown to cause a severe recessive form of Charot–Marie–Tooth disease [59,60].

A range of additional classes and GST-like enzymes have been specifically defined in other evolutionary orders, including the Delta and Epsilon classes in insects [61,62]. In plants, Phi, Tau, and Lambda classes have been described together with a dehydroascorbate reductase class that has not been assigned a Greek letter [63]. Numerous GSTs have been identified in different microbial species, and although many show sequence or functional similarities with mammalian GSTs, the class boundaries are indistinct [64–66]. The Zeta class is the most widely conserved class and has been reported in species from bacteria through to plants, fungi, insects, fish, birds, and mammals. The conservation of the Zeta class is probably because of its isomerase activity converting maleylacetoacetate to fumarylacetoacetate in the catabolism of tyrosine [6,54,67]. While the Zeta class has been both structurally and functionally conserved across evolutionary time, some GST classes have developed highly specialized functions while

maintaining structural conservation between species. Possibly the most striking functional divergence has occurred in the Sigma class GSTs that catalyze prostaglandin D₂ synthesis in mammals and act as lens crystallins in squids [68,69].

17.4.3 GST Function and Nomenclature

Historically, and in accordance with the accepted nomenclature system [70], GSTs have been assigned a number within each class (GSTA1-1, GSTA2-2, etc.) in order of their discovery. Since many of the duplication events that have generated the multiplicity of GSTs in specific classes have occurred independently in different species (see below), it unfortunately follows that GSTs that are members of a specific class and have been assigned the same number may have different catalytic functions and show significant differences in their regulation in different species. For example, human GSTA3-3 has specific steroid isomerase activity and potentially plays a significant role in steroid hormone synthesis [5]. However, mouse GSTA3-3 is an efficient conjugator of aflatoxin B1 and has low steroid isomerase activity [71]. This functional difference in the GSTA3-3 isoenzymes in humans and mice accounts for the relative resistance of mice to aflatoxin carcinogenesis compared with humans. In contrast, the GSTA4-4 isoenzymes are paralogous in mice and humans, as they both have high specific activity in the conjugation of 4-hydroxynonenal an endogenous product of lipid peroxidation [10,11]. As a result of these differences, care should always be taken in ascribing GST function on the basis of nomenclature.

17.5 CYTOSOLIC GST GENES

A summary of the human cytosolic GSTs and their corresponding genes is provided in Table 17.1. GST classes may include multiple genes and the number of enzymes expressed in each class can vary between species. For example, there are two Pi class and three Theta class GSTs in mice but only one Pi and two Theta class GSTs in humans. In humans, the genes that encode GSTs from each class are grouped into clusters on different chromosomes, indicating the role of gene duplication in generating the diversity that is apparent within many GST classes. A similar arrangement generally occurs in mice and rats but the mouse *GstA3* gene is known to occur on chromosome 1 and has been separated from other Alpha class genes that are located on chromosome 9 (Board and Liu, unpublished data) [72]. The GST genes in humans are relatively small in size most falling between 3 and 17 kb in length and are composed of five to nine exons (Table 17.1).

17.5.1 Human GST Pseudogenes

There are many GST pseudogenes within the human genome. Some are intronless and are derived from reverse transcripts of mRNA that have been inserted randomly in the genome. Intronless GST genes have been reported in *Drosophila melanogaster*, [110] but there is no evidence that any reverse-transcribed GST sequences in the human genome are transcribed and translated into protein. In contrast, there are numerous pseudogenes and gene fragments within the different classes of gene clusters [73,102]. These are further evidence of the on going role of gene duplication and gene conversion

TABLE 17.1 Human Glutathione Transferases and Their Genes

Enzyme					Gene		References
Class and Designation	Accession Number	Locus	Chromosome Band	Exons	Gene Size (kb)	Gene ID	
<i>Class Alpha</i>							
GSTA1-1	NP_665683	<i>GSTA1</i>	6p12.2	7	12.5	2,938	73–76
GSTA2-2	NP_000837	<i>GSTA2</i>	6p12.2	7	13.5	2,939	73,74,76–79
GSTA3-3	NP_000838	<i>GSTA3</i>	6p12.2	7	13.1	2,940	11,73,74
GSTA4-4	NP_001503	<i>GSTA4</i>	6p12.2	7	17.4	2,941	11,73,80,81
GSTA5-5	NP_714543	<i>GSTA5</i>	6p12.2	7	14.4	221,357	73,82
<i>Class Mu</i>							
GSTM1-1	NP_666533	<i>GSTM1</i>	1p13.3	8	6	2,944	47,83–87
GSTM2-2	NP_000839	<i>GSTM2</i>	1p13.3	8	16	2,946	86,88,89
GSTM3-3	NP_000840	<i>GSTM3</i>	1p13.3	9	7.1	2,947	86,90,91
GSTM4-4	NP_671489	<i>GSTM4</i>	1p13.3	8	9.4	2,948	86,92–94
GSTM5-5	NP_000842	<i>GSTM5</i>	1p13.3	8	6	2,949	86,95
<i>Class Pi</i>							
GSTP1-1	NP_000843	<i>GSTP1</i>	11q13.3	7	3.1	2,950	96–101
<i>Class Theta</i>							
GSTT1-1	NP_000844	<i>GSTT1</i>	22q11.23	5	8.2	2,952	53,102–104
GSTT2-2	NP_000845	<i>GSTT2</i>	22q11.23	5	3.8	2,953	102,105
GSTT2B-2B		<i>GSTT2B</i>	22q11.23	5	3.8	653,689	102,106
<i>Class Zeta</i>							
GSTZ1-1	NP_665877	<i>GSTZ1</i>	14q24.3	9	10.9	2,954	6,54
<i>Class Omega</i>							
GSTO1-1	NP_004823	<i>GSTO1</i>	10q25.1	6	12.5	9,446	12,107,108
GSTO2-2	NP_899062	<i>GSTO2</i>	10q25.1	7	30.6	119,391	107,108
<i>Class Sigma</i>							
GSTS1-1/ HPGDS	NP_055300	<i>HPGDS</i>	4q22.3	6	44.3	27,306	109

in the evolution of diversity within the class-specific GST gene clusters in different species. The Alpha class in humans provides an example of this diversity and the problems that can be associated with differentiating genes and pseudogenes. Normally, it is evident that a gene is functional if a corresponding mRNA can be identified. However, if a gene is only expressed transiently in highly specialized cells, it can be difficult to detect the complementary DNA (cDNA). Several studies have noted a number of pseudogenes and gene fragments within the human Alpha class gene cluster on the short arm of chromosome 6 [73,74]. One gene sequence putatively identified as *GSTA5* appeared to be complete with no frame shifts, premature stop codons, deletions or insertions, and acceptable intron/exon boundaries [73]. Although no evidence of the expression of this gene has been found, a recent study has shown that if the gene is driven by a heterologous CMV promoter, fully spliced mRNA and catalytically active enzyme can be expressed in HEK-293 cells [82].

17.5.2 Alternative Splicing of GST Transcripts

Alternative splicing of GST mRNAs is a potential source of variation that is significant in some other drug and xenobiotic metabolizing enzymes such as the UDP-glucuronosyltransferases but does not appear to generate functional diversity among the mammalian GSTs. Although alternative transcripts of human *GSTM4* [92] and *GSTT1* [111] and mouse *Gsto1* and *Gsto2* [107] have been reported, and bioinformatics studies on expressed sequence tags readily detect alternative splicing in other GSTs [112], there is no evidence at this stage that alternative transcripts give rise to variant GST proteins in mammals. In mice, several alternative *Gsto2* transcripts containing different 5' noncoding exons have been detected in EST databases and there are significant differences in the transcript size in different tissues suggesting that alternative splicing in 5' noncoding regions may be important in regulating expression in particular tissues or conditions [107]. In the parasitic nematode *Onchocerca volvulus*, alternative splicing of the Omega class GST Ov-GST3 may be dependent on a stress response [113]. In contrast to humans and mice, alternative splicing has been exploited extensively in insects to generate functional diversity that has implications in the development of insecticide resistance. Alternative splicing in the GST family has been reviewed recently by Wongsantichon and Ketterman [112].

17.6 GENETIC POLYMORPHISM OF HUMAN GST GENES

In addition to the variation in the GST family inherent in the number of classes and the variable number of gene loci within each class, a further layer of heterogeneity results from deletions and single nucleotide polymorphisms (SNPs). Genetic polymorphism can occur in coding and noncoding regions and can significantly modify gene expression and the function of the expressed enzymes. Genetic polymorphism can therefore result in individual differences that can have an impact on drug and xenobiotic metabolism. Some polymorphisms are common and are found in most populations, while others are rare and can be restricted to specific ethnic groups. A survey of PubMed reveals in excess of one thousand papers investigating the possible association of specific GST polymorphisms with a wide range of disorders that may be influenced by variations in the metabolism and disposition of drugs and other environmentally derived carcinogens and toxins. The validated polymorphisms in human GSTs that alter the amino acid sequence or have a direct impact on the expression, structure, and function of human cytosolic GSTs are provided in Table 17.2.

17.6.1 The Alpha Class

An excellent review of the genetic polymorphisms in the Alpha class GST genes has been published [114].

17.6.1.1 *GSTA1*. So far there are no validated SNPs in the coding region of *GSTA1* which cause an amino acid substitution. A single SNP (A375G) that is highly polymorphic in Europeans, Africans, and Asians has been detected, but it is synonymous and does not change the K75 residue [128]. Several linked SNPs (G-52A, C-69 T, C-115 T, T-567G, T-631G, and C-1142G) have been validated in the 5' noncoding region

TABLE 17.2 Characterized Polymorphisms in Human Cytosolic GSTs^a

Allele/Haplotype	Substitution or Deletion	Gene Frequency				Phenotype	References
		A	B	C	D		
<i>Alpha Class</i>							
GSTA1*A	Promoter	0.65	0.84	0.6	—	Wild-type allele	114
GSTA1*B	Promoter	0.35	0.16	0.4	—	A at −52 causes a significant reduction in hepatic expression	114
GSTA2*A	P110 S112, K196 E210	≈0.3	—	≈0.3	—	Wild-type allele	114
GSTA2*B	P110 S112, K196 A210	0.3	0.18	0.08	—	No observed effect	114
GSTA2*C	P110 T112, K196 E210	0.3	—	0.57	—	Reduced hepatic expression in Caucasians	114
GSTA2*D	P110 S112, N196 A210	0	0	0	—	Not observed so far	114
GSTA2*E	S110 S112, K196 E210	0.01	0.11	0.05	—	Low activity with CDNB and organic hydroperoxides; increased activity with azathioprene	114
GSTA3*A	I71	0.85	1	1	—	Wild-type allele	115
GSTA3*A	L71	0.15	0	0	—	Diminished activity with CDNB; no change in activity with Δ ⁵ -androstene-3-17-dione	115
GSTA4	—	—	—	—	—	No coding region SNPs validated	—
GSTA5	—	—	—	—	—	No coding region SNPs validated	—
<i>Mu Class</i>							
GSTM1*A	K173	0.5	0.07	0.16	—	Wild-type allele	47
GSTM1*B	N173	0.04	0.17	0.12	—	No functional difference	47
GSTM1*C	S85	0	0	0.07	—	Not characterized	116
GSTM1*O	Gene deletion	0.46	0.76	0.72	—	Associated with response to chemotherapy and susceptibility to cancer	47
GSTM2	—	—	—	—	—	No coding region SNPs validated	—
GSTM3*A	G147 V224	0.89	0.26	0.66	—	Wild-type allele	117
GSTM3*B	Intron deletion	—	—	—	—	—	118

GSTM3*C	G147 I224	0.11	0.73	0.34	—	Increased catalytic efficiency	117
GSTM3*D	W147, V224	—	—	—	—	—	117
GSTM3*E	W147 I224	—	0.016	—	—	Low specific activity	—
GSTM4	—	—	—	—	—	No coding region SNPs validated	—
GSTM5	—	—	—	—	—	No coding region SNPs validated	—
<i>Pi Class</i>							
GSTP1*I105	I105	0.43	0.81	0.66	—	Wild-type allele	119
GSTP1*V105	V105	0.57	0.19	0.34	—	Difference in stability and activity with carcinogenic diolepoxides	119,120
GSTP1*A114	A114	—	0.99	0.93	—	Wild-type allele	119
GSTP1*V114	V114	—	0.01	0.07	—	—	119
<i>Theta Class</i>							
GSTT1*O	Gene deletion ^a	21.8%	64%	23.7%	9.7%	Associated with carcinogen activation	121
GSTT1*B	P104	—	—	0.01	—	Causes deficiency, occurs in Sweden	122
GSTT1*N43	N43	0.01	0	0	0	Unstable	116
GSTT1*M65	M65	0	0	0	0.008	Unstable	116
GSTT1*N141	N141	—	—	—	—	No effect on activity	123
GSTT1*stop	Nucleotide (G412) deletion	0	0	0	0.008	Deficiency	116
GSTT1*I169	I169	0.13	0	0	0	Not characterized	116
GSTT1*K173	K173	—	—	—	—	Unstable, low activity	123
GSTT2*A	M139	—	—	0.98	—	Wild-type allele	102
GSTT2*B	I139	—	—	0.02	—	Function not studied	102
GSTT2p	Gene deletion	—	—	0.53	—	Lowers GSTT2 transcription	106
<i>Zeta Class</i>							
GSTZ1*A	L8, K32 R42, T82	—	—	0.086	—	Resistant to inactivation by DCA	124
GSTZ1*B	L8, K32 G42, T82	—	—	0.285	—	—	124
GSTZ1*C	L8, E32 G42, T82	—	—	0.473	—	Wild-type allele	124

(continued overleaf)

TABLE 17.2 (continued)

Allele/Haplotype	Substitution or Deletion	Gene Frequency				Phenotype	References
		A	B	C	D		
GSTZ1*D	L8, E32 G42, M82	—	—	0.156	—	—	124
GSTZ1*E	P8, E32 G42, T82	—	—	—	—	Unstable	124
<i>Sigma Class</i>							
GSTS1	IVS2+11	—	—	—	—	Occurs in Japanese	125
<i>Omega Class</i>							
GSTO1*A	A140 E155	0.92	0.84	0.67	0.77	Wild-type allele	108
GSTO1*B	D140	0.08	0.17	0.34	0.23	No functional change	108
GSTO1*C	ΔE155, K208	0.04	0	0.03	0.03	Unstable protein	13,108
GSTO1*D	ΔE155, E208	—	0.11	0.01	—	Unstable protein	13
GSTO1*E	Y32	0	0	0.01	0	Inactive protein	126
GSTO1*F	V236	0	0	0	0.03	Unstable protein occurs in South America	127
GSTO2*A	N142	0.15	0.73	0.69	0.74	Wild-type allele	108
GSTO2*B	D142	0.85	0.27	0.31	0.26	No functional change	108
GSTO2*C	I41	0.01	0	0	0	Stable	108
GSTO2*D	Y130	0.01	0.02	0	0	Unstable	108
GSTO2*E	I158	0	0	0.01	0	Unstable	108

A, African; B, Asian, predominantly Chinese; C, Caucasian; D, Mexican/South American.

^aData shown as percent homozygote nulls.

and analyzed in different haplotypes [129–131]. The most significant 5' SNP appears to be a G-52A substitution in an SP1 response element where the G allele increases reporter gene expression around fourfold in HepG2, GLC4, and Caco-2 cell lines [73]. The homozygous presence of this allele in the proposed *hGSTA1**A haplotype (G-52, C-69, and T-567) was associated with fourfold higher levels of GSTA1-1 protein in the liver than in *hGSTA1**B (A-52, T-69, and G-567) homozygotes [129]. While this finding indicates that the G-52A has a significant effect on GSTA1-1 expression in the liver, similar differences were not found in the pancreas [132], suggesting that the effect of this polymorphism on gene expression may be tissue specific. The frequency of the *hGSTA1**A haplotype containing the G-52 allele ranges between 0.6 and 0.85 in African, Asian, and Caucasian populations.

17.6.1.2 *GSTA2*. A total of four nonsynonymous SNPs that result in the substitutions P110S, S112T, K196N, and E210A have been detected in the coding sequence of *GSTA2* [128,133,134]. These SNPs occur in five haplotypes termed *GSTA2**A–*GSTA2**E (Table 17.2). However, the K196N substitution that gives rise to the *GSTA2**D haplotype has only been observed in two EST database sequences and has not been confirmed in any population studies [133]. Consequently, it is unclear if this variant exists or is the result of a DNA sequencing error.

The E210A and S112T substitutions that are represented in haplotypes *GSTA2**B and *GSTA2**C do not appear to have any significant effect on catalytic activity [128]. In contrast, the rare P110S substitution that occurs in haplotype *GSTA2**E has diminished activity with several substrates, including CDNB and organic hydroperoxides, but elevated activity toward 4-nitrophenylacetate [133,134]. A recent study has shown that the *GSTA2**E variant has very high catalytic efficiency with azathioprene [135]. Azathioprene is used clinically in the treatment of cancer and some autoimmune disorders and a low activity allelic variant of thiopurine methyltransferase has been associated with toxicity resulting from the accumulation of high levels of 6-mercaptopurine [136]. The inheritance of the *GSTA2**E variant may also cause the liberation of high concentrations of 6-mercaptopurine and contribute to azathioprene toxicity.

Quantitative studies on *GSTA2*-2 protein in human liver samples have shown that the presence of the T112 substitution (*GSTA2**C) causes a significant reduction to levels that are around 25% of those associated with the other haplotypes [134]. The cause of the apparently poor expression of the *GSTA2**C subunit is not clear. The possibility that the T112 substitution causes protein instability or sensitivity to degradation seems unlikely but has not been tested. It seems more likely that the SNP is linked to another SNP that directly affects gene expression.

17.6.1.3 *GSTA3*. *GSTA3*-3 in humans plays a highly specialized role in the isomerization of Δ^5 -3-ketosteroids in the synthesis of testosterone and progesterone. So far only a single SNP in the coding region of *GSTA3* has been validated and characterized [115]. This SNP results in an I71L substitution and has only been identified in African subjects. Recombinant *GSTA3*-3 with the L71 substitution has diminished activity with a number of substrates, including CDNB and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole. The low activity results from a significant increase in the K_m for GSH. Interestingly, there was no difference in the variant enzyme's specific activity toward Δ^5 -androstene-3,17-dione and no difference in the K_m for GSH with this substrate. Additional studies revealed that the L71 variant has diminished heat stability.

17.6.1.4 *GSTA4*. Thus far no SNPs in the coding region of *GSTA4* have been validated and characterized. Several SNPs have been identified in the 5' noncoding region, but they do not appear to influence function [131].

17.6.1.5 *GSTA5*. Thus far no SNPs in the coding region of *GSTA5* have been validated and characterized.

17.6.2 The Mu Class

Polymorphisms in the Mu class GSTs have been previously reviewed by Tetlow *et al.* [117]. Because of the similarity of some of the Mu class GST gene sequences, there are a number of alignment errors in SNP databases and the number of real coding region missense SNPs is less than that predicted. For example, there is an erroneous identification of an S210T substitution in both GSTM1 and GSTM2. This has arisen as a result of the misclassification of GSTM1 sequences with S210 and GSTM2 sequences with T210 and vice versa.

17.6.2.1 *GSTM1*. GSTM1 deficiency is the one most extensively studied GST polymorphisms. This polymorphism was first identified as a genetically determined GST deficiency with a frequency approaching 50% by starch gel electrophoresis [47]. At the same time, ion exchange chromatographic studies on human liver also revealed the absence of a significant GST isoenzyme in some samples [137]. Subsequent research also reported the inability of monocytes from some individuals to conjugate *trans*-stilbene oxide to GSH and this proved to be a result of GSTM1 deficiency since *trans*-stilbene oxide is a specific substrate for GSTM1-1 [138,139]. The original starch gel electrophoretic analysis predicted that GSTM1-1 deficiency was the result of a gene deletion and this was confirmed later by Southern blotting studies [83]. The *GSTM1* null allele is very common and is the most frequent allele in most populations studied so far. An association between GSTM1 deficiency and lung cancer was first reported in a study that was the forerunner of hundreds of investigations into the occurrence of GSTM1 deficiency and a range of different cancers and other disorders [140]. There have been a number of reviews and meta-analyses of these studies [141–152]. Possibly the most clinically significant studies have found that GSTM1 deficiency has a significant impact on survival of childhood leukemia [153–157].

The *GSTM1* null allele appears to have resulted from an unequal crossing over event that deleted an 18-kb region that included the entire *GSTM1* gene [158]. A polymorphic duplication of *GSTM1* has been reported in the Saudi Arabian population [159]. This variation results in an increase in an individual's capacity to conjugate *t*-stilbene oxide and presumably other GSTM1-1 substrates. Another GSTM1 polymorphism also originally identified by starch gel electrophoresis [47] and subsequently shown to result from an N173K substitution where the K173 variant has been termed GSTM1*A and the N173 isoform has been termed GSTM1*B [160] (Table 17.2). Functional studies have indicated that this polymorphism does not significantly affect the activity with a range of substrates [161]. An A85S substitution with a minor allele frequency of 0.068 in Caucasian Americans has been reported [116]. This variant has not been extensively characterized but appears to be stable when expressed in COS-1 cells.

17.6.2.2 *GSTM2*. Despite extensive studies, no confirmed missense SNPs have been identified in *GSTM2*.

17.6.2.3 GSTM3. Two coding region missense SNPs have been described and characterized [117]. A G147W substitution has been identified as a rare variant in Chinese subjects and a V224I substitution is common in African, Asian, and European subjects. These coding region alleles can be combined into four possible haplotypes (GSTM3*A, G147, V224; GSTM3*C, G147, I224; GSTM3*D, W147, V224; GSTM3*E, W147, I224). The term GSTM3*B has previously been used to describe a deletion in intron 6 (see below). Functional analysis of recombinant enzymes indicates that the presence of I224 in the GSTM3*C haplotype corresponds with a threefold increase in the specific activity with CDNB as a substrate, a reduction in the K_m for GSH, and a large increase in the catalytic efficiency of the enzyme [117].

The term GSTM3*B has previously been used to describe a deletion in intron 6 [118]. GSTM3*B appears to be in linkage disequilibrium with GSTM1*A and it has been suggested that this linked haplotype may include an element that alters the expression of GSTM1 [118].

17.6.2.4 GSTM4. Although the SNP databases predict a large number of missense SNPs, none has been validated as they can be explained as misalignments of other GSTM sequences. A 5' proximal promoter SNP at nucleotide-79 has been reported to influence expression. The G allele has only 67% of the activity of the C allele [131]. It is not clear if this difference is relevant *in vivo*.

17.6.2.5 GSTM5. No validated SNPs have been characterized in the coding region. Several variant alleles contribute to six haplotypes that have been noted in the 5' proximal promoter but none has been associated with a significant change in expression [131].

17.6.3 The Pi Class

17.6.3.1 GSTP1. The Pi class is represented in humans by a single functional gene (*GSTP1*). Although the SNP database lists several missense SNPs, only two have been validated and extensively characterized. The Ile105Val and Ala114 Val substitutions were first identified in cDNA clones [96] and later identified as genomic variants [162] (Table 17.2).

GSTP1-1 has been extensively studied in relation to drug resistance [35], and the possibility that the common SNPs at codons 105 and 114 alter drug and xenobiotic metabolism and susceptibility to cancer and other diseases has been the subject of numerous investigations [163–167]. Several studies have noted differences in the activity of GSTP1-1 variants with the anticancer drugs thiotepa and chlorambucil [163,168–170]. Many epidemiological gene association studies have been reviewed elsewhere and have been the subject of extensive meta-analysis [142,143,171–173]. Because of their potential impact on drug metabolism and drug resistance, the catalytic functions of GSTP1 variants have been intensively investigated. Most attention has been focused on the I105V substitution since residue 105 contributes to the architecture of the hydrophobic substrate-binding site (H-site) [174]. GSTP1-1 is active with a range of carcinogenic diol epoxides derived from polycyclic aromatic hydrocarbons [175–179] and differences in the GSH conjugating activity of the V105 and I105 variants have been attributed to the volume of the residue in the H-site and its hydrophobicity. It has been suggested that differences in the catalytic activity of the I105 and

V105 variants toward carcinogenic diol epoxides may underlie the reported associations between these alleles and cancer susceptibility [176], although other reports have implicated other factors [179]. Although most studies have identified significant catalytic and stability differences between the I105 and V105 variants [175,176], there have been contradictory reports. For example, while Zimniak and colleagues [180] reported that the I105 variant was less stable than the V105 variant at 50°C, a separate study indicated that the I105 variant was the more stable at 45°C [181]. Both studies found that the heat stability was differentially affected by the presence of substrates so the extent of GSH binding may explain this discrepancy and suggests that intracellular conditions could differentially affect the stability of the two variant isoenzymes and this could be reflected in different *in vivo* capacities to conjugate certain xenobiotics and their metabolites. Similarly, Johansson *et al.* [181] noted significant structure activity differences that were substrate dependent. For example, with CDNB as a substrate, the I105V polymorphism has significant affect on K_m but not k_{cat} . In contrast, with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole or ethacrynic acid as substrates, the polymorphism affected both parameters. Clearly, further studies are required to fully understand the structure/substrate interactions that underlie the reported associations between *GSTP1* genotype, xenobiotic metabolism, and cancer susceptibility.

17.6.4 The Theta Class

In humans, there are two functional Theta class GST genes (*GSTT1* and *GSTT2*) located on chromosome 22q11 [102,105]. The locus is complicated because of the apparent duplication of *GSTT2* to generate a pseudogene (*GSTT2p*) that is transcribed [102].

17.6.4.1 *GSTT1*. Although several missense mutations have been predicted in the SNP databases, few have been well characterized. The *GSTT1**A and *GSTT1**B alleles result from a T104P substitution that is relatively rare in Scandinavian populations [122]. The insertion of proline in helix 4 appears to destabilize the protein to a level that was below immunological detection and causes GSTT1-1 deficiency [122]. A major resequencing study [116] identified 18 SNPs, including the substitutions D43N, T65M, and V169I as well as a single nucleotide deletion (G412) from exon 4. With the exception of the V169I substitution that occurred at a minor allele frequency of 0.126 in African Americans, the other variants occurred at a frequency of 0.001 or less. The nucleotide 412 deletion causes a frame shift and a premature stop codon and would be expected to cause a deficiency. When transfected into COS-1 cells, the D43N, T65M, and T104P substitutions caused low levels of immunoreactive protein expression [116]. Two other rare variants (D141N and E173K) reported in the NIEHS Environmental Genome Project database have been expressed in *Escherichia coli* and characterized [123]. Although the D141N substitution had little effect on activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane, ethylenediiodide, and 4-nitrobenzyl chloride, and appeared to have a normal thermal denaturation profile, it had low activity in an ethylene dibromide mutagenicity assay. In contrast, the E173K substitution caused significant instability and had no or low enzymatic activity.

A *GSTT1* null allele (*GSTT1**O) is common in most populations and results from a gene deletion [102,103,182]. Since it was shown that GSTT1-1 can activate dihalomethanes to mutagenic intermediates [183], there has been considerable interest

in determining if the presence or absence of GSTT1-1 influences susceptibility to cancer and other disorders [184]. Many of the studies have been the subject of review and meta-analysis [143,146–148,150,185,186]. In addition to the potential role of GSTT1-1 deficiency in cancer susceptibility, mismatches in the *GSTT1* genotype between organ donors and recipients are considered to be significant risk factors in transplant rejection and in autoimmune hepatitis [187,188].

17.6.4.2 *GSTT2*. Although several GSTT2 missense SNPs have been included in the SNP database, close examination reveals that most can be attributed to sequence differences between GSTT2 and *GSTT2p* (also termed *GSTT2b*), a transcribed pseudo-gene that has been disabled by splice site changes and inframe stop codons [102]. Only one relatively rare missense SNP causing a M139I substitution has been unambiguously identified [102]. This protein has not been functionally characterized, but molecular modeling suggested that the enzyme's function is unlikely to be modified. A relatively common deletion of *GSTT2b* has recently been identified [106]. This deletion appears to diminish the transcription of *GSTT2* and is in linkage disequilibrium with the polymorphic deletion of *GSTT1*. Several SNPs generating at least four observed haplotypes in the 5' proximal promoter have been reported [131]. Of these, a T-105 to C-nucleotide substitution appears to result in a significant decrease in expression, but it is not clear if this study was able to differentiate between *GSTT2* and *GSTT2p*.

17.6.5 The Sigma Class

The human Sigma class GST is also known as *hemopoietic prostaglandin D synthase* (HPGDS). No functional polymorphisms have been unequivocally identified in the coding sequence but two rare variants in intron 2 (IVS2 + 11A > C) and intron 3 (IVS3 + 13T > C) have been reported [125]. In a survey of asthmatic families, the intron-2 SNP was significantly transmitted to asthma-affected children [125].

17.6.6 The Zeta Class

17.6.6.1 *GSTZ1*. In humans, there is a single Zeta class GST gene (*GSTZ1*) and it is located at chromosome 14q24.3 [6,54]. GSTZ1-1 is also known as *maleylacetoacetate isomerase* and catalyzes the penultimate step in the degradation of tyrosine [7]. Although deficiencies of other enzymes in this pathway are known and cause disease of varying severity, no cases of GSTZ1-1 deficiency have been unequivocally identified. Several missense SNPs have been identified that result in L8P, K32E, G42R, and T82M substitutions [124,189]. So far five haplotypes have been identified in human population studies (Table 17.2), and characterization of recombinant proteins has revealed a number of significant functional differences [124,189,190]. Notably, the GSTZ1*A haplotype generates a protein with a G42R substitution that has high specific activity with the (R)-enantiomer of 2-chloropropanoic acid and lower specific activity with the (S)-enantiomer. In contrast, the products of the other haplotypes showed no enantiomer selectivity. In addition, the GSTZ1*A protein has low isomerase activity with maleylacetoacetate and is resistant to inactivation by dichloroacetic acid (DCA) compared to the other GSTZ1-1 variants [124,190,191]. The impact of the arginine substitution at codon-42 on the active site and the functional properties of GSTZ1a-1a have previously been discussed in detail [190]. The resistance of the GSTZ1a-1a variant to inactivation

by DCA may be of clinical significance since DCA has been used to treat lactic acidosis [192] and has recently been proposed as a novel anticancer agent [193–195]. The pharmacokinetics of DCA disposition is complex. Although the elimination of DCA is dependent on the activity of GSTZ1-1, DCA also acts as a mechanism-based inactivator of GSTZ1-1 [191,196]. Consequently, the resistance of the GSTZ1a-1a variant to inactivation is likely to shorten the half-life of DCA in individuals with this allele. A cDNA clone with a L8P substitution has been identified in an EST database and although this allele was not identified in a study of 101 Europeans [124], it may be present in other ethnic groups.

A total of 10 SNPs have been identified in a region extending 1.5 kb upstream of the GSTZ1 start of transcription in African and European subjects [197]. Of these SNPs, only two (–1002G >A and –289C >T) were associated with significant changes in promoter activity. Further studies are required to determine if SNPs influence *GSTZ1* expression *in vivo*.

17.6.7 The Omega Class

In humans, there are two functional Omega class GST genes (*GSTO1* and *GSTO2*) that are located on chromosome 10q24.3 and are separated by about 1.5 kb [13,108]. A review of the structure and function of the Omega class GSTs has previously been published [107].

17.6.7.1 *GSTO1*. Several studies have reported missense SNPs or deletions in *GSTO1*, including C32Y, A140D, delE155, E208K, and A236V (Table 17.2) [108,126,198,199].

The C32Y substitution is a rare variant reported in Caucasians which is reported to degrade rapidly and has a short intracellular half-life [126]. As C32 is the primary active site residue [12], this substitution might be expected to eliminate the thioltransferase, methylarsonate reductase, and phenacylglutathione reductase activities of GSTO1-1. However, it might be expected to improve the enzyme's capacity to conjugate GSH to compounds such as CDNB since an experimental C32A substitution has been reported to increase activity with CDNB [107]. This activity may be further enhanced because of the substitution to tyrosine which is the active site residue in the Alpha, Mu, and Pi class GSTs that have very high GSH conjugating activity with CDNB.

The A140D substitution is the most common missense polymorphism at this locus. This substitution does not appear to have a significant effect on activity with a range of substrates [13,200].

The deletion of E155 occurs at a low frequency in most populations that have been studied and results from the deletion of AGG from the 5' splice donor site of exon 4 and the recreation of a new functional splice donor site (AGGAG/GT to AG/GT) [108,201]. Recombinant protein produced in *E. coli* has increased specific activity with several substrates [13] but low heat stability [108]. Recent data suggest that protein with the E155 deletion may be unstable *in vivo* and the allele could result in GSTO1-1 deficiency in homozygotes. This conclusion is based on the observations that the T47-D breast cancer cell line is hemizygous for the delE155 allele and is devoid of GSTO1-1 protein and activity [201]. In addition, transformed lymphoblastoid cell lines from delE155 heterozygous subjects show only 50% of normal GSTO1-1 enzymatic activity [201]. Given the low frequency of the delE155 allele in Europeans, it is expected

that homozygotes would be rare. However, the frequency of the deletion is more common in Chinese and one apparent homozygote was noted in a small sample of normal Chinese subjects, suggesting that the expected deficiency of GSTO1-1 is not particularly deleterious [108]. The delE155 allele is in strong linkage disequilibrium with an E208K substitution. In Europeans, the delE155 allele is linked to the K208 allele in contrast to Chinese subjects where the delE155 allele is linked to E208 [13]. Studies on recombinant GSTO1-1 with a single E208K substitution without the E155 deletion indicate that this mutation does not significantly affect the enzyme's stability or catalytic activity [13].

An A236V substitution has been reported in population samples from Chile and Mexico [126,127]. Evaluation of the recombinant protein has shown that the A236V substitution causes a marked decrease in the specific activity with all substrates tested and a significant reduction in heat stability [127]. It is likely that this substitution would lead to a deficiency of GSTO1-1 in homozygotes but none has been identified or studied so far.

Linkage disequilibrium has been reported within the *GSTO1* gene and between *GSTO1* and *GSTO2* [126]. Linkage studies have also shown that *GSTO1* modifies the age at onset of Alzheimer's and Parkinson's diseases [202,203]. This association has not always been evident in smaller studies [204,205]. GSTO1 polymorphisms have also been associated with vascular dementia and stroke [206,207]. The mechanisms underlying these associations with neurological disease are not clear, but as GSTO1-1 has been implicated in the activation of the proinflammatory interleukin-1 (IL-1) β [208] and as there are numerous reports of IL-1 variants influencing risk of Alzheimer's and Parkinson's diseases, this may be a profitable area for further investigation [209–212].

17.6.7.2 GSTO2. GSTO2-2 is difficult to express in heterologous systems and has not been extensively characterized. The limited studies that have been undertaken have shown that it has very high dehydroascorbate reductase activity [13]. With 66 polymorphisms being detected so far, the GSTO2 locus appears to be very polymorphic [126]. While most variation is in the noncoding regions, four missense SNPs have been characterized (V41I, C130Y, N142D, and L158I) [13,108,126]. The N142D substitution is the most common and is found in all populations studied so far. This substitution does not appear to influence catalytic activity [13] or stability [126]. Similarly, the V41I mutation appears to be relatively stable, but its catalytic activity has not been assessed [126]. In contrast, the C130Y and L158I substitutions are rare and appear to be relatively unstable [126].

17.7 STRUCTURE AND FUNCTION OF THE CYTOSOLIC GLUTATHIONE TRANSFERASES

17.7.1 Crystal Structures

Crystal structures have been determined for representatives of the known mammalian GSTs and many animal, plant, and microbial GSTs. Protein Database (PDB) files for representatives of all the human GST classes and the related CLIC proteins are provided in Table 17.3. Modeling has shown that the human GDAP1 may be a member of the GST structural family, but a definitive crystal structure has not been reported [57]. All

TABLE 17.3 Representative Crystal Structures of Human Cytosolic GSTs and Related Proteins

GST	PDB File ^a	References
GSTA1-1	1GUH	216
GSTA2-2	2WJU	217
GSTA3-3	2VCV	217
GSTA4-4	1GUL	
GSTM1-1	2F3M	218
GSTM2-2	1HNA	219
GSTP1-1	18GS	220
GSTT1-1	2C3N	221
GSTT2-2	3LJR	222
GSTS1-1 ^b	2CVD	223
GSTZ1-1	1FW1	67
GSTO1-1	1EEM	12
CLIC-1	1KOM	20
CLIC-2	2R4V	21
CLIC-3	3FY7	224
CLIC-4	2D2Z	225

^aThese are representative structures and many additional structures with a variety of ligands are available in the PDB.

^bGSTS1-1 is also commonly known as *hemopoietic prostaglandin D₂ synthase* (HPGDS).

the catalytically active members of the GST family form dimers. While most GSTs form homodimers, there is some evidence that closely related subunits from within a class can form heterodimers. Heterodimerization is particularly evident between GSTA1 and GSTA2 subunits [77] and has been reported to occur between GSTM1 and GSTM2 subunits [213] *in vivo*, dimerization generally occurs within a class as the differences in structure of the dimer interface seems to limit the formation of interclass dimers. However, there is evidence for the formation of Pi–Mu heterodimers *in vitro* under mild nondenaturing conditions, suggesting that heterodimerization may occur *in vivo* in some circumstances [214]. Within the Alpha, Mu, and Pi classes and to a variable extent in other GSTs, the dimers are stabilized by “key and lock” interactions where a Phe or Tyr residue from one subunit is wedged into a hydrophobic pocket in the other subunit. There is evidence that at least in the Pi class, this stabilization indirectly affects the catalytic competence of the enzyme by stabilizing the loop following α -helix 2 that forms the GSH-binding “G”-site [215]. In this light, it is of interest that the Pi–Mu heterodimers reported by Pettigrew and Colman [214] displayed novel catalytic activity.

In vivo, members of the Pi and Mu classes can also occur in a monomer–homodimer equilibrium where the GST Pi or Mu monomers interact with other proteins such as JNK, ASK1, and Prx IV (peroxyredoxin IV) [14,15,226]. The binding of GST monomers to signaling proteins such as JNK and ASK1 highlight some novel noncatalytic roles for the cytosolic GSTs. The CLIC proteins are members of the cytosolic GST family that occur as monomers [18,20,21]. Although these proteins have no known catalytic activity, they can enter membranes and form ion channels [19,21,227,228].

Other studies have shown that they can modulate the activity of ryanodine receptor Ca^{2+} channels that are essential for muscle excitation–contraction coupling [18,229].

The archetypical “cytosolic” GST fold is composed of two domains (Figs. 17.1 and 17.2) [12,67,174,216,222,230]. The N-terminal domain that includes the GSH-binding site appears to have evolved from a thioredoxin/glutaredoxin ancestor and has a typical thioredoxin topology ($\beta\alpha\beta\alpha\beta\alpha$). The C-terminal domain is composed of a helical bundle that does not appear to have any clear evolutionary progenitor. In contrast, the mitochondrial GSTK1-1 appears to have arisen via a parallel evolutionary pathway, and although it manifests a thioredoxin-like fold, the $\beta\alpha\beta\alpha_n\beta\beta\alpha$ topology, differs significantly as the helical bundle is inserted between $\beta 2$ and $\beta 3$ [41,43]. This topology is shared with the bacterial disulfide bond-forming proteins (DsbA).

17.7.2 The Active Site and Catalysis

The active site can be subdivided into the “G”-site or GSH-binding site and the “H”-site that binds the hydrophobic substrate. The G-site primarily involves residues in the N-terminal domain (Fig. 17.2), but in some GSTs, the G-site can also involve residues from the second subunit [216]. The residues involved in binding GSH are generally well conserved across those GSTs that have enzymatic activity. In GSTs from the Alpha, Mu, and Pi classes, the G-site is relatively open and accessible. This has allowed the purification of these isoenzymes by GSH affinity chromatography [50,51]. In the Theta and Zeta classes, the G-site is more buried and this precluded their purification by affinity chromatography and delayed their discovery and characterization. In contrast to the G-site, the residues constituting the H-site are largely within the C-terminal domain (Fig. 17.2) and are variable across the GST structural family reflecting the diversity of the hydrophobic compounds that are substrates for the different isoenzymes. The primary active site residue responsible for catalysis is a tyrosine in the Alpha, Mu, Pi, and Sigma classes, a serine residue in the Theta and Zeta classes and a cysteine in

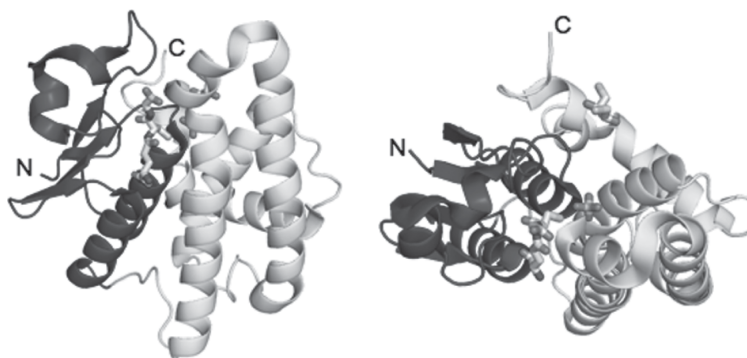


Figure 17.2 Two views of the crystal structure of a human cytosolic glutathione transferase GSTZ1 subunit (PDB code 1fw1) [67]. The N-terminal thioredoxin domain is shown in black, and the C-terminal helical domain is shown in gray. Glutathione, a sulfate ion, and dithiothreitol are represented in CPK stick format. The glutathione is in primary contact with N-terminal domain residues, and its thiol is positioned in the active site at the N-terminal of α -helix 1. The sulfate ion is coordinated by residues in the C-terminal domain and mimics the position of the second substrate. The dithiothreitol molecule forms a disulfide bond with Cys 205. (See color insert.)

the Omega class [12,67,107,216,222,231–233]. Tyr8 of human GSTA1-1 is conserved within the Alpha, Mu, Pi, and Sigma classes and its phenolic hydroxyl group is within hydrogen bonding distance of the GSH thiol. Mutation of Tyr8 to Phe caused a 90% loss of activity but was not absolutely essential for activity [216,231]. Subsequent mutation of Arg15 has confirmed that it also plays an important role in binding and activating GSH in the Alpha class where it is strongly conserved [234]. A conserved Ser residue appears to be important for catalysis in the Theta class GSTs; however, its precise role may vary with different substrates. Mutation of Ser11 in GSTT2-2 abolished its GSH peroxidase activity with cumene hydroperoxide and its GSH conjugating activity with ethacrynic acid but doubled its activity with 1-menaphthyl sulfate [232]. The Zeta class GST GSTZ1-1 is also known as *maleylacetoacetate isomerase* and undertakes the GSH-dependent isomerization of maleylacetoacetate to fumarylacetoacetate, the second last step in the catabolism of tyrosine. In addition, GSTZ1-1 catalyzes the GSH-dependent dehalogenation and oxygenation of α -haloacids such as DCA [190]. There are two adjacent serine residues and a cysteine residue (S₁₄, S₁₅, and C₁₆) in a highly conserved motif in the G-site of all Zeta class GSTs. Mutagenesis of these residues in human GSTZ1-1 found that only Ser14 is specifically required for catalysis despite its less favorable orientation toward the GSH thiol in the crystal structure [67,233]. The Cys16 residue is not essential for activity but does play a role in GSH affinity and binding [235].

The Omega class GSTs catalyze a range of reduction or thioltransferase reactions where cysteine could be expected to play a catalytic role. The determination of the crystal structure of GSTO1-1 shows GSH linked though a disulfide bond to Cys32 in the G-site and mutagenic studies confirmed that the thioltransferase and reductase activities are dependent on Cys32 [12,108,200]. Although GSTO1-1 does not normally catalyze conjugation reactions, mutation of Cys32 to Ala eliminates the thioltransferase and *S*-phenacylglutathione reductase activities but notably generates a novel GSH conjugation activity with CDNB [107].

The substitution reaction catalyzed by many GSTs has been well described [27,30] and is shown in the following equation:



The reaction proceeds from the initial lowering of the pK_a of GSH from around pH 9 in solution to around pH 6.5 when it is bound in the G-site. This promotes the deprotonation of the thiol to a highly nucleophilic thiolate anion. While some evidence suggests that the active site tyrosine and serine residues act as hydrogen bond donors that stabilize the GSH thiolate [30,234,236], there is additional evidence that the glutamyl α -carboxyl group of the bound GSH may accept the proton from the thiol group. The use of a decarboxylated analogue of GSH (4-aminobutyric-Cys-Gly) as a substrate for GSTA1-1 supported this proposal as the pK_a of the thiol increased from 6.7 to 9.2 and severely diminished the catalytic activity of the enzyme [237]. In addition, it has also been suggested that water molecules detected in the active site of some GST crystal structures could mediate the proton release [238,239]. A new model for GSH activation in GSTA1-1 has recently been proposed on the basis of QM/MM calculations. In this mechanism, an initial conformational change allows a water molecule to act as a bridge to assist in the transfer of the proton from the GSH thiol to the glutamyl α -carboxylate group [240,241]. Although this mechanism relies

TABLE 17.4 GST Knockout Mouse Strains

GST Gene	Phenotype	References
<i>GstP1</i> and <i>GstP2</i>	Increased skin tumorigenesis	246,247
	Resistant to acetaminophen toxicity	248
	Enhanced lung carcinogenesis	249
	Enhanced colon carcinogenesis	250
	Allergic response in the lung	251
<i>GstA3</i>	Susceptibility to aflatoxin B1	245
<i>GstA4</i>	Susceptible to oxidative stress	252
	Increased susceptibility to CCl ₄	253
<i>GstM1</i>	Decreased conjugation of DCNB	254
	Methemoglobinemia	255
<i>GstT1</i>	Model of human GSTT1-1 deficiency	256
<i>GstS1</i>	Delayed hypersensitivity	257
<i>GstO1</i>	Normal phenotype	258
<i>GstZ1</i>	Sensitive to tyrosine/phenylalanine	259,260
	Oxidative stress model	261

on a conformational change that may be specific for the Alpha class GSTs, the original studies with 4-aminobutyric-Cys-Gly showing the absolute requirement of the glutamyl α -carboxylate for catalysis obtained similar results with rat Alpha, Mu, and Pi class GSTs suggesting that the mechanism is not restricted to the Alpha class [242,243].

17.8 GST-DEFICIENT MICE

The physiological role of GSTs and their contribution to the disposition of drugs and carcinogens is increasingly being studied in GST knockout mice and has been recently reviewed [244]. A list of the knockout mice strains and experimental studies is provided in Table 17.4. Although these mice can provide novel insights into drug metabolism, care should be exercised in the interpretation of the data in relation to its relevance in humans as not all human GSTs have exact equivalents in mice [244]. For example, mouse GSTA3-3 has high activity with aflatoxin B1 and protects mice against aflatoxin carcinogenesis [71,245]. In contrast, human GSTA3-3 has low activity with aflatoxin but exhibits a significant steroid hormone isomerase activity that is not present in the mouse enzyme [5]. Inactivation of the *GstA3* gene in mice eliminates their natural resistance to aflatoxin and provides a novel model of human aflatoxin susceptibility [245]. In other cases, such as the Pi and Theta classes, where there are different numbers of isoenzymes in humans and in mice, and because of some redundancy in specificity, elimination of a single isoenzyme may not create an appropriate deficiency model.

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18 Amino Acid Conjugation: A Novel Route of Xenobiotic Carboxylic Acid Metabolism in Man

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18.1 Summary	1
18.2 Introduction	2
18.3 Mechanism of amino acid conjugation	2
18.4 Mitochondrial medium chain acyl-CoA synthetases (ACSM)	3
18.5 Mitochondrial acyl-CoA:glycine <i>N</i> -acyltransferase (GLYAT)	8
18.6 Life span aspects of amino acid conjugation	11
18.7 Conclusions	12
References	12

18.1 SUMMARY

Compared to most xenobiotic-metabolizing enzymes, knowledge of amino acid conjugation in terms of enzyme multiplicity, protein structure, and xenobiotic substrate selectivity is less advanced. Amino acid conjugation is a novel biotransformation pathway in man for a number of xenobiotic carboxylic acids, including arylacetic, aryloxyacetic, aromatic, and heteroaromatic acids. A notable example is salicylurate formation, which accounts for up to 85% of total salicylic acid clearance in humans.

Amino acid conjugation of xenobiotics involves two enzymatic processes that are localized in mitochondria. First, formation of a reactive xenobiotic–CoA thioester intermediate, catalyzed by an ATP-dependent acid:CoA synthetase (ACSM), and second, linkage of the activated acyl group via an acyl-CoA:amino acid *N*-acyltransferase (GLYAT) to the amino group of the acceptor amino acid. In humans, the principal amino acid utilized is glycine. Formation of a xenobiotic acyl-CoA thioester is an obligatory step in the overall process of amino acid conjugation. However, it is apparent that there are many more xenobiotics that form acyl-CoA thioesters than there are xenobiotics that are substrates for sequential metabolism by ACSM and GLYAT.

Although the active sites of the ACSMs and GLYATs clearly differ, detailed understanding of the metabolism of xenobiotic carboxylic acids via amino acid conjugation awaits the in-depth structural characterization of both proteins.

18.2 INTRODUCTION

The early pioneering work of Alexander Ure in 1841 laid the foundation for the field of amino acid conjugation by demonstrating that exogenously administered benzoic acid was metabolized to hippuric acid [1]. It was not until Dessaignes in 1845 determined that hippuric acid was a benzoic acid linked to glycine (benzoylglycine) that the first evidence of both a conjugation reaction per se and an amino acid conjugation reaction in humans was provided [2]. The future of the field seemed assured following the recognition that amino acid conjugation is the predominant route of metabolism of salicylic acid, with salicylurate (the glycine conjugate) formation accounting for 68–85% of the excretion of salicylic acid in urine. Although knowledge of conjugation reactions involving glucuronic acid, sulfate, and glutathione has advanced significantly, in relative terms, amino acid conjugation remains poorly understood and is limited to a number of arylacetic, aryloxyacetic, aromatic, and heteroaromatic acids.

For the majority of xenobiotic carboxylic acids, amino acid conjugation is a novel but minor metabolic pathway. However, rather than assignation in terms of importance to the bottom of the drug metabolism, enzymology ladder amino acid conjugation (or lack thereof) could be viewed as an evolutionary piece de resistance. Instead of exhibiting the substrate promiscuity of the cytochrome P450s (CYPs) or UDP-glucuronosyltransferases (UGTs), the relative substrate selectivity of the enzymes of amino acid conjugation has been very stable. This stability implies that the enzymes have a central role in the connectivity of biotransformation reactions, and hence, any disruption may have the potential to influence multiple pathways crucial to cellular integrity.

18.3 MECHANISM OF AMINO ACID CONJUGATION

A limited number of carboxylic acids are conjugated with an amino acid (principally glycine) before excretion. These include endogenous molecules (e.g., bile acids, branched chain fatty acids), environmental chemicals (e.g., herbicides), food preservatives (e.g., substituted benzoates), and drugs (e.g., salicylic acid and valproic acid (VPA)). In general, bile acid conjugation forming glycine and taurine conjugates is catalyzed by extramitochondrial (microsomal and peroxisomal) enzymes, while xenobiotic amino acid conjugation is mediated by mitochondrial enzymes. The latter is the focus of this chapter, and while much of our knowledge of amino acid conjugation both *in vivo* and *in vitro* is based on glycine as the acceptor amino acid, other amino acids are utilized in both man and other species. These include glutamine, glutamate, and taurine in man [3–5]; ornithine in birds and reptiles; and glutamine in ferrets, rabbits, and rats [6]. Formation of amino acid conjugates increases solubility and facilitates xenobiotic excretion in bile or urine.

In contrast to other conjugation reactions, amino acid conjugation is a coupled two-enzyme system. The general consensus is that amino acid conjugation proceeds through

- initial activation of the carboxylic acid moiety with ATP, generating an acyl adenylate and pyrophosphate;
- the reaction of the bound acyl adenylate with coenzyme A (CoASH) catalyzed by an ATP-dependent acid:CoA synthetase (AMP forming) to yield a “high energy” xenobiotic–CoA thioester intermediate;
- linkage of the activated acyl group via the action of an acyl-CoA:amino acid *N*-acyltransferase to the amino group of the acceptor amino acid with regeneration of CoASH (Fig. 18.1).

The formation of an acyl adenylate has not been proven unequivocally. However, multiple studies using proteins from liver and kidney of multiple species with an array of substrates indicate that the reaction follows a bi uni uni bi ping-pong mechanism. For example, using benzoate as the substrate, ATP binds first to the enzyme, followed in order by benzoate binding, pyrophosphate release, CoA binding, benzoyl-CoA release, and AMP release [7].

It is important to recognize at this stage that as a coupled enzyme reaction, a xenobiotic is first a substrate for the acid:CoA synthetase and then the xenobiotic–CoA conjugate is a substrate for the acyl-CoA:amino acid *N*-acyltransferase. Thus, specificity or selectivity of this metabolic route can be conferred at the level of either one or both enzymes. Furthermore, lack of amino acid conjugation does not necessarily infer lack of xenobiotic–CoA conjugation.

18.4 MITOCHONDRIAL MEDIUM CHAIN ACYL-CoA SYNTHETASES (ACSM)

An understanding of amino acid conjugation is predicated on the knowledge of the process of fatty acid activation and the commonality of the enzymes involved. Short-, medium-, and long-chain and very-long-chain fatty acids are activated to their corresponding acyl-CoA thioesters by different families of acyl-CoA synthetases (ACS). Since the fatty acid carbon chain length defines the selectivity of the different enzymes, the ACS superfamily has been subdivided into multiple subfamilies on the basis of fatty acid substrate selectivity. In biochemical terms, these enzymes were previously referred

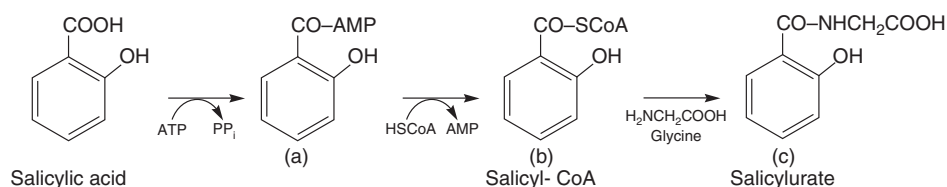


Figure 18.1 Salicylurate formation. (a) Formation of acyl adenylate, (b) formation of salicyl-CoA catalyzed by medium chain acyl-CoA synthetase, and (c) conjugation with glycine to form salicylurate catalyzed by acyl-CoA:glycine *N*-acyltransferase.

to as *ligases* because of their role in “ligating” a fatty acid to CoA. However, in view of the diversity of substrates utilized, both endogenous and exogenous, synthetase was adopted to describe the activity of the enzymes and acyl-CoA for the product [8]. The approved root symbol is ACS followed by a letter that specifies the carbon chain length of the fatty acid substrates, for example, S, short; M, medium; and L, long. The ACS nomenclature was introduced in 2004 for the medium chain ACS (ACSM) subfamily.

Short-chain fatty acids (C_2 – C_4) are primarily activated by the cytosolic short-chain ACSs, and these enzymes are not considered to contribute to xenobiotic metabolism. Long-chain fatty acids (C_{12} – C_{20}) are activated by members of the ACSL family and are present in the outer mitochondrial membrane, endoplasmic reticulum (ER), and peroxisomes. The microsomal ACSL catalyze formation of the CoA thioesters of nafenopin, clofibrate, and the R(-)enantiomers of the 2-arylpropionic acid non-steroidal anti-inflammatory drugs [9,10]. In general, the xenobiotic–CoA thioesters formed in the ER are not substrates for the mitochondrial acyl-CoA:amino acid *N*-acyltransferases. Likely explanations include the fact that xenobiotic–CoAs do not cross biological membranes readily, and they may also be poor substrates for the carnitine shuttle.

The most important enzymes in terms of amino acid conjugation are the medium chain ACSs (ACSM). The ACSMs are located in the mitochondrial matrix and activate C_4 – C_{12} fatty acids. These medium chain fatty acyl-CoA esters are then utilized either for β -oxidation or in anabolic pathways for the formation of cellular lipids. The commonality of involvement of ACSM in both activation of fatty acids and xenobiotics suggests that intramitochondrial formation of a xenobiotic–CoA has the potential to perturb mitochondrial function.

A purified beef liver mitochondrial enzyme (“Mahler’s enzyme”) was first described to catalyze CoA conjugation of C_4 – C_{13} fatty acids and their oxidized derivatives, benzoic acid, phenylacetic acid, and 2,4-dichlorophenoxyacetic acid (2,4-D) [11]. Subsequent studies, however, determined that salicylic acid and *p*-aminosalicylic acid were not substrates for “Mahler’s enzyme,” and mitochondrial “salicyl-CoA” and “propionyl-CoA” ligases were isolated from bovine and guinea pig liver, respectively [12–14]. For the next 20 years, progress was slow because of persistent methodological problems with lability of the mitochondrial ACS and hydrolysis of the xenobiotic–CoA conjugates from contaminating acyl-CoA hydrolases. In 1995, Vessey and Hu used column chromatography to purify three mitochondrial medium chain ACSs from bovine liver [15]. The three forms were designated XL-I, XL-II, and XL-III, and their apparent molecular masses were 54.7, 55.6, and 52.5 kDa, respectively. Partial sequencing of purified XL-I revealed regions of high homology with the essential hypertension SA protein, suggesting a role for the metabolism of endogenous carboxylic acids in the regulation of blood pressure [16]. Subsequent screening of a bovine cDNA library with oligonucleotide probes complementary to short sequences of the N-terminus of XL-I or XL-III resulted in the isolation of a 2065-bp cDNA encoding a protein of 577 amino acids (molecular mass, 64 kDa, XL-III) and an incomplete (75%) cDNA encoding XL-I. Comparison of the amino acid sequence of XL-III and the partial sequence of XL-I revealed an overall identity of 60%, suggesting a common ancestral gene [17]. A fourth bovine liver mitochondrial medium chain ACS (molecular mass, 65.5 kDa) was purified in 1996 by Kasuya *et al.* [18] and for convenience has been termed XL-IV in this chapter.

Additionally, ACSMs have been purified from mouse kidney [19] and mouse liver mitochondria [20]. In a comparative study, Kasuya *et al.* established that there was 48.0% amino acid identity among the mitochondrial ACSMs of mouse, rat, and bovine liver [20]. Furthermore, they reported that the mouse butyryl-CoA synthetase (SA protein) shared 74.2% amino acid identity with bovine XL-III. The latter is identical to a lipoate-activating enzyme that was purified from bovine liver mitochondria [21].

Two human liver mitochondrial xenobiotic/medium chain fatty acid ACSs kinetically distinct from the corresponding bovine liver XL forms were isolated in 1999: HXM-A (now designated ACSM2B) and HXM-B [22]. Subsequent cloning of human liver cDNAs determined that HXM-A was a 64-kDa protein exhibiting 56.2%, 58.7%, and 81.5% amino acid identity to MACS1, XL-III, and XL-I, respectively [23]. The recombinant protein (expressed in COS-7 cells) had greater activity toward benzoic acid than phenylacetic acid, which was consistent with the substrate selectivity of the purified HXM-A protein [22]. In 2001, Fujino *et al.* [24] characterized two medium chain ACSs (MACS1, now ACSM1; and SA, now ACSM3) located on human chromosome 16p. Both the proteins were localized to the mitochondrial matrix, and MACS1 appears to be the same as HXM-B.

18.4.1 Xenobiotic Substrates of ACSM

It is not surprising, given the high amino acid sequence identity between the mitochondrial ACSMs, that there is considerable overlap in terms of fatty acid and xenobiotic substrates. Tables 18.1 and 18.2 provide an overview of the *in vitro* substrate profiles of purified or recombinant bovine and murine (Table 18.1) and human (Table 18.2) ACSM proteins. It is difficult to compare both the substrate selectivity and relative enzyme activity of the ACSMs because the range of substrates varies between different studies, and different protein sources and preparations have been used. Although relative activities with each substrate vary enormously, common substrates for all bovine XL enzymes and murine ACSMs include hexanoic acid, octanoic acid, benzoic acid, 4-chlorobenzoic acid, and VPA. Salicylic acid is metabolized by XL-I, XL-II, and XL-III, with respective apparent K_m values of 2.4, 2.2, and 2.5 μM and respective V_{max} values of 1.2, 1.4, and 0.24 nmol salicyl-CoA formed per minute milligram of protein [25]. However, negligible activity was observed with XL-IV [26]. From the data detailed in Table 18.1, 1-naphthylacetic acid and 3-phenoxybenzoic acid are not substrates for XL-I and XL-II but are substrates for XL-III, XL-IV, and the murine liver and kidney forms of ACSM [15,18–20,24–27].

In contrast to the bovine and murine proteins, the substrate profile of the human forms HXM-A and HXM-B is poorly characterized (Table 18.2). Of the limited number of fatty acids investigated, substrate selectivity (C_4 , C_6 , and C_{10}) is similar to the bovine and murine enzymes. The only xenobiotic investigated was salicylic acid; K_m^{app} values for salicyl-CoA formation by HXM-A and HXM-B were 7.4 and 25 μM , respectively [22]. Formation of salicyl-CoA in humans is thought to be the rate-limiting step in clearance of salicylic acid to salicylurate, the latter accounting for the majority of total salicylic acid clearance in humans [28]. Salicylic acid clearance is greater in males than females due largely to enhanced salicylurate formation, but oral contraceptive steroid (OCS) use negates the gender difference in salicylic acid disposition parameters between males and OCS users [29]. At therapeutic doses, salicylurate formation

TABLE 18.1 Substrate Profile of Bovine, MACS1 and Murine Medium Chain Acyl-CoA Synthetases

Substrate	Bovine Liver				MACS1	Murine	
	XL-I	XL-II	XL-III	XL-IV		Kidney	Liver
<i>Fatty Acid</i>							
C ₃	✓	✓	Neg	✓	NA	NA	NA
C ₄	✓	✓	✓	NA	Neg	NA	NA
C ₆	✓	✓	✓	✓	✓	✓	✓
C ₈	✓	✓	✓	✓	✓	✓	✓
C ₁₀	✓	✓	✓	NA	✓	NA	✓
C ₁₂	Neg	Neg	✓	✓	✓	NA	NA
C ₁₆	×	×	×	NA	Neg	NA	NA
<i>Xenobiotics</i>							
Benzoic acid	✓	✓	✓	✓	✓	✓	✓
4-Chlorobenzoic acid	✓	✓	✓	✓	✓	✓	✓
DTA	×	×	✓	NA	NA	NA	NA
Ibuprofen	×	×	Neg	NA	NA	NA	NA
3-Methoxybenzoic acid	NA	NA	NA	✓	NA	✓	✓
1-Naphthylacetic acid	×	×	✓	✓	NA	✓	✓
1-Napthoic acid	×	✓	✓	NA	NA	✓	✓
3-Phenoxybenzoic acid	×	×	✓	✓	NA	✓	✓
Salicylic acid	✓	✓	✓	NA	NA	×	×
Valproic acid	✓	✓	Neg	NA	NA	Neg	Neg

✓, activity reported in nmol/min mg; ×, activity not detected; Neg, negligible activity; NA, not assayed; DTA, 2,4,6,8-decatetraenoic acid.

XL-I to XL-IV, kidney and liver ACSM column-purified proteins; MACS1, recombinant protein [24].

For further comprehensive data, see Refs 15,18–20, and 24–27.

is a saturable process, and this contributes to the dose-dependent nonlinear pharmacokinetics of salicylic acid [30,31]. Although the $V_{\max}$ value of the purified HXM-A protein was 0.7 nmol/min.mg [22], the catalytic efficiency calculated as k_{cat}/K_m is 0.006 $\mu\text{L}/\text{min.pmol}$ protein, suggesting that HXM-A is unlikely to be the sole enzyme responsible for salicyl-CoA conjugation *in vivo*.

In addition to the xenobiotics listed in Tables 18.1 and 18.2, mitochondrial ACSM catalyzes CoA conjugation of hypoglycin A [32], pivalate [33], 2-tetradecylglycidic acid (TDGA) [34], 3-mercaptopropionic acid [35], zomepirac [36], and 3-oxapentanoic and 3,6-dioxahexanoic acid, the carboxylic acid metabolites of the ethylene-glycol-based solvents 2-ethoxyethanol and diglyme, respectively [37].

18.4.2 Substrate Structure–Activity Relationships

As relatively few drugs and chemicals are activated to acyl-CoA thioesters, distinct physiochemical features of the xenobiotic presumably influence the initial conjugation reaction. Using a variety of aromatic and arylacetic acids and purified bovine liver ACSM, Kasuya *et al.* [18] observed high activity toward saturated straight medium chain fatty acids in contrast to aromatic acids. Investigation of benzoic acid derivatives showed that highest activity occurred with alkyl or alkoxyl substitutions in the para- or meta-position of the benzene ring, while ortho-substituted benzoic acids were not

TABLE 18.2 Substrate Profile and Kinetic Parameters for Acyl-CoA Conjugation by Human Medium Chain Acyl-CoA Synthetases HXM-A and HXM-B

Substrate	HXM-A	K_m (μ M)	V_{max} (nmol/ min.mg)	V_{max}/K_m (mL/ min.mg)	HXM-B	K_m (μ M)	V_{max} (nmol/ min.mg)	V_{max}/K_m (mL/min.mg)
<i>Fatty Acid</i>								
C ₃	✓	—	—	—	—	✓	—	—
C ₄	✓	800	27	0.03	✓	570	24	0.04
C ₆	✓	74	88	1.19	✓	34	118	3.47
C ₈	✓	—	—	—	✓	—	—	—
C ₁₀	✓	110	15	0.14	✓	20	8	0.4
C ₁₂	Neg	60	0.2	0.003	Neg	24	0.15	0.006
<i>Xenobiotics</i>								
Benzoate	✓	13	103	7.9	✓	21	9	0.4
Naphthylacetate	Neg	—	—	—	Neg	—	—	—
Nicotinate	×	—	—	—	—	Neg	—	—
Phenylacetate	✓	160	29	0.18	✓	143	2	0.014
Salicylate	✓	7.4	0.7	0.1	Neg	25	<0.1	<0.004
Valproate	✓	—	—	—	—	✓	—	—

✓, activity reported in nmol/min.mg; ×, activity not detected; Neg, negligible activity.
For further details, see Ref. 22.

activated to their corresponding acyl-CoA esters. Arylacetic acids, such as 1-naphthylacetic acid, exhibited similar activity to benzoic acid, while VPA, a branched medium chain fatty acid, exhibited negligible activity [18]. High activity (relative to hexanoyl-CoA formation) has also been observed for benzoic acids with methyl, methoxy, ethoxy, phenoxy, and *n*-pentyl and *n*-heptyl substituents [38].

Studies investigating the mechanism of valproyl-CoA toxicity found that α -fluorination of Δ^4 -VPA [39] and VPA [40] prevents the formation of the corresponding acyl-CoA derivative and, in the case of Δ^4 -VPA, prevents hepatic steatosis [39]. Similarly, lack of formation of acyl-CoA thioesters has been shown for α -fluoropalmitic acid, perfluorooctanoic acid, and perfluorodecanoic acid [41,42]. These data suggest that fluorine, an electron-withdrawing substituent, alters the molecular recognition by ACSM. As noted above, substitution of fluorine at the α -carbon in VPA prevents formation of Δ^4 -VPA acyl-CoA. This may be explained by the increased acidity of the carboxyl group due to the electronegativity of the fluorine atom (F-VPA, pK_a 3.55) in comparison to VPA (pK_a 4.80) [40]. Recently, a positive electron-deficient “patch” adjacent to the electron-withdrawing fluorine substituent has been identified on the electrostatic surface of Δ^4 -VPA [43]. This electrostatic feature of the fluorinated analog may account for the loss of molecular recognition by ACSM.

Recently, a crystal structure (3EQ6) of human ACSM2A with butyric acid bound in the active site has been reported [44], although full details of the structure appears not to have been published. Fifteen putative substrate-determining residues (SDRs) were identified: Phe-234, Asp-235, Ala-236, Trp-239, Thr-278, Ile-299, Ala-301, Gly-302, Ala-322, Tyr-323, Gly-324, Pro-325, Ile-330, Cys-331, and Lys-517. In addition to the 15 SDRs common to the ACS family of enzymes, an additional conserved histidine at position 210 was identified to be important in determining the chain length of medium chain fatty acid substrates, while disfavoring the binding of long-chain fatty acids [44]. Further site-directed mutagenesis studies are required to elucidate the importance of these residues in the binding of xenobiotics.

18.4.3 Inhibitors of ACSM

The bovine forms XL-I and XL-III show substrate-inhibition kinetics with salicylic acid, and although ibuprofen is a specific substrate for XL-III, it inhibits all three XL forms with K_i values in the range of 70–125 μ M [25]. Using a purified mouse liver ACSM and hexanoic acid as the substrate, Kasuya *et al.* demonstrated competitive inhibition by diflunisal (K_i 0.6 μ M), 2-hydroxynaphthoic acid (K_i 13.4 μ M), 2-hydroxydodecanoic acid (K_i 15 μ M), nalidixic acid (K_i 12.4 μ M), and salicylic acid (K_i 19.6 μ M) and mixed-type inhibition with enoxacin and ofloxacin with respective K_i values of 23.7 and 38.2 μ M [45]. Recently, it was demonstrated that while triacsin C is a potent inhibitor of ACSL ($K_i < 1 \mu$ M), this compound is a relatively weak inhibitor of HXM-A and HXM-B ($K_i > 100 \mu$ M) [46].

18.5 MITOCHONDRIAL ACYL-CoA:GLYCINE *N*-ACYLTRANSFERASE (GLYAT)

The second step in the overall process of amino acid conjugation in mammals most commonly involves transfer of the acyl group to the amino group of glycine. This

reaction is catalyzed by acyl-CoA:glycine *N*-acyltransferases (GLYAT, EC 2.3.1.13) located in the mitochondrial matrix. Kinetic studies with bovine GLYAT are consistent with a sequential bi–bi mechanism where the acyl-CoA substrate binds first, followed by addition of the amino acid, before dissociation of CoA, and the release of the peptide product as the final step [47]. GLYATs, which are principally found in the mitochondria of liver and kidney, differ from the bile acid:CoA amino acid *N*-acyltransferase (EC 2.3.1.65) that conjugates bile acids with either taurine or glycine. Following identification in beef liver mitochondria in 1953 [48], two glycine *N*-acyltransferases were purified from human liver mitochondria in 1976 [49] and 1994 [50]. One was specific for glycine and utilized either butyryl-CoA or benzoyl-CoA as acyl donors, while the second enzyme conjugated phenylacetyl-CoA or indoleacetyl-CoA with glutamine [49].

18.5.1 Xenobiotic Substrates of GLYAT

Specificity of the bovine liver GLYATs has been demonstrated. The “phenylacetyltransferase” protein exhibits activity toward phenylacetyl-CoA, phenoxyacetyl-CoA, and 2,4-D but not toward 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), while the “benzoyltransferase” protein conjugates both 2,4-D and 2,4,5-T [51]. However, both GLYATs exhibited very high K_m values for glycine (100 and 1000 mM) and slow catalytic rate constants with the phenoxyherbicide-CoAs [51].

Detailed *in vitro* studies of the substrate profile of human GLYAT are lacking. Mawal and Qureshi [50] reported K_m values of 58 and 84 mM for benzoyl-CoA and salicyl-CoA, respectively. In general, evidence of amino acid conjugation principally results from identification of conjugates in human urine (Table 18.3). Glycine conjugation has been observed for the histamine H_1 receptor antagonists astemizole [52] and brompheniramine [53], the insecticide permethrin [54], the antiplatelet agent triflusal [55], and the solvent *m*-xylene [56]. In contrast, a taurine conjugate of ibuprofen was identified in human urine [5].

18.5.2 Substrate Structure–Activity Relationships

Current data indicates that CoA thioesters, which are substrates for amino acid conjugation, exhibit varying affinities for GLYAT [57]. From studies of the reactivity of acyl-CoA thioesters with glutathione, a simple structure–activity relationship based on substitution of the carbon α to the acyl carbon and on the presence of an oxygen atom β to the acyl carbon has been proposed [58]. Clearly, structural aspects of the xenobiotic–CoAs influence reactivity, and this is most likely associated with the functionality adjacent to the carboxylic acid group. Although this is an interesting observation, it does not account for the selectivity of the GLYATs, and no studies to date have characterized the factors that determine the recognition of xenobiotic acyl-CoAs by GLYAT.

18.5.3 Glycine Conjugation *In Vivo*

Unlike the majority of metabolic pathways that follow first-order kinetics *in vivo*, glycine conjugation is readily saturable, and hence, metabolism of some carboxylic acids is dose dependent. Depending on the substrate, the rate-limiting step can be either formation of the acyl-CoA intermediate or acyl transfer of the amino acid. In

TABLE 18.3 Xenobiotics Metabolized via Amino Acid Conjugation as Either the Parent Acid or Carboxylic Acid Metabolite in Humans

Parent Compound	Carboxylic Acid Metabolite	Amino Acid Conjugate	References
Astemizole	4-Fluorobenzoic acid	2-[4-Fluorobenzoyl]amino]acetic acid ^a	50
	2-(4-Methoxyphenyl)acetic acid	2-[[2-(4-Methoxyphenyl)acetyl]amino]acetic acid	50
	2-(4-Hydroxyphenyl)acetic acid	2-[[2-(4-Hydroxyphenyl)acetyl]amino]acetic acid	50
Brompheniramine	3-(4-Bromophenyl)-3-pyridin-2-yl-propanoic acid	2-[[3-(4-Bromophenyl)-3-pyridin-2-yl-propanoyl]amino]acetic acid	51
Ibuprofen	NA	2-(2-[4-(2-Methylpropyl)phenyl]propanoylamino)ethanesulfonic acid	5
Permethrin	3-(2,2-Dichloroethenyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid	2-[[3-(2,2-Dichloroethenyl)-2,2-dimethyl-cyclopropane carbonyl]amino]acetic acid	52
Triflusal	2-Hydroxy-4-(trifluoromethyl)benzoic acid	2-[[2-Hydroxy-4-(trifluoromethyl)benzoyl]amino]acetic acid	53
<i>m</i> -Xylene	NA	2-[3-Methylbenzoyl]amino]acetic acid	54

Formation of the respective acyl-CoA thioester conjugate has not been demonstrated.

Abbreviation: NA, not applicable.

^aBenzoylaminoacetic acid is commonly referred to as *hippuric acid*.

Source: Adapted from Knights *et al.* (2007) [43].

addition to the kinetic characterization of ACSM and GLYAT, the two other factors that could result in saturation are the availabilities of the cosubstrates, coenzyme A, and glycine. Indeed, conjugation of benzoic acid and salicylic acid *in vivo* is concentration dependent but the mechanism differs. Formation of hippuric acid from benzoic acid is limited by the availability of glycine [6], while in humans, formation of salicyl-CoA is the rate-limiting step in the amino acid conjugation of salicylic acid [6,29].

Administration of exogenous glycine to rats confirmed that hippuric acid formation was limited by the availability of glycine [59]. Increasing the availability of the hepatic pool of CoA following dietary feeding with fenofibrate or bezafibrate led to an increase (8- to 10-fold) in hepatic CoA concentration and no change in benzoyl-CoA synthetase activity but a decrease in GLYAT activity [60]. Despite an increase in the available pool of CoA, the effect of fibrates was so complex that no conclusive evidence could be provided that the availability of CoA influenced glycine-conjugating capacity.

In humans, glycine conjugation of salicylic acid is capacity limited at therapeutic doses; however, oral administration of glycine does not “unsaturate” salicylic acid conjugation, suggesting that glycine availability is not a limiting factor [31]. Further studies conducted in 1969 in humans identified that formation of hippuric acid following oral administration of benzoic acid was also capacity limited. Concomitant administration of glycine markedly increased the rate of formation of hippuric acid. In contrast, prior administration of salicylic acid (1–3 g) had no measurable effect on hippuric acid formation [61]. The same study confirmed that salicylurate formation was capacity limited and that glycine availability had no measurable effect. However, administration of benzoic acid inhibited salicylurate formation, an effect not ameliorated by coadministration of glycine [61]. These data support the proposition that the availability of glycine is rate limiting for hippuric acid formation but not for salicylurate formation, while acyl-CoA conjugation is the rate-limiting step for salicylic acid but not for benzoic acid.

18.5.4 Inhibitors of Amino Acid Conjugation

As indicated, benzoic acid inhibits the elimination of salicylurate in man [61]. Since the activation step is rate limiting for salicylic acid and both are common substrates for the same ACSM, it is reasonable to assume that benzoic acid will inhibit salicyl-CoA formation *in vivo*. Indeed, benzoic acid does inhibit salicylic acid activation by bovine ACSMs *in vitro* [25]. However, salicylic acid also inhibits benzoic acid conjugation *in vitro* [25], and yet, in man, it does not appear to affect the formation of hippuric acid *in vivo* [62]. Ethanol is another proposed inhibitor of amino acid conjugation. Although ethanol was found to have no effect on the elimination of hippuric acid, it inhibited benzoyl-CoA conjugation [62]. In contrast, ethanol had no effect on either salicyl-CoA formation or the excretion of salicylurate [62].

18.6 LIFE SPAN ASPECTS OF AMINO ACID CONJUGATION

Human newborns have limited capacity to excrete *p*-aminobenzoic acid as a glycine conjugate [63]. Consistent with this *in vivo* finding, human liver and kidney homogenates from newborns have limited capacity to form glycine conjugates of benzoic and *p*-aminobenzoic acid [64,65]. Not surprisingly, wide variability in

hippuric acid excretion is observed in neonates and infants administered benzoic acid for the treatment of hyperammonemia [66]. This may be explained in part by immaturity of GLYAT, which appears to attain peak activity at 18 months of age [67]. However, salicylurate formation is detectable in fetal human liver slices [64]. To date, developmental maturity of the human ACSMs has not been investigated.

The capacity for glycine conjugation appears to decline with advancing age, and *in vivo* formation of hippuric acid from benzoic acid is known to decrease in the elderly [68]. Using human tissue homogenates from individuals aged 23–86 years, it was demonstrated that the capacity of the liver (but not the kidney) to form hippuric acid decreased slightly with age [69].

18.7 CONCLUSIONS

With the exception of salicylic acid, amino acid conjugation is a relatively minor pathway of metabolism of carboxylic acid xenobiotics. The limited number of xenobiotics that have been identified to form amino acid conjugates may reflect poor sensitivity of metabolite assays and also limited recognition of this metabolic route in humans. However, manipulation of this pathway is used clinically in the treatment of nonketotic hyperglycinemia and hyperammonemia in neonates and infants [66,70,71]. Additionally, the serum concentration of *p*-aminohippuric acid has been investigated as a prognostic test in hepatic failure [72,73], and 2-[[3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carbonyl]amino]acetic acid, the glycine conjugate of the pyrethroid insecticide permethrin, has been proposed as a urinary biomarker of human exposure [52]. These studies provide little in the way of mechanistic insight as availability of the cosubstrates, CoASH and glycine, and the substrate selectivity of ACSM and GLYAT can all impact the overall process of amino acid conjugation.

It is evident, however, that there are many more xenobiotics that form acyl-CoA thioesters, which are not substrates for amino acid conjugation, than there are xenobiotics that are substrates for both the ACSM and GLYAT. Limited data from structure–activity studies have alluded to the subtle nature of the active site of human ACSMs, but these studies have provided no information on factors influencing recognition of the xenobiotic acyl-CoA by GLYAT. Further elucidation of the critical structural aspects of substrates that confer amino acid conjugation and knowledge of the active sites of the enzymes involved, namely, ACSM and GLYAT, are essential for understanding the broader potential contribution of amino acid conjugation to xenobiotic disposition.

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1 ADME Profiling in Drug Discovery and Development: An Overview

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1.1	Introduction	1
1.2	What is ADME?	2
1.3	Factor affecting ADME	4
1.4	Issues in drug development	9
1.5	Adme assays in drug discovery	18
1.6	Adme studies in drug development	31
1.7	Summary and future perspectives	34
	Acknowledgment	35
	Abbreviations	35
	References	35

1.1 INTRODUCTION

Discovery and development of a new drug is a risky, a time-consuming, and an extremely expensive business. Recent studies revealed that it takes an average time of 12–14 years and costs more than one billion dollars to discover, develop, and approve a new drug in the United States. The advances in chemistry, high throughput screening, molecular and cell biology, and human genomics have resulted in a multi-fold increase in the number of new chemical entities (NCEs) for preclinical and clinical evaluations. The applications of parallel synthesis and combinatorial chemistry to expedite lead finding and lead optimization processes has shifted the chemical libraries toward poorer biopharmaceutical properties, that is, poor solubility and higher molecular weight (MW). It has been estimated that for every 5000 NCEs evaluated in a discovery program, only one is approved for market. At any point during the drug development process, a prospective drug may be terminated due to lack of *in vivo* efficacy, serious undesired side effects, and poor pharmacokinetic (PK) and absorption, distribution, metabolism, and excretion (ADME) properties. Therefore, efforts are being made to reduce attrition of drug candidates during the various stages of their development to bring safer compounds to market. The early refinement of these ADME properties has been regarded as an essential part of the drug candidate selection process.

In support of this need, and as a consequence of increased knowledge within the drug metabolism discipline, new approaches have been developed, which include extensive *in vitro* methods using human and animal hepatic cellular and subcellular systems, recombinant human drug-metabolizing enzymes (DMEs), transgenic animals and cell lines stably expressing human transporters, increased automation for higher throughput screens, sensitive analytical technologies, and *in silico* computational models to assess drug metabolism aspects of the NCEs [1–3].

The ideal drug candidate should be able to provide sustained exposure, undergo balance clearance pathways with multiple enzymes, and not interfere with DMEs and transporters. Therefore, the design of drugs with optimal potency, PK properties, and less toxicity is still challenging, given the opposing requirements for absorption and metabolism. In the past decade, multiple *in silico*, *in vitro*, and *in vivo* ADME tools have been developed and implemented in various stages of the drug discovery and development process to alert chemists of potential ADME issues in the clinic [1–3]. Although significant improvements have been made to reduce the failure rate due to human PK-related issues, the overall attrition rate has not been improved [4]. The reason for attrition has been shifted to efficacy and safety. However, the ineffective efficacy, safety, and PKs are all interrelated and assignment of failure to a distinct factor might be misleading. For example, the poor efficacy of a drug candidate might be due to extensive metabolism, and clearance to inactive metabolites arising from induction of DMEs and its toxicity might be due to formation of toxic metabolites and/or its prolonged and unnecessary exposure arising from inhibition of the DMEs and transporters. Therefore, quantitative prediction of human PK parameters still presents the single most difficult challenge in the design and advancement of drug candidates to the development stage. Nonetheless, it could be argued that high throughput ADME screens contribute to the selection of the right molecule with the best PK properties for success in humans [4]. The objectives of these screens are to optimize absorption, plasma clearance, metabolic stability, plasma protein binding (PPB), and ratio of metabolic to renal/biliary clearance and to minimize intestinal/hepatic first-pass metabolism, inhibition/induction of DMEs and transporters, metabolism by polymorphic DMEs, and the formation of reactive metabolites of the NCEs. In this chapter, we will attempt to discuss the various ADME profiling approaches in drug discovery and development process and the latest technologies of the selected assays.

1.2 WHAT IS ADME?

When a compound is administered intravenously, the dose is delivered directly into the systemic circulation. All other routes of administration are collectively termed *extravascular routes* (e.g., oral, parenteral, topical, rectal, buccal, and sublingual). For drugs, oral administration is the preferred dosing route due to convenience and to enhance patient compliance. Delivering a drug to its desired target receptor site after an oral dose involves several complex steps of ADME.

The “A” in ADME represents the absorption, the processes that involve transferring the drug from the gastrointestinal (GI) fluid across primarily the jejunum and the ileum segments of the small intestine into the portal blood system. Thus, a drug must pass through to one or more layers of membranes either by passive diffusion, facilitated passive diffusion, or active transport to reach the systemic circulation. Drug

has to cross epithelial or mucosal cell walls, endothelial or capillary cell walls (blood stream), and cellular plasma membranes to exert effect. The extent and rate of absorption are dependent on physicochemical properties, drug formulation, and anatomy and physiology of the site of absorption.

Solubility and permeability are the key determinants of a compound's absorption after its oral administration, and in ultimately determining "how much compound is available to the body," a term also called *oral bioavailability* (OBA). During development of a chemical series, it is very important to ensure that the series has optimal solubility and permeability to avoid inaccurate results not only in biochemical and cell-based *in vitro* biology assays, which are designed to assess compounds' potency to target biological receptors, but also in basic *in vitro* ADME assays, designed to optimize "drug-like" properties of a chemical series. Poor solubility and poor permeability are two major reasons for misinterpretation (or complete lack) of structure–activity relation (SAR) in chemical series as well as for a disconnect between biochemical and cellular assays. Lead optimization of poorly soluble and permeable compound, based on *in vitro* assay results, is extremely risky and can cause compound failure in later stages of the drug development, when a significant amount of resources have already been invested. A common guide that is routinely used by discovery teams to get an estimate of the desirable solubility and permeability for a chemical series is given by the maximum absorbable dose (MAD) [5]. MAD is a function of solubility and permeability and is shown in the following equation:

$$\text{Target solubility (mg/mL)} = \frac{0.015 \times \text{Target dose (mg)} (\text{MAD})}{\text{Permeability (min}^{-1})} \quad (1.1)$$

(assuming all the administered drug is completely absorbed, i.e., target dose = MAD).

In general, for a compound of average permeability and a projected clinical dose of 1 mg/kg, the aqueous solubility should be greater than 50–100 $\mu\text{g/mL}$ [6].

The "D" in ADME represents the distribution, that is, "the reversible transfer of a drug from one location to another within the body." The compound is distributed to different tissues of the body after entering into the systemic circulation (i.e., blood). In most cases, the volume of distribution at steady state (V_{dss}) is used to describe the distribution of a compound in the body. Distribution is generally uneven because of differences in blood perfusion, tissue binding, regional pH, and permeability of cell membranes. The distribution (which tissue and what rate?) of a drug is an important parameter in determining its pharmacological action. The acidic drugs such as warfarin and aspirin are highly protein bound and thus have a small apparent volume of distribution. The basic drugs (e.g., amphetamine and meperidine) are extensively taken up by tissues and thus have an apparent volume of distribution larger than the volume of the entire body.

The "M" in ADME represents the metabolism of a compound by enzymatic reactions. It is a biochemical process by which xenobiotics (e.g., drugs) are converted to more hydrophilic (water soluble) entities, which enhance their elimination from the body [7,8]. Metabolism occurs primarily in the liver and also can occur in other organs (e.g., lung, intestinal epithelial, and kidney). Drugs are metabolized in two different stages: phases I and II. Phase I, or functionalization, reactions include hydroxylation (aliphatic, aromatic, or nitrogen), epoxidation (aliphatic or aromatic), dealkylation (O–, N–, or S–), deamination, oxidation (N–or S–), reduction (nitro, disulfide, keto,

aldehyde, or olefin), and hydrolysis (amide, ester, carbamate, or epoxide). These reactions introduce or unmask a functional group (e.g., $-\text{OH}$, $-\text{CO}_2\text{H}$, $-\text{NH}_2$, or $-\text{SH}$) within a molecule to enhance its hydrophilicity. Phase II, or conjugation, biotransformations include glucuronidation, sulfation, methylation, acetylation, and amino acid (glycine, glutamic acid, and taurine) and glutathione (GSH) conjugation. Most phase II reactions result in a compound's concomitant increase in hydrophilicity and decrease in volume of distribution (V_{dss}), which together greatly facilitate its excretion from the body. The rate and extent of metabolism of a drug determines the dose of drug and the duration of drug effect.

The "E" in ADME represents the elimination (i.e., irreversible removal) of a compound by liver, kidney, and other organs from the body. After oral absorption, drugs are eliminated from the body via excretion as unchanged forms in urine and bile and/or metabolism followed by excretion. For a drug whose clearance is dependent on metabolism, its intrinsic metabolic stability dictates the unbound drug concentrations and the duration of pharmacological effects.

1.3 FACTOR AFFECTING ADME

1.3.1 Physicochemical Properties

Several physicochemical properties of chemical compounds are determinants of intestinal drug absorption, and Lipinski's rule-of-five is a good starter set of rules to predict good absorption [9]. The compounds with MW <500 , lipophilicity <5 (calculated $\log P$), total hydrogen bond acceptors <10 (acceptors being O and N), and total hydrogen bond donors <5 (donors being O-H and N-H groups) should have good oral absorption. It was proposed that when a compound violates two or more of these five rules, it will likely have poor intestinal absorption.

1.3.1.1 MW and Lipophilicity. One of the basic and simple parameters that affect a compound's absorption is its molecular size, which can be estimated fairly reasonably by its MW. Lower MW compounds generally have better absorption and lower biliary excretion. Another metric, the polar surface area (PSA), which reflects a compound's capacity to form H-bonds, is a critical determinant of permeability. It is proposed that compounds with $\text{PSA} > 140 \text{ \AA}^2$ will be absorbed $<10\%$ across the intestinal wall and hence possess poor bioavailability [10]. This relationship holds when compounds are primarily absorbed via a passive transcellular diffusion route and there is no involvement of active transporters.

Lipophilicity, commonly denoted as $\log P$, is defined as the ratio of the compound concentration between an organic phase and the aqueous phase at equilibrium. In general, a $\log P$ value of 0–3 is considered optimal for passive diffusion [11]. A $\log P$ value of <1 suggests that a compound will have good solubility by virtue of being hydrophilic but will have a poor permeability. Similarly, a $\log P$ value of >3 suggests that a compound is highly lipophilic and may possess low solubility or be prone to metabolism and/or biliary excretion.

1.3.1.2 Ionization Constant (pK_a). Ionization constant is a useful thermodynamic parameter to modulate the charge state and eventually solubility, permeability, and key

properties of an NCE. Therefore, accurate measurements of pK_a of an NCE allows for properly predicting and interpreting ADME properties. The ratio of the total drug, ionized plus unionized, in the aqueous medium and the organic phase is defined as the distribution coefficient, denoted as D and commonly reported as $\log D$ [11]. P (as in $\log P$) is a pH-independent parameter, while D is a pH-dependent parameter. In general, a $\log D_{7.4}$ value between -0.5 and 2 is considered optimal for oral absorption. Compounds with $\log D_{7.4}$ values <-0.5 have poor permeability, while those with $\log D_{7.4}$ values >2 are limited by poor solubility.

1.3.1.3 Solubility and Dissolution. Low solubility of NCEs remains a major drug development challenge since it not only causes aberrant results (artificially low potency) in *in vitro* cell assays [12] in the hit-to-lead stages but also can cause lack of efficacy in preclinical/animal models due to poor exposure, nonlinear PK, significant animal-to-animal PK variability, uncertainty in exposure prediction in humans, and formulation difficulties during clinical development. Significant time and revenues of research and development (R&D) are invested in developing a poorly soluble compound, via salt screens and formulation screens. Hence, it is highly desirable to avoid development of a poorly soluble compound from the early stages of drug development. Solubility is guided by two important parameters: lipophilicity and tightness of crystal structure, both of which are inversely related to solubility. Investigation of intrinsic solubility of poorly soluble marketed drugs revealed that several of these showed solvation-limited solubility (high lipophilicity), rather than solid-state-limited solubility (high energy of crystallization).

Several examples in the literature highlight medicinal chemistry efforts to improve solubility, via introduction of ionizable, N-containing basic groups [7,13,14], or disruption of planar crystal structure [15,16], as is shown in Fig. 1.1 for the selected literature examples.

Prodrug approaches have also been used to improve solubility of drugs such as that shown in Fig. 1.2. Fosamprenavir, a phosphate prodrug for the HIV protease inhibitor, amprenavir, showed a 10- to 1400-fold (in the pH range 3.3–7.4) improvement in solubility [17].

1.3.1.4 Permeability. Membrane permeability is not just critical for absorption across the biomembrane of the GI tract but also plays a very important role in its distribution to all other tissues of the body. Compounds can traverse the epithelial cell membranes via either (i) passive transcellular, (ii) passive paracellular, or (iii) active influx/efflux transporter-assisted routes. Majority of the neutral lipophilic drug candidates enter cells through the passive transcellular route, while hydrophilic or charged drugs do so via the passive paracellular route. Since the surface area of the tight junctions is about 0.01% of the small intestine surface area, the paracellular route of absorption is not a very efficient route as compared to the passive transcellular route, which takes place across the much larger surface area of the apical membrane of enterocytes' microvilli environment. Contribution of numerous transporters to drug uptake and efflux has also received a lot of attention in recent years.

Physicochemical properties of compounds alone sometimes fail to give insight into its permeability since there are various permeability pathways that involve specific transporters, which are essentially proteins that recognize the drugs in specific ways that cannot be predicted, based on physicochemical properties itself. In general, an increase

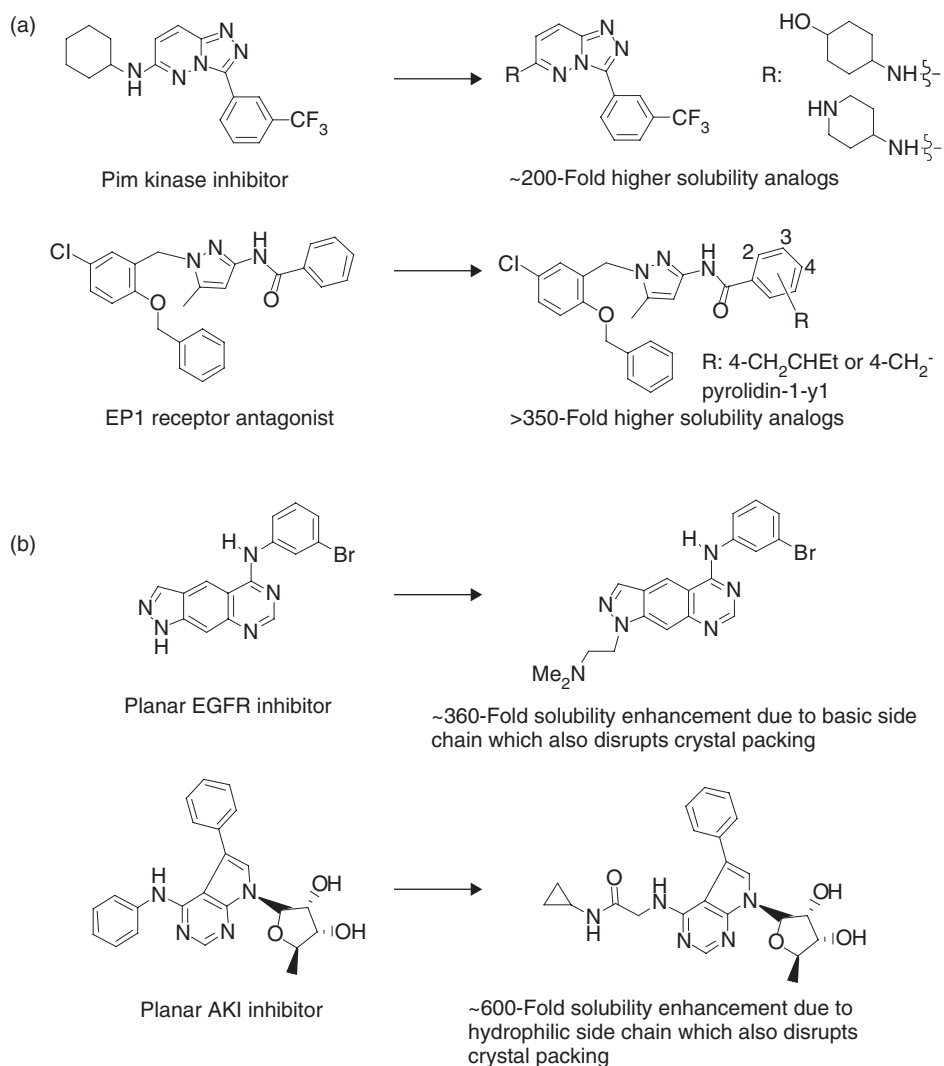


Figure 1.1 Literature examples of solubility optimization due to (a) introduction of ionizable groups and (b) disruption of crystal packing.

in lipophilicity, decrease in H-bonding, and reduction in polarity of a chemical series has been found to improve permeability of compounds [18–20] (Fig. 1.3). Sometimes, these changes do not always lead to an expected increase in the improvement of Caco-2 permeability as seen during structure optimization of a series of phenothiazine carboxylic compounds as novel histamine H₁ antagonist [21].

Attaining permeability of drug candidates across the blood–brain barrier (BBB) is a challenge in the development of central nervous system (CNS) drugs with only 2% of the CNS discovery compounds being able to cross the BBB to impart their therapeutic efficacy. Like Lipinski's rule-of-five, there are some predictive rules such as Clark's rule and Pardridge's rule, which are able to predict permeability across

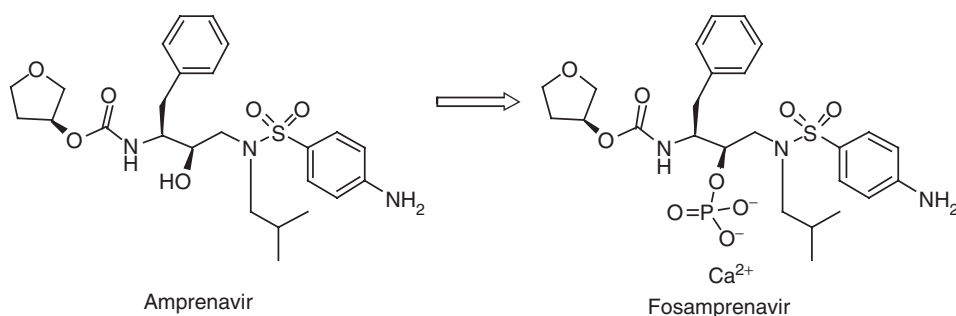


Figure 1.2 Overcoming poor solubility due to prodrug approach.

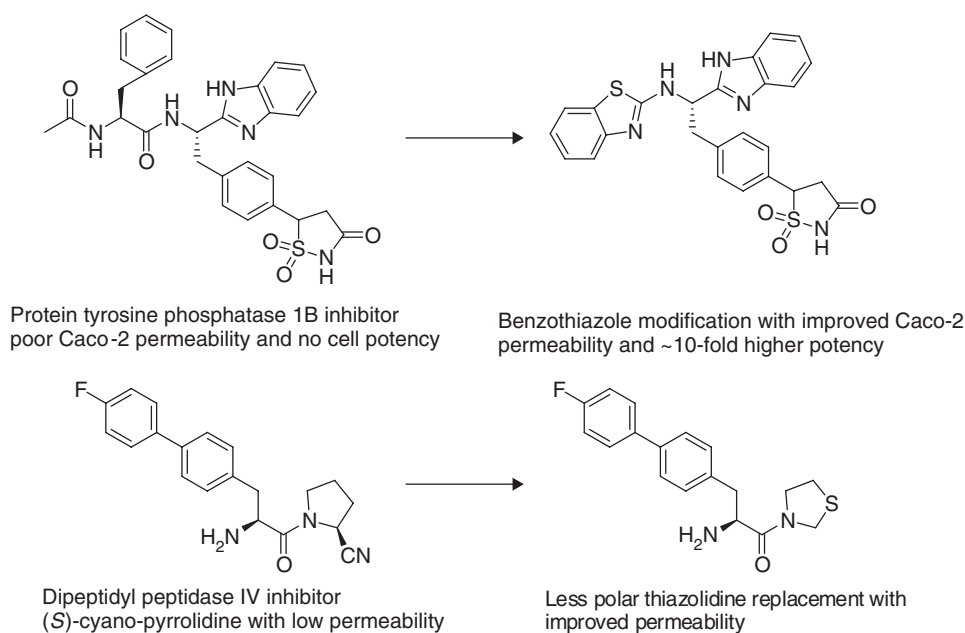


Figure 1.3 Literature examples of permeability optimization.

BBB with reasonable accuracy. The BBB membrane is negatively charged and this favors permeability of basic amine-containing compounds that are positively charged at physiological pH. Implication of charge can be seen in comparing indomethacin and trifluoroperazine. Indomethacin, which has a pK_a of 4.18 is negatively charged, while trifluoroperazine, which has a pK_a of 7.8, is positively charged at physiological pH, which results in inability of indomethacin to cross the negatively charged BBB [22]. Strategies that improve intestinal permeability, such as increased lipophilicity, decreased H-bonding, decreased polarity, also improve BBB penetrability. Uptake of morphine across BBB was found to be 32-fold higher than its M6-glucuronide, which has ~150-fold lower lipophilicity [23]. In another example, the substitution of one $-OH$ group in morphine by a $-OCH_3$ in codeine reduced the intermolecular H-bonding and lipophilicity and increased BBB penetration increased by 10-fold. Replacement of

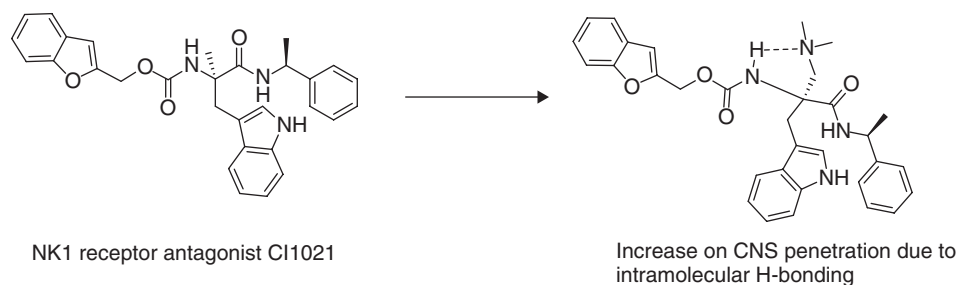


Figure 1.4 Introduction of intramolecular H-bonding to enhance BBB permeability.

both $-OH$ in morphine by acetates to form heroine resulted in a 100-fold increase in brain permeability [23]. Introduction of intramolecular H-bonding has been shown to be effective in making a compound more lipophilic, hence increasing CNS penetration (Fig. 1.4) [24].

In general, ways to improve solubility include (i) reduction of $\log P$ via introduction of ionizable groups, increase in H-bonding, increase in polarity, decrease in lipophilicity and (ii) decrease in crystal packing via introducing out-of-plane functional groups. However, some of these changes result in undesirable modulation of intestinal permeability which is favored by increased lipophilicity, reduced H-bonding (reduced PSA), and reduced polarity. It is often easier to fix solubility of compounds than permeability since solubility of a chemical series can be modulated over a million-fold range, while the difference between a poorly and highly permeable compound is only by ~ 50 -fold [5].

1.3.2 Metabolic Stability

The great majority ($>75\%$) of the marketed drugs are eliminated by metabolism. High metabolic liability usually leads to poor PK properties (high clearance, low exposure, short half-life, and low OBA), and therefore, it is desirable to have a metabolically stable compound, (except in cases of prodrugs) to ensure minimal variations in interindividual responses for its exposure and duration of action. Determination of metabolic stability, also commonly referred to as *in vitro* intrinsic clearance (Cl_{int}), is one of the key *in vitro* ADME parameters that is evaluated and optimized at a very early stage of discovery to transform a less desirable, metabolically unstable chemotype into one that has favorable Cl_{int} . Metabolic stability *in vitro* is also believed to predict hepatic clearance *in vivo*, which in turn is a major contributor to total body clearance of the majority of xenobiotics. High metabolic stability and hence low Cl_{int} are often desirable. In most cases, low Cl_{int} also leads to favorable PK properties such as good OBA and long half-life, which result in good exposure and desired duration of action, respectively. Compounds that possess high Cl_{int} are also more susceptible to drug–drug interactions (DDIs) and possess less-than-desirable exposure and duration of action required for therapeutic efficacy of a drug.

1.3.3 Plasma Protein Binding

PPB is believed to have a significant influence on the rate of drug diffusion between plasma and tissues (influx and efflux) [25] and therefore influence Cl and V_{dss} of

drugs. In general, it is desirable to avoid highly plasma protein bound drug since small changes in PPB of a highly bound drug can lead to significant fluctuation in its free fraction.

1.4 ISSUES IN DRUG DEVELOPMENT

1.4.1 Failure of *In Vitro*–*In Vivo* Correlation for Prediction of DDIs

In early stages of drug discovery, as actual human data are not available, results obtained from human subcellular fractions are extremely valuable in predicting the human PK parameters and assessing the potential of an NCE as a successful human drug candidate. But before a significant amount of effort is dedicated to optimize and modulate drug metabolism and pharmacokinetics (DMPK) properties of a new chemotype, it is very important to evaluate the ability of the *in vitro* ADME assays to predict the *in vivo* situation, that is, establish *in vitro*–*in vivo* correlations (IVIVCs) at very early stages of drug discovery. The following are some underlying reasons that are being made in predicting Cl_{hep} from Cl_{int} for the failure of IVIVCs [7]:

1. Assume that metabolism is the major route of Cl of the NCE.
2. Assume that liver is the primary organ responsible for the Cl (additional relevant *in vivo* clearance pathways, such as renal or biliary, that are not captured in *in vitro*).
3. Assume that cytochrome P450 (CYP)-mediated oxidative metabolism is the key metabolism pathway and contributions by other phase I and phase II enzymes, and hence DDI mediated by these, are nonexistent.
4. Failure to account for shift in major metabolic pathway *in vivo* as compared to *in vitro* (not predicted by *in vitro*).
5. Failure to account for formation of inhibitory (or inducing) metabolites *in vivo* (or vice versa).
6. Assume that Cl_{int} is similar in microsomes and hepatocytes *in vivo*.
7. Assume that enzyme expression and activities in isolated subcellular fractions is representative of those in the physiological milieu.
8. Assume that no uptake (not modeled by microsomes) or efflux transporters (not modeled by hepatocytes) are involved in drug uptake and Cl.
9. Failure to account for intestinal efflux.
10. Failure to account for effect of f_u in the intestine.
11. Uncertainty in quantitative effect of inhibitors toward intestinal clearance.
12. Failure to incorporate high PPB or concentration-dependent PPB of an NCE.
13. Failure to account for nonspecific binding of an NCE to microsomes, hepatocytes, or blood cells.
14. Failure to identify correct enzyme inhibition mechanism [reversible vs mechanism-based inactivation (MBI)] or inaccurate determination of k_{deg} in case of MBI.
15. Assume that the key kinetic parameters, such as K_i , k_{cat} , K_I , k_{inact} , k_{deg} , EC_{50} , and E_{max} , that are crucial in DDI assessment are similar *in vivo* to those determined *in vitro*.

16. Inaccurate determination of $f_m \times f_{m,CYP}$ (in the case of CYP-mediated DDI).
17. Effect of genetic polymorphism leading to interindividual variability in exposure, species, and gender difference in DME and transporter expression and activity.
18. Failure to quantitatively account for concurrent inhibition and induction by drugs (e.g., ritonavir and CYP3A4).
19. Lack of physiological scaling factors for transporters and DMEs other than CYPs [DMEs such as flavin monooxygenase (FMO), uridine glucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), and sulfotransferase (SULT) and transporters such as Pgp, Breast cancer resistance protein (BCRP), Multidrug resistance-associated protein (MRP), Organic anion transporter (OAT), and Organic anion transporter polypeptide (OATP)].
20. Inability of *in vitro* systems to evaluate complex interplay of DMEs and transporters.
21. Assume that no atypical kinetics between the NCE and the enzyme responsible for its Cl.

It can be simply summarized that due to species differences in the expression levels, tissue localization, substrate specificity and gene regulation of transporters and DMEs, and extrapolation of data from *in vitro* studies and *in vivo* preclinical species to predict drug disposition *in vivo* in humans is very challenging.

1.4.2 Projection of Human PK

Accurate projection of human PK parameters (Cl, V_{dss} , and F) and efficacious dose is extremely valuable in drug discovery and ensures that the NCEs with poor PK properties or safety concerns (due to high dose requirement) are not selected as potential drug candidates for development. Over the past two decades, numerous methodologies using the drug metabolism and disposition data from *in vitro* human tissues such as microsomes or hepatocytes and *in vivo* preclinical studies have been developed, each with its own advantages and disadvantages [26,27]. Using these techniques, an overall success rate of 60–80% of compounds in prediction of human PK parameters within twofold of actual human PK parameters [26–28] has been achieved. Recently, Hosea *et al.* [29] have shown that the either single-species *in vivo* data or *in vitro* human liver microsomes can quantitatively predict human *in vivo* PK within twofold of actual values by conducting a retrospective analysis of 50 proprietary compounds for which *in vitro*, preclinical PK data and oral single-dose human PK data were available. The success rate of 60–80% is good to rank order the NCEs in drug discovery but is insufficient to discriminate closely related analogs within a series [28]. In drug discovery, the ability to predict the human PK of an NCE from *in vitro* and *in vivo* models with a reasonable degree of accuracy (<twofold variation) is one of the most challenging task.

Human PK projection can be performed via some common methods (Fig. 1.5) [7]: (i) allometric scaling of preclinical *in vivo* PK parameters; (ii) *in vivo* Cl projection from *in vitro* Cl_{int} ; (iii) species invariant time methods such as equivalent time, kallynochrons (elementary Dedrick plot), apolysichrons, dienetichrons, or syndesichrons, each of which are some modifications of the elementary Dedrick plot method; and (iv) C_{ss} -mean residence time method. For human OBA estimation, the fraction of dose appeared into the portal vein ($F_g \times F_a$) of each preclinical animal species can be calculated, the average of which can be used to estimate human $F_g \times F_a$. This estimate

Simple allometry:	BW = body weight
	MLP = maximum life span
$Cl = a BW^b$	a = coefficient (log a is the y -intercept) of Cl
$V_{dss} = c BW^d$	b = exponent (b is slope of log-log plot of Cl and BW) of Cl
	c = coefficient (log a is the y -intercept) of V_{dss}
	d = exponent (b is slope of log-log plot of V_{dss} and BW) of V_{dss}

ROE used for Cl prediction:

if $0.55 < b < 0.7$ then simple allometry best (above equation)

if $0.71 < b < 0.99$ then $Cl \times MLP = a BW^b$

if $b \geq 1$ then $Cl \times \text{brain weight} = a BW^b$

Elementary Dedrick plot	Complex Dedrick plot	Dienetichrons
$Y = \frac{\text{Concentration}}{(\text{Dose}/BW)}$	$Y = \frac{\text{Concentration}}{(\text{Dose}/BW^d)}$	$Y = \frac{\text{Concentration}}{(\text{Dose}/BW^d)}$
$X = \frac{\text{Time}}{BW^{1-b}}$	$X = \frac{\text{Time}}{BW^{d-b}}$	$X = \frac{\text{Time}}{MLP \times BW^{1-b}}$

Calculation of OBA in human

$$f_a \times f_g = \frac{F_{PO, \text{animal}}}{\left(1 - \frac{Cl_{\text{hep, animal}}}{Q_{h, \text{animal}}}\right)}$$

$$F_{PO, \text{human}} = f_a \times f_g \times \left(1 - \frac{Cl_{\text{hep, human}}}{Q_{h, \text{human}}}\right)$$

Cl_{hep} = hepatic clearance
 Q_h = hepatic blood flow
 f_a = fraction absorbed
 f_g = fraction escaping gut first pass
 F_{po} = bioavailability

Figure 1.5 Various methods used to predict human pharmacokinetics.

can be combined with the predicted human Cl , obtained by methods outlined above, to yield the predicted human OBA. Human OBA values can further be used to estimate the predicted human exposure at a desired dose. It is highly advisable to use multiple scaling methods to obtain a range of values for each of the predicted PK parameters.

1.4.3 Prediction of Human Metabolites

Metabolism facilitates the removal of chemicals from the body. In general, metabolites are pharmacologically less active and less toxic than their corresponding parent compound. However, the biotransformation reactions can sometimes lead to undesirable consequences, such as too rapid drug clearance, formation of pharmacologically active metabolites, DDIs via inhibition or induction of DMEs, and/or formation of toxic metabolites. In addition, drug-metabolizing ability is age dependent, influenced by the genetics of the individual (polymorphisms), reduced by certain disease states and can be altered when coadministered with other drugs.

Knowledge of the metabolic site(s) of an NCE in early drug discovery is essential for selecting compounds with favorable PK credentials and aiding medicinal chemists in modifying metabolic “soft spots” and to design better, lower metabolic-clearance analogs. In development, elucidation of biotransformation pathways of a drug candidate by identifying its circulatory and excretory metabolites is vitally important to understand its physiological effects. In discovery, metabolite characterization is performed *in vitro* using hepatic subcellular fractions from human and multiple animals to guide species selection for safety assessment studies in preclinical development. The species that most closely resembles human with respect to metabolite profile are selected as the preferred species for toxicity evaluations. Metabolite identification is, therefore, very important in drug discovery for the NCE progression and should be incorporated at very early stages to eliminate structural liabilities in the later stages of drug development. Efforts directed to improve metabolic stability are represented by some selected examples from literature, as shown in Fig. 1.6 [7].

Structure elucidation of metabolites may also indicate the formation of reactive intermediates, which have the potential to bind to biomolecules and cause immune-mediated toxicity. Knowledge of formation of reactive metabolites in the early drug discovery stages is very helpful in minimizing the risk associated with reactive intermediates/metabolites. It is postulated that reactive metabolites bind to crucial proteins and result in either direct organ damage or the modified protein elicits a hapten-mediated immune response. A complex cascade of signaling results in unpredictable toxic responses, also called *idiosyncratic toxicity* or *idiosyncratic drug*

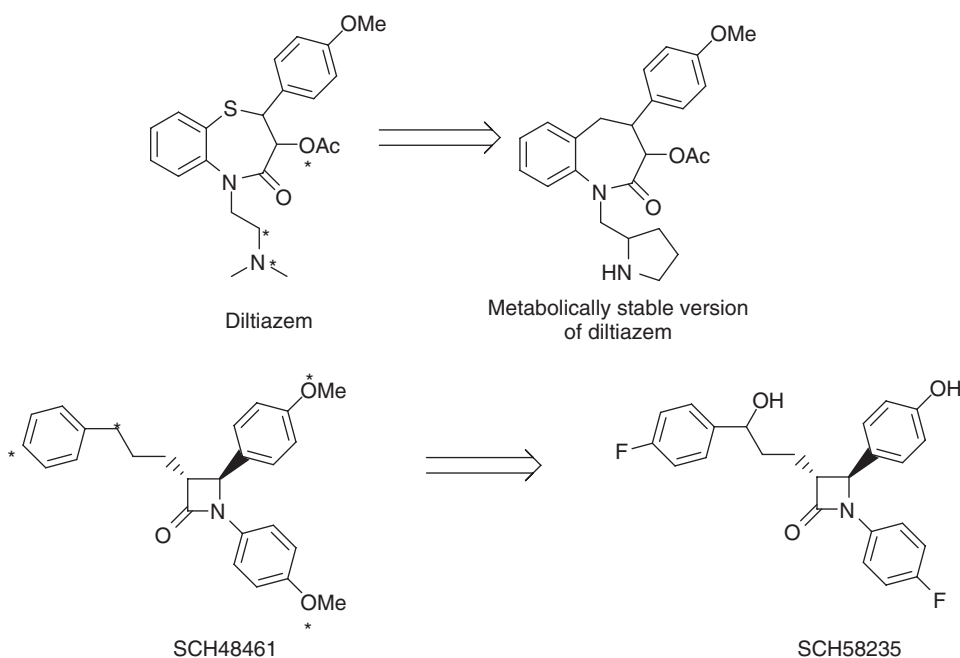


Figure 1.6 Examples to highlight structure optimization to improve metabolic stability: compounds shown on right side of the arrows are the metabolically stable version of the compounds on the left of the arrows. *Represents sites of metabolism that were blocked.

reaction (IDR). It is called idiosyncratic because the toxicity is not only unpredicted but also occurs in 1 of 1000 patients receiving the drug/NCE, thus making it virtually impossible to detect earlier at the preregistration phase. IDR risk is a significant liability and increases cost and risk of development of an NCE. It is therefore crucial to detect reactive metabolites very early on during the drug discovery phase and strategically remove the toxicophore moiety to avoid costly drug failures in development or worse, postmarketing. Structural characteristics of compounds forming reactive intermediates/metabolites have been extensively reviewed [7,30]. Compounds containing functional groups such as anilines, nitrobenzenes, benzyl/cyclopropyl amines, thiophenes, furans, thiazoles, sulfonylureas, hydrazines, carboxylic acids (those with α -protons), methylenedioxy, alkynes, terminal alkenes, halogenated hydrocarbons, and Michael acceptors [7,30] are all recognized to produce reactive metabolites.

In some cases, metabolites, due to structural similarity with their parent, could also be pharmacologically active, which results in disconnect between pharmacokinetics/pharmacodynamics (PK/PD) data. Several active metabolites have been developed and marketed as drugs. The examples of active metabolites of marketed drugs that have been developed as drugs include acetaminophen, fexofenadine, desloratadine, cetirizine, oxazepam, oxyphenbutazone, morphine-6-*O*-glucuronide, and minoxidil sulfate. Each of these drugs provides a specific benefit and has a better PK and/or safety profiles over the parent molecule.

1.4.4 Drug–Drug Interactions

Another major challenge in drug discovery is DDI. In recent years, several drugs have been withdrawn from the market in the United States and Europe due to serious adverse reactions as a result of significant DDIs [31]. DDIs are caused when the PK of one drug is influenced by a coadministered drug. The consequences of these DDIs can range from loss of therapeutic efficacy to the introduction of toxic effects. Most marketed drugs are eliminated from the body at least in part by metabolism, and many of the DMEs are inducible and can be inhibited; therefore, inhibition and/or induction of DMEs by one drug could have a significant effect on the disposition of another drug. The NCEs can either be perpetrators or victims of such interactions, and early evaluation of NCEs for their potential to function in either capacity is critical to the development of new drug candidates. The DDI risk is more profound if the victim drug has high intrinsic clearance or is selectively cleared via one major pathway. So, development of an NCE that is either itself a modulator of DME or affected by modulation of the DMEs, especially if the NCE also possesses high Cl_{int} , is not favorable. Therefore, inhibition and induction of DMEs by an NCE and the DME responsible for its metabolism are routinely assessed at very early stages of the drug discovery.

DDIs should not be viewed as solely undesirable as there have been several cases where PK of one drug has been modulated by another via a well-planned design to improve the exposure (and hence the efficacy) of the affected drug [7]. Kaletra is a coformulation of lopinavir and ritonavir, whereby ritonavir-mediated CYP3A4 inhibition results in higher plasma levels of lopinavir and boosts its anti-HIV protease activity. Ketoconazole (KTZ), a potent CYP3A4 inhibitor, is commonly used in combination with cyclosporine A (CsA) to enhance the immunosuppressive properties of

the latter as a result of increase in CsA exposure to due KTZ-mediated inhibition of CsA metabolism.

1.4.4.1 Drug-Metabolizing Enzyme Inhibition. Inhibition of enzymes can be reversible or irreversible and always results in reduction of intrinsic clearance of the pathway that is inhibited [32]. Reversible inhibition, also known as *direct inhibition*, can be typically classified as competitive, uncompetitive, noncompetitive, or mixed, with competitive inhibition being the most commonly observed pathway of inhibition. *In vitro* inhibition studies are valuable in assessing potency of an NCE as an inhibitor as given by $[I]/K_i$ ratio (details in Section 1.5.2). In addition to the $[I]/K_i$ ratio of the inhibitor, contribution of the inhibited pathway to the overall metabolism of an NCE, $f_m \times f_{m,CYP}$ (f_m = fraction of the NCE cleared via metabolism, $f_{m,CYP}$ = fraction of metabolism via CYP), enzyme interaction at the intestinal site and parallel routes of metabolism, all play a vital role in risk assessment due to potential DDI. In situations where an NCE can inhibit multiple CYPs with $[I]/K_i$ ratio >0.1 for each of the CYP-mediated pathways, a rank order approach is recommended for conduct of clinical DDI [7,33].

For MBI, *catalytic bioactivation of an NCE by an enzyme* is a *prerequisite*. This results in irreversible inhibition of the enzyme, and therefore, potency of both these types of inhibition increase over time (with preincubation). MBI of CYPs have been extensively investigated and the presence of functional groups [7,30] such as aniline, nitrobenzene, hydrazine, benzyl/propargyl/cyclopropyl amine, hydantoin, thioureas, thiazole, furan, thiophene, epoxides, methylene dioxy, methyl indoles, alkyne, isothiocyanate, and terminal alkenes, on an NCE warrants immediate and early assessment of inactivation potential of the NCE to avoid severe DDI liability in late stage development. It must be borne in mind that if an NCE does possess a structural alert, it does not automatically imply that it will be a potent inhibitor. Distinguishing MBI from simple reversible inhibitor is critical in predicting a clinical DDI, since applying a reversible inhibition model to an MBI may result in significant underprediction of a DDI risk.

The magnitude of a DDI is dependent on the degree of contribution of the clearance pathway that is modulated (inhibited or induced); higher the fraction of the dose metabolized by the enzyme that is modulated, greater is the magnitude of DDI. It is very favorable for an NCE to have multiple pathways of clearance, so that when one of these is affected, it is compensated for by the other pathways. Drugs that are metabolized by polymorphic enzymes (e.g., 2D6, 2C9, and 2C19) are prone to risk of either lack of drug efficacy (due to increased clearance of the drug in the rapid metabolizers) or increased toxicity (due to accumulation of drug in the poor metabolizers) and may warrant a dose adjustment based on the population genotype. Hence, identifying major pathways that are responsible for the clearance of an NCE is crucial in eliminating DDI risk later on in drug development [7]. A significant amount of work has been done in the area of the CYP enzyme phenotyping due to the availability of cDNA-expressed enzymes, normalization methods, and isoform-specific substrates and inhibitors (chemical and antibody) [34]. CYP phenotyping involves rigorous kinetic analysis of metabolite formation, to give an estimate of $f_{m,CYP}$, which represents *in vivo* contribution of a particular isoform(s) toward a biotransformation pathway of an NCE. The magnitude of the product of $f_{m,CYP}$ and f_m reflects the propensity of an NCE to be subjected to DDI. Higher the value of $f_{m,CYP} \times f_m$, higher will be the risk

Exposure change due to inhibition or induction of CYP-metabolism

$$\frac{AUC_{PO, \text{inhibitor}}}{AUC_{PO, \text{control}}} = \frac{f_{g, \text{inhibitor}}}{f_{g, \text{control}}} \times \frac{1}{\left(\frac{f_m f_{m, \text{CYP}}}{Cl_{\text{int, control}} / Cl_{\text{int, inhibitor}}} \right) + [1 - (f_m f_{m, \text{CYP}})]}$$

$$Cl_{\text{int, control}} / Cl_{\text{int, inhibitor}} = 1 + [I] / K_i \text{ for reversible competitive or noncompetitive inhibition}$$

$$= 1 + \frac{k_{\text{inact}}}{K_i} \cdot \frac{[I]}{k_{\text{deg}} \text{ for mechanism-based inhibition}}$$

AUC = exposure

f_g = fraction surviving gut first pass

f_m = fraction of total clearance due to all CYP-metabolism

$f_{m, \text{CYP}}$ = fraction of total CYP-metabolism catalyzed by the inhibited CYP isoform

$[I]$ = inhibitor concentration

k_{inact} = rate constant of CYP inactivation

K_i = half maximal inactivation rate

K_i = dissociation constant of enzyme-inhibitor complex

Exposure change due to inhibition of intestinal metabolism (for drugs that this pathway is significant)

$$\frac{AUC_{PO, \text{inhibitor}}}{AUC_{PO, \text{control}}} = \frac{Q_{gm} + f_u Cl_{\text{int, gm}}}{\left(Q_{gm} + \frac{f_u \cdot Cl_{\text{int, gm}}}{[1 + (I_{gm} / K_i)]} \right)} \cdot \left(\frac{1 + I_u}{K_i} \right)$$

I_{gm} = unbound inhibitor concentration in intestine
 I_u = unbound inhibitor concentration in liver
 Q_{gm} = blood flow to GI mucosa
 f_u = fraction unbound of inhibitor in plasma
 K_i = inhibition constant

Figure 1.7 Exposure change in the presence of inhibitor.

of DDI. Changes in exposure of an NCE in the presence and absence of a modulator (inhibitor or inducer) can be simulated by the equation shown in Fig. 1.7 [7].

Estimation of f_m can be most accurately made from a human radiolabel study. A good estimate can also be obtained from a single-dose phase I human study. In many projects, due to the absence of human data in early stages of development, estimates of f_m are not available and $f_{m, \text{CYP}}$ data are used to guide clinical DDI studies via a “rank order” approach [33].

Unfortunately, the assessment of the contribution by other enzymes such as FMO, SULT, and UGT are limited to qualitative evaluation only due to limitation of availability of cDNA-expressed enzymes, normalization methods, and isoform-specific substrates and inhibitors.

1.4.4.2 Drug-Metabolizing Enzyme Induction. DDI can also occur through induction of nuclear receptors and modulation of the transcription and the expression of DMEs and transporters. This results in elevation of enzyme levels or increase in activity, which leads to faster clearance of the NCEs. Faster clearance of an NCE leads to lower than expected exposure, as demonstrated by significant decrease in exposures of several drugs such as midazolam, amitriptyline, cyclosporine, digoxin, indinavir, irinotecan, warfarin, phenprocoumon, alprazolam, dextrometorphane, simvastatin, and ethinylestradiol in the presence of St. John’s wort, which has been shown to be a potent inducer of both CYP3A4 and Pgp [7]. Reduced exposure sometimes severely compromises therapeutic efficacy as seen with coadministration of cyclosporine (CYP3A4 substrate) and rifampin (potent CYP3A4 inducer) to organ transplant patients. Induction

of DMEs and transporters is regulated by a large family of nuclear receptors, such as aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), and peroxisome proliferator-activated receptor- $\alpha/\beta/\gamma$ (PPAR- $\alpha/\beta/\gamma$) [35]. Owing to regulation of several DMEs and transporters via common receptors, it is advised to monitor a few sentinel CYP isoforms that are indicative of activation of the principle nuclear receptors. CYP1A2, 2B6, and 3A4 induction are suggested to be indicative of activation of AhR, CAR, and PXR, respectively [36].

1.4.4.3 Interplay between Drug-Metabolizing Enzymes and Transporters. There are numerous examples in the literature where a DDI may be the result of modulation of a DME, as exemplified by the DDI between terfenadine-KTZ, fluvoxamine-astemizole, cisapride-erythromycin, statins-protease inhibitors, felodipine-nifedipine, codein-quinidine, β -blockers-ritonavir, and rifampin-cyclosporine/simvastatin/verapamil/midazolam. Sometimes it may be due to modulation of transporters such as those exemplified by digoxin-quinidine, probenecid-acyclovir/allopurinol/cephalosporins/ciprofloxacin, and talinolol/verapamil. More often the DDIs result from an intricate interplay of modulation of both DMEs and transporters. Gemfibrozil causes six- to eight fold increase in exposures of coadministered drugs such as cerivastatin and repaglinide (antidiabetic) not only due to inhibition of uptake inhibitor OATP1B1 but also due to inhibition of CYP2C8, CYP3A4, and Pgp. Increase in plasma and Cerebrospinal fluid (CSF) concentration of the HIV protease inhibitors ritonavir and saquinavir, due to coadministration with KTZ, has been attributed to potent inhibition of both CYP3A4 and Pgp by KTZ.

Transporters can also modulate the extent of metabolism of an NCE by affecting its exposure of an NCE to the DMEs [37,38]. Ability of transporters to modulate metabolism is also dependent on the Biopharmaceutics Classification System (BCS) class of the NCE under investigation [37,39]. Uptake or efflux transporters are unlikely to affect metabolism/exposure of class 1 (high solubility and high permeability) compounds, while uptake and efflux transporters are expected to modulate metabolism/exposure of NCEs, belonging to class 2 (low solubility and high permeability), class 3 (high solubility and low permeability), and class 4 (low solubility and low permeability).

1.4.4.4 Plasma Protein Binding. It is a common concern when the free drug concentration of a highly bound drug A increases due to PPB displacement by a strong binding drug B. The increased free drug A's concentration could potentially cause toxicity (due to more free fraction available to interact with target receptors) and may need dose adjustment. In addition to target-mediated efficacy/toxicity, PPB information is also helpful to estimate unbound/effective concentration of drugs that mediate inhibition of DMEs and transporters. Disease models are extensively validated in animals to progress a compound into development. *Unbound drug concentration* and therefore PPB across species is very informative in establishing safety margins and guiding selection of First in human (FIH) dose and human efficacious dose.

CNS drugs have been highlighted to be extremely sensitive to PPB and PPB-displacement situation. It must be recognized that BBB transporters, permeability, and protein binding to blood versus brain *all* play an equally important role in the rate and extent of drug uptake into CNS [40]. Increase in nonspecific binding in brain

tissue is considered to be a driving force to overcome effect of efflux transporters and therefore, Pgp substrates can still penetrate the brain because partition into brain tissue can provide a significant drug concentration gradient at the BBB to overcome effects of efflux transporters. Usually, increase in lipophilicity of a drug, not only increases its CNS penetration/permeability, but it also increases its nonspecific binding to brain tissue, resulting in lesser unbound/pharmacologically active drug available in brain for efficacy. Several examples of compounds that demonstrate high target affinity, high plasma concentration, and high brain/plasma (B/P) ratio, and yet fail to show efficacy, warrant extreme caution in optimization of the total B/P ratio only, for CNS drugs. CSF has been widely believed to be a surrogate for estimation of the unbound, pharmacologically active drug concentration in CNS [41]. Owing to a very low protein content of CSF, drug–protein binding in CSF is assumed to be negligible and CSF concentration is considered to represent the unbound drug in CNS, which is equal to the unbound drug concentration in plasma at steady-state equilibrium.

1.4.5 Polymorphism of Drug-Metabolizing enzymes

Many of the DMEs show genetic polymorphism, which can influence the levels of expression, enzyme stability, and catalytic properties [42–45]. Polymorphisms that have been discovered in the genes of CYP2C19, CYP2D6, UGT1A1, NAT, and thiopurine methyl transferase (TMT) are particularly good examples of the importance of knowing polymorphisms in DMEs and the challenges in the development of NCEs as new drugs. Genetic variability in DMEs is a significant contributor to the variability in human drug PK. Hence, knowledge of the enzymes involved in clearance of NCEs along with knowledge of polymorphisms that exist for these enzymes is critical to the development of safer drug.

1.4.6 Drug-Induced Toxicity

Drug-induced toxicity remains one of the major reasons for failures of new pharmaceuticals and for the withdrawal of approved drugs from the market. The combined *in vitro*, *in vivo*, and *in silico* computational models have been used to assess the toxicity of the NCEs. While using these approaches, many compounds that might have serious adverse reactions associated with them are effectively eliminated before reaching the clinical trials; some toxicities such as those caused by idiosyncratic responses, however, are not detected until the drug is in late stages of clinical trials or have become available to the market. One of the proposed mechanisms for the development of idiosyncratic drug toxicity is the bioactivation of drugs to form the reactive metabolites by DMEs (Section 1.4.3). Efforts are being made to detect these reactive metabolites in the early stages of drug development [46].

1.4.7 Metabolites in Safety Testing

In February 2008, Food and Drug Administration (FDA) issued the final Guidance for Industry: Safety Testing of Drug Metabolites. The guidance recommends that the exposure of human metabolites that exceed 10% of the parent drug exposure [ICH (International Conference on Harmonisation) revised to total drug-related material] at steady state should be equal or higher in at least one of the preclinical animal species be used for long-term safety assessment [47,48]. Additional toxicological testing on

metabolites that display higher exposure in humans than preclinical animal species may be required by direct administration of metabolites to the test animals. Therefore, metabolite safety qualification strategy needs to be established as soon as practical in drug development. There are a number of examples when human metabolism was quantitatively different from preclinical species.

1.5 ADME ASSAYS IN DRUG DISCOVERY

Multiple *in silico*, *in vitro*, and *in vivo* ADME studies have been developed and implemented in various stages of the drug discovery to weed out weak candidates and to select a compound with favorable PK properties for further development. Table 1.1 summarizes some of the commonly used ADME assays that DMPK scientists rely on to not only help in the iterative screening of compounds in discovery but also for risk mitigation as well as to gain insight into mechanism of certain ADME pathway or process. A list of useful guidances and white papers for the design and conduct of metabolism and DDI studies are described in Table 1.2 [47–55].

1.5.1 *In Silico*

Availability of experimental data, molecular descriptors (molecular size and shape, hydrophilic and hydrophobic regions, hydrophilic and lipophilic balance, and H-bond donors and acceptors), and statistical analysis methods (multiple linear, partial least squares, artificial neural nets, Bayesian neural networks, and clustered regression) are the three essential prerequisites for *in silico* modeling. *In silico* models for solubility take into account log *P*, melting point, crystal packing, intrinsic solubility, and ionization effects [56–60]. From the simple general solubility equation, to more complex models based on Monte Carlo simulations using 2D or 3D properties, and fragmental or atom-based or quantum-chemical methods have all been used to predict aqueous as well as Dimethyl sulfoxide (DMSO) solubility of compounds with fair accuracy. There are several programs (ACD/logs, SLIPPER, CSlogWS, KLOGS, CERIUS, and WSKOWWIN) available for these predictions and are highly recommended for solubility prediction in early discovery [61].

Molecular descriptors such as H-bond donors and acceptors, hydrophobic groups, aromatic rings, and basic nitrogen atoms are all important for the development of permeability and Pgp substrate models [61]. Computational Volsurf-based model [62], conformation-dependent, GRid-Independent descriptors (GRIND) descriptor method, and recent QSAR models [63] have all been valuable in aiding medicinal chemists to optimize permeability in drug discovery stages. Recent development in predictive models for BBB penetration has also gained significant popularity, which is also directed to gain better insight into transporters at the BBB junction [64,65]. Methods include 2D-QSAR techniques, built on pharmacophore modeling using Catalyst, DISCOtech, and 3D-QSAR techniques, using SYBYL, comparative molecular field analysis (CoMFA), and comparative molecular similarity index analysis (CoMSIA).

Composite QSAR models that incorporate solubility and permeability and programs (iDEA and GastroPlus) have been very useful to predict fraction of dose absorbed and bioavailability [66]. Bioavailability predictions are not very simple since it necessitates integration of absorption prediction (combination of solubility and permeability) and first-pass metabolism (gut and liver). QSAR models and adaptive fuzzy portioning

TABLE 1.1 List of Commonly Used ADME Assays Used in Various Stages of Drug Discovery and Development

Parameter	Type	Method	Throughput
Solubility	Kinetic	Nephelometry	High
		Direct UV	High
		Turbidimetry	High
	Thermodynamic	Equilibrium shake flask	Low
		Potentiometric	Low
Permeability	Passive diffusion	Artificial membrane-based (IAM and PAMPA)	High
		Cell-based (Caco-2, MDCK, LLC-PK1, and CHO)	Moderate
		Cell-based Calcein-AM	High
	Transporter assisted	Inverted membrane vesicle-based ATPase	High
		Cell-based (Caco-2, MDCK, LLC-PK1, CHO, HEK293, KB-V-1, and MCF-7)	Moderate
		Transient transfection (oocytes, CHO, and COS)	Moderate
		BMECs for BBB	Moderate
	Rate of absorption	Gene KO animal studies (<i>in vivo</i>)	Low
		<i>In situ</i> tissue (liver, intestine, and brain) perfusion	Low
		Intraduodenal and portal vein cannulated rats (<i>in vivo</i>)	Low
Metabolism	Clearance	cDNA-expressed enzymes	High
		Subcellular fractions (microsomes and S9)	High
		Cell-based (fresh or cryopreserved hepatocytes)	Moderate/high
	Enzyme Inhibition	cDNA-expressed enzymes	High
		Subcellular fractions (microsomes and S9)	High
		Cell-based (cryopreserved hepatocytes)	Moderate/high
	Enzyme induction	Liver slices	Low
		Reporter gene assay (PXR binding and transactivation)	High
		Cell-based (sandwich-cultured) hepatocytes	Low
	Reaction phenotyping	cDNA-expressed enzymes	High
		Subcellular fractions (microsomes and S9)	High
		Cell-based (cryopreserved hepatocytes)	High
	Metabolite ID	Subcellular fractions (microsomes and S9)	High
		Cell-based (cryopreserved hepatocytes)	High

(continued overleaf)

TABLE 1.1 (Continued)

Parameter	Type	Method	Throughput
PPB	N/A	Biological matrices (plasma, bile, and urine)	Moderate
		Biacore technology (fluorescence spectroscopy)	High
		Chromatography and capillary electrophoresis	Moderate
		Ultrafiltration	High
		Ultracentrifugation	High
		Equilibrium dialysis (traditional/rapid-RED)	Low/high
		Microdialysis (<i>in vivo/in situ</i>)	Low

TABLE 1.2 List of Some Useful Guidances and White Papers for Design and Conduct of Metabolism and DDI Studies

Guidance	Purpose	References
The conduct of <i>in vitro</i> and <i>in vivo</i> drug–drug interaction studies: a PhRMA perspective	DDI study design and conduct	49
The conduct of <i>in vitro</i> studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the PhRMA	TDI study design and conduct	53
PhRMA white paper on ADME pharmacogenomics	Polymorphic DME and transporters	55
Drug interaction studies—study design, data analysis, and implications for dosing and labeling	Design and conduct DDI	52
Drug metabolism/drug interactions in the drug development process: studies <i>in vitro</i>	Design and conduct <i>in vitro</i> metabolism and DDI	50
<i>in vivo</i> drug metabolism/drug interaction studies—study design, data analysis, and recommendations for dosing and labeling	Design and conduct <i>in vivo</i> metabolism and DDI	51
The International Transporter Consortium: A Collaborative Group of Scientists from Academia, Industry, and the FDA	Decision tree for transporter DDI assessment	54
Safety testing of drug metabolites	Safety assessment of metabolites	47
ICH guidance of nonclinical safety studies for the conduct of human clinical trials and market authorization for pharmaceuticals	Safety assessment of metabolites	48

(AFP) models have been observed to yield a 60–65% prediction at best [67,68]. *In silico* intestinal device (ISID) model has also been developed to model absorption for compounds that are dual substrates of CYP and Pgp [69].

QSAR models to predict drug metabolism have been extensively developed using various statistical techniques [MLR (Multiple Linear Regression), PLS (Partial Least Square), NBC (Naïve Bayes Classifier), k-NN, SOM (Self Organizing Map), RP (Recursive Partitioning), ANN (Artificial Neural Networks), and SVM (Support Vector Machines)] [70–72]. A recent MLR approach for human hepatic clearance prediction using *in vitro* experimental data and six molecular descriptors (MW, number of total atoms, number of aromatic rings, number of single bonds, TPSA, and SKlogP) yielded ~85% success [70]. Random Forest and Bayesian methods [based on MOE (Mixture of Experts) and E-state descriptors] to predict human microsomal stability have also yielded about 75–80% prediction success. Availability of X-ray crystallographic structures of mammalian CYP enzymes has prompted development of ligand- or CYP enzyme-based 3D-QSAR, quantum mechanics (QM), and pharmacophore models [73], which have predicted compounds' ability to be CYP substrates with reasonable accuracy. Softwares such as MetabolExpert, META, METEOR, and MetaSite are readily available to discovery teams for prediction of metabolic sites. Significant effort has been directed toward developing *in silico* models for CYP inhibition especially for CYP3A4 and 2D6, since these are the two isoforms that metabolize a vast majority of the marketed drugs. Several approaches such as RP ensemble method, PLS and ANN models, MLR analysis, NN, and Bayesian methods have been developed, some with as high as 99% prediction accuracy of CYP inhibitors. Pharmacophoric models and 3D-QSAR models (CoMFA and GOLPE/GRID) have also been used to predict CYP inhibitors with ~70% accuracy. It is interesting to note that for different CYP isoforms, different molecular descriptors are important, based on physicochemical nature of substrate binding at the CYP-active site. For example, molecular volume is important for CYP1A2 inhibition, while H-bond acceptor and hydrophobicity are more important for CYP2C9 models and MW, H-bonding capacity and number of aromatic atoms are more important for CYP3A4 models [74].

In silico modeling of CYP induction is very challenging due to the requirement of availability of crystal structure of nuclear receptors along with well-understood structural biology [75]. In addition, due to large binding pocket of some of the receptors, especially PXR, combined with the ability of the ligands to bind to several regions of the nuclear receptors further complicates modeling. Crystal structure of PXR, along with experimental data, has been used to build *in silico* PXR-binding model. Introduction of polar groups in the regions that show high affinity to the hydrophobic binding pocket of PXR, as well as making compounds more rigid to prevent binding, have been successful strategies, to synthesize compounds that do not bind to PXR. Pharmacophore modeling of hCAR also was able to model hCAR ligands with reasonable success.

In general, for CYPs and nuclear receptors, flexibility of their active sites, promiscuous nature due to which they accommodate very diverse ligands, the presence of multiple binding sites, binding modes, binding orientation, ability of ligands to alter protein structure, unavailability of unambiguous structural biology mechanism for some pathways, and the presence of multiple metabolic pathways have all contributed to make *in silico* modeling of CYP inhibition and CYP induction very challenging.

In silico metabolism prediction softwares such as Meteor and Metasite, coupled with several metabolite identification data processing softwares (offered by Agilent,

Applied Biosystems, and Thermo Scientific), have made biotransformation prediction for an NCE much easier. However, a strong organic chemistry background coupled with good expertise in liquid chromatography (LC)/tandem mass spectrometry (MS/MS) methodologies and data interpretation are the key prerequisites for successful metabolite identification.

1.5.2 *In Vitro* Methodology

1.5.2.1 Solubility. Solubility measurements can be of two different types—kinetic and thermodynamic [67]. For kinetic solubility, it is common to start with a stock solution of the compounds of interest in organic solvent, mostly DMSO, which is then diluted in buffer over a wide concentration range and solubility of compounds assessed via UV or optical light scattering methods. Kinetic solubility measurements are quite common in early discovery for rank ordering compounds and are most valuable for assessing solubility of compounds in a certain *in vitro* assay (biochemical or cellular) medium. Thermodynamic solubility measurements of NCEs are usually performed, in their solid state and hence this type of solubility measurements takes into account disruption of crystal lattices and dissolution process of the compounds. Although the gold standard for solubility measurements, thermodynamic solubility measurements are quite labor intensive and variations of a classical thermodynamic solubility assay are gaining popularity, one of these involves starting with DMSO stocks as in kinetic solubility measurements, but the DMSO is completely removed to dry the compound down to a powder form before adding the buffer [76].

1.5.2.2 *Permeability/Transporters.*

1.5.2.2.1 Cell-Based. Several cell-free and cell-based systems have been employed to investigate permeability and role of transporters in permeability of an NCE [61]. The cell-free parallel artificial membrane permeability assay (PAMPA) has been widely used as a high throughput, inexpensive, and simple tool to screen discovery compounds for their potential for passive permeability [77]. Caco-2 cell permeability is a widely used, “gold standard,” *in vitro* screening and investigative assay to rank order compounds in the discovery stages, for their intrinsic permeability across intestinal epithelium and to investigate basic mechanistic studies involving absorptive transport (paracellular vs transcellular transport and the presence of saturable active transporter) [78]. Madine–Darby canine kidney (MDCK) cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) 293 cells, porcine proximal tubular epithelial (LLC-PK1) cells, human metastatic breast cancer cells (MCF-7), and Multidrug resistance protein (MDR) human KB carcinoma (KB-V-1, human cervical carcinoma/epidermoid cell line) cells are some of the other commonly used cell lines, transfected with transporter(s) of interest, to perform diagnostic/mechanistic transporter studies [79]. Cultured brain microvessel endothelial cells (BMECs), which form a confluent monolayer and retain morphology and functional capacity of BBB, are very valuable in investigating transport mechanisms involving passive diffusion and polarized uptake/efflux across the BBB [80]. Double/triple transfected cell lines (most of the time with one uptake and another efflux transporter) such as MDCKII-BCRP/OATP-B, MDCKII-NTCP/BSEP, and MDCK II-BCRP/OATP2 are very useful in investigating synergistic effect of uptake and efflux transporters across intestinal epithelium and hepatobiliary membrane [79].

1.5.2.2.2 Membrane-Based. Membrane-based transporter assays are important tools to study transporter-mediated interaction/disposition of drugs [61]. ATPase assay is a plasma membrane-based simple, rapid, and high throughput assay that can assess whether a compound is a substrate or inhibitor of ATP-binding cassette ABC transporters [81,82]. The ATPase assay endpoint is release of inorganic phosphate due to adenosine triphosphate (ATP) hydrolysis. The release of inorganic phosphate is monitored via a simple colorimetric assay. However, this assay cannot be used for Solute carrier transporters (SLC) transporters since SLC transporters lack ATPase activity. Isolated membrane vesicles, such as the rat liver canalicular membrane vesicle (CMV) [83] and rat intestinal and renal brush-border membrane vesicles (BBMV) [84], are very useful for studying ABC transport. Inside-out vesicles present ATP-binding site to the NCEs being tested and substrates of ABC transporters are taken up in an ATP-dependent process across the vesicles.

1.5.2.2.3 Oocytes. *Xenopus laevis* oocytes, transfected with SLC transporters, have also been useful for studying SLC-transporter-mediated uptake studies. Recently, MDR1-mediated drug transport has also been investigated in this model [85]. Hepatic disposition of irinotecan and its glucuronide metabolite and the effect of OATP1B1 polymorphism on its uptake were investigated using this model [86].

1.5.2.2.4 B-CLEAR. B-CLEAR hepatocytes model has been very useful in investigating biliary excretion of compounds—primary sandwich-cultured human hepatocytes (SCHHs), used in this model, permit the canalicular network to be formed and maintained [87]. Since the uptake and efflux transporters are present in the SCHHs, this model is very valuable not only for biliary excretion prediction of compounds but also for interspecies differences in hepatobiliary disposition (rat vs human), hepatobiliary—transporter-mediated DDI prediction and hepatobiliary toxicity.

1.5.2.3 Metabolism. Since liver is considered to be the primary site of metabolism for the majority of the xenobiotics and endogenous compounds, subcellular fractions, intact cells, and tissue slices, derived from liver, are powerful *in vitro* tools and are routinely used in the pharmaceutical industry to assess the metabolism of NCEs [7]. Subcellular fractions (liver microsomes, microsomes with cDNA-expressed metabolic enzymes, and S9-homogenates) from a variety of rodent and nonrodent animal species, along with human, are commercially available, easy to handle, and amenable to long-term storage. Liver microsomes are the most commonly used *in vitro* system and although they contain enzymes located only in the endoplasmic reticulum, such as CYP, FMO, UGT, and esterases, microsomes are viewed as a very good system for assessing oxidative potential of NCEs. cDNA-expressing microsomes have the advantage of expressing high concentration of one particular enzyme/isoform and are often used when assessing the DDI potential of NCEs. S9 homogenates, although have the advantage over microsomes of containing both oxidative and conjugative enzymes, have not attained as much popularity as the microsomes due to relatively lower enzyme activity levels. Predictability of *in vivo* clearance from the subcellular-fraction-derived *in vitro* Cl_{int} is often questionable because of the need to fortify the subcellular fraction with supraphysiological proportions of cofactors [e.g., NADPH (Nicotinamide adenine dinucleotide phosphate), NADPH reductase, $MgCl_2$, and cytochrome b5]. In spite of the mixed success of *in vivo* Cl_{int} predictions from subcellular-metabolism-derived data (more discussed in the IVIVC section later), it is well accepted that in the early

stages of drug discovery, the subcellular fractions play an extremely important role in rank ordering compounds. Intact cells (cryopreserved and fresh hepatocytes) and tissue slices have certain distinct advantages over subcellular fractions in that they possess intact cellular morphology and are therefore believed to mimic the *in vivo* situation more closely than subcellular fractions. It must be remembered that cryopreserved hepatocytes, although mimics compound uptake through the cell membrane as in an *in vivo* situation, however, due to internalization of efflux transporters, compound efflux is not captured. They also do not need any added cofactors to facilitate metabolism, and metabolism studies can be carried out over much more extended time periods than with the subcellular fractions. However, handling and storage of intact cells and especially tissue slices are also lot more complicated than subcellular fractions, which are by far the most commonly used *in vitro* tools to evaluate metabolism of NCEs in drug discovery stages. Several excellent reviews have been published recently to compare the advantages and disadvantages of these various *in vitro* models and overview of experimental conditions required.

1.5.2.3.1 Prediction of Human PK. Metabolic stability at the early discovery stages is commonly expressed as either percent parent compound remaining after incubation for a certain period of time (up to 30 min for microsomes and up to 2/4 h for hepatocytes), but it is highly advisable to translate the percent parent remaining to Cl_{int} (Eq. 1.1, Fig. 1.8). Commonly used methods to calculate Cl_{int} involves determination of either the *in vitro* half-life ($t_{1/2}$) or by calculating the enzyme kinetic parameters; maximum velocity of the enzymatic reaction (V_{max}), and substrate concentration yielding the half of V_{max} (K_m), as shown in Equation (2) in Fig. 1.8 [7].

To gain a better physiological perspective of Cl_{int} , it is further converted to Cl_{hep} via several different methods, such as the well-stirred (Eq. 3, Fig. 1.8) or venous equilibrium model, sinusoidal perfusion or parallel tube model, and physiological-based dispersion mode. In most cases, when the NCE in question is a low clearance compound ($E < 0.25$), Cl_{hep} predicted from either well-stirred or parallel tube models will not be much different. However, for a high clearance compound ($E > 0.75$), Cl_{hep} predicted from parallel tube model yields a higher value than that from a well-stirred model. Hence, it is prudent to take into consideration the nature of the model with high clearance compounds. Once the predicted Cl_{hep} is calculated, the values can be expressed as percentage hepatic blood flow to classify compounds as low ($< 25\%$, Q_h), moderate ($25\text{--}75\%$, Q_h), and high ($> 75\%$, Q_h) Cl. With the recent surge of knowledge in transporters, the traditional Cl_{hep} equation has been modified to incorporate the transporter contribution to total hepatic clearance (Eq. 5, Fig. 1.8).

In early stages of drug discovery, metabolite identification is routinely performed in liver subcellular fractions, cDNA-expressed enzymes, or hepatocytes. Indeed, the results of a recent study suggested that all these systems reliably predicted human excretory and circulating metabolite profiles [88]. Although it is highly preferable to maintain low NCE concentration ($1\text{ }\mu\text{M}$) in incubation mixture to mimic physiologically relevant conditions, analytical difficulties in detection of metabolites that are formed in low quantities force most metabolite identification reactions to be performed around $10\text{ }\mu\text{M}$ or higher (depending on the extent of metabolism of the NCEs). At supraphysiological incubation conditions of most met Identification (ID) studies, the biotransformation pathways can sometimes be different from *in vivo* situation. In majority cases, this difference is mostly quantitative rather than qualitative—in other words,

Calculation of Cl_{int}

$$Cl_{int} = \frac{0.693}{in\ vitro\ T_{1/2}} * \frac{mL\ incubation}{mg\ microsomes} * \frac{45\ mg\ microsomes}{gm\ liver} * \frac{X\ gm\ liver}{kg\ BW} \quad (1)$$

$X = 20(\text{human}); 25(\text{dog, Pig}); 30(\text{Cyno}); 45(\text{Guinea pig, rat}); 87.5(\text{Mouse})$

$$Cl_{int} = \frac{V_{max} [S]}{K_m + [S]} \text{ at } S \ll K_m, \text{ simplifies to } Cl_{int} = \frac{V_{max}}{K_m}$$

(Involvement of one enzymes)

$$Cl_{int} = \frac{V_{max1} [S]}{K_{m1} + [S]} + \frac{V_{max2} [S]}{K_{m2} + [S]} \text{ at } S \ll K_m, \text{ simplifies to } Cl_{int} = \frac{V_{max1}}{K_{m1}} + \frac{V_{max2}}{K_{m2}} \quad (2)$$

involvement of two enzymes

Calculation of Cl_{hep} via Well-stirred model

$$Cl_{hep} = \frac{Q_h Cl_{int}}{Q_h + Cl_{int}} \quad (3)$$

(No protein binding)

$$Cl_{hep} = \frac{Q_h \left(\frac{1}{B/P} \right) (f_{u,serum}/f_{u,microsome}) Cl_{int}}{Q_h + \left(\frac{1}{B/P} \right) (f_{u,serum}/f_{u,microsome}) Cl_{int}} \quad (3)$$

(Plasma and microsome protein binding and blood:plasma partitioning)

$$Cl_{hep} = \frac{Q_h f_{u,blood} (Cl_{int}/f_{u,microsome})}{Q_h + f_{u,blood} (Cl_{int}/f_{u,microsome})} \quad (3)$$

(Blood and microsome protein binding)

Equation for extraction ratio

$$E = \frac{f_{u,plasma} Cl_{int}}{Q_h + f_{u,plasma} Cl_{int}} \quad (4)$$

$$E = \frac{f_{u,plasma} Cl_{influx} Cl_{int}}{Q_h Cl_{efflux} + Cl_{int} (Q_h + f_{u,plasma} Cl_{influx})} \quad (4)$$

Incorporating transporters

Calculation of Cl_{hep} incorporating transporters

$$Cl_{hep} = \frac{Q_h f_{u,plasma} Cl_{influx} (Cl_{int,sec} + Cl_{int,met})}{Q_h (Cl_{efflux} + Cl_{int,sec} + Cl_{int,met}) + f_{u,plasma} Cl_{influx} (Cl_{int,sec} + Cl_{int,met})} \quad (5)$$

Cl_{int} = intrinsic clearance

Cl_{hep} = hepatic clearance

$[S]$ = substrate concentration

V_{max} = maximum reaction velocity

K_m = substrate concentration that results in half of maximum velocity

Q_h = hepatic blood flow

$f_{u,blood/serum/plasma}$ = fraction unbound in blood/serum/plasma

B/P = blood:plasma partition ratio

Cl_{influx} = intrinsic clearance of influx

$Cl_{int,sec}$ = intrinsic clearance of biliary secretion

$Cl_{int,met}$ = intrinsic clearance of metabolism

Cl_{efflux} = intrinsic clearance of efflux

Figure 1.8 Methods to calculate Cl_{int} and Cl_{hep} .

even at artificially higher NCE concentrations, commonly used for met ID studies, the profile of metabolites would not alter significantly (in most cases), although their relative amounts and the enzymes involved in their formation may vary significantly. Very sensitive analytical LC/MS/MS- or LC/NMR-based methods have revolutionized for metabolite identification, quantitation, and characterization [89–97].

1.5.2.4 Reactive Intermediate Trapping. Reactive metabolites such as electrophilic intermediates or radicals are unstable, usually formed in small amounts, and rapidly react with nucleophiles and can, therefore, easily be missed in routine metabolism studies. Hard electrophiles react with basic groups in DNA and lysine residues in proteins (“hard nucleophiles”), while soft electrophiles react with the thiol groups of GSH and cysteine residue in proteins (“soft nucleophiles”). Radicals do not covalently bind to proteins but abstract a hydrogen atom from macromolecules. Direct detection of these reactive intermediates has proven almost impossible both *in vitro* and *in vivo* due to their inherent reactive nature. The basic methods to trap the reactive intermediates have been well established and have been used for screening of new chemical entities in a high throughput manner in early drug discovery. Majority of reactive metabolites generated are “soft” and are trapped by the endogenous thiol GSH and *N*-acetyl cysteine, while the “hard” electrophiles are trapped by the hard nucleophiles such as cyanide, methoxylamine, and semicarbazide. The trapped adducts are detected and identified by LC/MS/MS and Nuclear Magnetic Resonance (NMR).

In addition to commonly used GSH and Cyanide (CN) trapping of reactive intermediates, some of the recently emerging methods to measure “biochemical markers” of toxicity, resulting from reactive intermediate formation is also gaining popularity [98]. For example, increase in alanine transaminase level as an indicator of hepatotoxicity, induction of GSH transferase and quinone reductase as indicators of cells stress, and measure of mitochondrial function for cell toxicity have been proposed as methods that could be used for risk assessment toward reactive metabolite-mediated IDRs. Use of gene chips to explore gene expression patterns to identify individuals susceptible to IDRs has also been proposed. However, as with other existing methods, quantitative risk assessment and its clinical relevance remains a challenging hurdle for all these methods. Covalent binding assessment has also been extensively used as the gold standard for reactive intermediate screening [99]. However, expert opinion in this area emphasizes that the *in vitro* covalently binding studies are best used to *retrospectively* investigate mechanism of toxicity when encountered in clinical setting and is valuable in identifying imminent bioactivation pathways of NCEs and rank ordering NCEs in discovery stages. However, efforts to quantitatively link the degree of covalent binding to clinical toxicity should not be attempted *prospectively* [7,100–102].

1.5.2.5 CYP Identification/Mapping. In early discovery phases, a qualitative idea of the major route of elimination contributing to the total clearance of an NCE is sufficient. To tease out contribution of FMO- and CYP-mediated metabolism, it is common to incubate microsomes at 50°C for 2–3 min, as FMOs, but not CYPs, are thermally labile enzymes which can be denatured by incubating the reaction mixture in the absence of NADPH at 50°C.

During the late discovery stage, a little more rigorous method called *enzyme mapping* is implemented. Enzyme mapping helps identify the DMEs that are responsible for the overall clearance of an NCE. In early discovery stages, mapping is most commonly

done via the use of commercially available recombinant human enzymes (CYP, UGT, FMO, and NAT). Major caveat of this method is that since recombinant enzymes do not accurately represent physiological expression and specific activities of the enzymes, the results observed in recombinant enzymes are unable to give relative contribution of an enzyme to the total metabolism of the NCE *in vivo*.

When NCE is moved further into late stage discovery or early development, a more rigorous CYP phenotyping is performed where a more quantitative estimation of contribution by a CYP isoform to total NCE clearance is evaluated via a stepwise integrated approach [7,34,103].

- Step 1.* Assessment of metabolite formation of an NCE in a pooled Human liver microsomes (HLM) (20–50 donors to reduce intersubject variability) and ruling out non-CYP-mediated pathways.
- Step 2.* Enzyme kinetic parameters $V_{\max}$ and K_m for each major biotransformation pathway are determined under linear conditions (time and protein) and careful analysis of resulting Eadie–Hofstee plots (rule out multiple CYPs).
- Step 3.* Effect of CYP-isoform-specific chemical inhibitors on the particular biotransformation pathway (in HLM) is assessed (with $[S] \leq K_m$ of the NCE and $[I]/K_i > 10$ of the CYP-specific inhibitor).
- Step 4.* CYP-isoform-specific inhibitory antibodies are also used to evaluate relative contribution of different CYP isoforms toward NCE metabolism to complement data from chemical inhibitors (step 3).
- Step 5.* Relative activity factor method or intersystem extrapolation factor method are also utilized to gain an insight into relative contribution of the CYPs.

Sometimes other methods, such as, total normalized rate method, correlation analysis, or use of genotyped liver (in case where polymorphic enzymes are involved) have all been reported [34,103].

1.5.2.6 CYP Inhibition. Inhibitory potential of an NCE is routinely determined in early compound optimization stages by a simple, model-independent, IC_{50} determination assay in liver microsomes or cDNA-expressed protein [104,105]. Use of liver microsomes is more common in the case of CYPs which have specific/highly selective probe substrates and inhibitors available, while use of cDNA-expressed enzyme is more common in the case of UGT, NAT, SULT, and GST, for which isoform-specific/selective substrates and inhibitors are not well established and/or validated. Use of human hepatocytes-plasma system to predict inhibition-based DDI has also been demonstrated [106]. IC_{50} determination, in *in vitro* assays, is usually performed at one substrate concentration ($S \leq K_m$), and a wide range of inhibitor concentrations to monitor the decrease in reaction velocity with increasing inhibitor concentration. An NCE is classified as potentially high, medium, or low DDI risk based on whether its IC_{50} value for a particular enzyme is <0.1 , $0.1-1$, or $>1.0 \mu M$, respectively. IC_{50} values should be compared to maximum predicted NCE plasma concentration ($C_{\max}$) at steady state and only if IC_{50} values are greater than $C_{\max}$, a potential DDI risk *in vivo* can be anticipated. Drawback from risk prediction based on IC_{50} values is that IC_{50} values are heavily dependent on assay-dependent parameters such as the nature of sub-cellular fraction (pooled microsomes, cDNA-expressing microsomes, and hepatocytes); concentrations of the substrate, inhibitor, and enzyme; incubation time; solvent effect;

choice of marker substrate used especially for CYP3A4 and 2C9; solubility limit of inhibitor and substrate; and extent of protein binding of inhibitor.

A more rigorous assessment of inhibitor potency, usually performed for late stage discovery compounds, is via determination of K_i , which requires thorough kinetic analysis of a specific biotransformation pathway of interest. Eadie–Hoftsee (or Lineweaver–Burke) plots are generated using multiple substrate and inhibitor concentrations and the mechanism of inhibition (competitive, noncompetitive, uncompetitive, or mixed) is assigned based on model-fit of the data, defined by statistical criteria. K_i value, which unlike the IC_{50} value, is a more intrinsic number, is substrate independent, and yields a superior ranking method than IC_{50} determination. Evaluating an NCE's inhibitory potential involves assessment of its $[I]/K_i$ ratio, where $[I]$ represents the mean C_{max} (peak plasma) of inhibitor at steady state, after highest proposed clinical dose and K_i is the dissociation constant of the enzyme–inhibitor complex. An inhibition is considered high, medium, or low risk based on whether its $[I]/K_i$ ratio is >1 , $0.1–1$, or <0.1 , respectively.

1.5.2.7 CYP Induction. Reporter gene assays, such as the PXR–nuclear receptor binding assay and PXR-transactivation assay are rapid and high throughput assays are used to evaluate an NCE's potential for CYP induction. Induction has also been studied in other human hepatocytes cell lines such as HepG2, Fa2N-4, BC2, HepaRG, human intestinal cell line LS174T, and breast cancer cell line MCF-7 [79]. Each of these cell lines have their limitations, which need to be thoroughly considered before using these cell lines for predicting enzyme induction. Primary SCHHs model is deemed as the “gold standard” by DMPK scientists. Under optimal experimental/culture conditions, primary human hepatocytes maintain physiologically relevant receptors, DMEs, and transporters, all under their regulatory control and hence is a very good model to concurrently evaluate metabolism, inhibition, and induction. It has been observed that induction results obtained with primary hepatocytes cultures correlate well with clinical results. Readers are advised to refer to these excellent reviews that discuss in detail *in vitro* methodologies and optimization of experimental conditions such as culture format, number of donors, culture medium, confluence number, culture period, and so on, for studying enzyme induction with primary cultured hepatocytes. Endpoint readout in induction studies usually involves a combination of measuring the mRNA levels or protein levels or activity of the target enzyme(s) [36,107,108].

Frequently, in early discovery stages, a simple rank order approach based on C_{max}/EC_{50} ratio (C_{max} , maximum plasma concentration of inducer after administration of the highest anticipated clinical dose; EC_{50} , concentration of inducer where 50% of maximal induction is observed) is used. A high, medium, or low risk is associated to whether the C_{max}/EC_{50} ratios are >1 , $0.1–1$, or <0.1 , respectively. A more reliable method, called the *relative induction score* (RIS) method is also used to better predict clinical DDI based on *in vitro* induction data (Fig. 1.9) [108]. RIS takes into account the maximum induction response in addition to C_{max} and EC_{50} . The RIS method has also been modified to incorporate an additional No adverse effect level (NOAEL) term (Fig. 1.9), which is the highest concentration at which no induction is observed for an NCE. Generally, the DDI guidance by FDA recommends that NCEs that increase a particular isoform's enzyme activity by 40% of the positive control should be regarded as an inducer for that particular enzyme (assay done in freshly isolated or attachable cryopreserved hepatocytes in *in vitro* systems from at least three human donors).

Relative induction score	$E_{\max}$ = Maximim induction response
$\frac{E_{\max} \cdot C_{\max}}{EC_{50} + C_{\max}} = \text{Effect}$	EC_{50} = Inducer concentration that yields half maximum induction response
	$C_{\max}$ = Maximum un bound plasma concentration of inducer
	$f_{u, \text{plasma}}$ = Fraction unbound of inducer in plasma
$\frac{E_{\max} \cdot C_{\max} \cdot f_{u, \text{plasma}}}{EC_{50} + C_{\max} \cdot f_{u, \text{plasma}}} \times \frac{NOEL}{C_{\max} \cdot f_{u, \text{plasma}}} = \text{Effect}$	$NOEL$ = No adverse effect level

Figure 1.9 Calculation of RIS.

1.5.2.8 DDI Prediction in Transgenic Mice. There has been a significant recent interest in the use of genetically modified mouse models to assess DDI potential and toxicity prediction [109]. Double transgenic mouse (expressing human CYP3A4/PXR, CYPs 3A4/2D6, and OATP1B1), knockout (KO) mouse (specific drug disposition enzyme- and transporter-null mouse and SOD2 null mouse), humanized mouse (mouse expressing human CYPs PXR and PPAR), chimeric mouse with humanized liver have truly revolutionized ways to investigate the mechanism of human-specific biotransformation and toxicity [7]. However, *quantitative* prediction of clinical DDI and toxicity in human from these models is yet to be thoroughly established.

1.5.2.9 Plasma Protein Binding. The “gold standard” methods for PPB are equilibrium dialysis, ultrafiltration, and ultracentrifugation [110–112].

1.5.2.9.1 Equilibrium Dialysis. Its simple setup involves two chambers, usually plasma and buffer, separated by a dialysis membrane (MW cutoff) and an NCE (known volume and concentration) is introduced in the plasma chamber. Equilibrium between the two chambers is allowed to establish, whereby concentration of *free* NCE should be same across both sides of the membrane via simple diffusion. Quantitation of an NCE in the buffer side and the plasma side gives an estimate of percentage free NCE. This generates rapid, accurate, and quantitative data, with simple experimental setup. Caution should be exercised to minimize high membrane binding, volume shift due to osmosis, and Gibbs–Donnan effects.

1.5.2.9.2 Ultrafiltration. The assay principle is to separate a small volume of protein-free phase from plasma, containing known NCE concentration, by filtration through a semi-permeable membrane, and measure the concentration of unbound NCE in the filtrate. Also it provides the rapid, accurate, and quantitative data with simple experimental setup. Attention should be paid to membrane binding, sieve effects, Gibbs–Donnan effects, along with temperature and pH control.

1.5.2.9.3 Ultracentrifugation. This method involves application of a high centrifuge force ($100,000 \times g$ or more) to a drug solution (no membrane is required) for a prolonged period (e.g., 15 h) to sediment the protein to the bottom of the tube. The unbound drug concentration in the protein-free supernatant is measured. Although this technique is devoid of membrane-related issues or change in total drug concentration, sedimentation of drug molecules, floating lipid layer, extensive experimental setup and time, along with the need to control temperature and pH may pose as hurdles.

1.5.2.9.4 Ex Vivo PPB. *Ex vivo* PPB refers to PPB determination of a compound, via conventional PPB methods, in blood samples obtained after dosing the animals or individuals with the compound (most of the time, done with [^{14}C]-labeled compound). *Ex vivo* PPB values have been shown to yield slightly higher values than *in vitro* PPB determination [113] as reported for celecoxib. It is desirable to evaluate *ex vivo* PPB, especially in cases where a compound yields significant amount of metabolite *in vivo*. Metabolites, due to their more polar nature than the parent drug, are usually less plasma protein bound than the parent drug but an *ex vivo* evaluation in these cases captures any interaction between drug and metabolites at physiologically relevant concentration/therapeutic doses.

1.5.3 In Vivo

1.5.3.1 Absorption. OBA is defined as the fraction of drug that reaches systemic circulation after oral administration of a drug. It is therefore heavily dependent on how efficiently the drug is absorbed after an oral dose. For orally administered drugs, fraction of dose absorbed systemically F (also defined as *OBA*) is dependent on the fraction absorbed F_a , fraction escaping gut metabolism, F_g , and fraction escaping hepatic metabolism, F_h , given by the following equation:

$$F = F_a \times F_g \times F_h$$

Knowledge of fraction of dose absorbed is very informative for orally administered drugs. Although very accurate information about fraction of dose absorbed is obtained with radiolabeled compounds, yet availability of radiolabeled compound is very limited in early discovery stages and simple mechanistic studies with unlabeled compounds gives a reasonable estimate of this parameter [11,114]. For example, comparison of dose-normalized AUC between oral and intraportal vein dosing gives a reasonable estimate of $F_a \times F_g$, fraction of dose absorbed into portal vein, after an oral dosing (assuming hepatic clearance remains same between the two routes of dosing). $F_a \times F_g$ can also be estimated after oral dosing of a compound and comparison of compound concentration (dose-normalized AUCs) in portal vein versus systemic circulation. Fraction of dose escaping hepatic metabolism F_h can be estimated from either comparing dose-normalized AUCs after intraportal and intravenous (IV) dosing or after an oral administration comparing compound exposure in portal vein versus in systemic circulation (portal vein vs mesenteric artery).

KO and mutation of transporter expression in rodents is a widely used strategy to understand involvement of the silenced or mutated transporters in drug disposition in humans [115] and to explain transporter-mediated toxicity to certain xenobiotics. For example, studies in *Npc1l1*-null mice revealed the essential role of this transporter in cholesterol uptake across intestines, while *Dmt 1*-mutated rats showed that this is an important transporter for intestinal absorption of iron. Other KO models have also been valuable in investigating role of OAT, OCT, MRP2, MRP3, MRP6, ABCA1, BCRP, MDR1, MDR2, and MDR3 in hepatobiliary, renal, and brain disposition and toxicity of xenobiotics. Concurrent double and triple-transporter gene KO mice, such as *Mdr1a/b/Mrp2* KO [116], *Bsep/Mdr1a/Mdr1b* KO [115], and *Mdr1a/Mdr1b* KO [117,118], have also been very useful in understanding the role of multiple transporters in drug disposition.

Metabolite identification of *in vivo* samples is usually done in biological matrices such as plasma, urine, bile, and feces. Ion suppression from a variety of endogenous compounds in the biological matrices is a commonly encountered hurdle in *in vivo* metabolite identification and leads to drastic decrease in MS signal intensity of parent and its metabolites. A strong expertise in sample preparation and novel sample introduction into the LC/MS/MS [e.g., Ultra Performance Liquid Chromatography (UPLC) and nano-flow techniques] tremendously improves success in detection and identification of *in vivo* metabolites. There are a multitude of very good articles discussing several of these modern analytical strategies involved in metabolite identification and are highly recommended for further reading [89–97].

1.6 ADME STUDIES IN DRUG DEVELOPMENT

As described in Section 1.5, most of the ADME studies in drug discovery are conducted using cold material. However, ADME studies in drug development are generally conducted using radiolabeled (^3H or ^{14}C) material [119]. The use of radiolabeled material offers a unique mode of quantification of total drug-related molecules.

1.6.1 Absorption

Determination of the amount of drug-related material absorbed proves to be quite cumbersome using unlabeled compound. The use of a radiotracer allows an accurate quantitation of drug and drug-related material that is absorbed and this technique has been employed in studying absorption for several years. The radiolabeled version of the drug is administered orally and intravenously to intact or bile-duct cannulated animals and humans. Urine, bile, plasma, and feces are collected for as long as is necessary to obtain a full recovery of radioactivity [120]. Several methods have been then used to determine the fraction absorbed (F_a).

1. Calculating the percentage of cumulative excretion of radioactive drug-related material in urine and bile from bile-duct animals following oral administration as

$$\text{Absorption}(F_a) = \frac{\% \text{total dose excreted in urine and bile}}{100}$$

2. Measuring the amount of unchanged drug in feces after oral administration from intact animals using as

$$\text{Absorption}(F_a) = 1 - \frac{\% \text{dose of parent drug excreted in feces}}{100}$$

3. Comparing the amounts of total radioactivity excreted in the urine after oral and IV dose as

$$\text{Absorption}(F_a) = \frac{\% \text{dose excreted in urine after PO dose}}{\% \text{dose excreted in urine after IV dose}}$$

4. Comparing the exposure (AUC) of total radioactivity after oral and IV dose as:

$$\text{Absorption}(F_a) = \frac{\text{AUC}_{\text{total radioactivity after PO dose}}}{\text{AUC}_{\text{total radioactivity after IV dose}}} \times \frac{\text{dose IV}}{\text{dose PO}}$$

The extent of absorption of lasofoxifene was investigated in the intact and bile-duct rats after oral administration of single dose of [^{14}C]lasofoxifene [121]. The cumulative excretion amounted to 1.72% in the urine and 95.0% in the feces of intact rats. While in a separate bile-duct cannulated study in rats, 83% of the administered radioactivity was excreted in the bile following oral administration of a single dose of [^{14}C]lasofoxifene. These data suggest that at least 85% of dose (percentage recovery in urine and bile) was absorbed and that the fecal excretion in intact animals likely reflects excretion in the bile rather than incomplete absorption.

Barbhaiya *et al.* [122] and Shukla *et al.* [123] have demonstrated the differences in the extent of absorption of drug-related material and the systemic bioavailability of nefazodone in humans and dogs after oral and IV administration of [^{14}C]nefazodone. Plasma was analyzed for nefazodone and its metabolites, and plasma, urine, and feces were analyzed for total radioactivity. The bioavailability of nefazodone was estimated to be 14% and 15% in dogs [123] and humans [122], respectively (Table 1.3). The recovery of total radioactivity in urine and feces was similar after Oral (route of dosing) PO and IV administration for both dogs and humans, indicating complete absorption of the drug after oral administration (Table 1.3). The low bioavailability of nefazodone was due to first-pass metabolism rather than poor absorption.

1.6.2 Distribution

Drug regulatory agencies require that tissue distribution studies should be conducted with laboratory animals (rats) as a prerequisite for projecting tissue exposure to radioactivity before administration of radioactive drugs to humans. The objectives of this study are to determine the radioactivity in each organ of interest after administering the radiolabeled drug to animals. The concentration of drug-related material (total radioactivity) is determined by whole body autoradiography/whole body autoradioluminography (WBAL) or liquid scintillation counting of tissues of interest.

TABLE 1.3 Mean Total Excretion of Radioactivity and Exposure (AUC) of Nefazodone in Dogs and Humans following Oral and IV Administration of [^{14}C]Nefazodone

	Analyte	Dose (mg/kg)	Route	AUC0-inf	% Dose Excreted	
					Urine	Feces
Dog	Radioactivity	10	IV	25,642	24.9	61.1
		10	Oral	23,997	27.7	59
	Nefazodone	10	IV	6023	—	—
		10	Oral	829	—	—
Human	Radioactivity	5	IV	—	51.6	20.1
		20	Oral	—	49.1	24.1
	Nefazodone	5	IV	160	—	—
		200	Oral	1327	—	—

Prakash *et al.* [121] examined tissue distribution in male and female pigmented rats (Long–Evans) using WBAL following administration of a single oral dose of [^{14}C]lasofoxifene. [^{14}C]Lasofoxifene-related radioactivity distributed rapidly in the rat with most tissues achieving maximal concentrations at 1 h. Tissue exposure was similar in male and female rats, with the greatest exposure in the uvea. The GI contents had the highest concentrations of [^{14}C]lasofoxifene radioequivalents following oral administration. Half-life of radioactivity was longest in the uvea (124 h) and shortest in the spleen (~ 3 h). Radioactivity became undetectable in some tissues by 8 h postdose. At 168-h postdose, radioactivity could only be measured in the intestinal contents and uvea. The projected human exposure to ^{14}C radioactivity is estimated by direct extrapolation from the exposure data obtained from the rat quantitative whole body autoradiography (QWBA) tissue distribution study [124–126].

1.6.3 Metabolic Profiling and Identification

In drug development, the metabolite profiles of new drug candidates are generally accomplished by mass balance and excretion studies in which radiolabeled drugs are administered to laboratory animals and humans. The biological fluids are collected, analyzed for total radioactivity, and evaluated for a quantitative profile of metabolites. Metabolite profiling of samples is generally conducted using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and radiometric detector. Metabolite quantification is performed by measuring radioactivity in the individual peaks that are separated on HPLC, using a radiometric detector or collecting fractions followed by counting in a scintillation counter.

As an example, the metabolic fate of torcetrapib a cholesteryl ester transfer protein (CETP) inhibitor was investigated in rats, monkeys, mice, and humans following oral administration of a single dose of [^{14}C]torcetrapib [127,128]. More than 28 metabolites were identified in all species, and they were products of oxidation and conjugation pathways [127]. The metabolites identified in humans were detected at least in one preclinical species [128].

In some cases, oxidative cleavage such as N-dealkylation or hydrolysis of the ester and amide could result in splitting of a molecule into two large fragments. In these cases, it is useful to label both sides of the molecule either with one isotope at two different positions or a mixture of two radioisotopes. This strategy has been successfully used in *in vitro* studies of a GABA_A receptor partial agonist [129] and *in vivo* Absorption-metabolism-excretion (AME) studies of torcetrapib [128], ziprasidone [130,131], and gemopatrilat [132].

1.6.4 Excretion

Excretion studies in preclinical animal species used for long-term safety assessment and human volunteers with the aid of the radiolabeled drug provide the total fate of drug-related material. One of the primary objectives of these studies is to demonstrate that the administered dose is readily eliminated from the body, ideally after exerting the intended therapeutic effect. By quantitative collection of the excreta for sufficient time, it not only provides valuable information on mass balance, route, and extent of excretion of the administered radioactive dose but also provides critical information on the clearance mechanism of the drugs.

Several studies describing the procedure for determining the excretion of drugs using [^{14}C]- and [^3H]-labeled compounds have been published [128,130,131]. We have described a mass balance and excretion study of torcetrapib in rats, monkeys, and mice by oral administration of [^{14}C]torcetrapib, labeled either at the trifluoromethyl-3,4-dihydro-2*H*-quinoline moiety or at the benzylic carbon of the bis-trifluorophenyl ring [128]. The administered radioactive dose was quantitatively recovered in all species. Excretion of the radioactivity was rapid and nearly complete within 48 h after dosing. In the rats and monkeys, the majority of dose was excreted in feces, while in the mice; the dose was recovered equally in urine and feces. In the separate studies using bile-duct cannulated rats and monkeys, only <10% of the administered radioactivity was recovered in bile. This suggested that a major portion of dose excreted in the feces of rats and monkeys was mainly due to unabsorbed dose. There were no discernible gender differences in the excretion pattern of radioactivity in these species after oral administration of [^{14}C]torcetrapib [128].

1.7 SUMMARY AND FUTURE PERSPECTIVES

Drug discovery is an extremely time-consuming, expensive, and challenging process, where success is heavily dependent on very thorough understanding of chemistry and mechanism of metabolism of NCEs in humans. Unavailability of data in humans in early drug discovery stages makes it imperative to have well-validated, rapid *in vitro* ADME tools, in complement with relevant *in vivo* preclinical animal models, to accurately predict ADME/PK of an NCE in humans to minimize risk of failure in late stages of development. With advancement of technology, there is a plethora of *in silico* and *in vitro* assays available to model and evaluate each pathway of the complex ADME process such as involvement of transporters and DMEs, possible risk associated with their inhibition or induction, implications of enzyme polymorphism, insight into mechanistic biotransformation pathways, and reactive intermediate formation. It must be borne in mind that each of these *in silico* modeling techniques and *in vitro* assays and systems (e.g., recombinant enzymes, microsomes, hepatocytes, and membrane vesicles) have their limitations, depending on the experimental conditions tested (e.g., types of molecular descriptors in the case of *in silico* models and substrate concentration, buffer, incubation time, pH, and protein concentration, in the case of *in vitro* assays) and results must be carefully interpreted during ADME optimization of an NCE. Preclinical animal models should also be very carefully evaluated since there is significant species and gender difference in biotransformation pathways of NCEs, not only due to differential expression and activity of the transporters and DMEs but also in animal physiology and genetic polymorphism. Designing the correct *in vitro* and *in vivo* experiments and intuitively interpreting and integrating all ADME/PK data are challenging but key steps toward risk mitigation of an NCE in a clinical setting. More often than not, even with the most sophisticated modeling and the best designed experiments, extrapolation of animal data to human fails due to highly species-specific expression levels, tissue localization, substrate specificity and gene regulation of transporters and DMEs, and differences between human and animal physiology. To address these issues, IV microdose and pharmacologically active oral dose strategies for exploratory phase I studies should be employed.

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ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
CYP	Cytochrome P450
DDI	Drug–Drug Interactions
DME	Drug-Metabolizing Enzymes
DMPK	Drug Metabolism and Pharmacokinetics
ER	Extraction Ratio
FMO	Flavin Monooxygenase
GI	Gastrointestinal
IDR	Idiosyncratic Drug Reaction
IVIVC	<i>In Vitro–In Vivo</i> Correlation
LC	Liquid Chromatography
MAD	Maximum Absorbable Dose
MBI	Mechanism-Based Inactivation
MRM	Multiple Reaction Monitoring
MRT	Mean Residence Time
MS	Mass Spectrometry
MW	Molecular Weight
NAT	<i>N</i> -Acetyl Transferase
NCE	New Chemical Entity
OBA	Oral Bioavailability
PAMPA	Parallel Artificial Membrane Permeability Assay
PK	Pharmacokinetics
PK/PD	Pharmacokinetics/Pharmacodynamics
PPB	Plasma Protein Binding
R&D	Research and Development
QWBA	Quantitative Whole Body Autoradiography
SAR	Structure–Activity Relation
SULT	Sulfotransferase
TMT	Thiopurine Methyl Transferase
UGT	Uridine Glucuronosyl Transferase

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2 Anatomical and Physiological Parameters that Influence Gastrointestinal Absorption

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2.1	Summary	1
2.2	Introduction	2
2.3	Overview of the alimentary canal	2
2.4	Vascularity	10
2.5	Dimensions of regions of the gastrointestinal tract	16
2.6	Motility and transit times	20
2.7	Luminal contents	24
2.8	Enterohepatic recirculation	28
2.9	Conclusions	29
	Acknowledgments	30
	References	30

2.1 SUMMARY

The rate at which ingested compounds are absorbed into the bloodstream from the gastrointestinal (GI) tract and extent to which they are absorbed are governed by the physical–chemical properties of the ingested compounds, the presence and identity of other substances in the GI tract, and the anatomical and physiological characteristics of the GI tract. Humans and laboratory animals share the same basic blueprint for the GI tract, and this chapter describes the organs of the GI tract, the microscopic structure of the GI luminal epithelium, and the flow of blood and lymph from the GI tract. However, there is remarkable variability across species in the absorptive surface area of the GI tract, the time it takes for materials to move through the GI tract, and the nature of the GI contents. By understanding these differences among species, scientists can better predict the pharmacokinetics of a substance in a species or extrapolate pharmacokinetic data from one species to another.

2.2 INTRODUCTION

GI absorption entails the passage of materials from the lumen of GI tract (alimentary canal) to the bloodstream. Absorption of nutrients and essential minerals are crucial for a normal, healthy life. Understanding which materials are absorbed, in what specific portions of the alimentary canal, at what rate, and by what mechanisms is important to nutritionists, pharmacologists, and toxicologists. In all cases, scientists want to know how substances pass from the lumen to the bloodstream. Information about the process of absorption and factors that affect it are essential for modeling absorption kinetics and predicting the biodistribution of a substance within a given animal or extrapolating the findings between species (e.g., from rats to humans). Physiologically based pharmacokinetic models and biologically based models are valuable tools for predicting or extrapolating absorption, but they require qualitative—as well as quantitative—knowledge about the biological systems under study.

The rate and extent of absorption of orally ingested compounds are influenced by both properties that are intrinsic to the ingested substances themselves and factors that are associated with the milieu of the alimentary canal and its absorptive surface [1–4]. The most significant intrinsic factors that affect absorption are the physical and chemical properties of a substance, including its lipid solubility, ionization state, and molecular weight or size. These intrinsic factors are independent of the test species. Absorption of a substance is also extensively affected by the presence of other materials within the GI tract, including food eaten before or concomitantly with ingestion of the substance, or in the case of some experimental studies, the vehicle or solvent in which the substance is administered.

It is emphasized that the parameters discussed herein are those that influence the crossing of materials from the GI lumen into the bloodstream of the GI tract. From the major absorptive areas of the GI tract, blood passes by means of the hepatic portal system to the liver, where absorbed materials are subject to possible metabolism or storage. This process is termed the *first-pass effect* because it effectively reduces the concentration or amount of material absorbed from the GI tract that enters the systemic circulation. Thus, while information about absorption of materials from the GI tract is the first step in determining potential biological effects, this does not always provide accurate measures of concentrations found at target tissues that are distant from the GI tract [5].

The factors that contribute most to the interspecies differences in GI absorption are the anatomy and physiology of the GI tract of the species under consideration. Before discussing the differences among species, however, it is important to review their similarities.

2.3 OVERVIEW OF THE ALIMENTARY CANAL

The overall organization of the GI tract of humans is comparable to those of most test species. Detailed information concerning the anatomy and physiology of the GI tract in animals and humans can be found in various sources [6–10] and has been summarized therefrom. Figure 2.1 depicts the regions of the alimentary canals for humans and rats. The alimentary canal is an open-ended tube that extends through the body from the mouth to the anus. One can envision the lumen as a tunnel of the

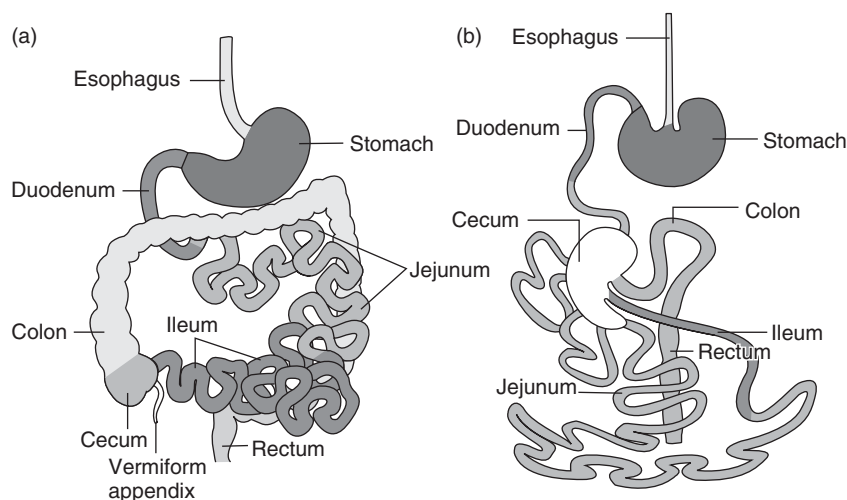


Figure 2.1 Alimentary canals of primates and rodents. In the primate (human depicted in panel a), note the eccentric position (left side) for the entry of the esophagus into the stomach. The portal into the small intestine is nearly horizontal. The small intestine is divided into duodenum (the short initial segment), jejunum, and ileum. The ileum empties into the large intestine. The first part of the large intestine (the cecum) has the largest diameter; the vermiform appendix is attached near its proximal end. Note that the diameter of the large intestine decreases as material traverses caudally. The rodent GI tract (rat depicted in panel b) shares the same overall organization as the human's, but with a few important differences. Note that the esophagus enters the stomach at a central location and that the entry into the duodenum faces cranially. The relative lengths of the small intestine differ from those of the human in that the jejunum makes up nearly the entire small intestine. The cecum of the rat is extremely large and lacks the presence of a vermiform appendix. Note that figures are not drawn to scale. *Source:* Reproduced from DeSesso JM, Jacobson CF. *Food Chem Toxicol* 2001;39:209–228, with permission.

external environment that runs through the body and the wall of the alimentary canal as the interface between the environment and the circulatory system [11,12]. The main functions of the alimentary canal are the digestion of food, the absorption of nutrients and other substances, and the propulsion of material through the digestive tract.

The mouth provides access to the alimentary canal. There, ingested materials are crushed and mixed with saliva by chewing (mastication). While enzymes found in saliva initiate the breakdown of carbohydrates and fats, saliva importantly acts as a lubricant for the ingested material. When a bolus of masticated material attains an appropriate consistency, it is propelled by swallowing (deglutition) into the pharynx.

The pharynx is a conduit that is shared by the respiratory and digestive systems. In humans, the pharynx has an extensive anterior opening that connects to the nasal cavity and mouth in its upper half; inferiorly, the pharynx connects to the esophagus and larynx. For the purposes of this discussion, *pharynx* refers only to the combined oral and laryngeal regions because under normal conditions, the nasopharynx does not participate in ingestion or swallowing. In the rat, mouse, and rabbit, the pharynx is divided into a respiratory region (an elongated nasopharynx) and a digestive region (analogous to the human's laryngopharynx). There is no oropharynx in these species

because the epiglottis rests against the palate and separates the nasal cavity from the oral cavity (see Ref. 13 for a comparison of the oral and upper respiratory regions of rats and primates). The inferior end of the pharynx adjoins the esophagus, a hollow muscular tube capable of considerable expansion. Little digestion or absorption occurs in the pharynx and esophagus; rather, these structures serve to rapidly transfer ingested material from the mouth to the stomach.

The stomach is a capacious muscular organ that mixes the ingested material with additional secretions to facilitate digestion. The stomach of rodents and lagomorphs has two grossly discernible regions: the forestomach and glandular stomach. The two are separated by a limiting ridge, which prevents these species from vomiting (Fig. 2.2a). The forestomachs of rodents and lagomorphs serve as an entry from the esophagus and are lined by stratified epithelium. The forestomach of rats and mice (but not rabbits) contains numerous bacteria that are important in their digestive processes. The epithelium that makes up the forestomach surface in these species resembles the

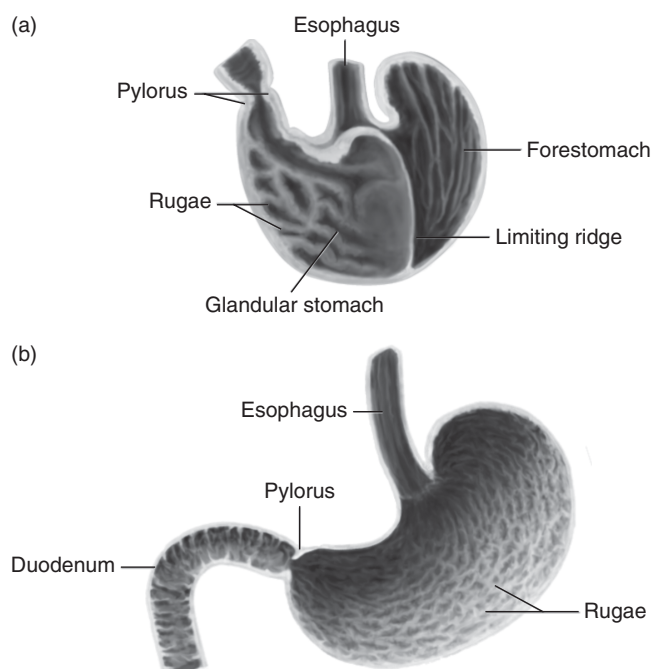


Figure 2.2 Interior structures of rodent and primate stomachs. The interior of the rat stomach (a) exhibits two distinctive regions separated by a prominent limiting ridge, which precludes the rat from vomiting. Food enters the stomach at the forestomach, a nonsecretory region with a hardy epithelium. The portion of the stomach that exits to the duodenum is the glandular stomach, which is lined by a delicate secretory epithelium and exhibits prominent folds (rugae) that are noticeable when the stomach is empty. Entry of food into the duodenum is regulated by a prominent muscular sphincter, the pylorus. The human stomach (b) differs from that of the rat in that the entire lining is secretory and there is no forestomach or limiting ridge. The human stomach has numerous rugae. *Source:* Reproduced from DeSesso JM, Jacobson CF. Food Chem Toxicol 2001;39:209–228, with permission.

mucous membrane of the oral cavity. The other portion of the stomach, the glandular region, contains acid-secreting glands. In dogs, pigs, and primates (including humans), the stomach is a single chamber that is secretory in nature and contains mucus- and acid-secreting cells. The human condition is depicted in Fig. 2.2b. The predominant gastric secretion in all species considered herein is rich in hydrochloric acid. In humans, the daily volume of gastric secretions can reach 1.5–2 L [4]. Gastric secretions act to continue the disintegration of food particles and to denature proteins. After mixing in the stomach for as many as several hours, small boli of the ingested material (now termed *chyme*) pass through the pyloric sphincter to the small intestine, where digestion is essentially completed and the majority of absorption takes place.

The small intestine is the major site for absorption of nutrients, water, electrolytes, and xenobiotics. The small intestine exhibits three unequally sized portions: the duodenum, the jejunum, and the ileum. These sections differ in length from species to species. The proximal part of the small intestine, the duodenum, receives secretions from numerous sources, including

- the pancreas—high in volume and rich in digestive enzymes and bicarbonate,
- the liver—bile (discussed in Section 2.7.2), and
- enteric (Brunner's) glands, located in the wall of the small intestine—rich in bicarbonate that neutralizes acid from the stomach.

The volume of these secretions in primates, dogs, and pigs more than doubles that from the salivary glands and stomach and serves to make the chyme watery. In contrast, the chyme of rodents remains rather pasty [4]. In humans, the majority of absorption occurs in the duodenum and the proximal half in the jejunum. In rodents and lagomorphs, absorption occurs more uniformly along the duodenum and entire jejunum. At the distal end of the ileum, chyme passes into the large intestine.

In primates, the large intestine is divided into the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anal canal. The other species discussed herein have similar divisions, except that, because of the absence of a true pelvis, they do not have a region designated as the sigmoid colon. The first portion of the large intestine, the cecum, has disparate functions among various species. In primates, fluids and salts that remain after completion of intestinal digestion are absorbed in the cecum. Rabbits, which are herbivores, have a much larger cecum that contains large amounts of bacteria that help digest cellulose. The ceca of carnivores such as dogs use strong muscle contractions to reverse the propulsion of the chyme and bring it back from the more distal regions of the large intestine to mix with newly arrived chyme from the ileum. As the chyme moves through the cecum and the remainder of the colon, it is mixed with intestinal bacterial flora. In fact, most nutrient processing in the large intestine is due to bacteria; little processing occurs as the result of intestinal mucosal/secretory activity. Substances formed as a result of bacterial metabolism that have properties favorable for absorption may be taken up from the large intestine. Water and electrolytes also continue to be absorbed from the large intestine. The progressive absorption of water from the chyme–bacterial matrix results in the formation of solid feces, which are eventually propelled to the rectum and stored until expulsion during defecation.

2.3.1 The Nature of the Protective and Absorptive Interfaces

Histologically, the GI tracts of humans and most test species are quite similar [14]. Detailed descriptions of the luminal surface of the alimentary canal can be found in textbooks of histology [15–17] and in various reviews [2,4,18]. Briefly, the alimentary canal is lined from the mouth to the anus with a mucosa that functions as the first barrier to entry of materials from the GI tract into the body. The mucosa is composed of an epithelium that is superimposed over a thin layer of loose connective tissue (lamina propria) in which reside blood capillaries and lymphatic vessels. The epithelial cells of the mucosa (discussed in further detail below) are joined together by tight junctions throughout the alimentary canal. The regions of the alimentary canal can be categorized by function: conduits that propel ingested substances through the canal (mouth, pharynx, esophagus, lower rectum, and anus) and regions that operate in digestion and absorption (stomach, small intestine, and all but the distal large intestine).

The mucosae of the conduits are usually a moist, stratified squamous epithelium (five to seven layers of cells) with a poorly vascularized lamina propria. In some conduit regions that are subjected to physical stress (such as the mouth), the epithelium may be keratinized. Conduit regions generally exhibit flat luminal surfaces with few irregularities that would increase the mucosal surface area. The mucosae of conduit regions are not well adapted for absorption due to their multiple layers of epithelial cells, reduced surface areas, and relatively sparse vascularization.

The mucosae of the absorptive regions differ markedly from those of the alimentary conduits. Absorptive mucosae exhibit simple columnar epithelium (i.e., a single cell layer) with prominent, well-vascularized laminae propriae. The mucosa in the absorptive regions of the alimentary canal is further characterized by modifications of the mucous membrane that increase the absorptive surface area (Fig. 2.3). These modifications include (i) large folds of the mucosal membrane (*plicae circulares*, also known as *valves of Kerckring*) that are present in some species, (ii) fingerlike projections of mucosal membrane called villi, and (iii) numerous projections from the luminal surface of the epithelial cell membrane to form a brush border (microvilli). This increased surface area of the mucosa is conducive to the transfer of substances from the lumen to the vascular system.¹

While the epithelia of the absorptive regions include diverse cell types, the predominant cell type important in the process of absorption is the enterocyte (Fig. 2.4). As previously noted, enterocytes are columnar epithelial cells that are joined to adjacent cells at the luminal surface by tight junctions. Absorption usually requires passage through the epithelium, the lamina propria, and the walls of blood or lymph capillaries. There are certain normal conditions that allow substances to traverse the junctions between epithelial cells [19–21]. Further, this process can be exacerbated in some disease states, for example, cholera [22]. The apical cell membrane of an enterocyte from the small intestine possesses an estimated 3000–7000 microvilli, which greatly increase the surface area available for absorption [11,23]. There are fewer microvilli on

¹The mucosa of the esophagus forms longitudinal folds and the wall of the stomach has ridges (*rugae*); however, these surface modifications serve to allow expansion during swallowing and after a meal, respectively, rather than increase absorption. Also, some epithelial cells in the stomach have microvilli, although these are secretory in function rather than absorptive. Thus, the surface modifications in the esophagus and stomach do not increase the *absorptive* surface area.

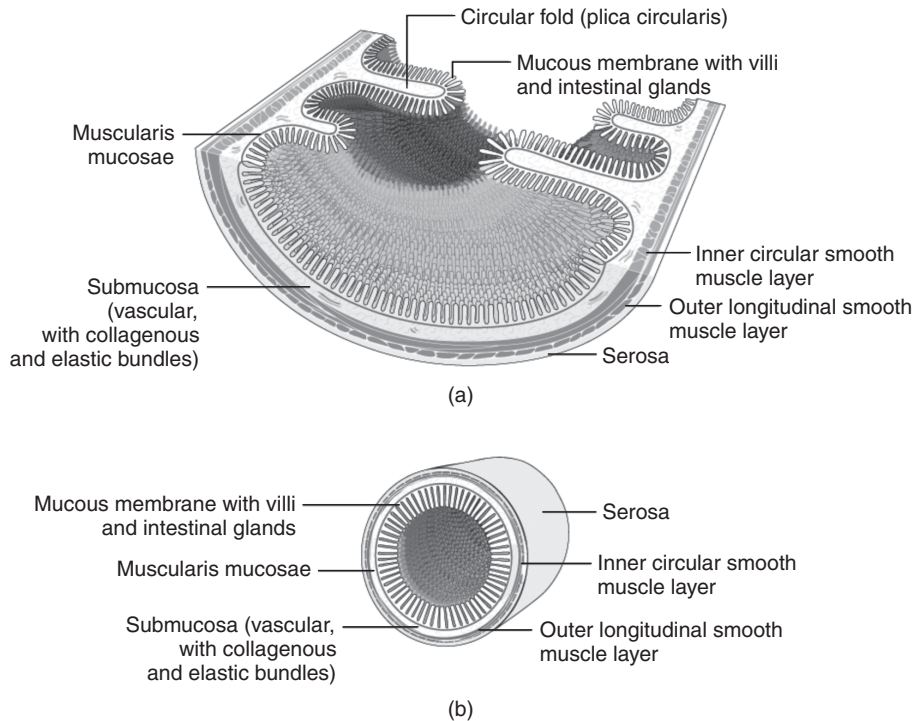


Figure 2.3 Cross sections of proximal small intestines of primates and rodents. Luminal surface modifications in (a) human and (b) rat. The surface areas for absorption from the lumina of the small intestines of rats and humans are greatly increased by the presence of millions of tiny fingerlike projections (villi). In the rat (b), which has a thick, chalky chyme, the villi project into the lumen from the cylindrical intestinal wall. In humans (a), who have a watery chyme, the absorptive surface area is further amplified by the presence of folds of luminal epithelium (plicae circulares) that intrude into the lumen, thereby allowing many million more villi to come into contact with chyme. (See also Fig. 2.7 to appreciate the difference in blood supplies to areas with many vs few intestinal folds.) *Source:* Reproduced from DeSesso JM, Jacobson CF. *Food Chem Toxicol* 2001;39:209–228, with permission.

enterocytes of the large intestine. Absorptive epithelial cells rest on a basement membrane and possess a carbohydrate-rich glycocalyx coat on the surface of the microvilli.

The lamina propria in absorptive regions of the alimentary canal possesses a rich supply of blood capillaries and lymphatic vessels. When villi are present (Fig. 2.5), the vascularized lamina propria makes up the core of each villus. The dilated, blind-ending lymphatic capillaries occupy the center of the villus; the blood capillaries form a meshwork of vessels beneath the basement membrane. Absorption may be augmented where fenestrations (small holes) in the capillary endothelial cells or pores in enterocyte membranes are found.

In some absorptive regions of the alimentary canal, such as the small intestine, a thin layer of smooth muscle, muscularis mucosae, lies within the lamina propria of the mucosa. Contractions of the muscularis mucosae appear to assist in the rhythmic movements of the villi that agitate the layer of intestinal secretions and chyme that are in contact with the epithelium (the unstirred layer) and thus promoting absorption.

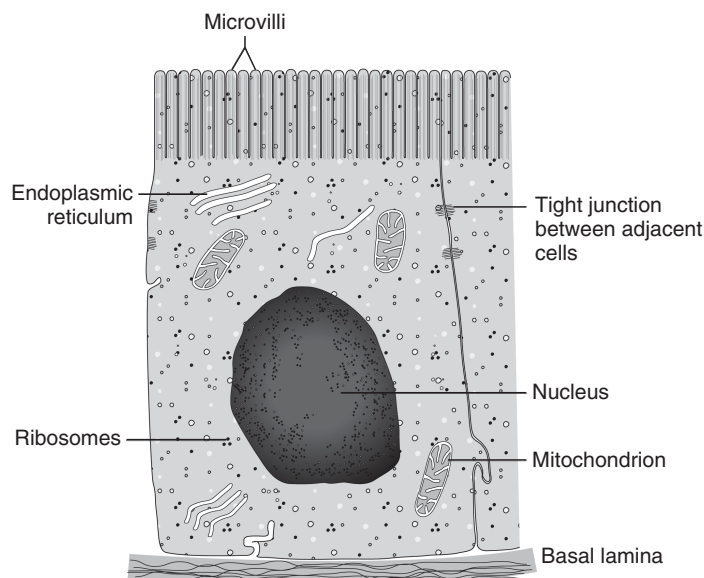


Figure 2.4 A typical intestinal epithelial cell (enterocyte). Enterocytes are high cuboidal to low columnar epithelial cells that rest on a basal lamina. Enterocytes are joined firmly to adjacent cells by tight junctions. The nucleus occupies the basal portion of the cell. The apical surface exhibits numerous microscopic projections (microvilli) that project into the lumen and give the appearance of a “brush border” when seen with a light microscope. Microvilli greatly increase the available absorptive surface area for the enterocyte. Nearly all substances taken up from the intestinal lumen traverse the cytoplasm of an enterocyte. Lipids and fats are absorbed at the base of the microvilli, traverse the cytoplasm, and exit the enterocyte at its lateral aspect, beneath the tight junctions. In contrast, amino acids, triglycerides, and carbohydrates are absorbed throughout the length of the microvillus. These substances traverse the cytoplasm and exit the enterocyte at its base. Under certain conditions, solvent drag (or convection) can be a paracellular route that takes advantage of partial separations of the tight junctions between intestinal cells to allow the passage of large amounts of water and small solutes. *Source:* Reproduced from DeSesso JM, Jacobson CF. *Food Chem Toxicol* 2001;39:209–228, with permission.

2.3.2 Sites of and Barriers to Absorption

Throughout the length of the alimentary canal, the luminal epithelium is both a barrier to the entry of undesirable luminal contents (such as bacteria and undigested materials such as cellulose) and a facilitator in the absorption of advantageous substances. Detailed discussions of these topics can be found elsewhere [3,4,11,20,24]. While the systemic absorption of orally ingested materials takes place predominantly in the small and large intestines, limited absorption occurs at other sites as well. The most important nonintestinal absorption site is the stomach, which can take up nonionized, lipophilic molecules of moderate size. Compared to that of the intestines, gastric absorption is limited by the comparatively small epithelial surface area, relatively large volume, and the brief amount of time that substances are in contact with the stomach epithelium. Neither the characteristics of the oral/pharyngeal/esophageal epithelium nor the transitory time that substances are in contact with the mucosa of those regions favors absorption. A small amount

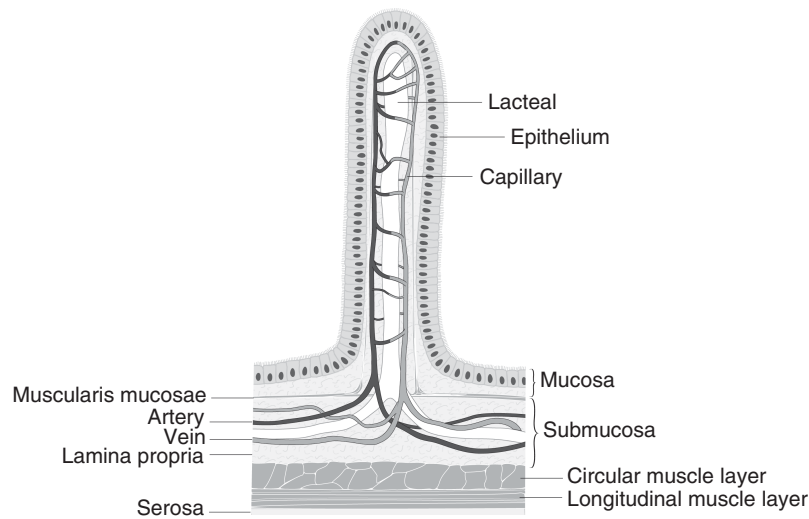


Figure 2.5 Structure of an intestinal villus. The luminal surface of the entire intestinal tract, including each villus, is lined by enterocytes. Note the fuzzy appearance of the brush border on the surface of the villus. The deep structure of the villus is characterized by a large, blind-ending lymphatic vessel (the lacteal), which absorbs higher molecular weight substances, including fats. The lacteals are tributaries to the intestinal lymphatic channels that coalesce at the cisterna chyli, the origin of the thoracic duct. Materials transported in the chyle (fatty fluid) bypass the liver and avoid the first-pass effect (see Fig. 2.10 to appreciate the route of lymphatic drainage from the GI tract). Note the small plexus of blood capillaries that surround the lacteal. In addition to the vascular structures, the villus contains loose connective tissue and small amounts of smooth muscle (muscularis mucosae). The muscularis mucosae wiggle the villi back and forth in the liquid layer of the lumen adjacent to the intestinal cells, thereby increasing the efficiency of absorption. *Source:* Reproduced from DeSesso JM, Jacobson CF. *Food Chem Toxicol* 2001;39:209–228, with permission.

of absorption occurs sublingually (at the underside of the tongue and the floor of the mouth beneath the tongue) and across the buccal membranes; these routes are exploited in the delivery of certain pharmaceuticals (e.g., nitroglycerin and isosorbide dinitrate) [25].

Mechanisms that allow for the passage of materials from the lumen to the bloodstream include (i) passive diffusion across the epithelial membrane, (ii) facilitated (carrier-mediated) diffusion, (iii) active transport, (iv) aqueous pores within the cell membranes or tight junctions, and (v) endocytosis (the transport of material into the cell by folding of the cellular plasma membrane inwards to form a vacuole). These mechanisms of transfer are localized in select portions of the alimentary canal, predominantly in the small intestine.

Most xenobiotic substances are absorbed in the intestines via diffusion across the luminal surface to gain access to the vasculature of the lamina propria. Passive diffusion follows Fick's law of diffusion [26,27]. Fick's law of diffusion states that bulk diffusion at steady state is proportional to the efficiency of exchange (membrane characteristics) and available surface area. Diffusion requires that a substance comes into intimate contact with the absorptive surface of the alimentary tract. Factors that affect passive

diffusion include the expanse of absorptive surface area and the amount of time the substance is in contact with that surface area. Thus, to be able to relate GI absorption in various animal species to humans, it is important to have an understanding of the dimensions of the absorptive regions of the alimentary canal, the available surface areas of those regions, and the transit time of ingesta through each subdivision of the alimentary tract across species. These topics are discussed in Sections 2.5 and 2.6.

2.4 VASCULARITY

The tissues of the alimentary canal are richly vascularized by both the blood and lymphatic systems. Absorbed substances that have passed through the enterocytes gain access to the interstitial fluid of the lamina propria. The interstitial fluid has two sources: fluid in chyme that leaves the lumen of the alimentary canal and an exudate of plasma derived from the capillaries in the lamina propria. The amount of plasma exudate in the lamina propria increases as a result of the physiological hyperemia that occurs when chyme is present in the alimentary tract. The blood and lymphatic vascular systems act as a sink for excess interstitial fluid: absorbed substances move from the interstitium to the vessels by Le Châtelier's principle, depending on the relative flow of fluids in the two systems and the anatomical arrangement and structure of the vascular beds (Figs. 2.6–2.8).

2.4.1 Blood Flow

The GI tract of mammals is richly supplied from three major arteries that emerge from the aorta. The major arteries vascularize the upper (stomach and duodenum), middle (jejunum, ileum, cecum, and the ascending and about half of the transverse colons), and lower (remainder of transverse and descending colons and the rectal regions) regions. Figure 2.6 is a diagram of the arterial supply to the rat intestinal tract. The human pattern is similar; textbooks of anatomy provide great detail for the human condition [10,28].

The perfusion rate for the blood vasculature is about 1000 times that of the lymphatic vasculature for most tissues [11]. Nevertheless, the resting lymph flow rate from the small intestine is measurable (~ 0.095 mL/min/100 g of intestinal tissue in humans [29]) and increases after meals. Indeed, when the intestine is absorbing at its maximum rate, the lymphatic vasculature transports about 20% of the absorbed fluid [29].

The blood capillaries are organized as a network (plexus) immediately subjacent to the basement membrane of the enterocytes. The degree of vascularity of this plexus is not constant along all regions of the GI tract (Fig. 2.7). A blood capillary comprises a single, thin endothelial cell layer and the surrounding basal lamina. The distance between the basal lamina and the capillary lumen is no more than a few micrometers. Some endothelial cells additionally have $500 \text{ \AA}$ ($0.05 \text{ }\mu\text{m}$) diameter openings (fenestrations) overlain only by the basal lamina; these fenestrations expedite absorption of water and various water-soluble materials [30]. Interestingly, the basal lamina appears to act as sieve; thus, molecules as small as albumin [$\sim 75 \text{ \AA}$ ($0.0075 \text{ }\mu\text{m}$) diameter] are excluded from absorption.

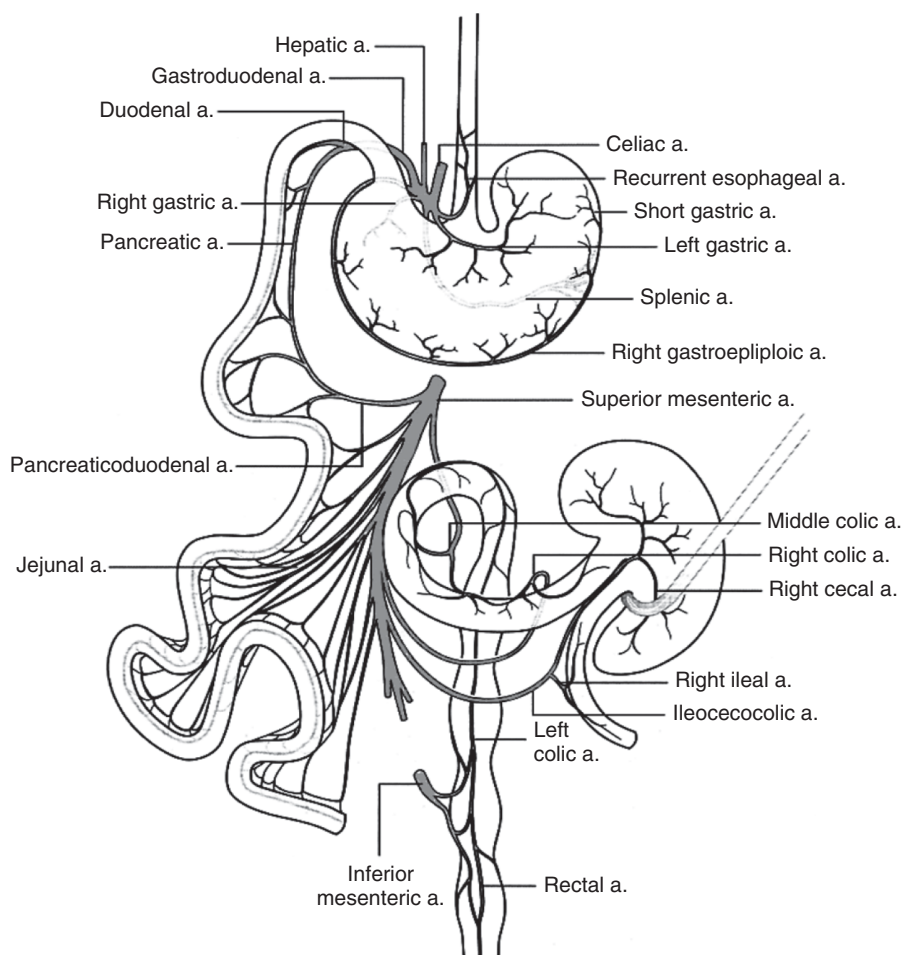


Figure 2.6 Arterial gastrointestinal supply in the rat. The rat arterial pattern is quite similar to that of the human (not shown). In both species, blood is provided by three major branches of the aorta: the celiac artery (which supplies the caudal portion of the esophagus, the stomach, the proximal duodenum, and portions of the pancreas), the superior mesenteric artery (which provides blood to portions of the pancreas, the caudal duodenum, the entire jejunum and ileum, the cecum, and the proximal half of the large intestine), and the inferior mesenteric artery (which furnishes blood to the caudal large intestine and most of the rectum). Note that the arteries enter the intestines along the margins of the organs and travel in tissue folds (mesenteries) that connect the intestines to the body wall. *Source:* Reproduced from DeSesso JM, Jacobson CF. Food Chem Toxicol 2001;39:209–228, with permission.

2.4.2 Lymphatic Flow

The lymphatic vessels are single saclike vessels (lacteals) located at the core of each villus. Their walls are made of a single endothelial cell layer, but the endothelial cells located here are thinner than blood capillary cells with no overlying basal lamina or fenestrations. Owing to apertures between adjacent endothelial cells, lymphatic vessels are permeable to even the largest chylomicrons [$\sim 6000 \text{ \AA}$ ($0.6 \text{ }\mu\text{m}$) diameter]

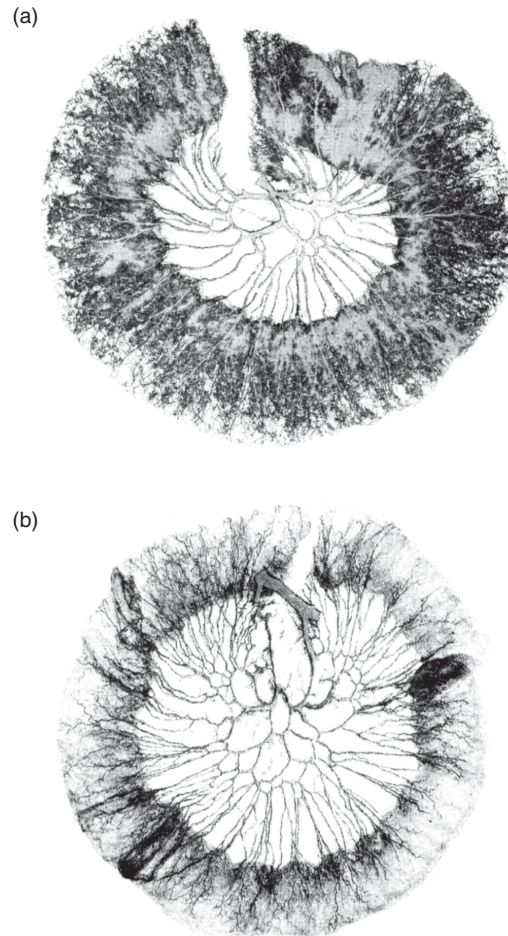


Figure 2.7 Blood supply in proximal versus distal small intestine. (a) Depicts the human proximal jejunum, which can be compared to (b), the distal ileum. The vascularity of these intestinal segments was visualized by injecting the superior mesenteric artery with colored gelatin, followed by clearing of the tissue in benzene. Note the much greater vascularity in the wall of the jejunum (a) versus that of the ileum (b), indicating the greater potential for jejunal absorption. This is a consequence of the presence of numerous plicae circulares in the proximal portion of the human small intestine. *Source:* Drawn after a preparation by M. C. E. Hutchinson [Warwick and Williams, 1973, p. 1285]; Reproduced from DeSesso JM, Jacobson CF. *Food Chem Toxicol* 2001;39:209–228, with permission.

emulsion droplets of resynthesized triglycerides, reesterified cholesterol, and phospholipids] [11,31].

2.4.3 Distinctions Between the Vascular Systems

The anatomical differences between the two vascular systems result in segregated routes of absorption for water-soluble materials versus chylomicrons. Water-soluble materials

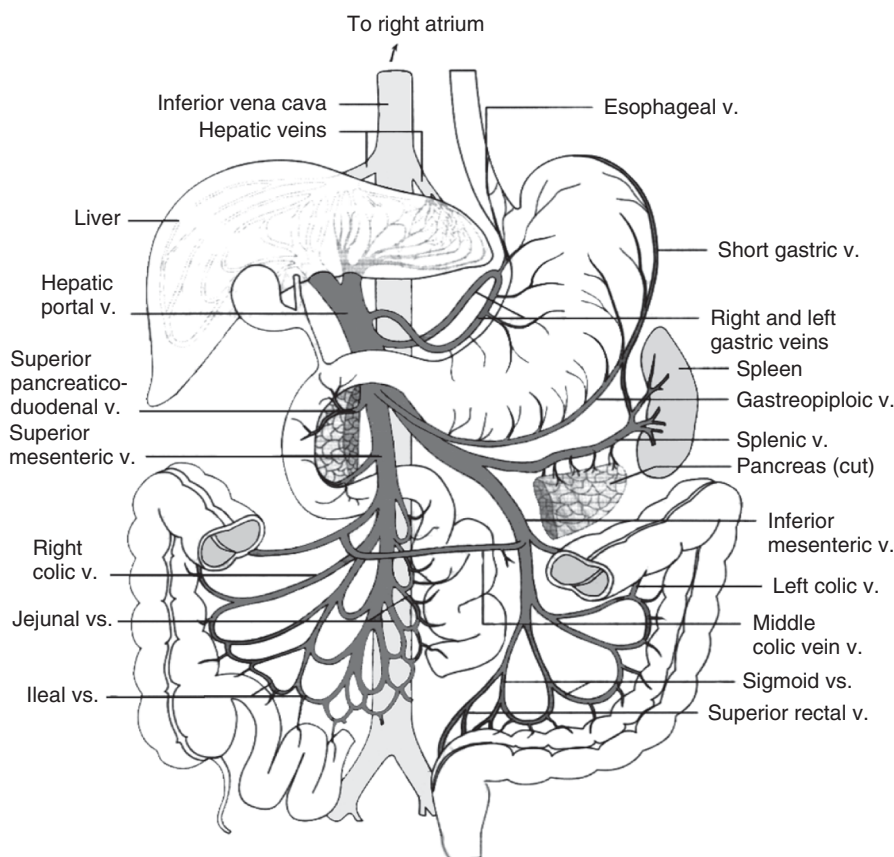


Figure 2.8 Human hepatic portal system. Blood draining from the stomach (via the gastric veins) and the entire small and large intestines (via the superior and inferior mesenteric veins) enters the large hepatic portal vein, which delivers the blood to the liver before the blood returns to the heart by way of the inferior vena cava. This vascular arrangement ensures that all materials absorbed into the blood capillaries of the gastrointestinal tract pass through the capillaries/sinusoids of the liver, where materials can be removed or metabolized before entering the general circulation. This is the anatomical foundation for the phenomenon known as the *first-pass effect*. Source: Reproduced from DeSesso JM, Jacobson CF. Food Chem Toxicol 2001;39:209–228, with permission.

are taken up by the blood vascular route, whereas lipophilic substances associate with chylomicrons and, therefore, follow the lymphatic route [20,32]. Amphipathic substances exhibiting moderate lipophilicity partition between chylomicrons and the aqueous route [12,33].

While both vascular systems serve as pathways that eventually return fluid to the systemic circulation, their routes of return have important differences. Blood vasculature from the gastroenteric organs (stomach, small intestine, and most of the colon) form into veins that ultimately become the hepatic portal vein. The hepatic portal vein drains to the liver, where the blood disperses into the sinusoids of the liver and comes

into intimate contact with the liver tissue. Enzymes within the hepatocytes can biotransform xenobiotics to either more or less biologically active substances, concomitantly reducing blood levels of the parent compound. As previously noted, this phenomenon is termed the *first-pass effect*. To leave the liver, the blood follows vessels that re-coalesce into the hepatic veins, which drain into the inferior vena cava en route to the heart. Thus, water-soluble substances absorbed from the gastroenteric organs first pass through the liver before entering the systemic circulation. The venous return of the hepatic portal systems for humans and is illustrated in Figs. 2.8 and 2.9, respectively.

High molecular weight and lipophilic substances in the lymphatic fluid draining from the stomach and intestines follow the lymph channels to the saclike cisterna chyli, which is the origin of the thoracic duct (Fig. 2.10). From the thoracic duct, lymph returns to the systemic circulation at the juncture of the left subclavian and left

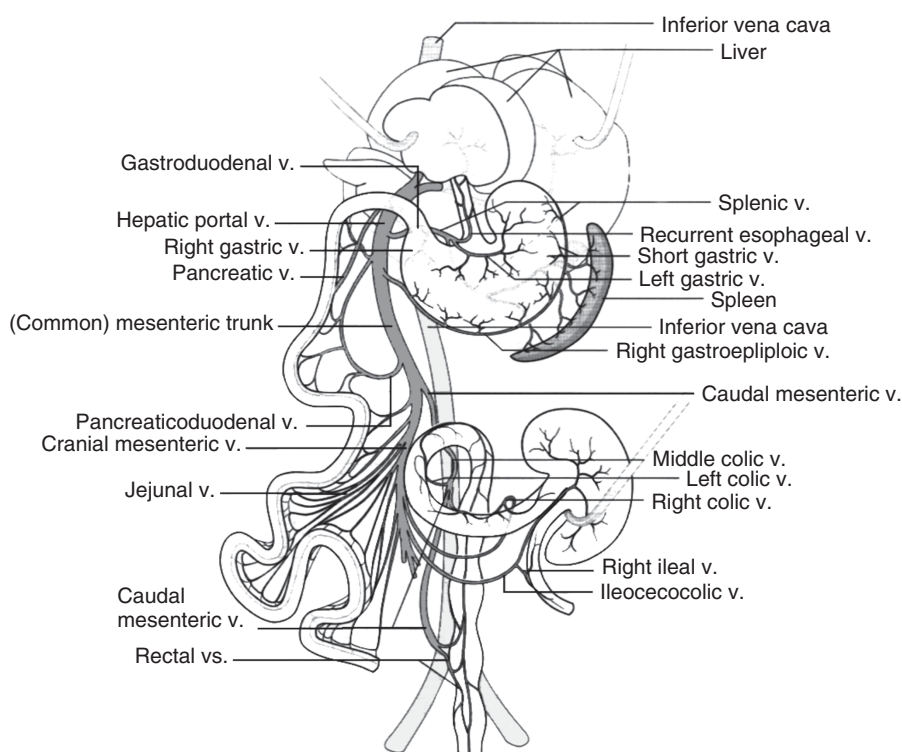


Figure 2.9 Rat hepatic portal system. The pattern of venous return from the gastrointestinal tract in rats exhibits some variability; therefore, for the purposes of this illustration, a common pattern of venous return is shown. In general, the venous return is similar to that of humans in that blood draining from the intestines eventually coalesces into the hepatic portal vein, which obligatorily passes through the liver before entering the inferior vena cava. Note that due to the postural difference between humans (upright) and rats (prone), the names of the analogous mesenteric veins of the rat are the cranial and caudal (rather than superior and inferior) mesenteric veins. *Source:* Reproduced from DeSesso JM, Jacobson CF. Food Chem Toxicol 2001;39:209–228, with permission.

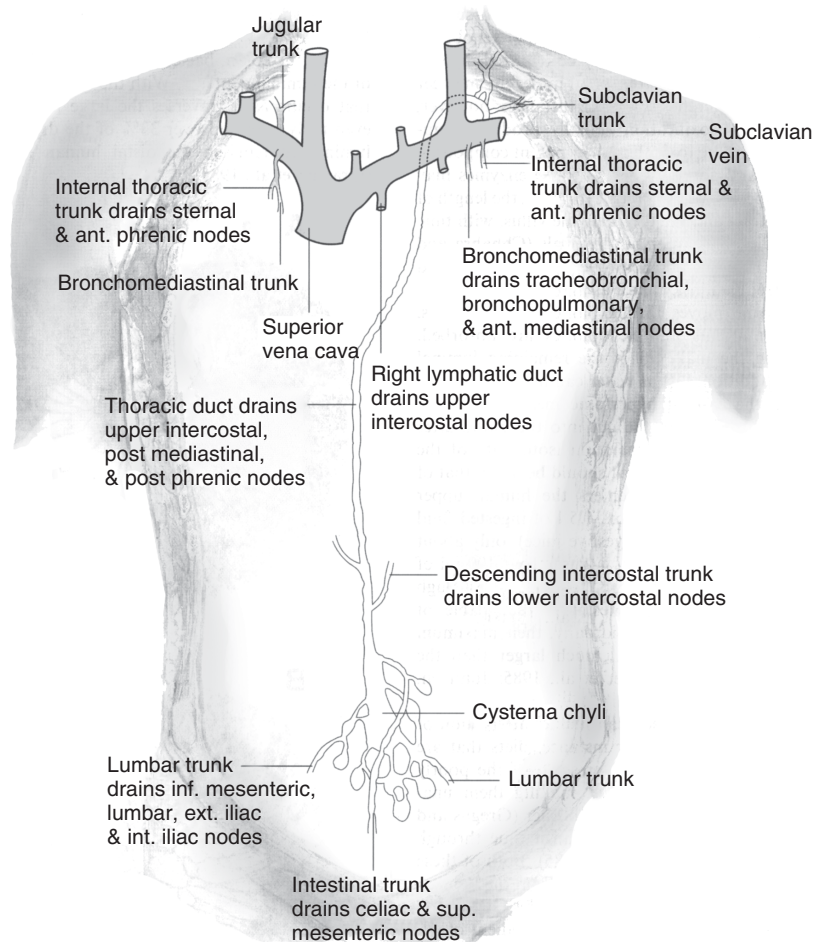


Figure 2.10 Human thoracoabdominal lymphatic system. Some high molecular weight and fatty materials absorbed from the intestinal tract make their way into the lacteals and follow the lymphatic channels through the cisterna chyli to the thoracic duct, to eventually enter the general circulation at the point where the left internal jugular and left subclavian veins join to form the brachiocephalic vein. From this point, the blood and lymph pass directly to the right atrium of the heart. Thus, materials absorbed into the lymphatics bypass the liver and do not undergo first-pass metabolism. Rats have a similar arrangement of lymphatic vessels, and consequently, materials absorbed into the rat lymphatic system also avoid first-pass metabolism. *Source:* Reproduced from DeSesso JM, Jacobson CF. *Food Chem Toxicol* 2001;39:209–228, with permission.

internal jugular veins in the root of the neck, thereby circumventing the liver and first-pass metabolism. The first capillary bed traversed by substances that are transported from the alimentary canal into the lymphatic system is at the lungs. Interestingly, any substance (regardless of its molecular weight or lipophilicity) that is absorbed into the bloodstream from the oral cavity (i.e., sublingually or buccally) returns to the heart by way of the superior vena cava, thereby also evading first-pass metabolism.

2.5 DIMENSIONS OF REGIONS OF THE GASTROINTESTINAL TRACT

While the basic organization of the alimentary canal of all mammals is comparable, there are great disparities in the dimensions of some GI structures, including the lengths and surface areas of the various subdivisions. In addition, the “environmental” conditions (e.g., pH and fluidity of the chyme and number and type of bacterial flora) within the lumen of the various subdivisions differ greatly among species. These differences, and their comparison to the human condition, must be taken into account when selecting an animal model for drug disposition studies and when extrapolating the results of studies from animal to human.

The portions of the GI tract considered in the remainder of this document are those involved in absorption of ingested materials, including the stomach, small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, ascending colon, transverse colon, descending colon, and rectum).

2.5.1 Linear Dimensions

This section concentrates on the lengths and percentages of total length of the small and large intestinal regions of the GI tract for seven species (mouse, rat, rabbit, dog, pig, monkey, and human). The data are presented in Table 2.1. The lengths for subdivisions of each region are reported, when available. The data were assembled from multiple sources and, consequently, are presented as ranges when sources disagree.

Inspection of Table 2.1 finds wide ranges in the reported lengths of various portions of the intestinal tract among species. These discrepancies may be due to variations in measurement methods as discussed by Snyder *et al.* [23] and Choi and Chiou [34]. For instance, if the investigators used intubation procedures, their measurements could be too short, either because the intestines tend to gather on the tube or because the propensity of the tube to stray from the center of the lumen by cutting corners. Post-mortem measurements may be too long because of the loss of smooth muscle tone in the intestine, which leads to flaccid intestines. If the intestines were fixed, imprecise measurements are likely due to the shrinkage that is attendant to the fixation process.

Potential artifacts notwithstanding, Table 2.1 shows that the rat cecum, which is a primary site for microbial digestion [43], makes up ~26% of the length of the large intestine, whereas the cecum accounts for only about 5% of the large intestinal length in humans. Interestingly, the rabbit has the longest cecum among the species considered herein. Rabbits are the only herbivores among the subject species. Microbial fermentation of dietary fiber occurs in the rabbit cecum and is the main source of rapidly absorbed volatile fatty acids [44]. Of the species for which data are available, none possesses intestinal length measurements that are proportional to those of the human.

2.5.2 Surface Areas

While comparison of the absolute and relative lengths of the subdivisions of the intestinal tracts reveals some interesting differences among these species, the lengths do not provide adequate information about the surface areas available for absorption. For instance, as noted by DeSesso and Jacobson [4], while the human small intestine is only about 5.5 times the length of the rat small intestine, the human surface area is more than 250 times that of the rat due to modification of the interior surface of the intestine as discussed below.

TABLE 2.1 Comparison of Lengths of the Intestinal Tract and Its Major Subdivisions in Seven Different Species

Region of Intestinal Tract	Mouse			Rat			Rabbit			Dog			Pig			Monkey			Human		
	Length (cm)	% of total	% of Sub-division	Length (cm)	% of Total	% of Sub-division	Length (cm)	% of Total	% of Sub-division	Length (cm)	% of Total	% of Sub-division	Length (cm)	% of Total	% of Sub-division	Length (cm)	% of Total	% of Sub-division	Length (cm)	% of Total	% of Sub-division
Duodenum	5.3 ^a	—	—	9.5–10 ^b	8	—	—	—	—	25 ^{c,d}	6	—	—	—	—	—	—	—	25 ^e	4	—
Jejunum	46.2 ^a	—	—	90–135 ^b	90	—	—	—	—	360	90	—	—	—	—	—	—	—	260 ^e	38	—
Ileum	—	—	—	2.5–3.5 ^b	2	—	—	—	—	15 ^{c,d}	4	—	—	—	—	—	—	—	395 ^e	58	—
Total small intestine	45	~75	—	125 ^b	83	—	339–398 ^{c,f}	61	—	~400 ^{c,g,h}	83	—	1500–2000 ^{c,g,h}	70–85	—	—	—	—	680 ^e	81	—
Cecum	3.4 ^a	—	—	5–7 ^b	26	—	41–61 ^{c,g,i}	—	28	5–8 ^{c,d,g}	~11	—	20–30 ^{c,g,h}	—	—	—	—	—	7 ^e	5	—
Colon	10.0 ^a	—	—	9–11 ^b	42	—	114–165 ^{c,g,i,j}	—	72	25–60 ^{c,d,g,h}	~81	—	400–500 ^{c,g,h}	—	—	—	—	—	93 ^e	60	—
Rectum	—	—	—	8 ^b	32	—	—	—	—	5 ^d	~8	—	—	—	—	—	—	—	55 ^e	35	—
Total large intestine	—	~25	—	25 ^b	17	—	—	39	—	~82	17	—	350–850	15–30	—	—	—	—	155 ^e	19	—
Total intestinal tract	—	—	—	150 ^b	—	—	582 ^{g,h}	—	—	482 ^{c,g}	—	—	2350 ^{c,g}	—	—	—	—	—	835 ^e	—	—

^aOgiolda *et al.* [35]; intestinal segments unfixed.

^bHebel and Stromberg [8]; anatomical lengths as reported in DeSesso and Jacobson [4].

^cKararli [9].

^dEvans [40].

^eSnyder *et al.* [23]; anatomical lengths as reported in DeSesso and Jacobson [4].

^fPappenheimer [36].

^gStevens [37].

^hDressman and Yamada [41].

ⁱSnipes [38]; formaldehyde fixed segments.

^jSnipes *et al.* [39]; Bouins solution fixed segments.

Source: Reproduced from DeSesso JM, Williams AL. Annu Rep Med Chem 2008;43:353–371, with permission [23,42].

The small intestinal surface area is important because the vast majority of substances that are absorbed in the GI tract do so in the small intestine, where they gain access to the bloodstream by means of passive diffusion. As noted by DeSesso and Jacobson [4], the absolute surface area of the human small intestine is more than 3800-fold greater than the surface area of the stomach. This enormous difference in surface areas helps explain why gastric absorption of substances is generally small when compared to intestinal absorption. Similarly, because of the modest surface area of the human large intestine (0.35 m^2 , which is less than 0.15% that of the small intestine), the large intestine tends to play a minor role in absorption compared with the small intestine.

Because the available surface area is a dominant factor in passive diffusion, Table 2.2 presents the smooth luminal surface areas of the small intestine for seven species, including humans. Various authors calculated smooth luminal values by conceiving of the small intestine as a cylinder and using the length and diameter measurements. As noted in Table 2.1, the values listed therein should be considered estimated measures. As previously mentioned, the rate and extent of absorption is increased by expansion of the absorptive surface area. The absorptive surfaces of the small intestine in the subject species exhibit modifications that increase the surface areas by a combined factor of 100–750 depending on the species, as shown in Table 2.2 and illustrated in Fig. 2.11.

TABLE 2.2 Calculated Total Small Intestinal Surface Areas for Seven Species

Species	Body Weights (kg)	Smooth Luminal Surface Area (m^2)	Fold-Increase Factors			Combined Multiplication Factors	Estimated Total Surface Area (m^2)
			Plicae	Villi	Microvilli		
Mouse	0.032	0.004 ^a	(1) ^b	(5) ^b	(20) ^b	100	(0.4)
Rat	0.3	0.016 ^a	1 ^c	5 ^c	20 ^c	100	1.6
Rabbit	2.6–3.9	0.087–0.096 ^a	1 ^d	5.7 ^e	24 ^f	136.8	11.9–13.1
Dog	10.7–12.6	0.099–0.14 ^{a,g}	1 ^d	10 ^h	25 ⁱ	250	24.75–35
Pig	47	1.4 ^g	1 ^d	6 ^j	(20–25) ^k	120	(168–210)
Monkey	2.7–3.0	0.091–0.11 ^{b,l}	(3) ^m	(10) ^m	(20) ^m	600	(54.6–66)
Human	70	0.42 ^a	3 ^c	10 ^c	20 ^c	600	252

^aValues as reported in Pappenheimer [36].

^bExtrapolated from values for rat.

^cFrom DeSesso and Jacobson [4].

^dValue based on the absence of plicae in the small intestine.

^eValue based on comparison of villi lengths between rabbits and humans as reported in Thomson and Snyder *et al.* [24,49].

^fAs cited in Westergaard and Dietschy [50].

^gValues as reported in Chivers and Hladik [48].

^hOn the basis of the same size villi as humans as reported in Paulsen *et al.* and Snyder *et al.* [24,51].

ⁱAs reported in Kararli [9].

^jOn the basis of the comparison of villi lengths between pigs and humans as reported in Shirkey *et al.* and Snyder *et al.*

^kExtrapolated from values reported for other animal species [24,52].

^lValues as reported for cynomolgus monkeys.

^mExtrapolated from values for human.

Source: Reproduced from DeSesso JM, Williams AL. *Annu Rep Med Chem* 2008;43:353–371, with permission [42].

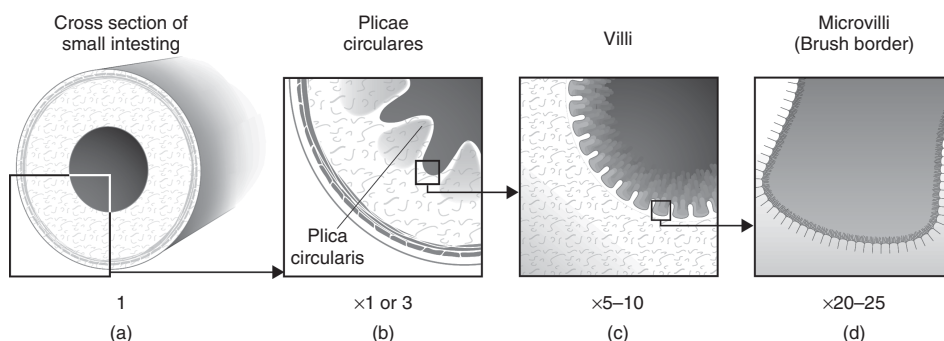


Figure 2.11 Increases in absorptive surface area by anatomic modifications of the intestinal wall. (a) The absorptive surface area of the intestine is depicted as that of a cylinder having a nominal value of 1. Modifications that increase the surface are shown to the right. (b) Folds of mucosa (plicae circulares) are represented. In species that have plicae circulares (monkey and human, among the species considered herein), the surface area is increased approximately threefold. (c) Fingerlike villi are shown projecting from the surface of the intestinal mucosa throughout the length of the intestine. The dimensions (length and width) of the villi vary for each species, as does the number of villi per square millimeter of mucosal surface. The villi increase the absorptive surface area by 5- to 10-fold. (d) The brush border on the surface of individual enterocytes is illustrated. The brush border is composed of microvilli, which further increase the absorptive surface area by 20- to 25-fold. *Source:* Reproduced from DeSesso JM, Williams AL. *Annu Rep Med Chem* 2008;43:353–371, with permission.

The greatest increase in absorptive surface area due to these modifications occurs in humans. Among the species listed in Table 2.2, only monkeys and humans possess plicae circulares, which increase the luminal surface area approximately threefold. Intestinal villi increase the luminal surface area by a factor ranging from 5 to 10. The range in values is due to interspecies variations in the dimensions of the villi (e.g., length, width, and density of the microvilli), as well as changes in dimensions within the different regions of the small intestine [8,23]. That is, the architecture of the villi changes as one progresses distally through the intestine. For example, villi in the duodenum tend to be broader than those of the ileum [45,46], resulting in a gradient of decreased surface area as one moves distally through the small intestine [47]. This gradient is even greater in those species (monkey and human) that possess plicae circulares (which also tend to be larger and more numerous in the proximal intestine). Microvilli, which account for the brush border, further increase the absorptive surface area by a factor of 20–25.

2.5.3 Relative Surface Areas

Animals of dissimilar sizes have different basal metabolic rates and, consequently, have different requirements for caloric intake. Thus, direct comparisons of the absolute surface areas of the GI tracts among various species are not straightforward. One way to normalize the surface areas of the stomach, small intestine, and large intestine across species is to consider these surface areas relative to the total body surface areas of different species. The metabolic rates of most mammals are proportional to body

surface area [53,54]; consequently, the relative surface area serves to normalize the effective absorptive surface based on the metabolic requirements of a given species. The surface areas of the GI tract are presented as both absolute surface area and relative surface area in Table 2.3. (The relative surface area is the absolute surface area of a GI tract region divided by the body surface area.)

Humans are the largest species analyzed in Table 2.3; thus, it is not surprising that they have the largest absolute small intestinal surface area. It is noteworthy, however, that when the basal metabolic rates of the various species are taken into consideration, humans also have the largest relative surface area. The impact of the differences in surface areas on absorption of substances can be appreciated by comparing the relative surface areas of the human small intestine to that of the rat. The human relative surface area is approximately four times greater than that of the rat (Table 2.3). Since the amount of a substance that crosses the enteric mucosa is determined by its flux (amount of mass per unit surface area per unit time), the impact of the increased relative intestinal surface area in humans on absorption is twofold. First, substances that are equally well absorbed in both rats and humans are likely to be absorbed more quickly in humans (i.e., exhibit a higher rate of absorption). Second, substances that are poorly or incompletely absorbed by both species are likely to be absorbed to a greater extent by humans.

It should be noted that the values in Table 2.3 can be altered by physiological changes that occur in response to nutritional challenges. For example, during times of starvation or disease (such as diabetes [58]) or during normal physiologic stress brought on by pregnancy, the mucosa of the rat stomach undergoes hyperplasia [59]. In pregnancy, the mucosal surface area of the small intestine increases as gestation progresses due to a temporary, but progressive, increase in villi height [60]. Further, this condition of increased absorptive surface area is maintained throughout lactation [61]. Changes in effective surface areas also occur as animals age. The villi of the small intestine decrease in height during old age [62–64], leading to a decrease in absorptive surface area, and, consequently, reduced efficiency in the absorption of nutrients and other materials from chyme (see further discussion under Section 2.6).

With respect to the often cited concept that metabolic rates of species are proportional to their surface area raised to the two-thirds power [65], it is interesting that the mucosal surface area of the small intestines among nonruminant eutherian mammals increases approximately in proportion to the 0.6 power of body mass [36].

2.6 MOTILITY AND TRANSIT TIMES

As previously noted, absorption of materials from the intestinal tract is determined not only by the total available surface area but also by the amount of time that substances are in contact with the absorptive epithelium. Most of the alimentary canal is invested with at least two layers of smooth muscle. The muscle fibers of the inner layer are arranged circumferentially relative to the lumen, while the outer layer is arranged parallel to the long axis of the canal. The coordinated, rhythmic contractions of these muscle layers produce intestinal motility, which affects the absorption of substances from the intestinal tract. First, intestinal motility thoroughly mixes the chyme to aid in its digestion. Second, it continually agitates the chyme, pushing it against the enterocyte brush border, thus, aiding in absorption. Finally, intestinal motility propels the chyme

TABLE 2.3 Comparison of Absolute Surface Areas and Surface Areas Relative to Resting Metabolic Rates of the Gastrointestinal Tract and Its Major Subdivisions in Seven Different Species

	Mouse		Rat		Rabbit		Dog		Pig		Monkey		Human	
Body weight (kg)	0.032		0.3		2.6–3.9		10.7–12.6		47		2.7–3.0		70	
Body surface area (BSA; m ²) ^a	0.0066		0.03–0.06		0.23		0.39–0.78		—		0.3		1.8	
Region	Absolute SA ^b (m ²)	SA relative to BSA (%) total)	Absolute SA (m ²)	SA relative to BSA (%) total)	Absolute SA (m ²)	SA relative to BSA (%) total)	Absolute SA (m ²)	SA relative to BSA (%) total)	Absolute SA (m ²)	SA relative to BSA (%) total)	Absolute SA (m ²)	SA relative to BSA (%) total)	Absolute SA (m ²)	SA relative to BSA (%) total)
Stomach	0.00035 ^c	0.05 (0.1%)	0.00062 ^d	0.01–0.02 (0.0%)	—	—	0.0344–0.0426 ^e	0.04–0.11 (0.1%)	0.016 ^f	—	0.0143–0.0306 ^g	0.048–0.10 (0.0%)	0.053 ^h	0.029 (0.0%)
Total small intestine ⁱ	0.4	60.61 (99.0%)	1.6	26.67–53.33 (97.9%)	11.9–13.1	51.74–56.96	24.75–35	31.73–89.74 (99.3%)	168–210	—	54.6–66	182–220 (99.8%)	252	140 (99.8%)
Total large intestine	0.0036 ^j	0.55 (0.9%)	0.034 ^k	0.57–1.13 (2.1%)	0.1606	0.7	0.023–0.245 ^{e,f}	0.029–0.63 (0.5%)	0.5142	—	0.0687–0.0933 ^g	0.23–0.31 (0.1%)	0.35 ^h	0.19 (0.1%)

^aAs reported in Derelanko, 2000.

^bSmall intestinal surface areas reported as calculated in Table 2.

^cOgiolda *et al.* 1998; mean bw = ~43 g; unfixed tissue.

^dDerived from Snipes, 1997; mean bw = 47.5 g; formaldehyde fixed segments; large intestinal value based on surface areas of cecum and colon combined.

^eJarvis and Whitehead, 1980; as reported in DeSesso and Jacobson, 2001.

^fYoung *et al.*, 1991; as reported in DeSesso and Jacobson, 2001.

^gDerived from Snipes, 1997; mean bw = 3.6 kg; formaldehyde fixed segments; large intestinal value based on surface areas of cecum and colon combined.

^hChivers and Hladik, 1980; values from 2 dogs (bw = 10.68 & 12.55 kg).

ⁱDerived from Snipes, 1997; mean bw = 13.75 kg; formaldehyde fixed segments; large intestinal value based on surface areas of cecum and colon combined.

^jChivers and Hladik, 1997; values based on single pig (bw = 47.98 kg); formaldehyde fix segments; large intestinal value based on surface areas of cecum and colon combined.

^kDerived from Chivers and Hladik, 1980; values for Cynomolgus monkeys (bw = 2.7–3.1 kg); unfixed tissues.

Source: Reproduced from DeSesso and Williams, Ann Rep Med Chem 2008; 43: 353–371, with permission.

TABLE 2.4 Range of Gastric Emptying Half-Times (in Hours) as Reported for Seven Species of Animals

Species	Liquids	Particulates
Mouse	----	1.23 ^a ----
Rat	—	—
Dog	1.5 ^b	1.5 ^b
Rabbit	1.3 ^b	12 ^b
Pig	2 ^b	10 ^b
Monkey	0.4–0.5 ^c /2.4 ^d	—
Human	0.2 ^e	0.5–3.4 ^{a,f}

^aSchwarz *et al.* [70].^bValues reported in Kararli and Stevens [10,37].^cKondo *et al.* [71]; measurements from fasted cynomolgus monkeys.^dKondo *et al.* [72]; measurements from fed cynomolgus monkeys.^eGranger *et al.* [11].^fDegan and Phillips; measurements for males and females, 19–45 years of age.Source: Reproduced from DeSesso JM, Williams AL. *Annu Rep Med Chem* 2008;43:353–371, with permission [42,73].

through the GI tract in a net aboral direction (peristalsis), the rate of which influences GI transit times. In addition, the thin layer of smooth muscle in the lamina propria (muscularis mucosae) undulates the intestinal villi, thereby churning the unstirred layer of fluid that is associated with the brush border.

Transit time is the amount of time that elapses as a bolus of food or chyme traverses a particular region of the alimentary canal. In the mouth, transit time is determined by voluntary control of the length of time that is spent chewing food. When the food bolus passes to the pharynx and esophagus, transit time is governed by gravity and peristalsis. In humans, transit time through the pharynx and esophagus is about 6 s. Once in the stomach, transit times depend on the nature of the contents (Table 2.4 [12,66]). In the fasted state, the half-time for saline emptying from the human stomach is 12 min, while the gastric transit time for a full meal is about 4 h [11]. Generally, the various dietary constituents of a meal leave the stomach at varying times, with carbohydrates emptying first, proteins next, and fatty substances last. Liquids drunk with a meal often bypass the solids and enter the duodenum quickly [11]. Table 2.5 presents the ranges of transit times through various portions of the GI tract for each of the subject species. Transit times tend to be shortest in the stomach and longest in the large intestine. Additionally, liquids typically pass through the stomach more quickly than solids. Ingestion of a fatty meal, however, will delay significantly the emptying of stomach contents to the duodenum [10,24]. The fasting/fed state of an individual can also affect transit time, as demonstrated by the great differences in gastric emptying half-times for liquids given to fasted versus fed monkeys. For instance, after prolonged fasting the orocecal transit time is extended, probably because the body needs to absorb additional calories from the ingested material [67]. The amount of fiber in the diet not only influences transit time but also interferes with the access of some substances such as cholesterol to the absorptive surface [68,69].

Physiological status also influences transit time. For example, life stage (e.g., puppy vs adult) influences transit time in dogs. The mean orocecal transit time decreased as large breed puppies grew (Great Dane and Giant Schnauzer). This did not occur, however, in smaller breeds (Miniature Poodle and Standard Schnauzer). It is believed

TABLE 2.5 Range of Gastrointestinal Transit Times (Hours) Through Different Portions of the Gastrointestinal Tract as Reported for Seven Species of Animals

Species		Oro-Cecal				Large Intestine		Total	
		Stomach		Small Intestine		Liquid	Solid	Liquid	Solid
		Liquid	Solid	Liquid	Solid				
Mouse		—		—		—		—	
Rat		0.7–2.1 ^a		2.6–3.3 ^a		15.5 ^b		38.9 ^c	
Dog		1.7 ^d		—		18.5 ^e		27.0–37.4 ^{e, f, g}	
				----14.3 ^e ----					
Rabbit				----9.1 ^h ----		9.6 ^h		18.1–20.6 ^h	
Pig		0.8–	1.0–	3.9–	3.7–4.3	24.9–	35.6–	29.1–	40.5–
		0.9 ⁱ	1.3 ⁱ	4.4 ⁱ	ⁱ	41.3 ⁱ	44.4 ⁱ	46.6 ⁱ	49.7 ⁱ
Monkey				----1.8 ^j /2.3–2.5 ^k ----		—		—	
Human	Child	1.1 ^l		7.5 ^l		17.2–40 ^{l, m}		—	
	Young	1.0–	1.9–	3.8–	3.4–	35.0 ⁿ		57.3–58.2 ^r	
	adult	1.6 ^{d, n}	2.3 ⁿ	3.9 ⁿ	3.5 ⁿ				
		1.1 ^{o, p}		1.8–8.0 ^{l, o, q}		17.5 ^l			
	Mature	0.8–	1.3–	2.0–	3.0–	33.5–61.5 ⁿ			
	adult	1.1 ⁿ	1.8 ⁿ	4.2 ⁿ	3.8 ⁿ				

^aTuleu *et al.* [74]; measurements from fed rats.^bEnck *et al.* [75]; measured by the insertion of dye in the cecum.^cSastry and Rao [76].^dLui *et al.* [77]; liquid transit in fasted Beagle dogs and young adults (21–29 years of age).^eHernot *et al.* [78]; measured in Standard Schnauzers.^fBurrows *et al.* [79]; measured in Beagle dogs.^gHernot *et al.* [80]; measured in French Bulldogs, English Cocker Spaniels, and Standard Schnauzers.^hGidenne and Ruckebusch [81].ⁱWilfart *et al.* [82]; measurements derived from pigs with average weight of 33 kg.^jKondo *et al.* [71]; measurements from fed cynomolgus monkeys.^kKondo *et al.* [72]; measurements from fasted cynomolgus monkeys.^lFallingborg *et al.* [83]; determined in children 8–14 years of age.^mWagener *et al.* [84]; determined in children 4–15 years of age.ⁿGraff *et al.* [85]; determined in adults 20–30 years of age (young) and 38–53 years of age (mature).^oEwe *et al.* [86].^pFallingborg *et al.* [87].^qDegan and Phillips [73]; measurements for males and females, 19–45 years of age.^rCummings *et al.*, 1978; males only.

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that the decrease is due to the shorter gastric emptying times in large breeds during growth rather than to a faster rate of transport through the intestine [89,90]. In humans, orocecal transit times are significantly longer among older people compared to young adults, but transit time in the large intestine increases significantly only among women [85]. Because the dimensions of intestinal villi change such that the available absorptive surface area decreases with age [91], the increased transit time may be a compensation that improves (or attempts to realize) sufficiency of nutritional absorption. This same group of investigators reported that the overall GI transit time was significantly longer in women than in men at all ages.

2.7 LUMENAL CONTENTS

2.7.1 Physical and Chemical Nature of the Contents

The nature of the luminal contents in all species is altered as the contents traverse the alimentary canal. This alteration is due to the physical dispersion of ingested solid matter by chewing and the muscular activity of the stomach, the mixing of the ingested matter with imbibed fluid and digestive juices, the action of enzymes on GI contents, the absorption of fluid and materials from the lumen, and the addition of bacterial flora to the contents. There is a dramatic difference in the nature of the chyme that enters the duodenum of rats compared to that of humans [4]. Rat chyme contains many bacteria and has the consistency of a moist paste when entering the duodenum, whereas human chyme is watery and its bacterial content is negligible. The chyme of mice and rabbits is quite similar to that of the rat. The chyme of dogs, pigs, and monkeys is more similar to human chyme than to that of the rat. As chyme moves through the distal portion of the small intestine in all species, it begins to acquire a more viscous consistency, especially at the distal end of the ileum.

The daily volumes and constituents of digestive fluids secreted (under neuronal and hormonal control) by the various portions of the digestive tract and adnexal organs vary greatly based on the species and its physiological status. Estimates of these vary among publications, but the values summarized by Kararli [9] and DeSesso and Jacobson [4] are useful starting points for understanding the differences among species. The quantity and characteristics of these secretions can be altered in response to luminal contents. In addition to the intestinal secretions, water can move from the blood into the duodenum in response to a hypertonic meal, in order to maintain the isotonicity of luminal contents with that of plasma [11,12,24].

The pH of the luminal contents also varies widely among species (Table 2.6). As a general rule, however, the pH of luminal contents is the lowest in the stomach and increases as the chyme progresses distally through the GI tract, approaching neutrality. This modification in pH as the chyme travels through the GI tract is accomplished through the secretion of various acidic and alkaline fluids. In most regions of the digestive tract, the secretions are slightly alkaline, and the luminal contents exhibit a pH of 7–8 as chyme approaches the distal small intestine. The stomach is the lone exception to this general statement. The secretion of acid by the gastric mucosa results in acidification of chyme. In carnivores (including humans, pigs, and dogs) as well as the rabbit (an herbivore), the pH of the stomach is quite low (as low as 1–2 in humans), whereas the gastric pH of rodents (rats and mice) is only slightly more acidic (pH 3–5) than that of the skin. The moderate pH in the stomach of rodents is a consequence of the requirement for microfloral digestion of ingested material that begins before entry to the small intestine [9]. Animals with very acidic stomach contents begin the digestion of proteins therein and have no requirement for a large bacterial colony in their stomach. When chyme enters the duodenum, it is quickly neutralized by the alkaline (bicarbonate) secretion of the Brunner's glands [92]. The pH of chyme affects the ionization state of certain molecules and, therefore, can affect absorption.² Despite

²The GI tracts of most elderly humans exhibit the same pH characteristics as do those of younger adults, but there are notable exceptions. Some seniors exhibit elevated gastric pH or even achlorhydria (the inability to secrete gastric acid; Dressman *et al.*, 1989). Achlorhydria is often accompanied by delayed gastric emptying times.

TABLE 2.6 Range of pH Values Reported for Different Portions of the Gastrointestinal Tract for Seven Species of Animals^a

Species	Stomach		Small Intestine ^b			Large Intestine		
	Anterior	Posterior	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum/Feces
Mouse	4.5	3.1	—	—	—	—	—	—
Rat	4.3–5.1	2.3–4.0	6.5–7.1	6.7–6.8	7.1–8.0	6.4–7.2	6.6–7.6	6.9
Dog	1.5–5.5	1.5–3.4	6.2	6.2–7.3	7.5	6.4	6.5	6.2
Rabbit	1.9–2.2	1.9–2.2	6.0–6.1	6.8–7.5	7.2–8.0	5.7–6.6	6.1–7.2	7.2
Pig	1.6–4.3		6.0	6.2–6.9	7.5	6.3	6.8	7.1
Monkey	4.7–5.0	2.3–2.8	5.6–6.0	5.8–6.0	6.0–6.7	4.9–5.1	5.0–5.9	5.5
Human	1.5–5.0		5.0–7.0	6.0–7.0	7.0–7.4	5.7–5.9	5.5–7.5	6.5–7.0

^aThe range of values reported here are from the following Refs. 10,44,77,87, and 93–95.

^bSome studies reported pH values for segments 1, 3, 5, and 7 of the small intestine. For the purposes of this table, segment 1 was designated the duodenum, segments 3 and 5 were designated the jejunum, and segment 7 was designated the ileum.

Source: Reproduced from DeSesso JM, Williams AL. *Annu Rep Med Chem* 2008;43:353–371, with permission [42].

its status as a carnivore, the pH of the GI tract of the dog presents some unique characteristics compared with humans. For instance, the fasting intestinal pH in dogs is higher than that of humans (7.3 in the dog vs 6.0 in humans [77]).

In addition to the pH gradient that exists along the linear axis of the GI tract, a pH gradient also exists from the center of the lumen moving radially toward the epithelial surface. The pH of the lumen is more acidic than the pH of contents at the epithelial surface. This is a function of the unstirred layer of water that is associated with the brush border [4] and the alkaline secretions of the intestinal epithelia [96]. Furthermore, this gradient may affect the rate of uptake of various substances, especially xenobiotics.

The contents of the alimentary canal are also altered by many enzymes found in the secretions of the digestive tract, integrated into or adhered to the cell surface membrane of small intestinal cells, and present within small intestinal cells. Many of these enzymes work to digest carbohydrates, proteins, and fats into readily assimilated molecules necessary to sustain life. Others are involved in the metabolism of xenobiotic compounds. Such compounds may be oxidized, hydrolyzed, and conjugated with large, polar molecules in order to facilitate their excretion. In some instances, however, this attempt at detoxification actually produces compounds that are more toxic than their parent compound. The presence and activity of all these enzymes may vary among species, with age and sex, along the length of the digestive tract and the height of the villus, with diurnal rhythm and diet, and among individuals [33,97–99].

The makeup of the lumenal contents is also changed through the progressive absorption of fluids, electrolytes, nutrients, and xenobiotics as chyme moves through the GI tract. Just as water can flow from the blood to the lumen in the case of hypertonic meals, water can rapidly be absorbed from the gut into the blood after hypotonic meals—again to maintain isotonicity of the lumenal contents with plasma. It should be noted that of the 8–9 L of fluid that enters the human upper digestive tract each day (~1.5 L of ingested fluid plus ~7 L of secreted digestive juice), only ~1 L enters

the large intestine and only about 100 mL of water is found in the daily output of feces [4].

2.7.2 Bile and Bile Salts

With regard to interspecies differences in secretions into the intestinal tract, it is important to note that among the subject species in this chapter, the rat is the only one that lacks a gallbladder. While the rat is unique in this respect among typical experimental animals, other common vertebrates, such as pigeons and horses, also lack gallbladders. Species that lack gallbladders do not concentrate bile; rather, bile flows into the lumen of the duodenum on a continuous basis. This means that bile continuously enters the rat duodenum as a dilute solution as it is made. By way of comparison, humans secrete from the gallbladder 2–22 mL of bile per kilogram body weight each day [41]. In contrast, rats secrete 48–92 mL of bile per kilogram body weight each day [20].

The daily flow rate of bile into the duodenum (normalized by body weight in kilograms) for five of the subject species is shown in Table 2.7. The table also lists the concentration of bile salts within the bile for five of the seven subject species. There is no clear relationship between the size of the species and its bile flow rate. Interestingly, the highest rate is found in the herbivorous rabbit.

Bile is a complex mixture of organic and inorganic materials of which the various bile acids are the major component. Bile acids are an extensive group of molecules that share a structural similarity to cholesterol but exhibit differences with regard to substituent side groups. Table 2.8 presents selected physical and chemical properties of bile and bile acids for six of the subject species in this chapter. Inspection of the table reveals that porcine bile has a bile acid composition and other properties that are most similar to human bile. Note that conjugation of bile acids to glycine or taurine is not an excretory pathway, but rather a means to enhance the enterohepatic recirculation of bile acids (see discussion in Section 2.8).

Interestingly, the composition of bile in hamsters, which is not considered in this chapter, is most similar to that of humans [100]. It is also noteworthy that rabbits have the most lipophilic bile of the animals considered, but they are unique in that about 80% of their bile acids are formed by bacterial flora [101].

TABLE 2.7 Comparison of Flow Rates and Composition of Bile from Seven Different Species^a

Species	Bile Flow Rate (mL/day/kg)	Total Bile Salts (mmol/L)
Mouse	—	—
Rat	48–92	17–18
Rabbit	130	6–24
Dog	19–36	40–90
Pig	—	—
Monkey	19–32	22
Human	2.2–22.2	3–45

^aData from Kararli [9].

Source: Reproduced from DeSesso JM, Williams AL. *Annu Rep Med Chem* 2008;43:353–371, with permission [42].

TABLE 2.8 Bile Acid Characteristics in Various Species

Species	Gallbladder	Lipophilicity of Bile Acid Pool	Bile Acids						Preferred Conjugate Molecule
			CA	DCA	CDCA	HCA	HDCA	MCA	
Rat	Absent	Very low	XX ^a					X ^b	Taurine
Mouse	Present	Very low	XX					X	Taurine
Rabbit	Present	Very high	X	XX					Glycine
Dog	Present	Low	XX	X					Taurine
Pig	Present	Moderate	X		XX	X	X		Glycine/ taurine
Human	Present	Moderate	X	X	XX				Glycine/ taurine

Abbreviations: CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; MCA, muricholic acid.

^aPredominant bile acid.

^bPresent.

Source: Data from Hofmann *et al.* (2010), Hofmann (2009), and Hofmann and Roda (1984) [100–102].

2.7.3 Bacterial Flora and Coprophagy

Bacterial flora populates much of the GI tract in all species and contributes to the lumenal contents. The mouth and pharynx have a rich and diversified bacterial population, although oral bacteria are not important in absorption. In rodents, large numbers of microorganisms are found in the stomach and intestines. In humans and rabbits, however, because of the low pH of gastric contents, microorganisms are virtually absent in the stomach and proximal small intestine. In fact, large numbers of bacteria are not encountered until chyme reaches the distal ileum and large intestine [11,94,103,104]. Bacteria are capable of metabolizing substances found in chyme and, thus, can alter constituents found in the luminal contents, which is particularly important in the case of ingested medicines and xenobiotics [105]. Differences between humans and rats with regard to the numbers and types of bacteria, as well as to their geographical location within the digestive tract, affect the site and extent of absorption of some substances [11,106]. Once chyme reaches the colon, bacteria are a major component of the lumenal contents. With the dehydration of chyme that occurs as it traverses the large intestine, bacteria eventually make up over 33% of the dry weight of the lumenal (fecal) content in the distal human large intestine [11].

Many species, especially rodents and rabbits, coprophagize (ingest feces) to aid the digestive process and to ensure absorption of essential components of their diets. This is exemplified by the rabbit, which has a unique digestive metabolism for a monogastric animal [107,108]. Rabbits defecate two types of fecal pellets: hard and soft [107,109]. The soft pellets are known as *cecotrophs* and are the pellets that are consumed by rabbits [110]. Cecotrophs are high in protein content and vitamin B complex [107]. Coprophagy also helps maintain a balanced, healthy population of flora. Impairment of coprophagy can result in deficiency of vitamins, as has been noted for over 80 years [111].

2.8 ENTEROHEPATIC RECIRCULATION

As previously stated, the hepatic portal system involves blood flow from the capillaries of the small intestine that drain to the hepatic portal vein, which flows into the hepatic sinusoids before reaching the heart. The movement of venous blood from the intestine along this specific route is referred to as the *enterohepatic circulation* (Fig. 2.12).

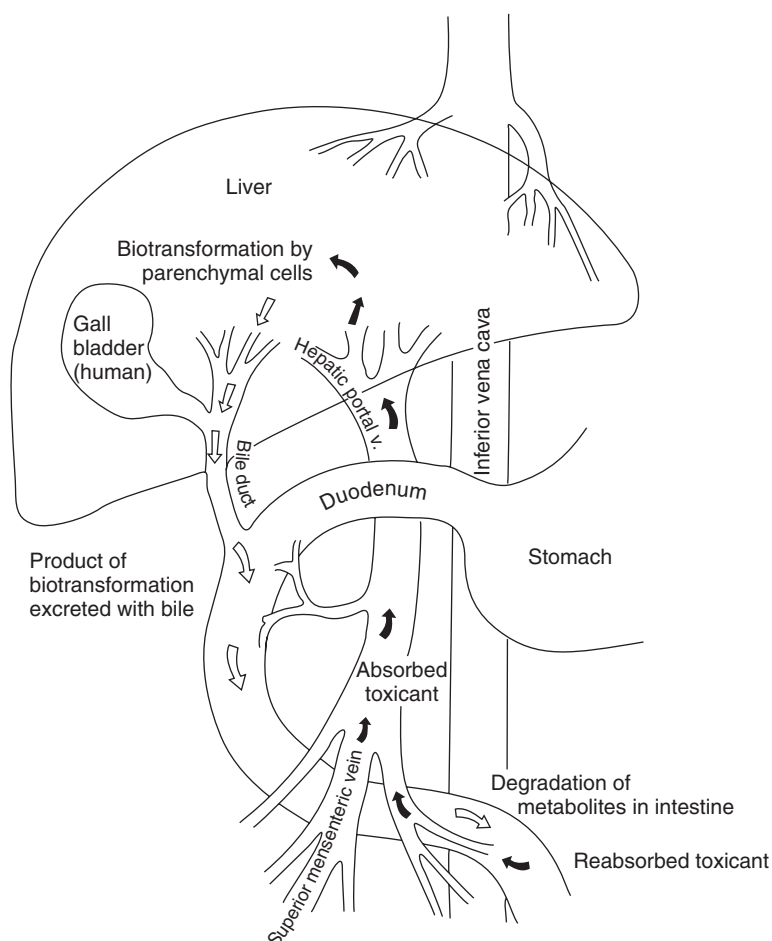


Figure 2.12 Enterohepatic recirculation. This cartoon illustrates the route followed by substances undergoing enterohepatic recirculation. Substances are absorbed from the lumen of the small intestine into the blood capillaries and are carried to the liver by the hepatic portal vein system (black arrows). Within the liver, the substances can be biotransformed by enzymes present in the hepatic cells, often being oxidized and/or conjugated. The biotransformed substances are subsequently excreted into the bile, which collects into the bile duct to empty back into the lumen of the duodenum (open arrows). There, the biotransformed substances may be acted on (usually deconjugated) by digestive enzymes, or, more commonly, by intestinal flora, to re-form the original substances that can then be reabsorbed into the intestinal capillaries, allowing the preceding steps to be repeated. *Source:* Reproduced from DeSesso JM, Jacobson CF. Food Chem Toxicol 2001;39:209–228, with permission.

Many absorbed substances are biotransformed by the hepatic parenchymal cells. Bio-transformation products tend to be larger and more polar than the parent compounds, which favors their excretion into the bile. Bile is eventually released into the duodenum. Because large, polar molecules tend not to be reabsorbed from the intestine, bile serves as a primary route of excretion for these molecules into the feces. Under certain conditions, however, some substances may be reabsorbed from the GI lumen, usually due to bacterial degradation of the metabolites in the intestinal lumen. Reentry of these substances into the enterohepatic circulation may be followed by another round of bio-transformation, followed by re-excretion into bile and then bacterial degradation and re-uptake. Enterohepatic recirculation can markedly affect the disposition of certain substances and is greatly affected by species-specific composition of bile acids [102].

A substance experiencing enterohepatic recirculation is able to maintain, or even increase, its systemic blood concentration, prolong its half-life in the blood, and even intensify its therapeutic or toxic effects. Alternatively, the compound may remain within the enterohepatic circulation (i.e., does not reach the systemic circulation), being absorbed from the intestine, taken to the liver, and excreted in bile over and over again. In such a case, systemic blood levels remain low, and any toxic effects outside the GI/enterohepatic tract are diminished, while effects within the tract may be increased [112].

2.9 CONCLUSIONS

Several parameters require consideration in any attempt (i) to define and model the events involved in the GI absorption of nutrients and nonnutrients and (ii) to extrapolate the results of these efforts between species. These include anatomical and physiological features of the GI tract (surface area, vascularity, transit time/motility, and enterohepatic recirculation). The physicochemical properties of the substance of interest and of the GI contents must also be considered. Variations in these parameters among different organs of the GI tract and among species affect the sites and rates of absorption, as well as the distribution and metabolism of the absorbed material. By far, the greatest amount of experimental information regarding GI absorption is available for the rat. Owing to similar small intestinal transit times, it is likely that the total amount of material absorbed by the small intestine after oral administration is similar between humans and rats, as has been concluded by others [113]. However, the rate at which ingested materials are absorbed differs between the two species. This is largely a function of the greater amount of surface area found in the proximal portion of the small intestine of humans. This led to the conclusion in our previous paper [4] that humans are likely five times more efficient at absorbing ingested materials than rats. This conclusion compares favorably to the report of Pappenheimer [36], who found that perfused jejunal segments of normal human subjects absorb fluids at a rate that is 5–10 times greater per unit area of mucosa than that of laboratory rats. It is also of interest to note that the oral absorption of a series of 43 drugs in dogs, which share many of the same physiological characteristics as humans, was found to differ significantly from the human [114], whereas the absorption of these substances in rats was much closer to that of humans [113]. The reasons for this remain unclear.

Not all parameters will be important for all chemicals, but a definitive knowledge of the role that each plays in the absorption of a particular substance of interest should

allow a quantitative description of the absorption process. Unfortunately, in many cases, the data necessary to evaluate the importance of these parameters is not available, and experiments must be done to measure their effects on absorption. Only by doing so can we effectively understand and accurately model the kinetics of ingested compounds. While the information provided in this chapter is not able to answer the question of which species is the most suitable model to study GI absorption, it is hoped that it will be useful to investigators who must select an animal model in which to test the pharmacological and toxicological effects of compounds for the purpose of extrapolating the findings to humans.

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3 Oral Absorption: from Physiology to Regulatory

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3.1	Summary	1
3.2	Fundamentals of oral bioavailability	2
3.3	Oral absorption in drug discovery	3
3.4	Tools and methods to assess drug intestinal absorption	14
3.5	FDA and intestinal absorption	17
	Acknowledgments	19
	References	19

3.1 SUMMARY

When developing an oral drug, the drug discovery team's goal is to balance the physicochemical and molecular properties that enable a molecule to reach its final target, while maintaining sufficient clinical efficacy and acceptable toxicity.

First, solubility, dissolution rate, lipophilicity, pK_a (negative logarithm of the acid dissociation constant, K_a), and molecular weight (MW) are major physicochemical properties driving the absorption potential of a drug. Indeed, a molecule should be in a solution to permeate the intestinal membranes, and the rate at which the molecule gets into the solution impacts its ability to get absorbed.

Second, after oral administration, a drug encounters physical barriers before it reaches the blood stream. These barriers are the physiology of the gastrointestinal (GI) tract and various GI fluids.

Intestinal absorption consists of the sum of multiple processes. The drug's permeability is a major determinant of its ability to be absorbed in the intestine. Molecules within the "rule of 5" chemical space are usually absorbed through a passive process. However, intestinal transporters could either hinder or promote a molecule's absorption. As an example, the multidrug resistance efflux transporter, MDR1 (P-gp/ABCB1) has been shown to limit oral absorption of low permeable molecules. On the other hand, the peptide transporter PepT1 can promote intestinal absorption of peptidomimetic molecules. Finally, the small and large intestine express metabolic enzymes. Therefore, intestinal metabolism can limit a drug's oral absorption and lead to drug–drug interactions (DDIs).

A multiplicity of tools exists to study intestinal absorption; these range from *in vitro* systems to *in vivo* experimentation. These tools provide data with different degrees of granularity and with distinct human absorption predictability.

Finally, the food and drug administration (FDA) has developed guidelines to assess molecules' properties in terms of intestinal absorption and potential of DDI.

3.2 FUNDAMENTALS OF ORAL BIOAVAILABILITY

In pharmacokinetics, absorption is a process by which a given dose extravascularly (EV) reaches the systemic circulation. The dose could be given orally, subcutaneously, intramuscularly, or any other way different from a direct injection into the vascular system. The term oral “*bioavailability*” (F) is a parameter that is used in pharmacokinetics to quantify the ability of a compound dosed orally to reach the systemic circulation, after surviving any first-pass extraction. Operationally, the systemic F can be determined as described by the following equation: $F = \frac{AUC_{p.o.}}{AUC_{i.v.}} \cdot \frac{Dose_{i.v.}}{Dose_{p.o.}}$.

In this equation, $AUC_{p.o.}$ refers to the area under the curve (AUC) derived from an oral administration and $AUC_{i.v.}$ refers to the intravenous (i.v.) administration. Conceptually, F quantifies the success with which a compound overcomes the potential barriers to reach the systemic circulation. A compound with $F = 1$ (or 100%) indicates that a given oral dose produces an identical systemic exposure to that observed in the corresponding i.v. dose. In this case, the complete dose reaches the systemic circulation, the compound completely overcomes any barriers, and absorption is complete.

Furthermore, On the other hand, $F = 0.5$ (50%) indicates that in transit from the oral administration site to the systemic circulation, half of the compound is lost; in this case, the oral dose to systemic concentration relationship indicates that the oral dose must be twice that of an equivalent i.v. dose to achieve a similar systemic exposure.

Furthermore, two other parameters are often used to describe absorption: C_{max} , which represents the maximal concentration observed following oral dosing and T_{max} , which is the time at which C_{max} is observed. C_{max} gives an insight into acute exposure maximums that can be achieved following oral administration, and T_{max} provides an insight into the timescales of absorption—both of which can be critical for the determination of a safety margin.

Some advantages of the oral (p.o. for per os which means literally “by mouth” in Latin) dosing route are that it is the most convenient, well-tolerated, patient compliant, and cost-effective route of drug administration. A significant disadvantage of p.o. administration includes the multiplicity of complex processes that determine a compound's absorption from the gut into the systemic circulation. As a consequence, the processes governing absorption is a significant source of inter- and inpatient variability in a compound's pharmacokinetic profile [1].

For oral administration, oral $F(F_{oral})$ can be defined as follows:

$$F_{oral} = f_a \cdot F_g \cdot F_h \cdot F_l$$

where f_a is the fraction of the dose absorbed from the gut. Physical processes typically determine this process. The terms F_{gut} , F_h , and F_{lung} are the bioavailability of the compound in the intestine (g), liver (h), and lung (l), respectively (F_l is typically 1,

and) is generally ignored—lung is usually not a barrier to oral administration). In addition to the compound's physicochemical properties, biochemical activities, such as transport and metabolism, hinder a compound's ability to be orally bioavailable. The equation accounts for the various barriers that a compound may encounter en route to the systemic circulation from the gut. It is clearly seen that a lack of f_a or bioavailability in any one of the organs will yield $F_{\text{oral}} = 0$ and, subsequently, the absence of systemic exposure.

3.3 ORAL ABSORPTION IN DRUG DISCOVERY

After oral administration, a drug encounters physical barriers before it reaches the blood stream. These barriers are the physiology of the GI tract and various GI fluids. The drug's chemical structure and physicochemical properties determines its ability to penetrate these barriers and reach its final target.

3.3.1 Role of Physicochemical Properties

Multiple processes determine the exposure of a drug after oral administration, and each of these processes is mainly driven by the drug's physicochemical properties.

3.3.1.1 Solubility. Solubility is a critical parameter for absorption because the drug must be in solution to permeate the GI membrane. Solubility is the maximum concentration a solid compound reaches in a solvent matrix at equilibrium and is altered under various conditions. Solubility is mainly driven by physicochemical properties, chemical structure, dissolution rate, and the solvent matrix used. Therefore, in a lead optimization stage, we can improve solubility by modifying the molecule's physicochemical properties and chemical structure.

The most efficient and frequent strategy used by medicinal chemists to increase the solubility is to introduce ionizable groups. Others include reducing lipophilicity, introducing hydrogen bonding and polar groups, reducing MW, and introducing out-of-plane substitutions. Introducing ionizable groups comes at a cost, since this may hinder permeability.

Beyond the ionization state, other physicochemical parameters, which influence the molecules' solubility, are MW and lipophilicity. Lipophilicity is the tendency of a compound to partition into a nonpolar lipid matrix versus an aqueous matrix. Lipophilicity is commonly determined using $\log P$ from octanol/water partitioning. A trend has been shown with lipophilicity where, on average, the higher the $\log P$, the lower the solubility [2]. While neutral molecules have been shown to be more poorly soluble compared to ionizable molecules, when $\text{clog } P < 3$, the average solubility of neutral molecules approaches the average solubility of ionizable molecules. The same trend has been shown with MW: as MW increases, solubility decreases [2].

The ionizability of a molecule is driven, in turn, by its $\text{p}K_a$ and the pH of the matrix in which the molecule is in solution. For acids, as pH decreases, there is a greater concentration of neutral molecules and lower concentration of anionic acid molecules. The reverse is true for bases (Fig. 3.1). In addition, from a physiological stand point, the luminal pH changes along the GI tract and in the presence of food;

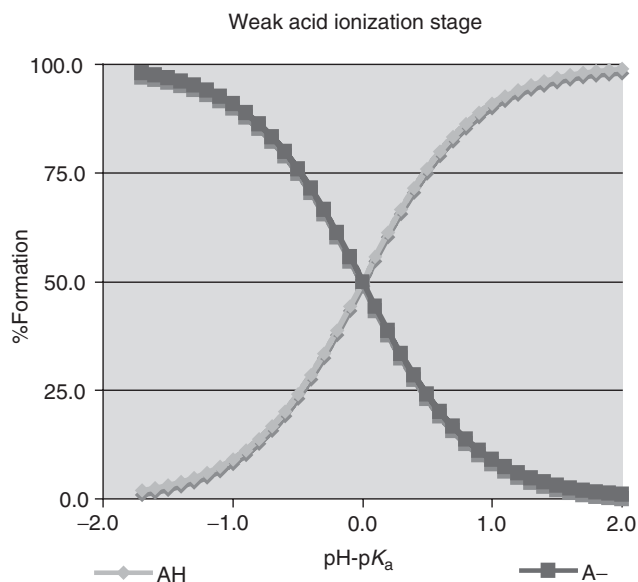


Figure 3.1 Percent formation of the ionized form of a weak acid over a pH range. (See color insert.)

therefore, solubility of acids and bases will vary in an opposite way, if their solubility is mainly driven by their ionization stage.

The drug discovery team will have to determine whether solubility or permeability is the rate-limiting step, and adjust the project strategy accordingly. Indeed, if solubility were the limiting absorption, introducing ionizable moiety would be the strategy of choice.

3.3.1.2 Dissolution Rate. The dissolution rate is the rate at which the molecule dissolves into a solvent from a solid form. Therefore, a molecule with a high dissolution rate will dissolve into solution quickly, leading to a quick absorption phase and increasing its chance to be absorbed within the GI transit time while its solubility remains constant. The dissolution rate depends on the particle size and compound physical and salt form. Reducing the particle size increases the surface area of the solid in contact with the solvent, which increases the dissolution rate.

The most frequent physical form in drug discovery is amorphous. An amorphous solid has no specific organization of molecules, whereas a crystal is a highly organized set of molecules. The amorphous form is often more soluble and less stable than the crystalline form. Therefore, caution is required when dosing poorly soluble amorphous compounds orally since these can precipitate in the GI tract to a more stable and less soluble crystalline solid form, leading to even poorer absorption.

To increase the dissolution rate, a salt form can be developed. Salts can stay in solution in a supersaturated state and delay the compound's precipitation. However, salts of weak acid or base can precipitate because they have converted to the free acid or base, leading to reduced intestinal absorption.

In conclusion, understanding the compound physical form and the size of the particles will help the team to understand if the dissolution rate is limiting molecules'

absorption. Furthermore, the team will determine whether additional formulation work could improve the molecule's dissolution rate.

3.3.1.3 Bioavailability. Lipinski *et al.* showed that particular physicochemical properties are associated with high or low oral bioavailability. They established the famous “rule of 5” that predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the MW is >500 g/mol and the calculated $\log P > 5$ [3].

Oral exposure is determined not only by the absorption through membranes of the GI tract but also by the extent to which organs just after absorption are able to extract these orally administered drugs. Therefore, gut and hepatic metabolism are key players in determining drug oral bioavailability. Varma *et al.* analyzed a set of 309 drugs where bioavailability, fraction absorbed (f_a), fraction escaping intestinal extraction (F_g), and fraction escaping hepatic extraction (F_h) were known. They determined which physicochemical property influences these parameters and how medicinal chemists and pharmacokineticists could play with these properties to enhance molecules' bioavailability [4]. They showed that f_a decreases with increasing MW (>500), polarity ($c \log D > -2$), polar surface area ($>125 \text{ \AA}^2$), total H-bond donors and acceptors (>9), and rotatable bonds (>12). Indeed, such properties limit the capability of small organic molecules to traverse lipid membranes. Molecules with a $\log P$ ranging from 1 to 3 are considered to be highly permeable. However, Varma *et al.* noted that high lipophilicity does not necessarily have a detrimental effect on f_a . Their analysis showed that the numbers of free rotatable bonds are negatively related with all three processes leading to a dramatic effect on bioavailability. They also noted that physicochemical properties that favor high f_a tend to be also associated with high rates of metabolism. For example, enough lipophilicity is needed to ensure good membrane penetrability, but too much will cause high hepatic and potentially intestinal extraction owing to metabolism.

This ionization state analysis of the compounds in the database indicated that bases are relatively less bioavailable, although they showed higher f_a than acids and neutrals, evidently because of higher first-pass effect [4]. Indeed, they explained that the higher first-pass effect of basic molecules can be attributed to their affinity for metabolic enzymes and relatively lower protein binding.

3.3.2 Paths to Intestinal Absorption

3.3.2.1 GI Tract Physiology. After oral dosing, the compound first encounters the buccal mucosa, where it can be absorbed. However, buccal or sublingual absorption is generally negligible. The drug is then ingested via the esophagus and arrives at the first part of the GI tract: the stomach. In the stomach, the drug is mixed with gastric acids, pepsinogen, and mucus secretions. Most drugs have limited stomach absorption since the stomach surface area is relatively small (about 1 m^2 [2]), the blood flow perfusion rate is low (150 mL/min) and the gastric emptying time is rapid ($0.5\text{--}1 \text{ h}$). Therefore, although the stomach's low pH (Table 3.1) favors absorption of acidic compounds, the absorption of acids is, in fact, faster in the small intestine. The small intestine consists of three consecutive sections: duodenum, jejunum, and ileum. The pH of each section increases successively and this progression of pH creates a gradient from the stomach to the ileum (Table 3.1). Absorption in the small intestine is greater because

TABLE 3.1 pH of the Gastrointestinal Tract Under Fed and Fasted State

GI Tract Section	pH, Fed	pH, Fasted
Stomach	3–7	1.4–2.1
Duodenum	5.2–6.2	4.4–6.6
Jejunum	5.2–6.2	4.4–6.6
Ileum	6.8–8	6.8–8
Colon	5.5–7	5.5–7

of the larger surface area (200 m [2]), the high blood flow perfusion rate (1 L/min) and the lengthy transit time (2–4 h). Since absorption is greater in the small intestine, the gastric emptying time can be a controlling step in the speed of drug absorption.

In the second part of the duodenum, the sphincter of Oddi allows the gall bladder to secrete bile into the GI tract. Bile is produced by the liver and stored in the gall bladder in humans, monkeys, dogs, and mice. Rats lack a gall bladder and this results in a continuous bile flow into the gut rather than the acute release of bile on gall bladder contraction. Bile, with its detergent-like properties, facilitates the solubilization and chemical breakdown of lipids, which explains why the bile is secreted in the presence of lipids in the duodenum. The presence of peptides and amino acids in the duodenum activates the secretion of pancreatic enzymes: amylases, lipases, and proteases. The pancreatic enzymes can hydrolyze some molecules that contain hydrolysable functional groups, resulting in the deactivation or activation of the drug in the GI tract. In addition, the pancreas secretes bicarbonates ion, which neutralize acid stomach secretions.

At the ileocecal junction, the small intestine connects with the large intestine. Although the large intestine transit time is long (7–20 h), drug absorption is limited mainly because of its small surface area (0.25 m [2]) and its lack of villi.

3.3.2.2 Permeability. Permeability is the rate at which a drug passes through a biological membrane and plays a major role in oral drug's bioavailability. Different mechanisms of membrane permeation exist: passive diffusion, active uptake and efflux, endocytosis, and paracellular flow.

3.3.2.2.1 Passive Diffusion. The predominant mechanism of absorption for commercial drugs is passive diffusion [3]. However, new chemical entities with physicochemical properties outside of the “rule of 5” might alter this trend.

Passive diffusion does not require energy and is driven by concentration gradient. Fick's law of diffusion best describes the parameters driving passive permeability (Eq. 3.1):

$$\frac{dQ}{dt} = \frac{DAK}{h} (C_{GI} - C_p) \quad (3.1)$$

dQ/dt = rate of diffusion

D = diffusion coefficient

K = lipid–water partition coefficient of drug in the biologic membrane

A = surface area of the membrane

h = membrane thickness
 C_{GI} = drug concentration in the GI tract
 C_p = drug concentration in the plasma

1. $(C_{GI} - C_p)$ represents the difference between free drug concentrations in the lumen and the free drug concentrations in the plasma. Because of the high GI blood flow rate, the drug is rapidly diluted into the blood stream after permeating the GI membranes. The high degree of drug dilution and the relatively high drug dose given orally (in the milligram range) are the bases for a large concentration gradient between the intestinal lumen and the blood stream.
2. K represents the lipid–water partitioning coefficient of a drug across the theoretical GI membrane. The physicochemical properties, such as, $\log P$, of the molecules significantly impact this parameter.
3. A represents the surface area of the GI membrane accessible to the drug. The larger the surface area of the GI tract, the faster the drug permeates. This explains why the duodenum that has the largest surface area is the principal GI region involved in drug permeation.
4. h represents the thickness of the theoretical GI membrane. It is assumed that it is a constant across the different GI regions.
5. D represents the amount of a drug that diffuses across a membrane for a given unit area when the concentration gradient is unity.

K , A , h , and D are constants for a given molecule with a given oral formulation. These parameters define the permeability of a drug and are summarized in one equation (Eq. 3.2). Furthermore, since the free plasma concentration is extremely low, C_p is considered negligible. Therefore, Equation 3.3 can be used as a surrogate for the Flick's law. This equation demonstrates that passive drug permeation through the GI membrane is a first order process.

Permeability equation derived from Equation 3.1:

$$P = \frac{DAK}{h} \quad (3.2)$$

$$\frac{dQ}{dt} = P(C_{GI}) \quad (3.3)$$

In addition to taking the free drug concentration into consideration, the ionization state of the molecules needs to be considered. Since a majority of molecules permeating through the membrane are in a neutral form, the efficacious gradient concentration is based on free neutral compound concentrations. Therefore, pH of the lumen and pK_a of the molecules impact the degree of gradient concentration. As an example, without taking blood flow into account, the amount of neutral form of an acidic compound in the duodenum lumen is much higher than that in the plasma, further driving the gradient concentration in the direction toward greater intestinal drug permeation. However, a formal electrical charge can be highly delocalized and therefore be less of a barrier than believed, especially when lipophilicity is sufficiently high.

3.3.2.2.2 Paracellular Permeation. Paracellular permeation and endocytosis are less frequent mechanisms of intestinal absorption. The paracellular pathway refers to the passage of drugs through the intercellular spaces. The paracellular pathway is governed by tight junctions (TJs). TJs or zonula occludens constitute the major rate-limiting barrier toward the paracellular transport of drugs. The dimensions of the paracellular space are between 10 and 30–50 Å, suggesting that solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route. Transepithelial electrical resistance (TEER), measuring paracellular ion flux *in vitro* and reflecting the tightness of the intercellular junctional complex, are calculated on the basis of the serosal surface area rather than the real surface area of mucosa. As presented in a previous paragraph, the latter can vary dramatically depending on the section of intestine. In the small intestine, mucosal surface area is greatly amplified by villus projections and undulations. When TEER data are corrected for differences in mucosal surface area, the permeability of small intestinal and colonic epithelium is determined to be virtually identical [5,6]. Therefore, paracellular absorption is more likely to occur in the small intestine not because of leakier TJs but because of a larger mucosal surface area.

3.3.2.2.3 Endocytosis. Endocytosis is a constitutive process observed in most mammalian cells for the uptake of macromolecules, for example, lectins. It requires metabolic energy and it is a slow uptake mechanism resulting in a fusion of endocytic vesicles with lysosomes containing high levels of enzymatic activity. Endocytosis may involve specific receptors, for example, vitamin B12 receptor [7]. Endocytosis of compounds is believed to be minimal in the small intestine and is not a quantitatively significant mechanism for drug absorption in the intestine.

3.3.2.3 Effect of Food. Food can impact the drug intestinal absorption through several mechanisms, such as delay in gastric emptying, stimulation of bile flow, changes in GI pH, alterations in luminal metabolism, or interactions of the drug with food [8]. The extent of the food effect is dependent on the meal characteristics such as calorie content (low vs high calorie meals), nutrient composition (protein, carbohydrate-rich, or high fat meals), volume, temperature of the meal itself, and fluid ingestion.

Food also increases blood flow to the liver; therefore, changes in first-pass extraction may cause differences in bioavailability between the fed and fasted state. For compounds with saturable first-pass extraction, the bioavailability will increase with food intake, whereas the opposite will occur if hepatic enzymes are not saturated during first pass.

In addition to physiologic considerations, factors such as drug and food nonspecific binding, sequestration, or chemical instability can cause drug–food interactions. As an example, if a drug chelates with ions present in the ingested meal, drug dissolution, and/or absorption may be reduced. Furthermore, the meal itself may pose a physical barrier that prevents drug diffusion to the site of absorption resulting in decreased bioavailability. Also, drug instability, as a result of acid degradation, may be exacerbated by prolonged gastric residence after food ingestion.

For highly lipophilic drugs or large MW macromolecules, lymphatic uptake can be increased by the presence of a high fat meal, thereby lowering plasma drug levels.

Since the effects of food depend on the physicochemical properties of the drugs as well as its absorption and metabolism pathway, the extent of food effects are not

always predictable. However, it is well known that food effects on bioavailability are generally the greatest when the drug is administered shortly after a meal is ingested and when meals are high in total calorie and fat content. Hence, the FDA recommends the use of high calorie and high fat meals to study the effect of food on the bioavailability and bioequivalence of drugs. Furthermore, qualitative prediction of food effect is often possible based solely on the Biopharmaceutical Classification System (BCS) class of the drug. Gu *et al.* [9] were able to categorize 80% of a set of 92 drugs as having negative, positive, or no food effect based simply on their dose, solubility, and permeability.

3.3.2.4 Prodrug. A prodrug is any compound that undergoes biotransformation before exhibiting its pharmacological effects. Prodrugs can thus be viewed as drugs containing specialized nontoxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule. The major objectives of prodrug design are to improve chemical stability, solubility, oral absorption, metabolic stability and duration of action, tolerability, and tissue distribution.

A prodrug can be classified as carrier-linked or as bioprecursor. A carrier-linked prodrug contains a temporary linkage of a given active substance with a transient carrier group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic cleavage. A bioprecursor prodrug does not have a linkage to a carrier group, but results from a molecular modification of the active principle itself. This modification generates a new compound, the active principle, which can be transformed metabolically or chemically.

A prodrug activation occurs enzymatically, nonenzymatically, or sequentially (an enzymatic step followed by a nonenzymatic rearrangement). As much as possible, it is desirable to reduce biological variability, hence the particular interest in nonenzymatic reactions such as hydrolysis or intramolecular catalysis.

3.3.3 Intestinal Transporters

The activity of intestinal transporters is now widely accepted as an important determinant of oral drug absorption. Certainly, one major transporter has become a key area of research that continues to grow; the efflux pump ABCB1, also known as *P-glycoprotein* (P-gp) or MDR1. Because MDR1 can play a role in limiting oral absorption of certain drugs [10–12], this transporter has emerged as a potential determinant of drugs' oral bioavailability.

3.3.3.1 Efflux Transporters. Efflux transporters are present on many biological membranes, including the villus tip of the apical brush border membrane of gut enterocytes, and actively cause efflux of drugs from gut epithelial cells back into the intestinal lumen. Following oral administration, intestinal efflux transport processes present a significant barrier toward drug absorption. Efflux transporter proteins are from the ATP-binding cassette (ABC) family. Major transporters that have been shown to play a significant role in drug absorption are MDR1, and breast cancer resistance protein (BCRP; ABCG2), but MDR1 is the most characterized and well known [13].

3.3.3.1.1 P-glycoprotein/MDR1 Overview. Borst *et al.* [14,15] have postulated several likely physiological functions of MDR1. MDR1 protects against the entry of exogenous toxins ingested with food, evidenced by expression in the small intestine,

colon, and blood–tissue barrier sites. It also precludes entry of toxic compounds in the central nervous system and testis [16,17]. Recently, evidence has been reported to show how MDR1-mediated efflux can make intestinal secretion a potential mechanism for drug elimination [18,19]. It is well established that the substrate specificity of MDR1 is quite broad with respect to both chemical structure and size. The structural diversity of MDR1 substrates (and inhibitors) is so broad that it is difficult to define specific structural features that are required for substrates/inhibitors of MDR1. However, some of the properties that are shared by many MDR1 substrates include the presence of a nitrogen group, aromatic moieties, planar domains, molecular size ≥ 300 Da, presence of a positive charge at physiological pH, amphipathicity, and lipophilicity [20–24].

MDR1 possesses multiple drug-binding sites [25], and these sites are located in the middle of the lipid bilayer [26]. Since MDR1 removes substrates directly from the membrane, the primary determinant of substrate specificity is the ability of the drug to interact with the plasma membrane and the secondary determinant would be the ability of the drug to interact with one of the binding sites. This could explain the broad substrate specificity of MDR1 as well as why nearly all MDR1 substrates are lipophilic. Shapiro *et al.* [27] demonstrated that MDR1 possesses at least three positively cooperating drug-binding sites, an H site selective for Hoechst 33342 and colchicine, an R site selective for rhodamine 123 and anthracyclines, and another site for progesterone.

The nature of an interaction between two MDR1 substrates or a substrate and inhibitor may be unique. Therefore, caution must be exercised when trying to extrapolate how the substrate/inhibitor may interact with an untested substrate/inhibitor.

3.3.3.1.2 Impact of MDR1 on Absorption. During absorption, MDR1-mediated efflux activity can potentially attenuate the overall bioavailability of its substrates by multiple mechanisms. It can attenuate the rate at which its substrates permeate from gut across intestinal enterocytes (where MDR1 is located on apical membrane) into blood, thus, potentially delaying absorption time, reducing $C_{\max}$ and possibly reducing total exposure (AUC). Additionally, MDR1 efflux during intestinal absorption may enhance intestinal metabolic elimination, thus indirectly reducing the amount of compound able to reach the bloodstream [cf. “Intestinal Metabolism” (Section 3.3.4) paragraph below].

It has been shown through studies with *mdr1a* (–/–) mice and in clinical trials, that the mean absorption time, AUC and $C_{\max}$ following oral administration of MDR1 substrates is affected by directed efflux activity of MDR1 in the intestine [28–30]. For example, the plasma AUC values determined for a 10 mg/kg dose of paclitaxel observed in *mdr1a*(–/–) mice were indeed several times higher following oral administrations compared to values obtained in wild-type mice [31]. Oral bioavailability of MDR1 substrates such as tacrolimus, cyclosporin, and talinolol are known to be incomplete and variable in the clinic, as these are regulated by intestinal MDR1 and modulated by coadministered drugs, genetic polymorphisms, and disease states. In humans, duodenal MDR1 mRNA content was significantly correlated with the AUC and $C_{\max}$ of oral talinolol [32]. Also, the mRNA levels of MDR1, but not CYP3A4, correlated well with the concentration/oral dose ratio and the oral dosage of tacrolimus [33]. Long-term St John’s wort treatment decreased talinolol AUC with a corresponding increase in intestinal MDR1 expression, suggesting that St John’s wort has a major inductive effect on intestinal MDR1 [34].

MDR1-mediated efflux activity is an important determinant of digoxin oral absorption and this has been observed from DDI between digoxin and quinidine, and digoxin and rifampicin. Digoxin absorption is not influenced by first-pass metabolism and any changes to digoxin absorption are thought to be due to changes in the actions of MDR1. The interaction between orally administered quinidine and digoxin results in a dramatic enhancement in digoxin $C_{\max}$ and AUC [35–38]. Conversely, treatment with the MDR1 inducer rifampicin, has been shown to decrease digoxin $C_{\max}$ and AUC in humans [39]. In fact, it was shown that intestinally expressed MDR1 (induced by treatment with rifampicin) closely correlated with digoxin AUC in a negative fashion.

However, it has been shown that the *in vivo* intestinal absorption of highly soluble and highly permeable MDR1 substrates is dominated by their high permeability, and MDR1 plays a minimal role in the intestinal absorption of these compounds [40]. Cao *et al.* [40] reported that Verapamil intestinal permeability did not correlate with any gene expression patterns in the intestine, and suggested that MDR1 plays a minimal role in the *in vivo* intestinal absorption because of Verapamil's high permeability.

Therefore, for highly permeable compounds, a relatively high fraction absorbed can be expected when dissolution in the GI tract is not the rate-limiting step. On the other hand, for highly soluble and low permeable MDR1 substrates, MDR1 limits intestinal absorption in the distal segments of the small intestine but plays a minimal role in the proximal intestinal segments because of significant lower MDR1 expression levels in this region [41]. In conclusion, it is important to note that MDR1 efflux activity does not always predict a compound's absorption profile. Absorption is a highly complex multifactorial process in which MDR1 can play a part. The magnitude of the effect of MDR1 efflux activity on a compound's absorption profile, ultimately, depends on the MDR1 activity in combination with other critical factors such as solubility, permeability, and metabolism.

3.3.3.1.3 Other Efflux Transporters. In addition to MDR1, MDR related protein (MRP), also plays an important role in multidrug resistance of cancer therapy, and in affecting the behavior of other drug substrates. The MRP proteins are a relatively large protein family consisting of at least nine members. The amino acid sequences for MDR1 and MRP2 show only 15% similarity [42]. Other differences in their protein structure include different numbers of transmembrane domains (12 for MDR1 and 17 for MRP) and different orientation of their N-termini.

MRP2, previously referred to as *canalicular multispecific organic anion transporter* (cMOAT), is an important ATP-binding cassette transporter and consists of 1541 amino acid residues, encoded by ABCC2 gene. This apical membrane bound transport protein is highly expressed in the canalicular membrane, and is also present in the intestine, kidney, and the blood–brain barrier (BBB) [43–47].

BCRP, also known as *mitoxantrone resistance protein* (MXR), is the product of the ABC half-transporter gene ABCG2. BCRP expression is high in placenta and haematopoietic stem cells, while low in small intestine, colon, hepatic canalicular membrane, breast, venous, and capillary endothelium [48,49]. The physiological functions of BCRP include the extrusion of porphyrins from haematopoietic cells and hepatocytes, as well as the secretion of vitamin B2 (riboflavin) and possibly other vitamins (such as biotin and vitamin K) into breast milk [50]. BCRP shares some substrates as well as inhibitors with MDR1 and MRPs. BCRP has been reported to affect oral absorption, distribution across BBB and blood–placenta barrier, and hepatobiliary

elimination as well as milk secretion (see chapter titled Predicting Human Biotransformation Pathways of this volume and summary by Xia *et al.* [51]). Sulfasalazine low absorption from the small intestine is mainly due to MRP2 and BCRP but not MDR1. Although these efflux transporters hinder sulfasalazine intestinal absorption they enable its colonic targeting and therapeutic action [52]. Dahan *et al.* [53] have also shown that the combined effect of MDR1 and MRP2, but not BCRP, dominates colchicine transepithelial transport, leading to complete coverage of the entire small intestine and makes the efflux transport dominate the intestinal permeability process.

3.3.3.2 Uptake Transporters. Solute carrier (SLC) transporters are localized in the GI barriers. SLC transporters do not require ATP and transport the drugs according to their concentration gradient, thereby improving the intestinal absorption of a wide range of drugs [54]. Drug transporter-relevant SLC members in the intestine at the apical surface of epithelial cells include SLC organic anion transporter families (OATP subfamilies; gene SLCO), SLC peptide transporter family (PepT1; gene SLC15A1) and organic zwitterion/cation transporters (OCTNs; gene SLC22) [55]. While the impact of PepT1 on drug absorption is well established, the clinical significance of intestinal SLCs and OCTNs in drug absorption needs to be confirmed and established. Therefore, we will focus this paragraph on PepT1 transporter.

3.3.3.2.1 PepT1 Overview. The expression of PepT1 was found primarily in the small intestine, particularly in the duodenum [56,57]. The physiological substrates for proton-coupled peptide transporters are mainly cationic, anionic, or zwitterionic di- and tripeptides. Free amino acids and larger peptides are excluded. A peptide bond in the narrower sense is not an essential structural requirement for a substrate [58].

The uptake of PepT1 substrates is mediated by a proton gradient at the apical surface of epithelial cells, the system is known as a *proton-dependent cotransport system* [59]. Indeed, an inward proton gradient is established at the brush border membrane by the Na^+/H^+ exchanger, and then the influx of protons back into the epithelial cells is coupled by PepT1 to transport its substrates [60]. PepT1 has generally been characterized as a low affinity/high capacity transporter with a wide variety of compounds as substrates. Drug molecules transported by PepT1 include β -lactam antibiotics such as penicillins and cephalosporins [61,62], the anticancer agent bestatin [63], angiotensin-converting enzyme (ACE) inhibitors such as captopril, and the ester prodrugs enalapril and fosinopril [64]. Prodrugs of acyclovir and L-dopa can also be recognized and transported by PepT1 [65,66].

3.3.3.2.2 Impact of PepT1 on Absorption. It has been well established in recent years that PepT1 is a major player in the intestinal absorption of β -lactam antibiotics allowing their oral administration [60,67]. The transport protein accepts many β -lactam antibiotics as substrates because of its steric resemblance to the backbone of physiologically occurring tripeptides.

After oral administration, most angiotensin-converting enzyme (ACE) inhibitors such as captopril, enalapril, and lisinopril exhibit 30–100% absorption, likely because these molecules sterically resemble di- or tripeptide structures, thereby sharing an intestinal transport pathway [68,69]. However, Brandsch *et al.* [58], applying the two-electrode voltage clamp technique to *Xenopus laevis* oocytes expressing PepT1, concluded that the oral availability of ACE inhibitors that generate tiny inward currents

and display $K_i > 15$ mM cannot be explained by their interaction with the intestinal peptide transporter, especially at pharmacological doses, indicating other routes for absorption.

The human intestinal peptide transporter appears to be an effective target for increasing intestinal absorption of some small molecules. It is attractive as a prodrug delivery target because of its high capacity, broad substrate specificity, high expression in the intestinal epithelium, and low occurrence of functional polymorphisms [70,71]. Therefore, by using enzymatically hydrolyzable bonds in the preparation of PepT1-targeted prodrugs, it is possible to dramatically improve the systemic availability of poorly absorbed drugs, with limited systemic exposure to the intact prodrug. Han and Amidon described this general strategy as “peptide transport associated prodrug therapy” [72]. However, there are still relatively few examples of PepT1-targeted prodrugs in literature, with valacyclovir being the most widely studied [65]. Perkins and Abraham, at Eli Lilly, put in practice this approach and delivered the prodrug LY544344, demonstrating the utility of PepT1-targeted nonester prodrugs to overcome poor permeability and low bioavailability. This compound exhibits near-ideal prodrug properties, with good solubility and chemical stability, extensive and reproducible absorption across species, low concentrations of circulating nontoxic prodrug, and pharmacokinetic linearity across a wide dose range [73].

3.3.4 Intestinal Metabolism

The small intestine is the first site capable of metabolism of orally ingested xenobiotics. Therefore, it can play an important role in the first-pass metabolism of chemical compounds because both phase I and phase II metabolic enzymes are expressed [74,75]. Indeed, a number of therapeutic drugs such as cyclosporine [76], verapamil [77], and midazolam [78,79] undergo extensive intestinal first-pass metabolism.

Using Western blot analysis on microsomes prepared from mucosal scrapings from the duodenal/jejunal portion of 31 human donor small intestines, Paine *et al.* [80] demonstrated that CYP3A and CYP2C9 represent most intestinal cytochrome P450 enzymes, accounting for 80% and 15% of the total immunoquantified P450s, respectively. They also found that the expression of CYP3A4 varies along the length of the small intestine, decreasing from the duodenum to the distal ileum. The CYP3A4 content in the intestine is estimated to be <1% of that in the liver [80,81]. Furthermore, the greater overall weight of the human liver (1.5 kg) relative to that of the small intestine (0.7 kg), which when combined with the P450 concentrations and the microsomal protein contents provides for a greater overall metabolic capacity for the liver.

Interindividual variability in expression of phase I and II metabolic enzymes has been observed more frequently in small intestine compared to the same enzymes in the liver [82,83]. The mechanisms for the small intestinal variability in expression are not well known. One possibility is that the intestinal P450s are differentially induced by dietary and environmental factors [75].

As in liver, selective inhibition or induction of intestinal metabolizing enzymes either by dietary or environmental xenobiotics or by coadministered drugs has been identified as an important source of drug interactions and a contributor to variability in oral drug bioavailability [51,84,85].

Recent evidence has shown that MDR1 can play a role in determining the oxidative metabolism of its substrates that are also substrates of CYP3A. Indeed, several factors

have led to the observation that MDR1 and CYP3A4 may act in concert to determine the oral absorption and bioavailability of drugs [86–89]. In addition, MDR1 and CYP3A4 can be induced by many of the same compounds although it has recently been shown that these proteins are not coregulated [90]. It is well known that there exists a large degree of overlap between the broad substrate specificities of MDR1 and CYP3A4 [86], and it seems reasonable that the combined actions of MDR1 and CYP3A4 could contribute to the low oral bioavailability determined for many of these dual substrates. Studies involving cyclosporin A transport across Caco-2 cell monolayers have shown how the actions of MDR1 and CYP3A4 may act coordinately to enhance the attenuation of apical to basolateral transport of this drug. It was observed that cyclosporin A metabolism was much greater when the compound was transported in the apical to basolateral (absorptive) direction, than in the basolateral to apical (secretory) direction [91]. It was postulated that the reduction in the apical to basolateral flux of cyclosporin A, caused by apically directed MDR1 efflux, enhanced the exposure of the compound to CYP3A4, and thus a greater amount of metabolism was achieved [91].

In contrast to these findings, several pharmacokinetic models have been published that fail to predict an increase in CYP3A metabolism because of MDR1 efflux [92–95]. Recently, Badhan *et al.* [96] used a physiologically based model to confirm this hypothesis, demonstrating that intestinal drug metabolism is influenced directly by drug concentrations within the enterocyte, which is governed by the degree of MDR1-mediated drug efflux and drug permeability through the enterocytes. For drugs possessing apparent permeabilities in excess of 15×10^{-6} cm/s, increasing efflux ratio was predicted to result in an increase in CYP3A drug clearance by up to 12-fold in the distal intestine.

3.4 TOOLS AND METHODS TO ASSESS DRUG INTESTINAL ABSORPTION

A multiplicity of *in vitro* and *in vivo* tools are used to study intestinal absorption, providing data with different degrees of granularity and with distinct human absorption predictability.

3.4.1 *In Vitro* Tools

3.4.1.1 Permeability. One of the key parameters in predicting and understanding both GI absorption and the ability of a compound to cross cell membranes in general is permeability. For drug dosed orally, the membranes of most interest to the pharmaceutical industry are those in the GI tract, and for central nervous system (CNS) compound the, BBB. It is difficult to perform measurements on these complex membranes directly so a variety of *in vitro* models have been utilized over the years to gain an understanding and estimation of how a compound may cross biological membranes *in vivo* [97].

The most simplistic measure of permeability is lipophilicity. The general trend indicates that highly lipophilic molecules are likely to be more permeable. However, the scale of measurable lipophilicity is relatively compressed, which results in very little resolution of permeability for compounds that are structurally similar, and the process of crossing a membrane is too complex to be described by lipophilicity alone [98]. Thus, in an attempt to provide a more refined measure of permeability, cell monolayers

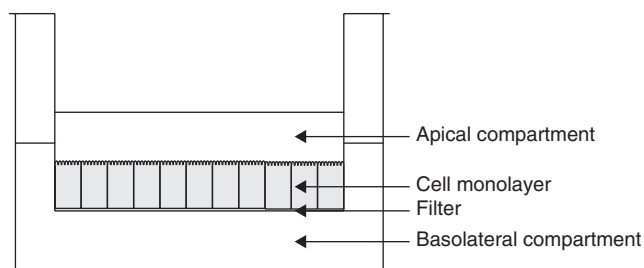


Figure 3.2 *In vitro* system used to determine drug permeability through a cell monolayer. (See color insert.)

grown on membrane supports were introduced in the 1990s [99]. The most common cell monolayer is the Caco-2 cell line, which is a polarized human colonic cancer cell line that forms integral monolayers and TJs when grown for ~ 20 days. These cells can be grown in 6-, 12-, 24-, and 96-well plate formats, which lend themselves well to robotic automation for both seeding and feeding, and permeability measurements [100,101]. Transcellular permeability is the movement of the compound through the cell itself and is often referred to as *passive permeability*; it is the parameter of most interest at the early screening stage. The typical experimental setup for a Caco-2 permeability experiment is to measure the apparent rate of permeability (P_{app} in 10^{-6} cm/s) of the compound in both an apical to basolateral (A to B) and a basolateral to apical (B to A) direction after incubation at 37°C for a given period of time (Fig. 3.2). The relative rates of permeability in the A to B and B to A directions indicate the compound's permeability and subsequent influx or efflux ratio (B to A/A to B). The advantage of Caco-2 is that it is a well-established technique in academia and industry for high quality permeability measurements, but the culture of the cell line is expensive and manually intensive when used as a screen for permeability.

MDCK (Madine-Darby Canine Kidney) is a dog kidney cell line that has also been applied to permeability measurements [102–104]. Although this cell line requires 3 days of culture compared to 15–21 days for most Caco-2 models, it is still relatively expensive and requires cell culture expertise. It is also less physiologically relevant, as this is a canine kidney cell line and generally the permeability measured is used to estimate intestinal absorption in humans.

The realization that the pharmaceutical industry required a cheaper but higher throughput measure of permeability resulted in the introduction of the parallel artificial membrane permeability assay (PAMPA). PAMPA utilizes artificial membranes designed to “mimic” biological membranes such as the GI tract and the BBB [105–108]. Extracted or synthetic lipids are solubilized in a solvent and dispensed onto a membrane support to form a lipid membrane. The amount of compound that moves from the donor chamber to the acceptor chamber after a period of incubation is measured, along with the amount left in the donor chamber.

These measurements and other parameters characteristic of the experimental setup are used to determine the effective permeability, P_e , and the retention factor, R_f [107]. PAMPA is a cost-effective screen because it is a physicochemical measure of permeability that is performed in a 96-well plate and is very amenable for automation. Despite the fact that PAMPA is another step further away from a physiologically relevant measurement for *in vivo* permeability or absorption, it can be used in the pharmaceutical

industry as a primary screen for measuring the intrinsic permeability of thousands of compounds per week [105,108]. PAMPA does not provide information about whether a compound is a substrate for active influx or efflux transporters that could be determined using the Caco-2 cell line. However, if this type of information is important then it can be determined at a later stage when the compound shows potential as a new drug candidate. PAMPA does aid the chemist, as the output can be a simple measure of permeability that allows for clearer structure – activity relationships (SARs) and easier interpretation when attempting to design a more permeable series.

3.4.1.2 Efflux Transporters. Some *in vitro* systems of note that are commonly used to both identify transporter substrates and study their effects on cellular disposition include the Caco-2 cell model and transfected MDCK cells [109–114]. The efflux transporters show a polarized expression in each model (localization to either apical or basolateral membrane) [115,116]. Taipalensuu *et al.* investigated the expression and interindividual variability in transcript levels of multiple drug efflux systems in the human jejunum and compared the expression profiles in these cells with that of Caco-2 cells. They showed that the transcript levels of 9 out of 10 ABC efflux transporters correlated well between jejunum and Caco-2 cells but BCRP exhibited a 100-fold lower transcript level in Caco-2 cells compared with jejunum. They concluded that Caco-2 cell line is a useful model of jejunal drug efflux, if the low expression of BCRP is taken into account [117].

MDR1 is a common efflux transporter expressed naturally in Caco-2 cells. However, the MDR1 levels are different in Caco-2 cells from lab to lab and in the gut wall of patients. These differences make it difficult to extrapolate effects seen *in vitro* to those *in vivo* when considering permeability. Shirasaka *et al.* [118] developed a method to estimate the permeability of MDR1 substrate drugs in human intestine from the expression level of MDR1 in cell lines, and thus the possibility to predict oral absorption of those drugs. Although Caco-2 cells express major intestinal ABC transporters, the high cost of sustaining these in culture for two to three weeks versus the three-day requirement for MDCK, make the latter an attractive model to assess compounds' affinity to MDR1 or any other efflux transporter.

3.4.2 *In Situ* and *In Vivo*

Although *in situ* models are seldom used in drug discovery setting, these models, for example, *in situ* single-pass perfusion of the rat intestine, can provide useful mechanistic information. The drug concentration in the intestine is known and controlled and the subsequent barriers a compound has to cross to reach the portal blood circulation are identical in the *in situ* and *in vivo* situation, which definitely contributes to the reliability of the *in situ* model. However, these are labor intensive and low throughput systems. Therefore, the drug discovery team relies on *in vivo* pharmacokinetic studies to give a sense of the absorption profile of their molecules. Rat is normally used to determine molecules' bioavailability and the fraction absorbed. Comparing data on 98 drugs, Zhao *et al.* [119] showed that rat absorption is similar to human absorption in terms of percentage of dose absorbed. Therefore, evaluation of *in vivo* absorption in rats could be used as an alternative method to predict the extent of intestinal absorption in humans following oral administration. To explain the good correlation between human and rat absorption data further, Cao *et al.* [120] determined the expression of

all known transporters and metabolizing enzymes in both human and rat duodenum and colon. They found a moderate correlation for the expression levels of transporters in the duodenum of human and rat and no correlation for the expressions of metabolizing enzymes. These findings provide the molecular mechanisms for the similarity and correlation of drug absorption and explain the lack of correlation in bioavailability between rat and human [120].

Chiou *et al.* [121] conducted a retrospective evaluation of using dog as an animal model to study the fraction of oral dose absorbed of 43 drugs in humans. The correlation between mean fraction of oral dose of drugs absorbed in humans and dogs is relatively poor; absorption is generally better in the dog. Although the dog is commonly employed as the nonrodent preclinical species for studying oral absorption in drug discovery and development, one should exercise caution in the interpretation of data obtained [121].

3.5 FDA AND INTESTINAL ABSORPTION

3.5.1 BCS Classification

The Biopharmaceutics Classification System (BCS) is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability (Amidon *et al.*) [122]. BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate release solid oral dosage forms: dissolution, solubility, and intestinal permeability. According to the BCS classification, drug substances are classified as follows:

- Class 1: High Solubility – High Permeability
- Class 2: Low Solubility – High Permeability
- Class 3: High Solubility – Low Permeability
- Class 4: Low Solubility – Low Permeability.

A drug substance is considered highly soluble when the highest dose strength is soluble in 250 mL or less of aqueous media over the pH range of 1–7.5.

The permeability class boundary is based indirectly on the extent of absorption, that is, the fraction of dose absorbed, of a drug substance in humans and directly on measurements of the rate of mass transfer across human intestinal membrane. Alternatively, nonhuman systems capable of predicting the extent of drug absorption in humans can be used (e.g., *in vitro* epithelial cell culture methods). In the absence of evidence suggesting instability in the GI tract, a drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose.

The BCS classification can be used to request a waiver from the FDA for *in vivo* bioavailability and/or bioequivalence studies for immediate release solid oral dosage forms.

3.5.2 FDA View on MDR1

Having developed guidelines for using *in vitro* metabolism studies to assess drug metabolism (specifically cytochrome P450)-mediated potential DDI, the FDA has initiated an effort to develop similar guidelines for transporter-mediated DDI (<http://www.fda.gov/oc/ohrt/transporter-guidelines.pdf>).

fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064982.htm). Reflecting on the availability of extensive literature and industry data on the role of MDR1 in DDI, the FDA has developed a more comprehensive guideline on MDR1-mediated DDI. The FDA acknowledged the role of other transporters in inhibiting or inducing drugs disposition but without proposing guidelines on how to study these transporters. The International Transporter Consortium (ITC) recently published a paper addressing this specific gap [123].

For details of the guideline and decision trees developed by the FDA, the reader is referred to the website: <http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064982.htm>.

The guideline discusses design of *in vivo* DDI studies including the choices of substrate and interacting drugs, labeling implications, decision trees for substrates and inhibition data, and how the *in vitro* assays should be run. To identify MDR1 substrates for *in vivo* drug interactions studies, the guideline proposes the following process (i) examining bidirectional transport of the test compounds in Caco-2 or MDR1-MDCK cell monolayers, (ii) selecting likely substrates based on an efflux ratio >2 , (iii) confirming MDR1 substrate activity by showing that specific inhibitors of MDR1 decrease the efflux ratio, and (iv) selecting compounds for *in vivo* MDR1-related interactions studies if transport of a test compound with an efflux ratio of >2 in these test systems is inhibited by MDR1 inhibitors.

To identify MDR1 inhibitors for *in vivo* drug interactions, the guideline proposes the following process (i) examining bidirectional transport of MDR1 probe substrates across Caco-2 or MDR1-MDCK cell monolayers, (ii) determining the ability of the test compound to decrease net flux ratio of a MDR1 probe substrate, (ii) determining K_i or $[I]/IC_{50}$ of the test compounds, and (iv) selecting compounds with K_i or $[I]/IC_{50} > 0.1$ for *in vivo* drug interaction studies with a MDR1 substrate such as digoxin.

3.5.3 Recommendations from the International Transporter Consortium

The ITC comprises industrial, regulatory, and academic scientists with expertise in drug metabolism, transport, and pharmacokinetics [123]. The consortium was formed to address transporter-related questions from pharmaceutical scientists involved in drug development. They identified which transporters, based on current knowledge, are well-established determinants of pharmacokinetics; discussed methodologies to characterize drug–transporter interactions using *in vitro* and *in vivo* studies; and proposed recommendations that are important for drug development scientists in guiding preclinical and clinical studies of transporter-mediated drug interactions [123].

The consortium focused its work on a set of seven transporter proteins based on compelling evidence that they are involved in drug absorption, disposition, and/or drug drug interaction (DDI): OATP1B1, OATP1B3, OCT2, OAT1, OAT3, MDR1, and BCRP. MDR1 and BCRP are transporters proteins playing a key role in the absorption of new chemical entities (NCEs). For details of the ITC recommendations and decision trees, the reader is referred to the ITC paper [123].

In summary, to identify MDR1 and BCRP substrates, the ITC broadened the FDA drug interaction guidance and decision tree to include both MDR1 and BCRP. The ITC also recommends that when an NCE is identified as MDR1 or BCRP substrates, preclinical and clinical information should be assessed to determine whether a clinical

in vivo DDI study is warranted. In particular, the relative contribution of the transporter-mediated pathway to the overall clearance of the drug is the primary determinant of whether an inhibitor will have a major effect on the disposition of the NCE.

A NCE is considered to be a potential MDR1 and/or BCRP inhibitor if the net flux ratio of a MDR1 (or BCRP) probe substrate is decreased in the presence of the NCE using a bidirectional transport assay. Zhang *et al.* [124] proposed that drugs exhibiting an $[I]_2/IC_{50} \geq 10$ should be evaluated to determine whether intestinal MDR1 or BCRP inhibition occurs *in vivo*. In that publication, $[I]_2$ is the theoretical maximal GI NCE concentration after oral administration and calculated as the highest clinical dose (mg) in a volume of 250 mL. Using this approach, a drug with MW of 500 Da and an *in vitro* IC_{50} value of 10 μ M will have a $[I]_2/IC_{50}$ value >10 at a dose of ~ 12 mg or greater. Recently, using data from 26 digoxin DDI clinical trial and applying statistical analyses of binary classification and receiver operating characteristic (ROC), Cook *et al.* [125] revised cutoff values and ratio of $[I]_2/IC_{50} > 5$ were identified to minimize the error rate, a reduction of false negative rate from 36% to 9%.

The ITC discussed the characteristics of an NCE that would trigger an *in vitro* interaction study with a specific transporter: the physicochemical properties of the drug and the organ(s) involved in its clearance. In addition to *in vitro* data, human pharmacokinetic property of an NCE is also important in assessing the likelihood of a clinical transporter-related DDI.

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4 General Principles of Drug Distribution

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4.1	Introduction	1
4.2	Background of drug distribution	2
4.3	Multiple physiochemical factors influence drug distribution	2
4.4	Physiological factors affecting drug distribution	7
4.5	The role of transporters in drug distribution	19
4.6	<i>In vitro</i> methodologies for determining drug distribution	23
4.7	<i>In vivo</i> methodologies for determining drug distribution	29
4.8	Calculating distribution	32
4.9	Clinical implications of altered drug disposition	37
4.10	Summary	41
	References	41

4.1 INTRODUCTION

Drug distribution describes the process by which a drug moves between the blood and various tissues (e.g., muscle and fat) or organs (e.g., brain). It is an integral part of ADME (absorption, distribution, metabolism, and excretion), which encompasses the complete fate of a drug when it enters the body and further background is provided in Section 4.2. Many factors can influence the ability of a drug to distribute in the body, including physiochemical and physiological factors, which are discussed in Sections 4.3 and 4.4, respectively. Uptake and efflux transporters also have an important role in drug tissue distribution as discussed in Section 4.5. Methods to investigate distribution continue to evolve and are employed *in vivo*, in both animal and human studies and in more sophisticated *in vitro* methods. Dosing of subjects with radiolabeled drugs is common and can be coupled with analytical techniques such as microdialysis, magnetic

resonance imaging (MRI), and positron emission tomography (PET). Classical and current *in vitro* and *in vivo* methods to investigate drug distribution are reviewed in Sections 4.6 and 4.7. Different models employed in the calculation of drug distribution from data are also outlined in Section 4.8. Finally, examples of the clinical implications of drug distribution are presented in Section 4.9.

4.2 BACKGROUND OF DRUG DISTRIBUTION

Drug distribution is an important part of a larger pharmacokinetic picture known as *absorption, distribution, metabolism, and excretion* (ADME), which has become integral in understanding and explaining drug effects. ADME is the interplay between absorption, distribution, metabolism, and elimination. Drug distribution describes the process by which a drug moves between the blood and various tissues (e.g., muscle and fat) or organs (e.g., the brain). Many factors can influence the ability of a drug to distribute in the body, including physiochemical and physiological factors and the contribution of transporters. When a drug is administered intravenously, or absorbed after oral dosing, it will rapidly circulate through the body in the bloodstream. A complete circulation of the blood typically takes about 1 min, and after this time, a drug will begin to distribute into tissues [1]. Factors that can affect the ability of the drug to distribute into tissues include water and fat solubility. A drug that is more water soluble will tend to stay in the blood or interstitial spaces, whereas a fat-soluble drug may concentrate in fatty tissues. Drugs can also be limited in their ability to penetrate into tissues depending on their ability to cross barriers, such as the blood–brain barrier (BBB) or placental barrier. Drug binding to plasma proteins can also affect its ability to distribute. Bound drug is not able to distribute into tissues; only the fraction unbound (free) moves into tissues and determines the pharmacodynamics or efficacy of the drug.

4.3 MULTIPLE PHYSIOCHEMICAL FACTORS INFLUENCE DRUG DISTRIBUTION

Cell membranes form the barriers between all the compartments of the body. There are four main ways in which small molecules and drugs can cross membranes and gain access to the different body compartments. First, they can diffuse directly through the lipid, they can diffuse through aqueous pores formed by special proteins in the membrane, they can combine with a transmembrane carrier protein, and, finally, they can transverse the membrane by pinocytosis. Of these four, diffusion through the lipid and transport via carrier-mediated mechanisms are the most important in terms of the disposition of drugs, including both paracellular and transcellular distribution. The aqueous pores in membranes are too small (ca. 0.4 nm) for most drugs to pass through, and pinocytosis is generally more important for the transfer of macromolecules (such as insulin) but not for small molecules. Significant advances in identifying and understanding transporters for xenobiotics have been made in recent years, and it is estimated that at least 5% of all human genes are transporter related [2]. This indicates the importance of transport across barriers in normal physiological processes. Transfer via carrier-mediated mechanisms is dealt with in more detail in Section 4.5, and this

discussion will therefore concentrate on factors that influence the passive diffusion of drug molecules through the lipid membrane.

4.3.1 Lipophilicity, pH, pK_a , and Ionization

The ability of drugs to cross membranes depends on their solubility in the membrane (determined by the partition coefficient of the drug between the membrane lipid phase and the aqueous phase) and the diffusion coefficient. There is therefore a close relationship between the lipid solubility of a drug and its ability to permeate a cell membrane. As a result, lipid solubility is a major determinant of the pharmacokinetics of a drug, and, for example, its absorption from the intestine, ability to transverse the BBB, and rate of renal elimination can to a large extent be predicted from its lipid solubility.

Many drugs are either weak bases or acids and can exist as both ionized and non-ionized forms, and as such their lipid solubility depends to a large extent on the pH of the environment. The dissociation constant, pK_a , can be determined by the Henderson–Hasselbalch equation, and this relationship can be used to predict the extent of ionization of weak acids and bases in different pH environments. For a weak base, the relationship is

$$pK_a = pH + \log \frac{[\text{ionized form}]}{[\text{nonionized form}]}$$

and for a weak acid,

$$pK_a = pH + \log \frac{[\text{nonionized form}]}{[\text{ionized form}]}$$

For both weak acids and bases, the ionized form is not generally lipid soluble, and cannot cross membranes, unless by a specific transporter mechanism. On the other hand, the lipid solubility of the nonionized form depends on the structure of the drug, and many nonionized drugs will permeate rapidly. However, there are exceptions to this and among them are polycationic aminoglycoside antibiotics, which as a group are not well absorbed orally and do not penetrate into the BBB or into most other body fluid compartments. Elimination of these drugs is almost exclusively by glomerular filtration, and 50–60% of a therapeutic dose is excreted unchanged by patients within 24 h [3].

Drugs have a wide range of pK_a values, and the values found among a selection of common drugs are depicted in Table 4.1. Where a pH difference exists between body compartments, then the extent of ionization of a drug would influence its ability to distribute within the body, with the ratio of ionized to nonionized drug being determined by the pK_a of the compound and the pH of the compartment fluid. An example of this is shown in Fig. 4.1, which illustrates the distribution of a weak acid, aspirin (pK_a 3.5), and a weak base, erythromycin (pK_a 8.8), between the stomach contents (pH 2), plasma (pH 7.4), and urine (at pH 6, which is the normal situation, and at alkaline pH 8). In this model, it is assumed that the nonionized drug will cross membranes and reach equal concentrations in each compartment, whereas the ionized drug will not cross at all. As a result, the total drug concentration in each compartment will be different, and an acidic drug will be trapped as an ion in the compartment with the highest pH. Theoretically, large concentration gradients can be built between compartments if there is a large difference in pH. However, in practical terms, this extreme situation does not

TABLE 4.1 pK_a Values for Some Acidic and Basic Drugs

Drug	pK_a value
<i>Bases</i>	
Chloroquine	10.8
Amphetamine	9.9
Atropine	9.9
Ephedrine	9.6
Desmethylinipramine	9.5
Propranolol	9.5
Chlorpromazine	9.3
Erythromycin	8.8
Dopamine	8.4
Morphine	7.9
Codeine	5.8
Diazepam	3.3
<i>Acids</i>	
Levodopa	2.3
Penicillins	2.7
Probenacid	3.4
Aspirin	3.5
Flufenamic acid	3.9
Warfarin	5.0
Phenobarbitone	7.4
Phenytoin	8.3

Bases are arranged in decreasing strength, acids are arranged in decreasing strength, with the strongest acids at the top, and the strongest bases at the top.

happen because fluids in body compartments are not static, and the constant flux of fluids between compartments reduces the concentration gradients. It is also of course unrealistic to assume that ionized drug is totally nonpermeant, and a small percentage will diffuse across membranes.

It is important to realize that for gastrointestinal (GI) absorption, a major overriding contributing factor is the enormous absorptive area of the villi of the small intestine compared with the smaller absorptive area of the stomach. For this reason, partition between different pH environments is not a major determinant of the site of absorption of drugs from the GI tract. Nevertheless, weak acids and bases are well absorbed from the intestine, while strong acids and bases are not, as discussed by studies such as that published in 1957 by Schanker and coworkers, which shows the absorption of a range of drugs across the rat [4] and human [5] stomach as a function of their pK_a values. They showed that strong acids, with pK_a of <2 , and strong bases, with $pK_a > 10$, are not well absorbed. The usefulness of the low oral bioavailability of a strong base is well demonstrated by the case of curare, a mixture of alkaloids found in various South American plants, used as an arrow poison by South American Indians. The effective constituent is tubocurarine, a quaternary-ammonium-containing compound, and the low

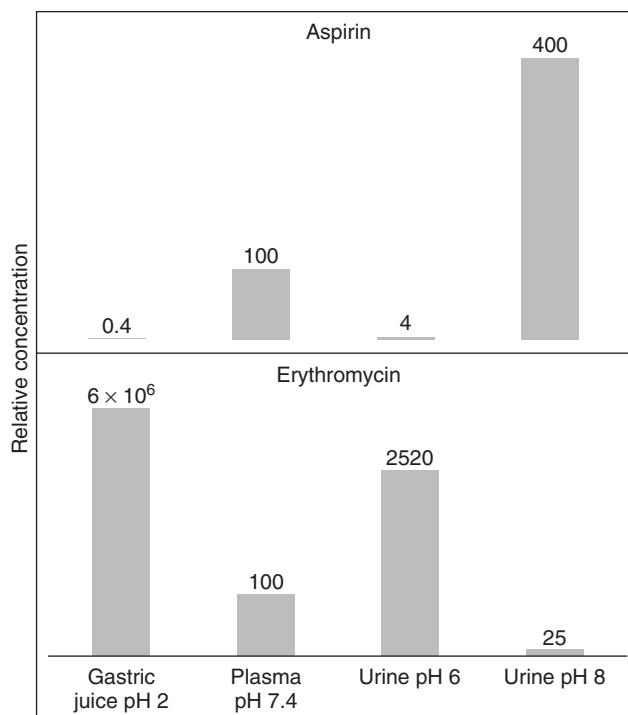


Figure 4.1 Theoretical partition of a weak acid (aspirin) and a weak base (erythromycin) between gastric juice, plasma, and urine (at the normal situation of pH 6 and at alkaline pH of 8). The numbers represent the relative partition compared with plasma, which has been taken as 100%, and illustrate the large differences in total drug concentrations that could result from the different fractions of ionized species present as a function of environment pH.

oral absorption of tubocurarine allowed it to be used safely in the hunting of animals for food. It was the earliest nondepolarizing neuromuscular blocking agent identified. Tubocurarine is not used clinically now in medicine being superseded by synthetic nondepolarizing blocking agents such as pancuronium, atracurium, and vecuronium, all of which are quaternary ammonium compounds with varying durations of action and low oral bioavailability [6].

The pH of the environment and the pK_a does, however, have important consequences for urinary excretion and for the distribution of drugs into the brain. If the urine becomes acidic then the excretion of weak acids is decreased, and that of weak bases is increased, and if urine becomes basic, this has the opposite effects. This means of enhancing the excretion of drugs by altering the pH of the urine has been used extensively to increase drug elimination in cases of overdose, particularly with aspirin and phenobarbitone, where alkalinization of the urine is an established clinical protocol to promote excretion of the weakly acidic drugs. This is discussed further in Section 4.9, with examples provided from the literature. In addition to altering urine pH, administration of sodium bicarbonate increases plasma pH and will increase the movement of weak acids from the central nervous system (CNS) into the plasma. In

overdose, administration of sodium bicarbonate would thus both increase the elimination of phenobarbitone and decrease its CNS toxicity. Conversely, the administration of a carbonic anhydrase inhibitor, such as acetazolamide, decreases plasma pH and causes weakly acidic drugs to become concentrated in the CNS, thus increasing their neurotoxicity.

4.3.2 Molecular Size

The rate of diffusion of a substance depends on its molecular size, the diffusion coefficient for small molecules being inversely proportional to the square root of the molecular weight. Although large molecules diffuse more slowly than small ones, because most drugs have molecular weights between 200 and 1000 kD, the effect of molecular weight does not have a major effect on their pharmacokinetics.

In terms of renal elimination, molecular weight is not an important factor as the glomerular capillaries allow molecules below a weight of about 20,000 kD to diffuse into the filtrate. Plasma albumin, with a molecular weight of about 68,000 kD, is virtually completely excluded, but most drugs with the exception of macromolecules cross the glomerulus freely. Molecular weight is, however, a major determinant controlling whether a chemical is excreted into bile. As a general rule, low molecular weight substances (<325 kD) are poorly excreted into bile [7]. Biliary excretion is regulated by the presence of the xenobiotic transporters present on the canalicular membrane of hepatocytes and has consequences for the biological half-life and toxicity of compounds. The role of transporters in drug distribution has been further discussed in Section 4.5.

4.3.3 Drug Delivery Systems

Particle size has a major effect on GI absorption, and this has been a factor in the standardization of formulations of pharmaceutical agents such as digoxin. For example, Lindenbaum *et al.* [8] demonstrated the marked variability in plasma concentrations seen in patients given four different tablet preparations of digoxin, and observations such as these contributed to the standardization of formulations. Digoxin has a low therapeutic index (maximum nontoxic dose/minimum effective dose), and control of circulating concentrations is crucial; problems with bioavailability contribute to unexpected clinical results [9], and capsule preparations proved to be more consistent than tablets [10]. The aim of clinical pharmacy is to deliver a drug for an appropriate time, within the therapeutic window, to a specific target, outside the systemic toxic concentration range. Conventional routes of administration, such as oral or injection, cannot always achieve this satisfactorily. Controlled release systems match the rate of drug delivery to its elimination, and thereby the concentration remains within the therapeutic range for most of a 24-h period. Today, such controlled release can be provided by a wide range of processes such as encapsulation, transdermal patches, bioadhesive systems, osmotic micropumps, microimplants, drug-eluting medical devices, and nanoparticles [11,12]. Polymers are employed to delay the dissolution of drugs and control their flux from the delivery system. This is usually achieved using a polymeric coating or matrix that degrades at a slower rate than the drug diffuses and releases. Copolymers of various ratios of lactic acid to glycolic acid [poly(lactide-co-glycolide)] are commonly used, the ratio controlling the rate of degradation [13]. The

development of delivery systems for small molecules, proteins, and DNA has taken an enormous step forward with advances in nanotechnology, and these are now making a significant contribution to the global pharmaceutical market. It is known that nanoparticles are taken up by cells more effectively than larger sized microparticles [14]. Nanodelivery systems can be designed with different compositions and biological properties, such as nanoparticles, dendrimers, nanocages, micelles, molecular conjugates, and liposomes, and these have been extensively investigated for drug and gene delivery systems [15]. The effect of particle size on the distribution of nanocarriers within the body has been established, and following intravenous administration, small particles (<30 nm) are eliminated by renal excretion [16], particles of 30–150 nm are taken up into the bone marrow, heart, kidney, and stomach [17,18]. Larger particles (>200 nm) are sequestered by the mononuclear phagocytic system and located mainly to the reticular endothelial system in the liver [19]. In addition, recently, the role of shape in the biodistribution of nanoparticles has been suggested [20]. In this context, Devarajan and coworkers [21] have described the effect of nanoparticle shape on *in vivo* distribution in rabbits and dogs. They have shown that irregularly shaped polymeric lipid nanoparticles evade macrophages and localize in the spleen opening up particle shape as a new possibility in controlling the disposition of nanoparticulate drug delivery systems.

4.4 PHYSIOLOGICAL FACTORS AFFECTING DRUG DISTRIBUTION

Drug distribution begins on absorption of a drug into the bloodstream. The various regions of the systemic circulation are perfused with blood through innumerable branching pathways, which are effectively arranged in parallel (Fig. 4.2) [1]. Distribution of drug occurs through the blood via the circulatory system. Blood is continually flowing from the heart to all areas of the body through arteries, to capillaries through organs and skin, reconverging into veins that return to the heart. The blood then travels to the lung for reoxygenation via the pulmonary circulation before returning to the heart to complete the process over and over again. Uninterrupted movement of blood is required for maintaining the supply of oxygen from the lungs and nutrients from the gut, as well as for the distribution of hormones, chemicals, including xenobiotics, water, and heat, and the delivery of waste for excretion [1]. The first stage of drug distribution is dependent on the cardiac output and regional blood flow to the various organs. Drug distribution is rapid to highly perfused organs, where the rate of blood flow is higher and these organs receive most of the drug, while distribution to adipose tissues, which are not so well perfused, is generally slower and drug concentrations are markedly less. Drug distribution is a reversible process in equilibrium so drug can move between organs and the circulation. Distribution of drug from systemic circulation to tissues is dependent on the interplay of the physiochemical properties of the drug such as lipid solubility, ionization, and molecular size (Section 4.3) and physiological factors such as binding to plasma proteins, rate of blood flow, susceptibility to passively diffuse, or to undergo active transport. It can be affected by local ionic and cellular conditions and special barriers such as biological membranes like the BBB and placental barrier. The drug exists in two forms in blood, the free form, which is active and available for biotransformation and excretion, or the bound form, which is usually pharmacologically inactive. The main physiological compartments, where drugs

are distributed, are the plasma (5% of body weight), interstitial fluid (16% of body weight), intracellular fluid (37% of body weight), fat (20% of body weight), specific organs (liver/kidney), as well as muscle, bone, and the CNS [22]. Additional factors can influence the ability of a drug to distribute, including the propensity of the drug to bind to plasma proteins. In the plasma, drugs generally bind to albumin although drugs can also bind to globulins (α , β , γ), α -1-acid glycoprotein (AGP) or lipoproteins. Since protein binding can have a large impact on drug distribution properties, Section 4.4.4 is devoted to covering this topic in more detail. The lipid solubility of the drug can also affect its distribution (Section 4.3.1). Additionally, the disease state itself can play an important role in determining the extent of distribution since disease-induced changes to many of the physiological parameters can markedly influence drug distribution. Many of these physiological factors are explained in further detail in subsequent sections.

4.4.1 Distribution Between Body Water

Drug distribution and dilution can occur between various organ compartments of water within the body and this information is sometimes useful for comparing the distribution of drugs with the volumes of the body water compartments by calculating a volume of distribution or V_d . The volume of distribution is a proportionality constant that relates the amount of drug in the body [dose (mg)] to the plasma concentration. $C_p = C_{p0}e^{-kt}$, where C_{p0} equals the initial plasma concentration and is a function of the distribution of the drug in the body:

$$V_d(\text{volume of distribution}) = \frac{\text{Dose (mg)}}{\frac{[\text{Plasma}] \text{mg}}{\text{mL}}}$$

V_d values can describe where drug is and do not necessarily correspond to real, physiological volumes. The V_d can reflect the ability of the drug to cross cell membranes (polarity), bind to plasma proteins (limits distribution), or bind to tissue proteins (increases distribution).

The amount of body water can be as much as 75% of the total body weight with the average being about 60% or around 44 L. This body water is distributed between four main compartments, the intracellular fluid (61%), interstitial fluid (27%), blood plasma (7%), and blood cell (5%) [22]. The V_d calculated for individual drugs can provide information on the extent of distribution between body water and tissues. Distribution between body water compartments is dependent on their volume and the barriers present, such as lipid membranes, binding to plasma proteins, and pH gradients that are encountered. Following entry into the body, regardless of route of administration, a drug has the propensity to be distributed into any one of these distinct body water compartments or to be sequestered within a cellular site. When a drug is administered directly to the bloodstream, it enters the plasma compartment. When a drug is absorbed, it passes through cell linings of the absorbing organ (skin, lung, or GI tract) into the fluids surrounding cells of the organ known as the *interstitial fluid*. The interstitial and intracellular fluids are in equilibrium with water and electrolytes, which move slowly into and out of cells in contrast to fast-moving blood. Drug can leave interstitial fluid by entering local tissue cells, entering blood capillaries (systemic circulation) or entering

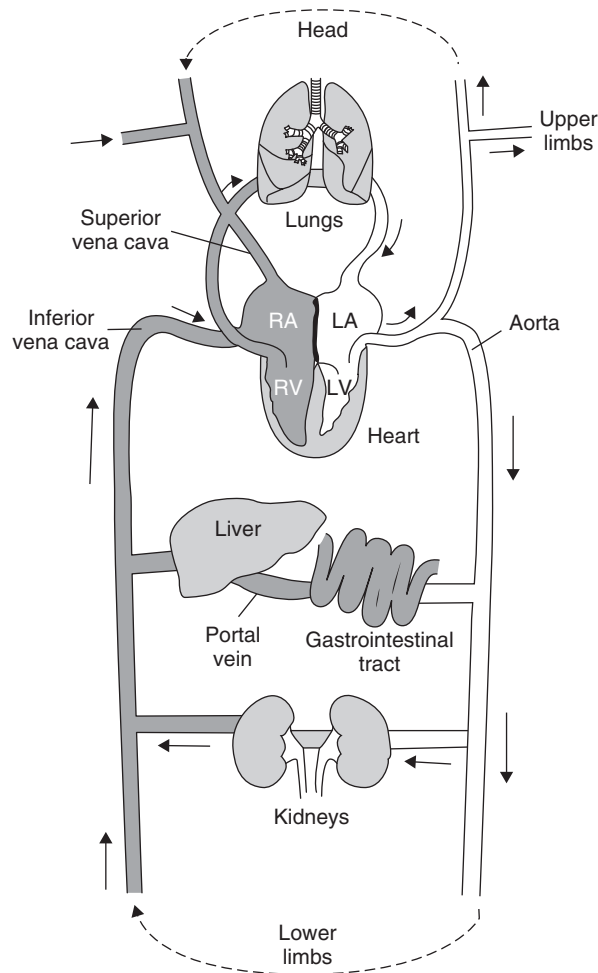


Figure 4.2 Simplified schematic of the circulatory system showing direction of flow from the inferior vena cava and superior vena cava to the right atrium. From the right atrium, the blood will enter the right ventricle where it is pumped into the lungs oxygenated and returned to the left atrium where it is pumped by the left ventricle to the rest of the body and returned to the right atrium through the IVC and SVC as depicted above.

the lymphatic system. Drug can also enter transcellular fluids by passing through an epithelium. Examples of transcellular fluids are cerebrospinal fluid (CSF), aqueous humor of the eye, contents of renal tubules, urine in the bladder, glandular contents, synovial fluid in joints, pleural fluids, peritoneal fluids, bile, saliva, and GI secretions. Figure 4.3 shows a schematic of drug distribution after various routes of administration through to elimination. While these compartments of water (intracellular fluid, interstitial fluid, blood plasma, and blood cell/transcellular fluid) are important in describing the distribution of drugs, compounds rarely associate solely within them, but most often distribute among several compartments, often binding to cellular components as described in Section 4.4.8.

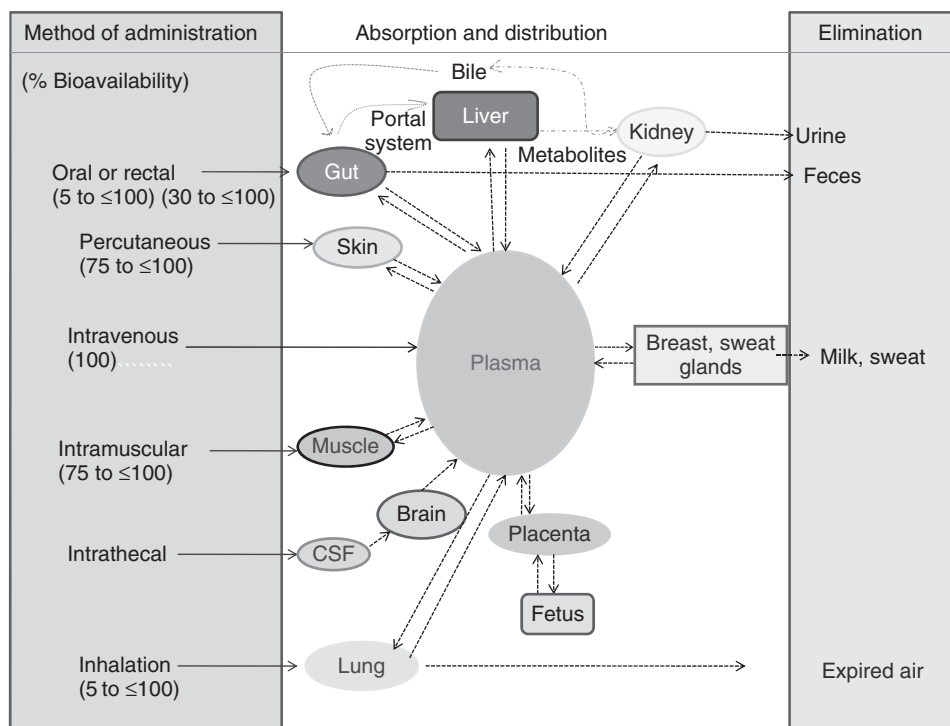


Figure 4.3 Pictorial representation of the disposition of a drug on administration. The theoretical bioavailability of the drug is highlighted below the route of administration on the left. Once the drug has been administered, it is absorbed and potentially distributed into various compartments of the body as detailed above. The methods for elimination will be dependent on a host of factors, including extent of distribution to eliminating organs such as the liver and kidneys. (See color insert.)

4.4.2 Drug Distribution Patterns

On entering the vascular system, a drug is distributed throughout body fluids, as described in Section 4.4.1, or into various tissues. The pattern of distribution that the drug exhibits reflects both the physiochemical nature of the drug and its ability to penetrate different membranes. The V_d can provide useful information on the type of distribution pattern displayed by a drug, and four different distribution patterns are recognized. Drugs exhibiting pattern 1 remain largely within the vascular system. This would encompass drugs such as dextran with a V_d in the region of 3–5 L, which approximates the volume of plasma [23]. Drugs that are distributed throughout the body water and not limited to the vascular system exhibit pattern 2 and demonstrate a V_d of around 30–50 L. Xenobiotics that exhibit this pattern are generally low molecular weight and water soluble. Examples of pattern 2 drugs would be ethanol (molecular weight = 46.07 g/mol), atenolol (molecular weight = 266 g/mol), and some sulfonamides (molecular weights typically <250 g/mol). Pattern 3 distribution evokes very large V_d values because of tissue partitioning, which may or may not be related to the site of action. Highly lipid-soluble compounds that can concentrate into fat tissue also tend to have higher V_d values. Most drugs follow a nonuniform distribution

pattern, also referred to as *pattern 4*, which is a combination of patterns 1–3 [23]. These drugs display different lipid/water solubility and varying abilities to pass through membranes. In some cases, this may result in higher concentrations within sites with higher rates of blood flow such as the kidney and liver, which can be reflective of the amount of drug being excreted. These drugs can have a V_d value within a wide range.

4.4.3 Rate of Blood Flow

The rate of distribution of a drug to organs is dependent on the blood flow rate to these regions. Highly perfused organs such as the heart, liver, lungs, kidneys, and brain reach equilibrium more rapidly because the rate of blood flow is higher. Areas where blood flow is lower such as skin, bone, and fat have a slower rate of equilibration. The rate of blood flow can also differ between rest and exercise and is dependent on physiological cues.

4.4.3.1 Rate of Blood Flow to Organs and Blood Perfusion Rate. Systemic flow is the culmination of flow through many parallel circuits supplying different organs and tissues. Since systemic and pulmonary circulation is one continuous circuit, the flow through the lungs is entirely dependent on the flow through the systemic circulation. Because they are integrated, both the systemic and pulmonary flow must each be equivalent to the volume pumped by each ventricle, which is the cardiac output. Cardiac output is the product of the stroke volume and the number of heartbeats per minute. In a typical 70-kg male volunteer at rest, the average stroke volume of the left ventricle is 0.08 L, while the average heart rate is 69 beats/min; therefore, at rest, cardiac output is about 5.5 L/min [1]. However, due to biological barriers, capillary permeability, and other physiological requirements, the quantity of blood flowing through each organ or region supplied by the systemic circulation is regulated. Table 4.2 outlines the cardiac output to various organs when a healthy, 70-kg man is resting. Cardiac output can increase with exercise or during emotional or physical stress, which can include illness, trauma, or disease. In contrast, if there is a decrease in stroke volume from dehydration or blood loss, there will be a decrease in cardiac output. Therefore, it should be considered that there are ranges to the outputs described in Table 4.2.

TABLE 4.2 Blood Perfusion Rates for Selected Organs

Organ	Perfusion Rate (mL/min/mL of Tissue)	Percentage of Cardiac Output
Skin	0.05	5
Bone	0.02	5
Muscle	0.03	15
Fat	0.01	2
Brain	0.55	15
Heart	0.7	4
Liver	0.75	20
Kidneys	4.5	24

Source: Extracted from Ref. 22.

4.4.4 Plasma Protein Binding

Plasma comprises ~55% of the total blood volume and is composed of water containing trace amounts of salts, minerals, and nutrients. Plasma contains a variety of proteins such as immunoglobulin and fibrinogen (for clotting); however, one of its main constituents is albumin, which comprises ~60% of the total plasma protein [24].

Among serum proteins, human serum albumin (HSA) and AGP play important roles in protein binding of many drugs, which is important in determining drug distribution throughout the body [25]. Albumin is basic, and therefore preferentially binds acidic and neutral compounds, while basic drugs can bind to AGP, which is acidic. Since albumin is a good carrier for acidic drugs, many drugs will circulate through the bloodstream bound to it. While drugs can bind to other plasma proteins, such nonspecific binding occurs to a much lesser extent. Binding of drugs to albumin, which enables circulation throughout the body, can also limit distribution to the surrounding tissues, since only the unbound portion is free to enter tissues from the bloodstream [26]. Since protein binding may retain and thereby determine the amount of drug within the central blood compartment, a higher degree of protein binding will produce a lower V_d . It has been shown that some medical conditions that alter the levels or binding properties of plasma proteins can affect the degree of plasma protein binding of drugs, and thus lead to drug interactions [27–29]. For instance, disease and stress can induce conformational changes in plasma or tissue proteins through synthesis of endogenous substances that can interfere with binding. Conjunctive tissue diseases, such as aging, prolonged bleeding, and starvation, are characterized by reduced plasma proteins, while heart infarction or liver morbidities can increase AGP levels, which can change binding profiles of basic drugs and affect distribution or pharmacokinetic and pharmacological parameters [27,28,30]. During pregnancy, albumin levels have been shown to decrease, thereby resulting in notable increases in unbound fractions for diazepam, valproic acid, phenytoin, phenobarbitone, salicylic acid, pethidine, lignocaine, dexamethasone, sulfafurazole, and propranolol [31,32]. A summary of pathological and physiological conditions that can affect protein binding through modulation of HSA, AGP, or lipoprotein levels can be found in Table 4.3 [33]. It is important to note that changes in the unbound drug fraction do not always result in proportional changes in clearance or distribution volume. Important contributors to the extent of concentration change will also be the extent of binding, type of clearance, the size of the distribution volume, administration route, and concomitant changes in intrinsic (cellular) clearance function caused, for example, by induction or inhibition of hepatic enzymes [28].

4.4.5 Red Blood Cell Partitioning

Although not as routinely discussed as plasma protein binding, red blood cell (RBC) partitioning is an important factor for determining V_d and clearance. Establishing the kinetic properties of drug binding to RBC enables appropriate selection of a biological fluid (whole blood, plasma, or serum) for analyzing drug concentration. This ensures more physiologically relevant PK parameters and improves the analytical sensitivity for drugs with K_b/p (whole blood-to-plasma ratio) or K_e/p (drug affinity to binding sites in erythrocytes relative to those in plasma) >2.0 . Chiral characteristics, lipophilicity, and molecular size are all important determinants of RBC partitioning. In some cases, partitioning can occur by passive diffusion (organic, anionic drugs, and nonelectrolytes), through aqueous channels by small hydrophilic compounds (<150 D), or, in

the case of lipophilic compounds, through dissolving into the lipid bilayer membrane. Binding of drug to RBC can occur to hemoglobin and [34] membrane or proteins within the cytosol such as carbonic anhydrase. Additionally, RBCs have the ability to metabolize some drugs, which can add another consideration to the disposition of drugs and the predictions of PK parameters. The rate and extent of RBC binding can

TABLE 4.3 Effect of Physiologic and Pathological Conditions on Plasma Serum Protein Levels

Physiologic and Pathological Condition	Protein					
	Albumin		AGP		Lipoprotein	
	Decrease	Increase	Decrease	Increase	Decrease	Increase
Age fetal			×			
Age geriatric	×			×		
Age neonate	×					
Bacterial pneumonia	×					
Benign tumor		×				
Burns	×					
Celiac disease				×		
Cirrhosis of liver	×					
Crohn's disease				×		
Cystic fibrosis	×					
Diabetes						×
Exercise		×				
GI disease	×					
Histoplasmosis	×					
Hyperthyroidism					×	
Hypothyroidism		×				×
Injury				×	×	
Liver abscesses	×					
Liver disease					×	×
Malignant neoplasm	×					
Multiple myeloma	×					
Myocardial infarction				×		
Nephrotic syndrome	×		×			×
Neurological disease		×				
Neurosis		×				
Pancreatitis	×					
Paranoia		×				
Pregnancy	×					
Psychosis		×				
Renal failure	×			×		
Rheumatoid arthritis				×		
Schizophrenia		×				
Severe malnutrition	×					
Stress				×		
Surgery	×			×		
Trauma	×			×	×	
Use of oral contraceptives			×			

Source: Extracted from Ref. 33.

be determined by *in vitro* methodologies where drug is added to suspended RBC in plasma or water containing plasma. Or by *ex vivo* methods where drug is administered to humans, samples are collected and RBC and plasma are separated through centrifugation after which levels of drug are measured in both biological fluids. In some cases, such as with neuroleptic drugs (butaperazine, haloperidol, and thioridazine), the RBC concentration is better correlated with therapeutic effect or dose than plasma concentrations and in the case of digoxin toxicity, RBC concentration better distinguishes between toxic and nontoxic drug levels than plasma concentrations [34].

4.4.6 Perfusion and Diffusion

The distribution of the free fraction of drug can be limited by tissue perfusion rate or by diffusion of the drug across barrier membranes. Only small molecules that are not bound to albumin can undergo diffusion because, when bound to albumin, the molecular weight of the complex is so high (>68,000 kD) that it limits diffusion [35]. However, this protein binding is not a static process and can be described with an on-off rate equation. Passive diffusion occurs when drug moves from an area of high concentration to an area of low concentration following the equation of Fick's law of diffusion [22]:

$$\text{Rate of drug diffusion} = \frac{-DKA(C_p - C_t)}{h}$$

In this equation, C_p is the concentration of drug in plasma, C_t is the concentration of drug in tissue, A is the surface area of the membrane, K is the lipid-water partition coefficient, D is the diffusion constant and is inversely proportional to the weight of the drug, while h is the thickness of the membrane. The negative sign denotes the net transfer of drug from inside the capillary lumen into tissues and extracellular spaces. In this manner, the diffusion is both spontaneous and temperature dependent. In the case of nonpolar, highly lipid-soluble drugs there can be a perfusion rate limitation. Being described as perfusion rate-limited assumes that on entry into blood circulation, the drug is free to distribute completely and instantly across membranes without diffusion barriers. In this case, it is limited only by the rate of blood flow to the organ. If drug distribution is limited by perfusion, the distribution equilibrium may take longer to achieve when perfusion is low and the partition coefficient is high. If the permeability coefficient is high, then the concentration of drug at the tissue must be high before equilibrium takes place. In the case of diffusion rate-limited distribution, as would most likely occur with polar drugs, the rate of distribution into tissue is a function of the permeability coefficient of the drug, the concentration gradient, the distance that the drug travels, the surface area for diffusion, the temperature, and the molecular weight of the drug since smaller lighter molecules will diffuse faster than larger heavier ones. Capillary permeability can also have a profound effect on drug distribution.

4.4.7 Capillary Permeability

The lining of endothelial cells that make up the blood vessels, the lymphatic vessels, and the inside of the heart can affect capillary permeability to drugs depending on how tight the junction between the cells is. The capillary endothelium has discontinuous junctions, which can make the capillary walls quite permeable [36]. For instance,

lipid-soluble drugs can pass through vascular endothelium rapidly, whereas water-soluble drugs will penetrate more slowly, and the rate is dependent on physiochemical properties such as molecular size. Additional physiochemical properties that can affect the rate of transfer across the capillary membrane are whether the drug is ionized and this depends on the pK_a of the drug and the pH of the blood as discussed in Section 4.3.1. Membrane permeability tends to restrict the transfer and distribution of drugs once they are delivered to the tissue. The other major factors that determine the rate of drug distribution is blood perfusion and the expression of transporters.

Two deviations to the typical capillary structure can result in variation from normal drug tissue permeability.

1. The renal and hepatic capillaries in the membrane of the endothelial cells, known as *fenestrations*, are larger, thereby increasing permeability that enables more extensive distribution of many drugs out of the capillary beds of the kidney and liver. Since both these organs serve as sites of elimination, this would serve to reduce the plasma concentration by acting as a mechanism of clearance (refer to Section 4.8).
2. An opposite phenomenon exists within the brain where capillaries seem to have relatively impermeable walls compared with normal vascular endothelium. This greatly restricts the permeability of molecules from blood to brain tissue, despite the cardiac output being relatively high.

The brain capillaries and the renal and hepatic capillaries are two important deviations from the normal capillary wall and both have a profound effect on drug distribution [36]. Capillaries within the brain have tight junctions and have endothelial cells that are so close together that they form the BBB. This limits the distribution of drugs into the brain, and generally only lipid-soluble drugs, or drugs that resemble endogenous substances and are actively transported, can enter the brain. In general, polar or ionized drugs cannot distribute in the CNS. In contrast, the capillary walls in the kidney and liver are very permeable since there are large gaps in the junctions between the endothelial cells and fenestrations through the cell cytoplasm allowing movement of drugs and plasma proteins. This enables rapid and extensive distribution of drugs and proteins out of the capillary bed, which is well suited for these organs since they play such an important role in drug elimination. Since the distribution of a drug to tissues can be dependent on the permeability of the capillaries at the site of distribution, Table 4.4 describes the various pore sizes of some of these barrier membranes. These

TABLE 4.4 Capillary Permeability

Barrier Membrane	Pore Diameter (Å)
Intestinal epithelium	4
Capillary endothelium	40–80
Muscle capillaries	60
Glomerular capillaries	75–100
Glomerular endothelium	1000
Liver capillaries	1000

barriers to distribution are described in more detail in Section 4.4.9. Other capillary beds with tight junctions that can limit distribution include those found in the testicles, eyes, and synovia [36]. Disease states such as inflammation, liver disease, and diabetes can affect capillary barriers and therefore disable or enable entry of drugs and thus change the normal distribution pattern observed in the healthy state [36].

4.4.8 Tissue Storage

As mentioned in Section 4.4.1, some drugs may accumulate in certain types of tissues and act as a reservoir, slowly releasing the drug into the bloodstream, thus keeping the blood level of the drug from decreasing rapidly and prolonging its effect. These drugs generally exhibit pattern 3 distribution, as described in Section 4.4.2, and tend to have very large V_d values. For example, long-term administration of the antimalarial agent quinacrine results in accumulation in the liver [37] and gentamicin binding to tissue results in accumulation in the kidney and vestibular system [38]. This phenomenon can be attributed to binding of drug to cellular constituents such as proteins, phospholipids, or nuclear proteins, as described above. An additional mechanism of accumulation within tissues can be attributed to active transport (e.g., liver enrichment of statins), which is covered in more detail in Section 4.5. Fat can also serve as a reservoir for many lipid-soluble drugs and since the blood flow to adipose tissue is only 2% of cardiac output, it serves as a rather stable reservoir. Drugs that accumulate in fatty tissues can leave the tissue so slowly that the drug can still be present in the bloodstream for days after dosing has ceased. An example of highly lipid-soluble xenobiotics that extensively distribute into fat are polychlorinated biphenyls and dicophane. Other examples of drugs stored in fat would be the lipid-soluble barbiturate thiopental, remifentanyl, and some other β -blockers [39,40].

Bone can also serve as a site of accumulation for drugs such as tetracycline antibiotics, other divalent metal-ion chelating agents, and heavy metals [41]. Drugs and other toxic agents such as lead or radium can accumulate into the bone crystal surface and incorporate into the crystal lattice where effects can persist long after exposure [42]. This type of accumulation and exposure can have therapeutic advantages for treatments aimed at osteoporosis. Bound drug in the bloodstream can also serve as a reservoir since the drug will be released as the unbound levels decrease. As an example, digitoxin and phenytoin can be sequestered by plasma proteins, which then act as a reservoir for drug [43].

4.4.9 Physiological Barriers to Drug Distribution

Epithelial and endothelial cells act as internal barriers, while external barriers include keratinized epithelium (skin), ciliated epithelium (lung), or epithelium with microvilli such as is found in the intestine. These types of cells are connected by zonulae occludens, which is a tight junction that creates an unbroken phospholipid bilayer [44]. There are also internal or blood–tissue barriers that are made up of endothelial cells joined via zonulae occludens [45]. In these cases, drug permeation occurs mostly in the capillary beds, for example, endocrine glands in the gut have fenestrations on endothelial cells, which allow for passage of small molecules, whereas the liver has large fenestrations (~ 100 nm), thereby allowing drugs to exchange freely between the blood and interstitium [45]. Cardiac muscle has high endo- and transcytotic activity,

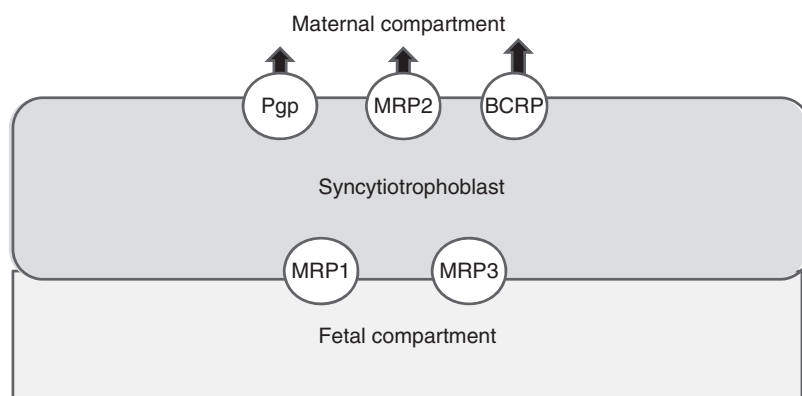


Figure 4.4 A depiction of xenobiotic transporter expression at the placental barrier where the expression of efflux transporters serves to protect the fetal compartment from exposure to substances that are substrates for the expressed transporters. *Source:* Extracted from Ref. 81. (See color insert.)

which enables drug transport via vesicles, whereas the CNS has endothelia that lack pores and possess only a little transcytotic activity, therefore limiting diffusion to small lipophilic drugs that can undergo transcellular passive diffusion [46]. This provides a barrier that is very restrictive and limits the types of drugs that can permeate.

4.4.9.1 Maternal:Fetal Barrier. The composition of the placental barrier changes over the course of pregnancy, but it consists of the chorion frondosum (fetal tissue) and deciduas basalis (maternal tissue), which are divided into cotyledons separating the maternal and fetal circulation [47]. It is composed of a single layer of polarized epithelial cells known as the trophoblast, which contains the syncytiotrophoblast, and the fetal capillary endothelia (refer to Fig. 4.4). Drug flux can occur across the apical (toward maternal) and basolateral (toward fetal) membranes of the syncytiotrophoblast [47]. The placenta is very well perfused and therefore not as impenetrable as often thought [48]; while most lipophilic drugs can readily cross by diffusion, there are a large number of efflux transporters present, similar to those expressed in the brain, allowing export of drugs out of the placenta. Uptake transporters are also present [49], which facilitate the transport of more hydrophilic nutrients. Although these mechanisms are covered in more detail in Section 4.5, it is important to note that the presence of efflux transporters can limit intracellular concentrations and hence exposure to the fetus, despite the ability of lipophilic drugs to readily diffuse. The physiochemical properties of the drug and whether it is a substrate for transport processes have a large impact on the actual fetal exposure. Physiological and physiochemical factors previously covered such as lipid solubility, extent of plasma binding, and degree of ionization of weak acids and bases are also important general determinants in drug transfer across the placenta.

4.4.9.2 Mammary Gland. The mammary gland represents an important barrier that limits drug exposure to a suckling infant. The functional unit for milk production and the milk–blood barrier is a single layer of epithelial cells in the alveoli, which are extensively proliferating during lactation [50]. The mammary gland is highly perfused

with a large surface area, and therefore distribution of drug to milk is generally similar to that of blood with very little lag time. However, there is high expression of transporters that can serve to limit the extent of exposure to drug. Additional factors that may influence drug distribution include the slightly lower pH of milk (7.2) compared with blood, thereby allowing ion trapping of weak bases [51]. There can also be a degree of binding to milk proteins and, since the milk fat content is high, lipophilic drugs may have large milk to serum concentration ratios due to partitioning into milk [52]. There is also evidence of transporter expression on the mammary epithelium, mostly for endogenous substrate transfer. While there is more extensive discussion of transporters in Section 4.5, it is worth noting that some members of the organic anion-transporting polypeptide (OATP) and multidrug resistance protein (MRP) families have been detected, while ABCB1 (MDR1) and ABCG2 (breast cancer resistance protein (BCRP)) have also been shown to play a role in drug accumulation in secreted milk [53,54].

4.4.9.3 Blood–Brain Barrier. The BBB serves to protect the brain from the contents of the CSF and blood, and it comprises a layer of endothelial cells sealed by tight junctions supported in terms of nutrition and communication by astrocytes [55]. Projections from astrocytes form a communication link between the capillary endothelium and the neurons of the brain, and lipids within the astrocyte cell walls and the tight junctions between the adjacent endothelial cells limit the passage of water-soluble molecules at the BBB. This is not totally impenetrable but slows the rate at which drugs can cross into brain tissue, while still allowing essential nutrients such as oxygen to pass through [56]. The expression of transporters at the apical membrane of the epithelial cells can also prevent distribution of drugs across the barrier and contribute to drug resistance phenotypes [57,58]. As discussed in Section 4.4.3, the rate of blood flow to the brain is quite high, since the percentage of the cardiac output distributed to the brain is around 15%. This enables some highly lipid-soluble drugs, such as certain anesthetics, and small lipophilic compounds, such as barbiturates and benzodiazepines, to diffuse rapidly into the CNS. The role of efflux transporters on the apical membrane can effectively serve to actively exclude a large variety of lipophilic drugs from the brain capillary endothelial cells, thereby limiting the distribution of these types of drugs. This can be beneficial in reducing adverse neurotoxic effects and may also reduce the treatment options for neurological disorders. Therefore, there may be therapeutic advantages to transporter modulation as a mechanism for distribution of novel therapeutics. In contrast to the BBB, the choroid plexus contains fenestrated endothelium, which allows for drug distribution across the choroid plexus [59,60]. The choroid plexus is a continuous layer of epithelial cells that function to control CSF homeostasis. CSF flows from the ventricles across the surfaces of the brain and spinal cord into the venous blood sinuses [61]. The CSF–blood barrier (Fig. 4.5) is composed of choroidal epithelial cells joined together by tight junctions at their apical surface. In this manner, the barrier prevents all but the most lipid-soluble drugs from entering the CSF [57]. This can result in profound differences in concentration of drug between the aqueous CSF and the CNS tissue [62–64]. Similar to the other barriers discussed, there is a large expression of various uptake and efflux transporters, including SLC22A8 (Oat3), SLC16A1 (MCT-1), SLCO1A2 (OATP1A2), ABCB1 [P-glycoprotein (Pgp)], ABCC4 (MRP4), ABCC1 (MRP1), and ABCG2 (BCRP), which profoundly affect drug distribution into the CNS and these are discussed in further detail in the following section [65].

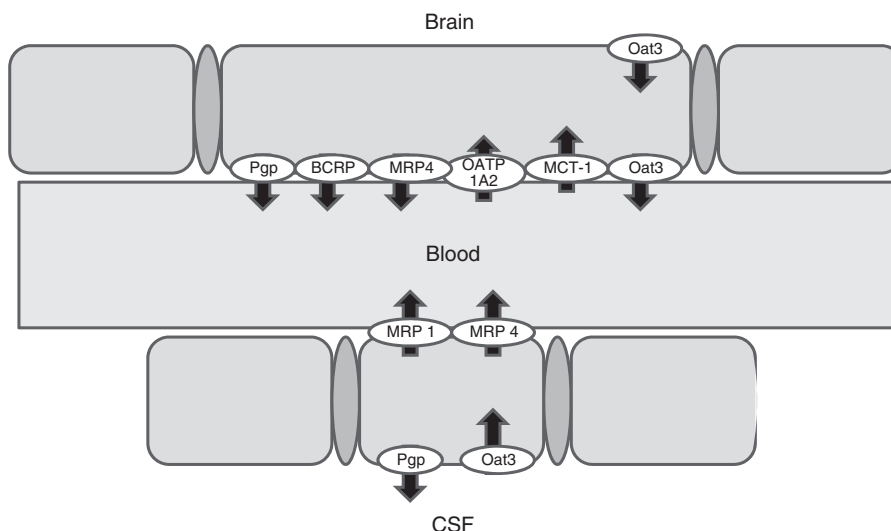


Figure 4.5 A depiction of xenobiotic transporter expression at the blood–brain barrier (BBB) and choroid plexus, cerebral spinal fluid (CSF)–blood barrier. As evidenced in detail above, the expression of efflux transporters is pronounced along the apical membrane of the BBB, thus limiting the exposure of substrates for these transporters into the brain. *Source:* Extracted from Ref. 64. (See color insert.)

4.5 THE ROLE OF TRANSPORTERS IN DRUG DISTRIBUTION

It has become increasingly recognized that most drugs are distributed across cellular membranes with the assistance of transporter proteins. These transporters have been identified in every living cell in varying amounts and are distributed differentially throughout tissues and barrier membranes [66–70]. It is thought that transporters may play an important role not only in distributing drug molecules to their effector site but also in distribution to almost every organ and tissue in the body [66]. This has been shown to contribute to both pharmacological and toxicological effects and to nonlinear pharmacokinetics.

4.5.1 Transporter Subtypes Involved in Distribution

Since the discovery of Pgp as an important contributor to efflux drug transport across membranes and its underlying role in multidrug resistance (MDR) [71], it has become more commonly accepted that transporters can have a large impact in drug disposition. The identification of various transporters has resulted in classification systems based on the method and direction of transport. Transporters that have been identified as requiring the direct binding and hydrolysis of ATP have been grouped into a superfamily of ABC (ATP-binding cassette) transporters. So far, there are at least 49 members of the ABC superfamily identified in humans and these are grouped into seven families and contain therapeutically important and well-studied transporters such as ABCB1, ABCC1, ABCC2, and ABCG2 [72]. Another important large family of transporters is the SLC (solute carrier) transporters of which there are as many as 50–55 gene families

and at least 362 members [73,74]. Included in this class are all the major classes of uptake transporters, including the OATPs and organic cation transporter (OCTs), which have both been implicated in drug–drug interactions [68]. Unlike the ABC transporters, the SLC transporters do not directly utilize an energy source [66]. Together the ABC and SLC transporters are increasingly being implicated in regulation of the passage of endogenous and exogenous chemicals across cellular and tissue membranes, thereby playing a pivotal role in drug distribution and subsequent disposition, efficacy, and toxicity [66]. An additional consideration in understanding the differential distribution of drugs through body compartments within a human population is the occurrence of single-nucleotide polymorphisms within the transporter molecules [75–77]. Since transporters clearly play an important role in drug distribution having an understanding of whether a drug of interest is a substrate/inhibitor of specific transporters can provide a more lucid interpretation of V_d and result in more predictive physiologically based pharmacokinetic (PBPK) modeling (refer Section 4.8.4). This field is rapidly evolving and expanding, therefore while a vast number of transporters have been identified and their respective tissue distribution has been described, it is understood that not all these transporters may result in clinically relevant drug–drug interactions. In 2007, an international transporter consortium was established, and in 2010, a review article was published recognizing the importance of transporters in drug disposition and making recommendations regarding the conduct of transporter studies and data analysis [78].

4.5.1.1 ABC Transporters. ABC transporters make up the largest class of membrane-bound proteins in biology and function by enabling active transfer of molecules across biological membranes, through binding and hydrolysis of ATP [66]. As stated earlier, there are currently at least 49 members within seven families (ABCA–ABCG), which are identified by the presence of a characteristic amino acid motif within the nucleotide binding domains (LSGGQ) [79]. A number of ABC transporters have been shown to be expressed at barrier membranes and are therefore considered important to drug distribution. Table 4.5 summarizes the expression of the ABC transporter mRNA in multiple tissue types in descending order of expression, while Table 4.6 summarizes the gene identification and the alias of the transporters that are involved in xenobiotic transport.

4.5.1.2 SLC Transporters. SLC transporters represent a basic and abundant mechanism by which endogenous and exogenous molecules are distributed across membranes [66]. SLC transporters do not directly access an energy source and they are able to function as uniporters (facilitated diffusion of substrates across membranes), symporters (transport of two compounds in the same direction at the same time), and antiporters (transport of two compounds in opposite directions at the same time) [66,68]. While the majority of the SLC transporters serve to facilitate transfer of highly specific endogenous substrates, a number of them can contribute to MDR and limit accumulation by transporting drugs into and out of cells [68]. As an example, multiple statin drugs are substrates for OATPs that are highly expressed in the basolateral membrane of hepatocytes and this contributes to their ability to accumulate into the liver [75]. Certain SLC transporters can function bidirectionally depending on substrate concentration gradients so they can play the role of efflux transporter. Many OCTs can function in this manner [68]. Often times the extent and direction of drug movement into organs, including the intestine and liver, is determined by the unique interplay between apically and

TABLE 4.5 ABC Transporter Expression in Various Tissues^a

Tissue	Expression of the 10 Most Abundant Human ABC Transporters in Various Tissues Ranked from Highest to Lowest									
Adrenal gland	ABCA1	ABCC3	ABCB1	ABCD1	ABCA8	ABCG1	ABCF2	ABCF1	ABCA6	ABCD3
Bladder	Not known									
Bone marrow	ABCB10	ABCF1	ABCD3	ABCF2	ABCC4	ABCE1	ABCB2	ABCC1	ABCC10	ABCB8
Brain	ABCA2	ABCA5	ABCF2	ABCD2	ABCF1	ABCD3	ABCE1	ABCC5	ABCB9	ABCB8
Colon	ABCD3	ABCC3	ABCB2	ABCC7	ABCA1	ABCF2	ABCA5	ABCE1	ABCD1	ABCB10
Heart	ABCA8	ABCF2	ABCC9	ABCD3	ABCF1	ABCE1	ABCA9	ABCA5	ABCB1	ABCD1
Kidney	ABCD3	ABCC4	ABCF2	ABCF1	ABCB1	ABCC2	ABCE1	ABCA2	ABCC3	ABCB8
Liver	ABCA6	ABCG8	ABCB11	ABCB4	ABCG5	ABCA1	ABCD3	ABCC2	ABCC9	ABCF1
Lung	ABCA6	ABCA1	ABCB2	ABCF1	ABCC4	ABCA8	ABCF2	ABCD3	ABCG1	ABCE1
Mammary gland	Not known									
Ovary	Not known									
Pancreas	ABCC7	ACF1	ABCA5	ABCD3	ABCE1	ABCF2	ABCC3	ABCD1	ABCC1	ABCC10
Peripheral leukocytes	ABCB2	ABCF1	ABCD1	ABCF2	ABCE1	ABCB3	ABCB10	ABCA2	ABCC1	ABCA1
Placenta	ABCD1	ABCF1	ABCA1	ABCB8	ABCF2	ABCG2	ABCG1	ABCB2	ABCD3	ABCC10
Prostate	ABCC4	ABCF1	ABCD3	ABCF2	ABCA5	ABCE1	ABCB2	ABCC1	ABCA1	ABCB10
Retina	Not known									
Salivary gland	ABCF1	ABCF2	ABCD3	ABCA6	ABCE1	ABCC7	ABCD1	ABCB2	ABCB10	ABCG1
Skeletal muscle	ABCF1	ABCF2	ABCA5	ABCD1	ABCD3	ABCC1	ABCC9	ABCA6	ABCB10	ABCA1
Small intestine	ABCD1	ABCD3	ABCF1	ABCG5	ABCB2	ABCF2	ABCG8	ABCB10	ABCC3	ABCG2
Smooth muscle	Not known									
Spinal cord	ABCA2	ABCD3	ABCA8	ANCF1	ABCA6	ABCA1	ABCE1	ABCA5	ABCB2	ABCB8
Spleen	ABCB2	ABCF1	ABCF2	ABCG1	ABCA1	ABCD1	ABCE1	ABCC1	ABCD3	ABCB10
Stomach	ABCD3	ABCF1	ABCC3	ABCF2	ABCE1	ABCB2	ABCA6	ABCA5	ABCC1	ABCA8
Testis	ABCF1	ABCF2	ABCD1	ABCB9	ABCE1	ABCC1	ABCD3	ABCA5	ABCA8	ABCC12
Thymus	ABCF1	ACA1	ABCB2	ABCD3	ABCF2	ABCC1	ABCG1	ABCD1	ABCA7	ABCC10
Thyroid gland	ABCF1	ABCA2	ABCF2	ABCA6	ABCD3	ABCA5	ABCE1	ABCA8	ABCC4	ABCA1
Trachea	ABCF1	ABCD3	ABCB2	ABCC1	ABCA1	ABCG1	ABCA5	ABCA8	ABCC4	ABCE1
Uterus	ABCF1	ABCF2	ABCA6	ABCD1	ABCD3	ABCA1	ABCG2	ABCB2	ABCE1	ABCB10

^aRef. 66.

TABLE 4.6 ABC Transporter Alternative Names and Distribution^a

Transporter Gene	Alternative Name	Distribution
ABCA1	ABC1	Ubiquitous
ABCA2	ABC2	Brain
ABCA3	ABC3, ABCC	Lung
ABCA4	ABCR	Rod photoreceptors
ABCA5	—	Muscle, heart, testes
ABCA6	—	Liver
ABCA7	—	Spleen, thymus
ABCA8	—	Ovary
ABCA9	—	Heart
ABCA10	—	Muscle, heart, testes
ABCA11	—	Stomach
ABCA12	—	Low in all tissues
ABCB1	MDR1, PGP	Adrenal, kidney, brain
ABCB2	TAP1	Ubiquitous, ER
ABCB3	TAP2	Ubiquitous, ER
ABCB4	PGP3, MDR3	Liver
ABCB5	—	Ubiquitous
ABCB6	MTABC3	Mitochondria
ABCB7	ABC7	Mitochondria
ABCB8	MABC1	Mitochondria
ABCB9	—	Heart, brain
ABCB10	MTABC2	Mitochondria
ABCB11	SPGP, BSEP	Liver
ABCC1	MRP1	Ubiquitous
ABCC2	MRP2	Liver
ABCC3	MRP3	Lung, intestine, liver
ABCC4	MRP4	Prostate
ABCC5	MRP5	Ubiquitous
ABCC6	MRP6	Kidney, liver
ABCC7	CFTR	Exocrine tissues
ABCC8	SUR	Pancreas
ABCC9	SUR2	Heart, muscle
ABCC10	MRP7	Low in all tissues
ABCC11	MRP8	Low in all tissues
ABCC12	MRP9	Low in all tissues
ABCD1	ALD	Peroxisomes
ABCD2	ALD1,ALDR	Peroxisomes
ABCD3	PMP70, PXMP1	Peroxisomes
ABCD4	PMP69, P7R	Peroxisomes
ABCE1	OABP	Ovary, testes, spleen
ABCF1	ABC50	Ubiquitous
ABCF2	—	Ubiquitous
ABCF3	—	Ubiquitous
ABCG1	ABC8	Ubiquitous
ABCG2	BCRP	Placenta, intestine
ABCG4	White2	Liver
ABCG5	Sterolin 1	Liver, intestine
ABCG8	Sterolin 2	Liver, intestine

^aRef. 80.

basolaterally expressed transporters. In this manner, transporters that result in vectorial drug transfer into the systemic circulation are referenced as *absorptive transporters*, whether they are efflux or uptake transporters. Transporters involved in the excretion of substrates from circulation are known as *secretory transporters*. Expression of these transporters on the canalicular membrane of hepatocytes further effects the disposition of drugs since substrates can be effectively eliminated from distribution through hepatic phase I and II biotransformation and subsequent efflux into bile. Table 4.7 summarizes the expression of the SLC transporter mRNA in multiple tissue types in descending order of expression, while Table 4.8 summarizes the gene identification and the alias of the transporters that are involved in xenobiotic transport.

4.5.2 Transporter Expression at Barrier Membranes

The expression of multiple ABC and SLC transporters in the apical and basolateral sides of epithelial and endothelial cells lining pharmacological barriers indicates their importance in protection and detoxification [70]. ABCB1 (PGP) is highly expressed in most barrier tissues, such as the BBB, testis, placenta, and endothelial cells of human cardiac vasculature. Additional transporters of importance include the ABCC family, which consists of the MRPs and ABCG2 (BCRP) and are also expressed in many of the epithelia and endothelia that constitute important barrier membranes. Transporter expression at some important barriers to distribution is shown in Figs. 4.4 and 4.5, which describe transporter location at the BBB and CSF–blood barrier and at the placental barrier, respectively. A number of transporters are also expressed at the blood–testis barrier where tight junctions between sertoli cells allow polarization and barrier formation, which protect the germ cells. These transporters include Pgp, GLUT1, 2, and 3, urea transporter, and potentially OCT1, 3, OCTN1, and 2. The latter are thought to contribute to accumulation of propranolol and imipramine in the testis [81], which may result in adverse effects in this tissue. Additionally, the role of transporters on the apparent V_d should be considered, since transporter-mediated uptake into hepatocytes can affect the extent of clearance and therefore the apparent V_d . An example of this would be cerivastatin, which is highly distributed to the liver both through uptake processes and cellular binding. This has been shown as a mechanism for drug–drug interaction between cerivastatin and CsA, since CsA acts as an inhibitor of cerivastatin uptake.

4.6 IN VITRO METHODOLOGIES FOR DETERMINING DRUG DISTRIBUTION

Some of the different parameters of drug distribution can be determined using *in vitro* methodologies. Well-established methods for evaluating plasma protein binding and determining the role of transporters as well as more recently reported methods are discussed in the following sections. A more detailed description of the transporters involved in drug distribution is given in Section 4.5.

4.6.1 Plasma Protein Binding

Plasma protein binding studies play an important role in the process of candidate drug selection and development. The amount of drug that binds to protein is dependent on

TABLE 4.7 SLC Transporter Expression in Various Tissues^a

Tissue	Expression of the 10 Most Abundant Human SLC Transporters in Various Tissues Ranked from Highest to Lowest									
Adrenal gland	SLC16A9	SLC40A1	SLC25A3	SLC25A37	SLC39A9	SLC24A6	SLC3A2	SLC30A1	SLC6A6	SLC25A44
Bladder	SLC25A6	SLC25A23	SLC25A3	SLC40A1	SLC25A5	SLC25A24	SLC8A1	SLC25A44	SLC25A37	SLC39A9
Bone marrow	SLC25A37	SLC4A1	SLC40A1	SLC16A3	SLC25A6	SLC25A39	SLC2A3	SLC25A3	SLC25A5	SLC30A6
Brain	SLC22A17	SLC25A23	SLC39A10	SLC25A6	SLC17A7	SLC25A39	SLC1A2	SLC25A44	SLC25A5	SLC12A5
Colon	SLC25A6	SLC25A5	SLC25A3	SLC39A5	SLC25A23	SLC39A9	SLC25A24	SLC16A3	SLC25A44	SLC30A6
Heart	SLC25A37	SLC8A1	SLC16A7	SLC40A1	SLC25A30	SLC25A23	SLC25A11	SLC25A6	SLC25A37	SLC25A5
Kidney	SLC39A5	SLC25A6	SLC25A5	SLC5A12	SLC7A7	SLC12A1	SLC27A5	SLC34A1	SLC3A2	SLC25A3
Liver	SLC22A17	SLC40A1	SLC2A2	SLC25A5	SLC13A5	SLC39A5	SLC38A3	SLC22A7	SLC25A37	SLC39A9
Lung	SLC40A1	SLC34A2	SLC6A4	SLC25A6	SLC2A3	SLC39A8	SLC16A3	SLC25A37	SLC25A24	SLC25A3
Mammary gland	SLC25A37	SLC25A37	SLC25A6	SLC40A1	SLC25A39	SLC25A44	SLC37A3	SLC12A2	SLC20A2	SLC16A3
Ovary	SLC25A6	SLC40A1	SLC30A6	SLC25A37	SLC16A9	SLC25A39	SLC25A5	SLC25A23	SLC25A24	SLC24A6
Pancreas	SLC25A6	SLC25A5	SLC39A5	SLC40A1	SLC25A3	SLC39A9	SLC39A8	SLC30A6	SLC30A1	SLC39A6
Peripheral leukocytes	SLC25A37	SLC16A3	SLC25A6	SLC2A3	SLC25A3	SLC25A44	SLC25A5	SLC40A1	SLC30A6	SLC7A7
Placenta	SLC2A1	SLC40A1	SLC25A6	SLC3A2	SLC39A9	SLC7A2	SLC30A6	SLC39A6	SLC25A5	SLC39A8
Prostate	SLC30A4	SLC39A10	SLC39A6	SLC25A6	SLC25A37	SLC39A9	SLC40A1	SLC14A1	SLC25A37	SLC25A23
Retina	SLC16A3	SLC25A6	SLC25A3	SLC23A2	SLC24A44	SLC25A5	SLC30A6	SLC16A14	SLC40A1	SLC25A23
Salivary gland	SLC25A6	SLC39A9	SLC25A5	SLC13A5	SLC25A3	SLC31A1	SLC5A5	SLC9A1	SLC25A23	SLC25A37
Skeletal muscle	SLC16A3	SLC25A37	SLC25A11	SLC25A4	SLC40A1	SLC25A23	SLC25A30	SLC19A2	SLC25A9	SLC25A6
Small intestine	SLC5A1	SLC25A6	SLC40A1	SLC25A5	SLC25A3	SLC39A5	SLC2A5	SLC39A9	SLC25A24	SLC13A2
Smooth muscle	SLC25A6	SLC25A23	SLC25A3	SLC8A1	SLC40A1	SLC25A5	SLC30A6	SLC12A2	SLC25A24	SLC25A37
Spinal cord	SLC22A17	SLC25A6	SLC39A10	SLC14A1	SLC16A9	SLC25A23	SLC39A9	SLC25A3	SLC39A6	SLC25A44
Spleen	SLC25A6	SLC16A9	SLC39A10	SLC25A5	SLC16A3	SLC25A39	SLC25A37	SLC30A6	SLC39A9	SLC40A1
Stomach	SLC25A6	SLC25A5	SLC25A3	SLC40A1	SLC25A37	SLC39A9	SLC16A3	SLC30A6	SLC25A23	SLC39A7
Testis	SLC25A37	SLC30A6	SLC16A3	SLC30A4	SLC30A1	SLC25A33	SLC40A1	SLC2A5	SLC16A9	SLC25A6
Thymus	SLC25A6	SLC25A5	SLC40A1	SLC25A37	SLC25A4	SLC25A8	SLC30A6	SLC39A9	SLC25A11	SLC16A3
Thyroid gland	SLC25A6	SLC5A3	SLC26A7	SLC5A5	SLC25A23	SLC25A44	SLC25A3	SLC30A6	SLC25A37	SLC40A1
Trachea	SLC25A6	SLC12A2	SLC16A3	SLC25A37	SLC40A1	SLC25A3	SLC25A5	SLC39A9	SLC16A9	SLC25A44
Uterus	SLC25A6	SLC40A1	SLC25A3	SLC25A5	SLC16A9	SLC39A9	SLC25A37	SLC25A23	SLC37A3	SLC30A6

^aRef. 66.

TABLE 4.8 SLC Transporter Alternative Names and Distribution^a

Transporter Gene	Alternative Name	Distribution
SLC22A6	OAT1	—
SLC22A7	OAT2	—
SLC22A8	OAT3	—
SLC22A9	OAT4	—
SLC21A2	Hpgt	—
SLC21A3	OATP-A	—
SLC21A9	OATP-B	—
SLC21A8	OATP8	—
SLC21A6	OATP-C	—
SLC21A11	OATP-D	—
SLC2112	OATP-E	—
SLC21A14	OATP-F	—
SLC22A1	OCT1	Small intestine, liver, kidney
SLC22A2	OCT2	Small intestine, kidney
SLC22A3	OCT3	Small intestine, liver, kidney
SLC22A16	OCT6	Testis, monocytes, lymphocytes, hematopoietic progenitor cells
SLC22A4	OCTN1	Brain, lymph nodes, testis, monocytes, CD4+-lymphocytes
SLC22A5	OCTN2	Brain, monocytes, CD4+-lymphocytes
SLC28A1	CNT1	—
SLC28A2	CNT2	—
SLC28A3	CNT3	—
SLC29A1	ENT1	—
SLC29A2	ENT2	—
SLC29A3	ENT3	—
SLC23A1	NCBT1	—
SLC23A2	NCBT2	—
SLC15A1	PEPT1	—
SLC15A2	PEPT2	—
SLC2A5	GLUT5	Small intestine
SLC2A1	GLUT1	—
SLC5A1	Sodium/gluc cotransporter	—
SLC25A6	ADP-ATP translocase	—
SLC25A5	ADP-ATP translocase 2	—
SLC25A3	Phosphate carrier protein	—
SLC25A37	Mitoferrin-1	—
SLC39A5	Zip5, zinc transporter	—
SLC39A9	Zip9	—
SLC19A9	MOT9, monoxcarboxylate transporter	—
SLC40A1	Ferroportin-iron	—
SLC14A1	Urea	—
SLC16A3	MOT4	—

^aRef. 53 and 66.

the concentration of protein, the concentration of free drug, and the drug's affinity for the binding sites of the protein molecule. Plasma protein binding studies allow the determination of the amount of unbound (free) drug concentrations in the blood. A low fraction unbound (f_u) value (e.g., $f_u < 0.1$) indicates that a compound is highly bound to plasma proteins. Plasma protein binding data should be considered with other *in vitro* data to determine the potential of a drug for efficacy in a clinical context. Low protein binding may be more favorable than high protein binding. However, factors such as solubility of the drug and nonspecific binding need to be considered when interpreting data.

These are basic concepts that are important to the understanding of the overall topic of drug distribution.

There are numerous *in vitro* methods for the determination of protein binding, including equilibrium dialysis, dynamic dialysis, ultrafiltration, ultracentrifugation, exclusion chromatography, and circular dichroism [82]. The three most widely used *in vitro* protein binding techniques in pharmaceutical research are those which allow high throughput, namely chromatographic separation, 96-well ultrafiltration, and 96-well equilibrium dialysis [83]. These are discussed further herein. The method of chromatographic separation, for example, using a HSA-immobilized column, separates multiple test compounds on the same column. This allows relative ranking by calculation of percent binding for each compound. This method is easy to set up and use and is relatively inexpensive. With the 96-well ultrafiltration method (an automated and rapid method for analyzing multiple compounds), percent binding is calculated. However, nonspecific binding of the analyte to the plastic housing or ultrafiltration membrane surface can affect the quality of the data. Nevertheless, it is frequently used in industry. The 96-well equilibrium dialysis method is the "gold standard" means of protein binding analysis [83]. Protein binding studies are traditionally performed with ^{14}C -labeled compounds. With the equilibrium dialysis method, the procedure is performed in a 96-well system. There is a membrane in each of the 96 wells with buffer solution added to one side of the membrane and an equal volume of plasma containing the test compound added to the other side of the membrane. The plate is incubated at 37°C with rotation for up to 24 h to allow the incubations to reach equilibrium. The long equilibration times sometimes required could be an issue if the analyte is not stable in plasma under these incubation conditions. Samples are removed from either side of the membrane for analysis by LC/MS/MS. The equation to calculate free fraction is as follows:

$$f_u = \frac{C_{\text{buffer}}}{C_{\text{plasma}}}$$

where C_{buffer} is the unbound compound concentration in buffer after dialysis, and C_{plasma} is the postdialysis plasma concentration [83]. This equation assumes that error added as a result of volume shift is negligible since potential error in the 96-well format is minimized.

The percentages of drug unbound (free) and bound to protein are calculated as follows [83]:

$$\% \text{Free} = f_u \times 100\% \text{bound} = (1 - f_u) \times 100$$

Recovery of test compound at the end of the study should theoretically be 100%. Any deviation from 100% may indicate binding of the compound to the dialysis equipment

or it could also indicate a solubility issue with the compound:

$$\text{Percentage recovery} = \frac{(\text{Buffer compartment [After dialysis]} + \text{Plasma compartment concentration [After dialysis]})}{(\text{Initial solution concentration [Buffer]} + \text{Initial solution concentration [Plasma]})} \times 100$$

Both plasma protein and RBC binding studies are conducted as a routine part of the ADME process. There are many representative examples of routine protein binding studies demonstrating both high and low binding compounds. However, one interesting example is the plasma protein binding studies conducted with radiolabeled dihydroartemisinin in rat and human plasma [84]. Dihydroartemisinin exhibits high binding capacity with both rat and human plasma proteins (76–82%). The concentration of total radioactivity in the plasma fraction is <25% of that in whole blood with total radioactivity distributed in RBCs about three- to fourfold higher than that in plasma, suggesting that the antimalarial potency of dihydroartemisinin (DHA) in treatment of blood stage malaria may relate to the high RBC binding.

4.6.2 Transporter Studies

Transporters and methods to investigate the role of transporters in the body has been discussed more extensively in the chapter titled *Solute Carrier (SLC) Family Transporters*. The objectives and strategies for conducting these types of studies will be related to their impact on drug development. The current methods for investigating protein binding, V_d , and other parameters are described in brief in the following sections.

4.6.2.1 Membrane-Based Assay Systems. There are several established *in vitro* methods to evaluate the role of efflux transporters in the distribution of a drug. The most simple of these methods is the use of “inside out” membranes and vesicles such as those from baculovirus-transfected Sf9 insect cells, which have been engineered to overexpress the efflux ABC transporters specifically. Substrates of the transporter are taken up into the vesicles in an ATP-dependent manner. The two main assays are the vesicular transport assay and the ATPase assay. The vesicular transport assay is a direct measure of transporter activity. Following incubation, vesicles can be separated from the incubation solution by rapid filtration through filters or nitrocellulose membranes. The test compound, trapped inside the vesicles, is retained on the filter. The amount of the transported molecule is determined by quantitative measurement with high performance liquid chromatography (HPLC), LC/MS/MS, radioactivity, or fluorescence measurement. This method is not suitable for lipophilic or adsorptive drugs; however, it can be used for “nonsticky” transporters regardless of basal ATPase activity. As an alternative assay method, ABC transporter membranes that are dependent on hydrolysis of ATP can be used with an ATPase assay to determine possible substrate or inhibitor properties of test compounds. The ATPase assay is an indirect measure of transporter activity. ATP hydrolysis yields inorganic phosphate, which can be measured by a simple colorimetric reaction. The amount of inorganic phosphate is directly proportional to the activity of the transporter. This assay system is simple to use and can be used in a high throughput manner. It is suitable for lipophilic drugs but not appropriate for use with transporters of high basal ATP activity such as MRP4, MRP8, bile salt export

pump (BSEP), and BCRP. This assay requires less transporter protein than the vesicle transport assay; however, high concentrations of compound are required.

The advantages of using membranes and vesicles are the simplicity of the system and the focus on a single transporter. When testing hydrophobic compounds, there can be a high degree of background binding of the compound to cell membranes in vesicle or cellular systems. However, these assays when used with other *in vitro* methods help the investigator to understand, more thoroughly, the relevant PK properties of new drugs.

4.6.2.2 Cell-Based Assay Systems. Cell-based transporter assays can determine if a test compound is a substrate or inhibitor of an individual transporter. Whole cell models have an advantage over membrane and vesicle assays as they can be utilized to study both uptake and efflux of test compounds. These systems can thus be used in studies to determine transport mechanisms. Bidirectional transport, namely apical-to-basolateral and basolateral-to-apical flux, can be measured to determine the transport of a test compound across the small intestine (Caco-2 cell line) or BBB. Caco-2 cells endogenously express numerous uptake transporters, which can make identifying uptake transporter substrates in these cells difficult. As an alternative approach, cell lines such as the epithelial cell line, Madin-Darby canine kidney (MDCK), can be specifically transfected to express recombinant uptake, efflux, or both transporters. For example, MDCK cells transfected with the MDR1 gene (MDCK-MDR1) express the efflux transporter, human Pgp. The MDCK-MDR1 cell line has superior transepithelial electrical resistance (TEER) values compared with Caco-2 and wild-type MDCK cells. Higher TEER creates a very tight cell system that allows for increased sensitivity in identifying Pgp substrates. Therefore, a transporter effect observed in the MDCK-MDR1 cell system may not necessarily be observed in the Caco-2 cell system. The lower TEER values of the Caco-2 cells create a more “leaky” system, which may be more representative of the human small intestine. Use of transfected cell lines such as MDCK-MDR1 allows focus on a single transporter and could help identify drug permeability issues early on in drug development.

Cells isolated from intact tissue (primary cells), such as hepatocytes isolated from liver tissue, can also be used to evaluate ADME properties of a compound, including the role of transporters in distribution. Primary cells are more difficult to maintain in culture than cell lines. In the case of hepatocytes, the sandwich culture method was developed to help maintain more *in vivo*-like properties such as cell polarity and shape and bile canaliculi reformation. One disadvantage of the cell culture systems is the static nature of the culture without “flow” properties. The different primary hepatocyte cell models such as suspension, monolayer, and sandwich culture formats are discussed in more detail in the chapter titled *Solute Carrier (SLC) Family Transporters*.

4.6.3 *In Vitro* Methods to Study the Brain Tissue Distribution

In vitro models of the brain have been developed for estimating unbound drug concentrations in the brain interstitial and intracellular fluids [85]. The method uses brain slices from rat and guinea pig for uptake studies and brain homogenate for binding studies. Agreement between *in vivo* and *in vitro* methods for estimating the unbound brain volume of distribution ($V_{u, \text{brain}}$) has been established by comparing *in vitro* data with microdialysis data *in vivo*. Microdialysis is discussed in more detail in Section 4.7.2.1.

The brain slice method is suitable for measuring tissue distribution of hydrophilic through to very lipophilic compounds in a high throughput manner [86] and measures the partitioning of drug between buffer and brain slice to yield, $V_{u, \text{brain}}$. The unbound drug concentration in the brain interstitial fluid, the most relevant measure of central drug exposure, can then be calculated from the measured total amount of drug in brain tissue using $V_{u, \text{brain}}$ as a conversion factor. Friden and colleagues have optimized their method and combined the use of brain slice and brain homogenate studies to define the intracellular to extracellular partitioning of unbound drug in the brain tissue [86,87]. Models to assess drug distribution in the BBB have been reviewed by Alavijeh *et al.* [82]. With respect to *in vitro* cell models of drug distribution in the brain, MDCK cells are considered a good predictor of BBB permeability as they have a high trans-membrane resistance causing tight junction between the cells, as exists in the BBB. Assessment of passive diffusion across the BBB and Pgp transport can also be studied with MDCK cells. The combination of the two parameters allows BBB permeation and active efflux out of the BBB to be modeled. However, the endothelial cells such as Caco-2 express a greater range of transporter molecules, which makes them much better suited for studies investigating different transporters within the BBB. *In vitro* methods assessing transport function in the liver and kidneys is discussed in the chapter titled *Solute Carrier (SLC) Family Transporters*.

4.7 IN VIVO METHODOLOGIES FOR DETERMINING DRUG DISTRIBUTION

Drug distribution can be difficult to determine *in vivo*, particularly in man where whole tissues or organs cannot be analyzed directly. Traditional methods, in addition to modern methods such as imaging and analysis, are discussed in the following sections. Many of these current techniques have been used in both animal and human studies. Most current tissue distribution studies consist of a combination of both traditional and modern methods.

4.7.1 *In Vivo* Studies in Preclinical Animals and *Ex Vivo* Organs

Drug distribution studies have been performed in preclinical species by collection and analysis of organs and techniques such as whole body autoradiography (WBA). Generally, a study would involve single oral or intravenous doses of increasing concentrations of radiolabeled drug being given to a preclinical species such as rat. Blood samples would be taken at time intervals from animals for pharmacokinetic analyses. For tissue distribution analysis, animals are euthanized at each time point and tissue and organ samples taken. Aqueous homogenates are prepared from most tissues and radioactivity measured in each. Feces and urine are collected from animals housed in metabolism cages and analyzed. Furthermore, blood, fat, the contents of the stomach, and contents of different sections of the intestine are also collected and analyzed. For example, radiolabeled dihydroartemisinin, a powerful antimalarial drug has been studied in rats [84] to determine pharmacokinetics, tissue distribution, and mass balance. Tissue distribution study methods are described in detail in the study by Xie *et al.* [84]. WBA is a drug distribution process using radiolabeled drug in live animals such as rat. It is a sensitive and quantitative method. The main advantage of WBA is that it

allows collection of maps of the spatial and temporal distribution of a drug in individual tissues and throughout the body. However, it is not possible to distinguish whether the radioactivity detected is from the drug or from its metabolites. Furthermore, it is limited in the identification of the distribution of more than one compound administered simultaneously. It is an invasive technique and analysis is postmortem, therefore a large number of animals are required.

In pharmacokinetic studies conducted in preclinical animals or in humans, the most common procedure is where a mixture of known amounts of labeled and nonlabeled drug are administered in experiments to evaluate the mass balance and tissue distribution of the drug. The total dose used in the radiolabel studies is usually a pharmacologically relevant dose. This dose can be determined from results of early efficacy studies. If a specific dose has not yet been established, a total dose of radioactivity could be 100 μCi , or less, providing that exposure of a specific tissue to the radiolabeled drug does not exceed radiation limits to avoid local radiation damage [87]. The most commonly used radioisotope is ^{14}C . It is highly stable and therefore no radiolytic or chemical decomposition of the compound occurs. Disadvantages of using radiolabeled compounds include the obvious potential health risk of radioactivity exposure to humans, the expense to synthesize and purify the radiolabeled compound, the time required for this process, and the further expense of correct handling and disposal of radioactive material.

There are also *ex vivo* and other *in vivo* models to study drug transport and V_d . *In vivo* models such as gene-knockout and transporter-deficient animal models are now used in drug distribution studies. Transporter knockout models can be used to establish the extent of the importance of the pathway removed by the knockout process to the overall clearance of a drug. In these knockout models, compensatory mechanisms may come into play by upregulation of other pathways involving alternative transporters and metabolizing enzymes, and this will complicate interpretation of results. Factors such as sex, species, strain, housing conditions, and diet can affect the outcome of these studies and also need to be considered when interpreting results and in cross-species comparisons [78].

There is particular importance in studying transporters in drug distribution not only due to the potential inhibition of a transporter by a comedication but also because of the genetic polymorphisms of transporters in the human population. Recently, matrix-assisted laser desorption ionization (MALDI) imaging mass spectrometry coupled with high definition histology has been used to follow the *in vivo* transport of unlabeled drugs within specific organ and tissue compartments [88]. The authors tracked and quantified the distribution of an inhaled reference compound, tiotropium, a bronchodilator used in the treatment of chronic obstructive pulmonary disease (COPD), within the lungs of dosed rats. Drug ion distribution patterns compared between adjacent tissue sections, showing tiotropium parent MS ions and fragmented daughter MS/MS ions, were dispersed into the lung and pleura, in a concentration gradient. For comparison, levels of drug measured in the lung compartments by a chemical extraction method were similar. Change in volume of distribution for any reason, such as a disease process, aging or genetic polymorphisms in transporter genes, for example, can lead to a significant change in drug efficacy or likelihood of toxicity, as well as a change in its half-life independent of a change in clearance. Comparing studies reported in the literature, Grover and Benet [89] concluded that the liver is a larger contributor to distribution volume than the kidneys, taking into consideration both uptake and efflux transporters.

4.7.2 Studies in Man

Classical methods for pharmacokinetic measurements were the major option to determine tissue distribution of a test compound in patients. In these studies, plasma levels of parent drug and metabolites are commonly determined following intravenous administration of a drug by infusion over time. For example, Fogli *et al.* [90] administered paclitaxel followed by gemcitabine to patients with non-small-cell lung cancer and were able to determine volume of distribution as well as other pharmacokinetic parameters to optimize their treatment regimens in phase I trials. In another interesting ADME study, the adipose tissue and plasma distribution of various antiretrovirals in HIV-1-infected patients was investigated [91]. Plasma samples were collected in a routine manner. Adipocyte samples were collected, as a by-product of liposuction plastic surgery in patients suffering from lipodystrophy. Protease inhibitor (PI) and nonnucleoside reverse transcriptase inhibitor (NNRTI) concentrations in plasma and adipocyte lysates were determined by HPLC assays coupled with either ultraviolet photodiode array or spectrofluorimetric detection. The concentrations of these compounds in adipocyte lysates indicated that in patients with effective nadir plasma concentrations of PI and NNRTI, PIs may diffuse into fat tissue. The authors concluded that NNRTIs have a high affinity for fat tissue and may accumulate there.

4.7.2.1 Microdialysis. The technique of *in vivo* microdialysis has become one of the major tools to sample endogenous and exogenous substances in extracellular spaces [92]. Microdialysis is a semi-invasive technique based on the passive diffusion of substances across a small semihollow fiber permeable membrane (a probe), connected to an inlet and outlet tubing with a small diameter to allow the interior of the probe to be perfused with a suitable carrier solution [93]. Although developed for use with intracerebral measurements, it is now used to measure drug disposition in many different tissues, organs, and biological fluids. One advantage is that the samples taken are generally stable due to the absence of enzymes in the sample. Therefore, samples can be analyzed after the procedure. Samples are commonly separated using liquid chromatography or capillary electrophoresis to isolate the drug and/or drug and its metabolites from endogenous compounds. Detection is usually by fluorescence, electrochemical, or mass spectrometry [93]. This method allows continuous monitoring of drug or metabolite concentrations over significant time periods. Extracellular concentration gradients caused by transport of the drug or perfusion differences can also be evaluated. Compared with other techniques, microdialysis does not cause fluid loss or pressure in the tissue being measured since the samples are replaced by fresh fluid. Cells, protein, and debris are excluded from the dialysate by the semipermeable membrane. This helps prevent enzyme degradation of the sample and maintains clean samples that can be analyzed without the need for a clean-up step.

The microdialysis technique was reviewed by Zhou and Gallo [92] and they discussed many examples where microdialysis has been used to measure tumor-specific drug concentrations compared with plasma drug concentrations, as an indicator of tumor response to chemotherapy. Microdialysis has also been used to measure the unbound drug concentration in biological fluids. This permits direct measurement of plasma protein binding *in vivo* [94]. Disadvantages include local damage to the tissue, circulation, and membrane barrier integrity. It has lower spatial and temporal resolution compared with some other techniques. If low flow rates are used in the experiment,

this technique may not be suitable for use with lipophilic compounds as they may stick to the probe.

4.7.2.2 Positron Emission Tomography. PET is a noninvasive technique that involves the patient being injected with a biologically active, radiolabeled drug (radiotracer) that localizes at targeted sites within the body. Radiation emitted from the site or sites of accumulation is detected by a bank of detectors to produce a three-dimensional image of the radiotracer of drug distribution. A positron-emitting radionuclide is used as the label in PET [95]. PET methods are very sensitive and allow determination of absolute radioactivity concentrations within tissues [96]. Rosso and coworkers [97] characterized the distribution of temozolomide, an important alkylating agent used in the treatment of brain tumors. These studies predicted normal brain and brain tumor temozolomide concentration profiles for different temozolomide dosing regimens allowing optimization of treatment schedules.

4.7.2.3 Magnetic Resonance Techniques. Two other imaging methods to measure drug disposition *in vivo* noninvasively are MRI and magnetic resonance spectroscopy (MRS).

MRI can be thought of most simply as the measuring and mapping of the magnetic properties of an object. Instead of measuring the magnetism point to point in the object, MRI can create an image that depends on the rates of magnetization for each point [95]. MRS produces spectra that depict individual changes in the concentrations of parent drug and metabolite for a sensitive volume. However, there is no simple means with MRS to measure absolute concentrations [96]. Compared with PET, MRS imaging has low spatial resolution and average drug concentrations are usually obtained from large regions of interest (e.g., whole brain).

Human blood-oxygenation-level-dependent (BOLD) functional magnetic resonance imaging (fMRI) is based on the fact that changes in the oxygen level in the blood can affect the fraction of hemoglobin in the deoxygenated state. This can be used to create image contrast [98], and the technique has been used to investigate how anxiolytics such as benzodiazepines affect amygdala functioning in healthy volunteers [99]. This technique is relatively novel and its range of applications is still being established.

4.7.2.4 Modeling. PBPK models are also used to predict ADME, including drug distribution. Some of these are discussed in detail in the study by Theil *et al.* [100] and mentioned elsewhere in this chapter. Mathematical models to reflect different aspects of drug distribution are described in the literature. Examples are models for plasma protein binding and how it affects rates of uptake of drugs to the brain [101] and models to predict the distribution of drugs delivered intracranially to the brain [111].

4.8 CALCULATING DISTRIBUTION

The relationship between the concentrations of a drug found in the different parts of the body with time after administration of a dose is known as *pharmacokinetics*, and an understanding of pharmacokinetics is crucial to enable both optimal and safe use of drugs in patients and new product development. Pharmacokinetic data are obtained by

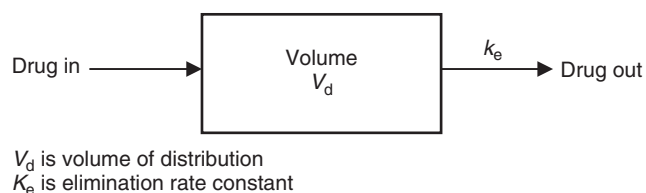


Figure 4.6 One-compartment model with a well-perfused central compartment.

measuring the concentration of drug in various tissues and body fluids over a period of time and fitting them to a mathematical model of the body.

4.8.1 One-Compartment Model

Although the movement of drug between the compartments of the body is a complex dynamic process, a simple analysis can be made by assuming that all the body compartments are in rapid equilibrium with a central compartment, which is usually interpreted as the blood, and that the concentration of a drug is constant throughout this compartment (Fig. 4.6). In this simple model, the body is considered to be a single well-stirred compartment, and it is assumed that if a drug is injected (e.g., intravenous injection) into this compartment, it will instantaneously distribute throughout it. Thus, the concentration of drug at zero time (C_0) can be calculated, and if C_0 is known, then V_d , the volume of distribution, can be calculated:

$$C_0 = \frac{D}{V_d}$$

where V_d is the volume of distribution and D the dose.

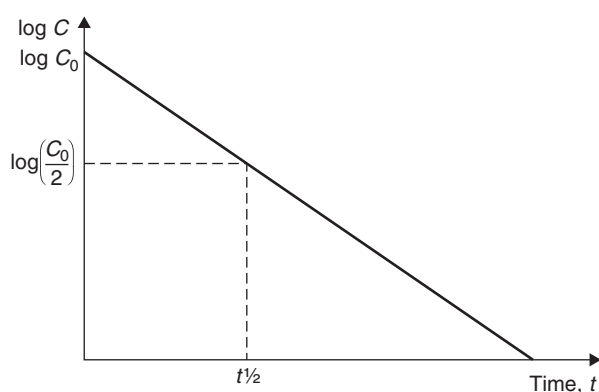
V_d does not represent the volume of an anatomical compartment, but links the total amount of drug in the body to its concentration in blood plasma. As described in Section 4.4.1, total body water is divided into the plasma water, the interstitial water, the blood cell/transcellular fluid, and the intracellular water, and the way in which a drug distributes between these will affect its plasma concentration. If the drug only distributes into plasma water (about 3 L in man), then its plasma concentration will be higher than if it distributed into all the extracellular water (about 14 L) or indeed total body water (about 40 L).

Although the V_d is a mathematical parameter, without absolute physiological meaning, it gives important information about a compound. A very high V_d value indicates that the drug is localized or sequestered in a storage site such as fat or bone. On the other hand, if the V_d is low, then it indicates that the drug is retained in the plasma (an example of various drugs and their corresponding V_d can be found in Table 4.9).

Clearance of the drug from the central compartment takes place at a rate determined by the elimination rate constant, K_{el} , which represents removal of drug by all processes from the biological system. For many drugs, a plot of log drug concentration versus time yields a straight line and indicates first-order kinetics where the rate of elimination is proportional to the drug concentration (Fig. 4.7). This means that the time taken for the plasma concentration of drug to halve will always be the same regardless of where on the curve the original value is taken. This time is referred to as the *half-life* of

TABLE 4.9 Values of Volume of Distribution for Selected Drugs

Drug	V_{ss} (L)
Humira (mAb)	5
Warfarin	8
Gentamicin	15
Phenytoin	50
Cyclosporine	300
Digoxin	400
Desipramine	3000
Quinacrine	40,000

**Figure 4.7** Theoretical log plasma drug concentration versus time curve for a one-compartment model.

the drug ($t_{1/2}$). The elimination rate constant is inversely related to the half-life of the drug:

$$K_{el} = \frac{0.693}{t_{1/2}}$$

The faster the clearance of the drug, the faster one half of the concentration of the drug will be cleared and the shorter will be the half-life. If the pathways of elimination of a drug are saturated (e.g., a cofactor may become rate limiting), then the elimination rate becomes independent of drug concentration, and the drug is being cleared as fast as possible. This is seen with the metabolism of acetylsalicylic acid [102] where the conjugation pathways are saturable and with ethanol metabolism where the cofactor, nicotinamide adenine dinucleotide (NAD), is the rate-limiting factor.

4.8.2 Two-Compartment Model

The simple single-compartment model assumes that the rates of absorption, metabolism, and excretion are all directly proportional to the concentration of drug in the compartment from which the transfer is occurring. However, the different organs of the body,

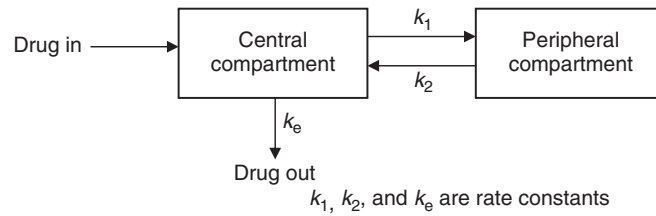


Figure 4.8 The two-compartment model with a central and peripheral compartment.

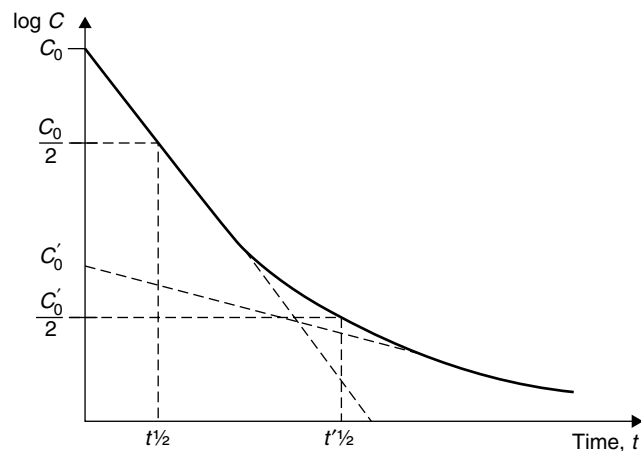


Figure 4.9 Theoretical log plasma drug concentration versus time curve for the two-compartment model.

for example, the brain, body fat, and muscle tissue, are very different in terms of their blood supply, permeability of their capillaries to drugs, and the partition coefficient for drugs, and all these differences affect the time course of drug distribution. A two-compartment model, which introduces a separate peripheral compartment to represent the tissues, in contact with the central blood compartment, resembles the real body situation more closely (Fig. 4.8). In this case, the plot of log drug concentration (in the central compartment, i.e., in the blood) shows two distinct slopes (Fig. 4.9). The first steeper slope represents the distribution of the drug from the blood to the tissues, which results in a rapid fall in the blood concentration, and the second slower phase is the elimination of the drug from the central compartment. The half-life for the slow phase provides an estimate of the K_{el} . If a drug is rapidly metabolized, then the two phases are not well separated.

The mathematical analysis of a two-compartment model is more complex than a one-compartment model, and in practical terms, drugs are not usually given intravenously. Where a drug is administered by a route other than intravenously, the plasma concentration profile will be a composite of absorption in addition to distribution and elimination. For a one-compartment model, the absorption rate constant (K_a) and the elimination rate constant K_{el} can be calculated by extrapolation of the appropriate part of the log dose curve (Fig. 4.10), but in the two-compartment model, absorption, distribution,

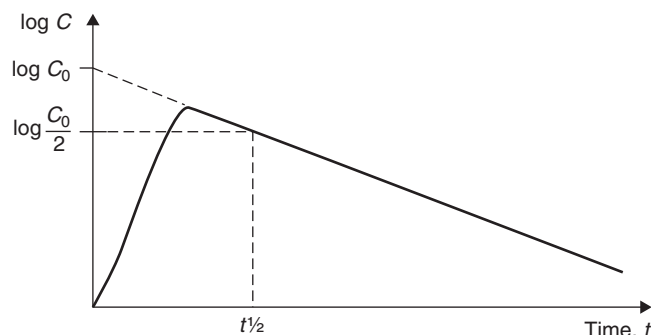


Figure 4.10 Theoretical log plasma drug concentration versus time curve for a one-compartment model with an initial absorption phase.

and elimination are occurring simultaneously and the curve is difficult to analyze satisfactorily. Pharmacokinetic data from orally administered drugs are also difficult to analyze. The administered dose of the drug may not be the same as the dose which is absorbed and available systemically. This is due partly to incomplete absorption from the GI tract and to first-pass metabolism mainly by the liver. The extent of first-pass metabolism by the liver can be extensive. For example, approximately 70% of the drug propranolol is metabolized before it reaches the systemic circulation when given orally [103]. The dose of a drug actually absorbed can be quantified by measuring the area under the plasma concentration versus time curve (AUC), which is a measure of the total body load of a drug. The oral bioavailability can be compared by

$$\text{Bioavailability} = \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{iv}}}$$

4.8.3 Hepatic Drug Clearance

The main means of removing a drug from the central compartment is by hepatic drug metabolism. The ability of the liver to eliminate a drug is related to the hepatic blood flow and the intrinsic clearance of the liver. When intrinsic clearance is much greater than hepatic blood flow, the liver metabolizes and extracts all the drug from the blood presented to it, and the higher the blood flow through the liver, the more drug will be extracted from it. On the other hand, if the intrinsic clearance is much less than blood flow, then the hepatic clearance is dependent on the rate of metabolism. These two extremes are called *flow-limited* and *metabolism-limited extraction*, respectively. Examples of the former are propranolol, and lignocaine, and of the latter antipyrine. The ability of the liver to clear a drug from the blood is dependent not only on the metabolic activity of the organ but also on factors that influence the hepatic blood flow, such as cardiac output and redistribution of blood during exercise.

4.8.4 Tissue Distribution Prediction Models for PBPK

PBPK modeling attempts to predict ADME parameters using mathematical modeling of *in vitro* and preclinical derived data. There are some main assumptions to generic PBPK models related to compound distribution. These include the following:

- Distribution is mainly a passive process with no significant active transport involvement.
- Distribution into tissues is rate limited primarily by site-specific blood flow.
- Each tissue is viewed as a well-stirred compartment.
- Permeability across the membrane does not hinder or limit the ability of compounds to distribute into tissues.

Several mathematical models to predict distribution of drugs into various organs and tissues have been proposed. Input data generally required for these models include both parameters related to the compound and to the system. Compound-related input parameters are $\log P$, pK_a , and blood plasma partitioning, while system-related parameters are the volume of lipids, phospholipids, and water in plasma and tissues, and the pH. The models aim to estimate the partition coefficients (K_p). Currently, it is common practice to use a number of the proposed models for predicting distribution parameters and compare them across known data sets to determine which model is most suitable. Five commonly used distribution models are described in Table 4.10. A major limitation with PBPK modeling predictions is lack of accounting for the presence of active uptake components, which can lead to a lack of correlation with *in vivo* data derived from preclinical species. Efforts to improve prediction models by incorporating transport parameters are on-going and are described in the literature [104,105].

4.9 CLINICAL IMPLICATIONS OF ALTERED DRUG DISPOSITION

Understanding the role of disposition in the pharmacokinetics of a drug is essential both for drug development and also for clinicians. The latter group must understand how the dosage of products is ascertained so that they can use the drug optimally in all groups of patients, appreciate that subgroups of patients may be at risk during therapy, and understand any limitations. This is particularly important for drugs with a low therapeutic index. An example of a group at risk is patients with deficient renal function. It is crucial to carefully monitor their response to drugs excreted predominantly by the kidney, for example, digoxin and aminoglycoside antibiotics, to avoid adverse effects.

4.9.1 Absorption

Absorption plays an important role in a clinical context and this can be illustrated, for example, in that the low oral bioavailability of many drugs limits their route of administration. For example, heparin is not absorbed from the GI track because of its size and charge, and it is therefore administered by injection, intravenously in emergency situations, and also routinely by patients at home subcutaneously [112]. On the other hand, treatment of *Clostridium difficile* infection resident in the gut by vancomycin has taken advantage of its poor GI absorption, which gives rise to high local concentrations in the colon [113].

4.9.2 Renal Elimination

Treatment of poisoning or drug overdose is often concentrated on minimizing absorption and also on increasing the elimination of the toxin. In many cases, the latter may

TABLE 4.10 Commonly Used Models for Predicting Distribution Parameters

Prediction Model (Reference)	Input Data	Output Data	Important Assumptions	Accurately Predicts	Mispredictions/ Additional Information
Poulin and Theil [106]	$\log P$, fup, pK_a (compound)	Partition coefficients Pt:p based on volumes of lipid, phospholipids water, and plasma proteins of each tissue	Lipid subcompartment mainly phospholipids, passive diffusion. Equation 4.1: homogeneous distribution; Equation 4.2: nonhomogeneous distribution	Acids, bases, and neutral compounds	Zwitterions and some acids and bases. Improvements to method have been published
Berezhkovskiy [107]	$\log P$, fup, pK_a	Same as Poulin model	Same as Poulin and Theil	Modified Poulin model	Additionally considers the exchange of drugs between compartments and peripheral drug elimination
Rodgers <i>et al.</i> [108]	$\log P$, fup, pK_a , BP	Same as Poulin model	Passive distribution and nonsaturating mechanisms. Electrostatic interactions between ionized compounds (bases) and anionic phospholipids (based on B:P). Interactions with intracellular neutral phospholipids and neutral lipids (based on olive oil–water partition). Consideration of interactions with intracellular neutral phospholipids and neutral lipids	Moderate to strong bases and improved prediction for zwitterions	Peripheral elimination, binding to tissue constituents other than phospholipids and when there is active transport

Willman <i>et al.</i> , PK-Sim [109]	$\log P$, f_{up} , pK_a , estimated RBC, membrane affinity	Same as Poulin model	Well-stirred system	Moderate to strong bases	<i>In silico</i> measured membrane affinity is used as a marker of lipophilicity
Schmitt [110]	$\log P$, f_{up} , pK_a , membrane affinity	Same as Poulin model but includes neutral lipids, neutral and acidic phospholipids, and proteins of each tissue	Lipid subcompartment consists of neutral lipids, neutral phospholipids, and acidic phospholipids	Class- independent model	Recommends use of membrane affinity to describe binding to phospholipids, if unknown can apply $\log P$ as an approximation. Accounts for electrostatic interactions between charged molecules at physiological pH with acidic phospholipids. Considers the difference in pH of tissues and plasma

be achieved by altering the pH of the urine. For example, acute aspirin or phenobarbitone poisoning is treated by alkalization of the urine. To achieve this, treatment with intravenous bicarbonate infusion is used and this may raise the urine pH between 7.5 and 8.1. As a result, the plasma half-life of aspirin has been shown to decrease from 19.4 h in control patients to 5.0 h in the treated group providing an effective means of removing the toxin [114]. Similarly, the renal clearance of phenobarbitone can be increased from 0.8 to 5.1 mL/h/kg in patients treated with sodium bicarbonate [115]. Although, theoretically, similar advantages may be gained from acidification of the urine for accelerating the clearance of basic drugs such as amphetamine or phencyclidine, there are significant risks associated with this procedure, such as acute renal failure and acid–base/electrolyte disturbances. Acidification of urine is therefore not recommended as a therapeutic method for treatment of overdose.

4.9.3 Transporter-Mediated Distribution

The distribution of many drugs is regulated by their uptake and efflux into body compartments and organs regulated by their affinity for various transporter systems as discussed in Section 4.5. Interference with transporter systems was used to clinical advantage during the World War II when penicillin was still in short supply. Doctors maximized its efficacy by coadministration of probenacid, which competitively inhibits its secretion from the renal tubules [116]. These transporters also of course transport endogenous ligands, for example, bilirubin, and indeed Campbell and coworkers [117] have suggested that the unconjugated hyperbilirubinemia induced during indinavir, rifamycin SV, and cyclosporine A therapy may be partly caused by competition and resultant inhibition of OATP1B1-mediated bilirubin uptake.

Coadministration of drugs is commonplace in pharmacy, and adverse drug–drug interactions can result in changes in both pharmacokinetics and pharmacodynamics. These interactions can occur during GI absorption, by interference with plasma protein binding, or with carrier-mediated transport (including hepatic or renal uptake and biliary or urinary excretion) or by metabolism. The latter mechanism is well established and has received a great deal of attention (reviewed by Campbell *et al.* [117] and discussed in detail elsewhere in this volume); however, the hepatic clearance of many poorly metabolized drugs has been found to be determined by hepatic uptake. Competition between different classes of drugs for the uptake transporter mechanisms is recognized as a mechanism responsible for several clinically significant drug–drug interactions. One of the classes of drugs involved in these interactions is the cholesterol lowering 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, commonly referred to as the *statins*. The major target site of action for these drugs is the liver and they are taken up into hepatocytes by the organic anion transporter polypeptide OATP family, as mentioned previously in Section 4.5.1.2. Coadministration of various drugs has been reported to increase the plasma concentrations of statins [118] and this can occasionally lead to severe side effects such as myopathy and rhabdomyolysis [119,120]. Cyclosporin A interacts with the statins that are not appreciably metabolized in the liver, and the interaction with cerivastatin [121] and pitavastatin [122] has been demonstrated to occur because the cyclosporine A inhibits OATP1B1-mediated uptake. Hirano and coworkers [122] have predicted the degree of inhibition of OATP1B1 in humans *in vivo* by relating the blood unbound fraction of the inhibitor, the concentration of the inhibitor present at the inlet to the liver, and the *in vitro* K_i value for

inhibition of transport by OATP1B1 (measured in cultured HEK293 cells) for several compounds. As a result of their calculations, they have suggested that cyclosporine A, rifampicin, rifamycin SV, clarithromycin, and indinavar would interact with pitavastatin in a clinical situation to increase the plasma concentration, affect its cholesterol lowering activity, and more importantly cause side effects. Caution should also be advocated with inhibitors of OAT-mediated transport such as cephalosporins [122]. The occurrence of drug–drug interactions through competition and inhibition of transporter mechanisms has been extensively reviewed by Shitara *et al.* [123], and they constitute a major contribution to clinical adverse drug reactions, especially when the incidence of polypharmacy is considered.

4.10 SUMMARY

All the parameters affecting distribution, that is, physicochemical and physiological factors, and the action of metabolism and transporters, act together to determine the volume of distribution of drugs, and an understanding of these processes leads to better decision making about drug development and therapeutic use by pharmaceutical scientists and clinicians. To improve this decision-making process, the way forward should be to develop better models for future use concentrating on *in vitro* methods that allow accurate extrapolation to the *in vivo* patient situation. Strategies should be in place to encourage development in the areas of safety evaluation of candidate pharmaceuticals and minimize attrition in the drug discovery process.

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5 ABC Drug Transporters and Their Impact on Drug Disposition/Drug Sensitivity and Resistance

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5.1	Summary	1
5.2	Introduction to the ABC family of transport proteins	2
5.3	Transport of small molecules by ABC proteins	7
5.4	Influence of ABC activity in drug resistance	13
5.5	Influence on adverse reactions and drug–drug interactions	17
5.6	Modulation of abc protein function	17
5.7	Transcriptional regulation of the ABC genes	19
5.8	Polymorphisms—effect on gene expression and protein function	20
5.9	Conclusions and future perspectives	27
	References	28

5.1 SUMMARY

Of the 49 members of the ATP-binding cassette (ABC) family of proteins, the majority harness the energy of ATP hydrolysis to facilitate a unidirectional efflux of substrates from the cytoplasm and inner leaflet of the lipid bilayer to the extracellular space. Each of these proteins share highly conserved amino acid sequences that permit their classification into the ABC superfamily. These motifs lie within the nucleotide binding domains (NBDs) and include the Walker A, Walker B, and Signature C motifs. Importantly, their structures differ substantially in the substrate binding regions, ensuring the transport of a wide range of substrates. A large number of small-molecule therapeutics have been identified as substrates of at least one ABC transport pump. P-glycoprotein (P-gp), which is encoded by the *ABCB1* gene; multidrug-resistance-associated protein 1 (MRP1), which is encoded by the *ABCC1* gene; and breast cancer resistance protein, which is encoded by the *ABCG2* gene, in particular, are known to confer a multidrug resistance (MDR) phenotype. Therapeutics vulnerable to efflux

by this family of proteins include the anthracycline antibiotics and tyrosine kinase inhibitors used in the treatment of cancer, antivirals used to treat HIV, antihistamines used in the treatment of allergies, and immunosuppressants used to prevent graft rejection. Despite their well-established role in the onset of drug resistance, reversal of the MDR phenomenon by targeting the proteins through molecular mechanisms such as small interfering RNA (siRNA) or small-molecule inhibitors such as verapamil, or the more specific second- and third-generation inhibitors such as dexverapamil and tariquidar, respectively, have yielded limited results. To date, the ABC transporters are not targeted in adjuvant therapy and MDR continues to hamper many chemotherapy regimes. Further confounding the role of these proteins in drug response is genetic variation, particularly, single nucleotide polymorphisms (SNPs). This variation, which is often evident in populations of distinct evolutionary history, may manifest itself either through differences in drug efficacy between populations or through the onset of adverse effects. While the effect of such genetic variation is often subtle, loss-of-function mutations in the ABC genes are also common and may lead to severe diseases. Examples include cystic fibrosis (CF) and Tangier disease, which result from mutations in *ABCC7* and *ABCA1*, respectively. Each member of the ABC superfamily contributes to drug resistance and/or drug response in a unique manner that is dependent on the protein location, the specific drug, and the genetic history of the individual. Within this chapter, the role of the ABC transport proteins on drug efficacy and resistance is explored. Efforts to disrupt ABC-mediated drug resistance are discussed, and the influence of polymorphisms and mutations on protein function is also assessed.

5.2 INTRODUCTION TO THE ABC FAMILY OF TRANSPORT PROTEINS

5.2.1 Classification of the ABC Family

The ABC superfamily is the largest family of membrane-bound proteins in biology. To date, 49 members have been identified, and although some have functions other than substrate transport across lipid bilayers, each possess key sequence motifs and ATP-binding domain organization that permits their classification into one of seven subclasses, ABCA–ABCG [1,2]. While many of the genes that encode the ABC proteins are scattered throughout the genome, several reside in close proximity to each other, most often encoding members of the same subclass. Furthermore, while some of the ABC transport proteins are expressed ubiquitously throughout the body, others remain localized to specific tissue types where they transport physiological substrates that are required by that particular tissue or organ. A complete summary of ABC localization and function is presented in Table 5.1.

5.2.2 Protein Structure and Function

In eukaryotic cells, the majority of the ABC transport proteins are responsible for a unidirectional, active transfer of substrates across lipid bilayers, from the cytoplasm to the extracellular space. In some instances, where ABC proteins are localized to the lipid bilayer membranes of intracellular vesicles such as lysosomes or endosomes, substrates are trafficked from the cytoplasm into the vesicular compartments for further processing or efflux into the extracellular space [12,13]. Members of the ABCE and

TABLE 5.1 Location of Genes Encoding Members of the ABC Superfamily of Proteins, their Cellular/Tissue Localization and their Physiological Substrates

Gene Name	Gene Location	Cellular/Tissue Localization	Physiological Substrate/Function	References
ABCA1	9q31.1	Brain and liver	Lipids (HDL and cholesterol)	1–4
ABCA2	9p34	Brain, monocytes	Steroid derivatives, lipids	
ABCA3	16p13.3	Lung (alveolar type II cells)	Pulmonary surfactant	
ABCA4	1p22.1-p21	Retina	Retinylidene phospholipid complexes	
ABCA5	17q24	Intracellular localization; skeletal muscle, kidney, liver, placenta	Unknown; possibly lysosomal substrates	
ABCA6	17q24	Ubiquitous	Unknown; possibly macrophage lipids	1–3, 5, 6
ABCA7	19p13.3	Myelolymphatic tissues, keratinocytes	Phospholipids, including phosphatidylcholine	
ABCA8	17q24	Heart, skeletal muscle, liver	Unknown	
ABCA9	17q24	Ubiquitous	Unknown	
ABCA10	17q24	Ubiquitous, particularly in the intestinal tract	Unknown; possibly lipid transport	
ABCA12	2q34	Keratinocytes, stomach	Lipids	
ABCA13	7p11-q11	Trachea, testis, bone marrow	Unknown	
ABCB1	7q21	Intestine, liver, kidney, placenta, Blood brain barrier (BBB)	Multidrug resistance	
ABCB2	6p21	All cells	Peptide transport	
ABCB3	6p21	All cells	Peptide transport	
ABCB4	7q21.1	Liver	Unknown	1–3, 5
ABCB5	7p14	Ubiquitous	Unknown	
ABCB6	2q36	Mitochondria	Iron transport	
ABCB7	Xq12-q13	Mitochondria	Fe/S cluster transport	
ABCB8	7q36	Mitochondria	Unknown	
ABCB9	12q24	Heart, brain	Unknown	2, 7, 8
ABCB10	1q24	Mitochondria	Unknown	
ABCB11	2q24	Liver	Bile salts	
ABCC1	16p13.1	Ubiquitous	Organic anion efflux	1–3, 5

(continued overleaf)

TABLE 5.1 (Continued)

Gene Name	Gene Location	Cellular/Tissue Localization	Physiological Substrate/Function	References
ABCC2	10q24	Liver, kidney, intestine	Organic anion efflux	
ABCC3	17q21.3	Pancreas, kidney, intestine, liver, adrenal gland	Organic anion efflux	
ABCC4	13q32	Prostate, testis, ovary, intestine, pancreas, lung	Nucleoside transport	
ABCC5	3q27	Ubiquitous	Nucleoside transport	
ABCC6	16p13.1	Liver, kidney	Unknown	
ABCC7	7q31.2	Exocrine tissue	Chloride ion channel	
ABCC8	11p15.1	Pancreas	Sulfonylurea receptor	
ABCC9	12p12.1	Heart, muscle	Unknown	
ABCC10	6p21	Liver, heart, kidney	Unknown	
ABCC11	16q11-q12	Ubiquitous	Unknown	
ABCC12	16q11-q12	Ubiquitous	Unknown	
ABCD1	Xq28	Peroxisomes; ubiquitous	Acyl CoA, VLCFAs	9
ABCD2	12q11-q12	Peroxisomes; ubiquitous	VLCFA CoAs	
ABCD3	1p22-p21	Peroxisomes; ubiquitous	LCFA CoAs	
ABCD4	14q24.3	Peroxisomes	Unknown	
ABCE1	4q31	Ovary, testes, spleen	Oligoadenylate binding protein	2,10
ABCF1	6p21.33	Ubiquitous	Not Applicable (NA)	2
ABCF2	7q36	Ubiquitous	NA	
ABCF3	3q25	Ubiquitous	NA	
ABCG1	21q22.3	Adipocytes, macrophages	Cholesterol and Phospholipids	2,11
ABCG2	4q22	Placenta, intestine, breast, liver	Toxin efflux, drug resistance	
ABCG4	11q23	Adipocytes, macrophages	Cholesterol and phospholipids	
ABCG5	2p21	Liver hepatocytes, enterocytes in small intestine and colon	Lipophilic cholesterol	
ABCG8	2p21	Liver hepatocytes, enterocytes in small intestine and colon	Lipophilic cholesterol	

Abbreviations: VLCFA, very-long-chain fatty acid; LCFA, long-chain fatty acid.

Source: Adapted from Ref. 2.

ABCF subclasses do not function as membrane-bound transporters but are instead involved in ribosome biogenesis and translation initiation and elongation [10,14]. As these two subclasses do not influence the pharmacokinetics of drugs, they are not discussed further in this chapter.

Functional ABC transport proteins possess a minimum of two transmembrane domains (TMDs) with each TMD containing four to eight transmembrane (TM) helices. These not only permit a passageway for substrate transport but also form a cavity for drug binding known as the ligand binding domain (LBD) [3]. Attached to the TMDs are cytosolic NBDs that facilitate the binding and hydrolysis of ATP. By harnessing the energy of ATP hydrolysis, active transport of substrates across lipid bilayers, and often against chemical gradients, may occur [5]. Although the specific mechanism of substrate transport has not been completely defined, it is believed that a conformational change in the arrangement of the TMDs occurs following NBD dimerization. This subsequently alters substrate binding affinity and opens up the LBD to the extracellular space [15].

The NBDs, unlike the TMDs, are highly homologous across the ABC superfamily and feature a number of sequence motifs essential for protein function. The Walker A motif and the A-loop, which is a single tyrosine residue ~25 amino acids upstream of Walker A [16], have both been shown to be essential for ATP binding and hydrolysis [5,17,18], while other sequences including Walker B, the D-loop, H-loop, Q-loop, and the Signature C motif (LSGGQ), which is unique to the ABC transport proteins [19], are believed to bind diatomic metals such as Mg^{2+} and contribute to the binding and hydrolysis of ATP via their arrangement during NBD dimerization [16,20]. ATP initially binds to only a single NBD before dimerization of the two NBD occurs [21]. During dimerization, ATP is sandwiched between the two NBDs, in a binding cavity composed of the Signature C motif and D-loop from one NBD and the Walker A, Walker B, A-loop, H-loop, and Q-loop from the other NBD [22].

A number of ABC transport proteins are known as *half-transport proteins* and comprise only a single TMD and NBD each (Fig. 5.1) These include ABCB2, ABCB3, ABCB6, ABCB7, ABCB8, ABCB9, and ABCB10, as well as the four members of the ABCD subclass and the five members of the ABCG subclass [3]. In these cases, transport function is only acquired after dimerization between two or three half-transport protein members of the same subclass. In some instances, a homodimer may form, such as with ABCD1 [23] and BCRP (breast cancer resistance protein) [24]. In other cases, however, heterodimer complexes will be formed before integration into the lipid bilayers. This protein interaction was shown to exist between ABCG5 and ABCG8 [25], and it is well established that ABCB2 forms a functional heterodimer with ABCB3 [3].

With the exception of those in the ABCE and ABCF subclasses, the remaining ABC proteins are known as *full transporters*, each possessing at least two TMDs and two NBDs (Fig. 5.1). Each member of the ABCA subclass possesses this general structure as does the well-established ABC transporter P-gp, which was originally discovered in 1976 in MDR Chinese hamster ovary (CHO) cells [26]. The crystallization of mouse P-gp, which features 87% sequence homology to human P-gp, highlighted many of the structural features that give rise to protein function. These included substrate “entry portals” that are open to the inner leaflet of the lipid bilayer and ligand binding cavity of ~6000 Å³ that houses 73 residues capable of substrate binding [27]. A smaller group of “full transporters” are all classified into the ABCC subclass feature an additional N-terminal TMD and linker region (Fig. 5.1). These include ABCC1, ABCC2, ABCC3,

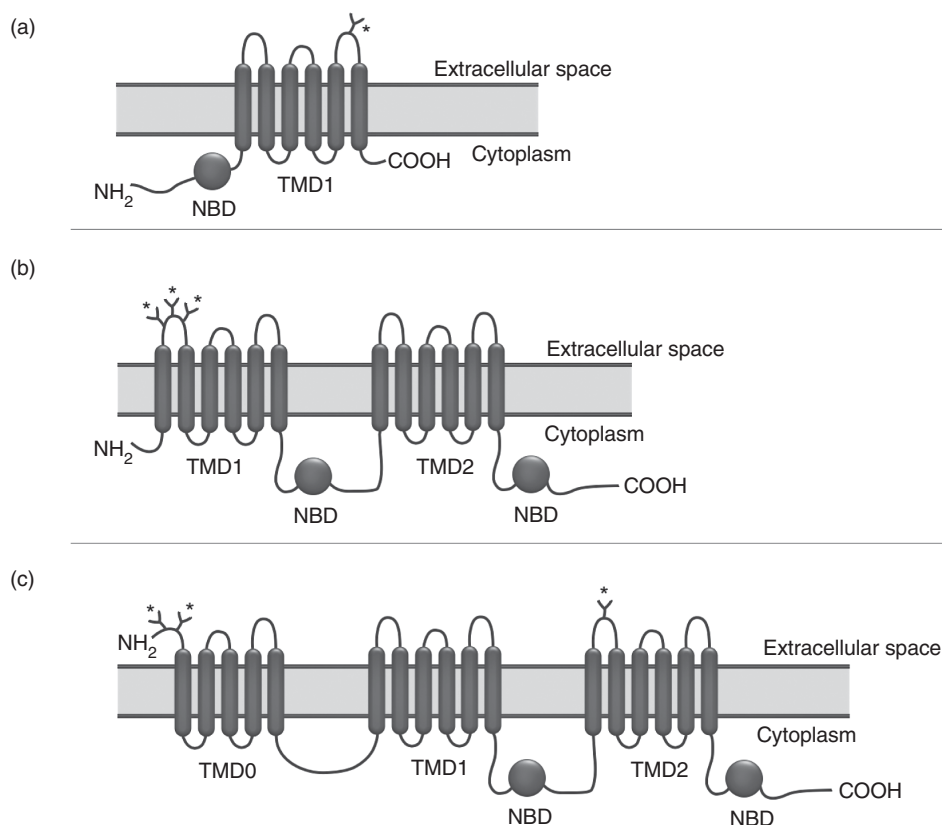


Figure 5.1 Schematic diagram showing the topology of the three major ABC transport proteins. (a) ABCG2 (BCRP) is a half transporter, comprising only a single transmembrane domain made up of six transmembrane helices and a single cytoplasmic nucleotide binding domain. (b) ABCB1 (P-glycoprotein) is a full transporter with two transmembrane domains and two nucleotide binding domains. (c) ABCC1 (MRP1) is also a full transporter but with an additional transmembrane domain (TM0) comprising five transmembrane helices. Each of these proteins contains glycosylation sites that are indicated by “*.” *Source:* Adapted from Ref. 5. (See color insert.)

ABCC6, ABCC8, ABCC9, and ABCC10. MRP1 is one of the most well-established ABC transport proteins possessing this structural property. Sharing less than 20% amino acid identity with P-gp [28], MRP1 features not only an additional TMD, which comprises five TM segments [29], but also a 13-residue amino acid sequence deletion between the Walker A and Walker B sequence motifs in the NBD1 [28]. The importance of this deletion was made evident when it was shown that insertion of the 13 residues from P-gp renders MRP1 inactive [30]. Although the additional cytoplasmic linker region (L_0) was shown to be vital for substrate transport, loss of TMD₀ does not greatly affect substrate transport [31]. Two other “full transporters” possessing an additional TMD are the sulfonyleurea receptors ABCC8 and ABCC9. These proteins function only in complex with their K^+ channel counterparts despite possessing three TMDs. Each protein remains in the cytoplasm until being activated by ATP/ADP, at which point hetero-octameric complexes are formed with the potassium channels Kir6.1 and Kir6.2,

respectively. It is in this position that ABCC8 and ABCC9 regulate the permeability of these potassium channels [32].

5.3 TRANSPORT OF SMALL MOLECULES BY ABC PROTEINS

5.3.1 Specificity of Substrate Binding

Most pharmacological substrates are transported by more than one ABC protein. As highlighted in Table 5.2, many anticancer drugs are vulnerable to efflux via four well-researched ABC drug transporters; P-gp, MRP1, MRP2, and BCRP. For example, the well-established topoisomerase poisons, etoposide and teniposide, are substrates of these three ABC drug transporters, while the taxanes, paclitaxel and docetaxel, are not known to be substrates of either MRP1 or BCRP, yet are transported by P-gp [5].

Generally, P-gp will transport neutral or positively charged hydrophobic molecules, whereas MRP1 is predominately an anionic transporter [51]. Binding and efflux of substrates is, however, selective, and the structural properties of substrates, in combination with the arrangement of amino acids within the LBDs, will determine this specificity. Great efforts have been made, with varying degrees of success, to establish the basis for selectivity in drug binding, particularly with respect to P-gp. Through analytical comparison of 100 known P-gp substrate structures, Seelig and colleagues attempted to determine the structural properties of P-gp substrates. A theoretical model was presented that was based on the spatial separation of essential electron-donor groups, and in this model, it was postulated that each hydrophobic substrate must feature two electron-donor groups $2.5 + 0.3$ Å apart, two electron-donor groups $4.6 + 0.6$ Å apart, or three electron-donor groups with the outer two groups separated by $4.6 + 0.6$ Å [52,53].

Alternative methods focused on mapping the drug substrate binding regions of P-gp, using photoaffinity analogs of established P-gp substrates. Such methods revealed several specific binding sites within the LBD. For example, [125 I]iodomycin, a derivative of daunorubicin, was found to be bound to amino acids 230–312 (located in TM helices 4 and 5 and the cytoplasmic loop joining them) in isolated hamster P-gp [54]. The prazosin analog [125 I]iodoarylazidoprazosin was found to bind to three sites (amino acids 248–312 in TM helices 4–5, 758–800 beyond TMD8, and 1160–1218 in the second NBD) following tricine/PAGE and HPLC separation of 125 I-labeled fragments and subsequent Edman sequencing [55]. Similarly, benzophenone analogs of paclitaxel were used to identify two binding sites at amino acids 683–760, which lie in TM helices 7–8, and 985–1088, which begin in TM helix 12 and end after the Walker A motif in the second NBD [56]. Similar studies have identified the binding sites of analogs of azidopine [57], dextriguldipine [58], cyclosporin A [59], and others [60].

Further evidence to indicate that multiple drug binding sites exist within a single LBD was reported following the crystallization of stereoisomeric QZ-59 compounds with mammalian P-gp. A range of distinct residues promoted drug binding, and although it was shown that the same residue may interact with molecules bound at different sites, the specific role of these residues in each interaction differs [27]. Two residues previously identified as being protected by MTS-verapamil labeling and therefore located in verapamil binding sites [61,62] were found also to interact with the QZ-59 compounds. A further 16 residues identified as interacting with the QZ-59

TABLE 5.2 Summary of Pharmacological Substrates of the Three Primary ABC Drug Transporters

Drug	Drug Class/Type	Indication	P-gp	MRP1	MRP2	BCRP
Vincristine	Vinca alkyloid	Cancer	+	+	+33	
Vinblastine			+	+	+ 34	
Doxorubicin	Anthracycline antibiotic		+	+	+ 35	+
Daunorubicin			+	+		+ ^a
Paclitaxel	Taxane		+			
Docetaxel			+		+ 36	
Etoposide	Epipodophyllotoxin		+	+	+ 35	+
Teniposide			+	+	+ 37	+
Topotecan	Camptothecin		+	+	+38	+
Irinotecan				+		+
Bisantrene	Anthracene		+			+ ^a
Mitoxantrone			+			+
Methotrexate	Folic acid analog			+	+ 39	+
Melphalan	Alkylating agent			+ ^b		
Cyclophosphamide				+ ^b	+ 40	
Imatinib mesylate	Tyrosine kinase inhibitor		+	+		+
Gefitinib			+	+		+
Flavopiridol	Cyclin-dependant kinase inhibitor					+
Ritonavir	Antiviral	HIV	+	+	+ 41	
Saquinavir			+	+	+ 41	
Nelfinavir			+			
Morphine	Opioid analgesic	Severe pain	+		+ 42	
Fexofenadine	Antihistamine	Seasonal allergic rhinitis. Urticaria	+		+ ^c 43	
Cimetidine	H ₂ -receptor antagonists	Duodenal and Gastric Ulcers	+			
Cyclosporin A	Immunosuppressant	Graft rejection	+		+ 44	

Tacrolimus		Adjunct to liver, kidney, lung, and heart allograft transplantation	+		
Amiodarone	Antiarrhythmics	Severe tachyarrhythmias	+	+ ^d 45	
Propafenone			+		
Felbamate	Anticonvulsant	Epilepsy	+		
Topiramate			+	– 46	
Lovastatin	Statin	Hypercholesterolemia	+ ^e	– 47	
Simvastatin			+ ^e	– 47	
Rosuvastatin				+ 47	+
Pravastatin				+ 48	+
Ondansetron	Antiemetics	Nausea	+		
Digoxin	Cardiac glycoside	Chronic heart failure and atrial fibrillation	+		
Ivermectin	Antinematodal	Onchocerciasis, intestinal strongyloidiasis	+		
Verapamil	Ca ²⁺ channel blocker	Hypertension, tachyarrhythmia, angina	+		
Nifedipine			+		
Diltiazem			+		
Trifluoperazine	Phenothiazine antipsychotic	Chronic psychotic disorders, anxiety, and alcoholism	+		
Chlorpromazine		Acute functional psychoses	+		
<i>Trans</i> -flupentixol	Thioxanthene neuroleptic	Chronic schizophrenia	+		
Propranolol	β-Adrenergic blocker	Hypertension, angina	+		

(continued overleaf)

TABLE 5.2 (Continued)

Drug	Drug Class/Type	Indication	P-gp	MRP1	MRP2	BCRP
Difloxacin	Quinalone antibiotic	Susceptible infections		+		
Erythromycin	Macrolide antibiotic		+		+ 49	
Gramicidin A	Antibiotic		+			
Ciprofloxacin	Fluoroquinolone antibiotic					+
Norfloxacin						+
Dexamethasone	Glucocorticoid	Corticosteroid responsive conditions	+		+ ^d 50	
Disulfiram	Alcohol deterrent	Chronic alcoholism	+			

Where information is not from Ref. 5, reference is included in the table. Blank cells indicate no information was obtained. “-” indicates MRP2 was confirmed as having no influence on the drug pharmacokinetics.

^aTransported by ABCG2R482T mutant only.

^bTransported as glutathione conjugates.

^cIn mice may be species specific.

^dStudy found ABCC2 transcription and expression increased as a result of drug exposure. Impact on transport is unclear.

^eLater studies suggest neither drugs are transported by MRP2 47. “+” indicates the drug is transported by the ABC protein.

Source: Adapted from Ref. 5.

compounds were also highlighted for their role in drug binding by an independent study that utilized arginine-scanning mutagenesis to determine residues important in drug translocation. By introducing arginine residues at each position in TM helices 2–12 of a P-gp processing mutant (G251V) and assessing their effect on protein maturation, the location of each residue in relation to possible drug binding sites was established. Several arginine substitutions located on the internal surface of the LBD were shown to reduce verapamil binding affinity 10-fold, while others promoted a 10-fold increase in binding affinity of rhodamine B, again highlighting that multiple binding sites exist within the LBD, promoting the selectivity of P-gp substrates [63].

Some examples of pharmacological agents that are transported by P-gp, MRP1, and BCRP are presented in Table 5.2.

5.3.2 Role of Glutathione in ABCC Subfamily Activity

Despite primarily transporting organic and anionic molecules, MRP1 transports a greater number of substrates than P-gp [64]. This phenomenon has been attributed to the essential role of glutathione (GSH) in drug transport mediated by members of the ABCC subfamily, particularly, MRP1 and MRP2. Although the exact mechanisms by which GSH aids in drug transport remains unclear, evidence suggests that the reducing tripeptide either binds to MRP1 and induces a conformational change to subsequently enhance the transport of hydrophobic compounds or is cotransported with the drugs [31]. Importantly, GSH is required not only for the MRP1-mediated efflux of therapeutics but also for the transport of several physiological substrates, including the well-established substrate of MRP1, leukotriene C₄ (LTC₄) [65]. Other endogenous molecules such as prostaglandin (PG) A₂, 4-hydroxynonenal, and the PGD₂ derivative 15-deoxy- $\Delta^{12,14}$ -PGJ₂ are also cotransported with GSH, in each case as GSH-conjugates [65].

GSH itself is a poor substrate of MRP1 and can only be transported in the presence of hydrophobic compounds. For example, vincristine, a vinca alkaloid used in the treatment of various cancers, cannot be transported by MRP1 alone. In the presence of GSH, however, both vincristine and GSH accumulate in vesicles expressing the transporter, indicating that GSH is cotransported with the drug [66] in a mechanism where both substrates rely on each other for transport. Studies using inside-out membrane vesicles isolated from MRP1-overexpressing cells indicate that without physiological concentrations of GSH present, MRP1 lacks the ability to transport many unmodified anticancer drugs [67,68]. Unlike the endogenous substrates of MRP1, however, there is no evidence to indicate that conjugates form between GSH and xenobiotic substrates. Instead it is believed that these agents are effluxed together with GSH in a simple cotransport mechanism or following conformational changes to MRP1, they are induced when GSH and the drug bind to the protein. Currently, little is known about the mechanism by which GSH and substrates induce conformational changes to MRP1. However, several studies have been carried out to determine possible substrate–GSH binding sites. Photoaffinity labeling techniques using derivatives of MRP1 substrates such as [H₃]LTC₄, iodoaryl-azido GSH (IAAGSH), and azidophenacyl GSH (APAGSH) have predicted the location of various substrate binding sites within the protein [69]. In the case of [H₃]LTC₄, it was suggested that substrate binding sites may exist within the lipid bilayer, possibly in the final two TM segments of TMD1 and TMD2 [69]. Site-directed mutagenesis studies have also revealed several sites in the TMDs of MRP1 that

may be important in the binding of GSH, such as Lys332 and His335 in α -helix 6 of TMD2 [70,71]. Using homology models to explore the influence of a range of proline substitutions in various TM α -helices of MRP1, it has also been shown that mutating Pro478 of TM9 to an alanine residue selectively reduces LTC₄ transport [72]. Similar studies aimed at determining substrate binding sites of MRP1 inhibitors showed preferential cross-linking of the radiolabeled photoactive compounds to segments within the third (COOH-proximal) TMD [73,74], suggesting that MRP1 has more than one drug binding site. It is also possible that MRP1 substrates such as GSH form individual, yet not necessarily unique, binding sites within a common binding pocket in the transporter [65].

Like MRP1, the mechanisms by which GSH facilitates MRP2-mediated drug transport is unclear. Early studies revealed GSH-stimulated vinblastine transport by rabbit MRP2 expressed in the insect cell line Sf9 [75]. Similarly a 10-fold increase in GSH was observed in the canine cell line MDCKII transfected with MRP2 versus wild-type cells, leading to the conclusion that MRP2 facilitates a low affinity transport of GSH [76]. Subsequent studies using MDCKII reported similar findings in the transport of vinblastine [34]. This study also found GSH to be cotransported with low concentrations of sulfinpyrazone; however, at high concentrations of the drug, transport of GSH was not observed, while transport of sulfinpyrazone continued [34]. It was hypothesized that two binding sites exist in MRP2, and at high concentrations, sulfinpyrazone will occupy both sites, preventing the cotransport of GSH, yet allowing sulfinpyrazone to be transported alone [34].

5.3.3 Influence of ABC Transporters on Drug Accumulation in Vesicles

Several ABC drug transporters localize to the lipid bilayer membranes of intracellular vesicles such as lysosomes and endosomes. From these locations, the drug transporters facilitate the active sequestration of various agents into the acidic compartments for drug efflux or redistribution away from the target. It was demonstrated that the ABC transport pump, ABCA3, localized to the lipid bilayers of lysosomes and multivesicular bodies where it conferred resistance to a range of cytotoxic agents including daunorubicin, mitoxantrone, etoposide, and vincristine. This drug resistance resulted from an ABCA3-mediated drug uptake into vesicles of low pH [12]. Earlier studies had also implicated the ABC transport proteins in the uptake of anticancer drugs into acidic vesicles of the membrane trafficking system. In 2003, Rajagopal and Simon provided evidence that in HeLa cells, MRP1, P-gp, and BCRP were not only localized to membranes of intracellular vesicles but their roles at these sites contributed to drug resistance phenotypes. Using a cell impermeable MRP1 inhibitor, it was highlighted that two separate pools of MRP1 were active within the cell and active sequestration of the drug by vesicular MRP1 alone was sufficient to confer drug resistance. In this study, doxorubicin was shown to colocalize in lysosomes with MRP1 and accumulation of the drug was prevented following pretreatment with verapamil, an ABC transport pump inhibitor [13,77]. In a separate study using HL-60 cells, it was reported that while the anthracycline antibiotic, doxorubicin, accumulated in acidic vesicles following passive diffusion of the lipid bilayer, and subsequent protonation of the compound, the zwitterionic compound, sulforhodamine 101, accumulated in the Golgi body following an MRP1-mediated uptake into the organelle [77].

5.4 INFLUENCE OF ABC ACTIVITY IN DRUG RESISTANCE

Although the ABC proteins have been shown to transport many therapeutic agents, with the cause of MDR being multifactorial, their influence on this phenomenon, especially *in vivo*, remains debatable. In a review of P-gp function in cancer, Ambudkar and colleagues suggested five points that should be met in order to declare an ABC transport protein responsible for drug resistance in a clinical setting. First, the protein should be expressed at a level comparable to that which is known, *in vitro*, to confer resistance. Second, this level should predict the degree of resistance, while in the third point, it was suggested that any increase in drug resistance during treatment should occur together with an increase in protein expression. The fourth point suggested that if the ABC protein is the sole contributor to resistance, then this resistance should be reversed with protein inhibition. Finally, it was suggested that any inhibitor would be advantageous as an adjuvant therapy [60].

To date, a total of 18 ABC transport proteins have been implicated in drug resistance [78]. Of these 18 proteins, only P-gp, MRP1, and BCRP have been identified on several occasions to contribute to the development of the MDR phenotype *in vivo*, and hence, greater attention will be paid to the role of these proteins in drug resistance. Although these proteins may confer resistance to drugs used in the treatment of many diseases, their influence on the efficacy of cancer therapy dominates the literature. As such, this chapter focuses on the role of the ABC proteins in cancer drug resistance.

5.4.1 ABCB1 (P-gp) and Cancer Drug Resistance

A large amount of clinical data correlates expression and function of P-gp with response to therapy in cancer patients. Two independent studies have drawn correlations between expression level of the protein and response to therapy in the treatment of breast cancer. One of these studies indicated that the presence of P-gp, which was increased 1.8-fold by prior exposure to chemotherapy, translated to a three- to fourfold greater chance of treatment failure [79], while the other reported a strong correlation between the extent of taxol and doxorubicin resistance in 359 resected breast cancer samples and the expression levels of P-gp [80]. Similar results were observed in nonsmall-cell lung carcinoma (NSCLC) where 68% of tumors that expressed P-gp were resistant to the drug paclitaxel, while those lacking the protein responded well to therapy [81]. In contrast, P-gp was overexpressed in only 2% of 67 metastatic glioma patients despite a high level of drug resistance observed in all patients. In this case, it was suggested that P-gp activity is just one mechanism contributing to the MDR phenotype [82]. Abe and colleagues supported these findings in 1998 reporting that only 4–23% of gliomas were P-gp positive. This study also highlighted that P-gp expression was higher in patients who had previously been exposed to chemotherapy, suggesting the protein played a role in acquired drug resistance [83]. P-gp has also been identified as a cause of drug resistance in leukemias and lymphomas, where expression of the protein is upregulated after exposure to chemotherapy. It is expressed in ~30% of all acute lymphoblastic leukemia (ALL) patients at diagnosis, and although studies have reported an association between its expression and patient response to therapy [84,85], these findings were contradicted by other studies [86–88].

5.4.2 ABCC1 (MRP1) and Cancer Drug Resistance

MRP1 is ubiquitously expressed throughout the body and as a result high levels of the protein have been identified in most cancer types. Unlike P-gp, MRP1 does not appear to contribute to MDR to any significant degree in metastatic breast cancer nor ALL. Despite this, MRP1 is associated with the development of MDR in a large number of tumor types including lung carcinomas and gliomas. The protein is regularly expressed in NSCLC where it correlates highly with a poor patient response to chemotherapy and overall survival [64,89]. These findings were described in a study that assessed MRP1 levels in 126 samples via immunohistochemistry. In this study, MRP1 levels were more highly expressed in stage 1 tumors versus late-stage tumors and were particularly high in patients who had not yet received chemotherapy, suggesting that the overexpression is inherent in NSCLC and not induced by chemotherapy exposure. It was also highlighted in the same study that although MRP1-positive tumors appear less aggressive, they are particularly resistant to chemotherapy [89]. Similar results have been reported in small-cell lung carcinoma (SCLC) where MRP1 expression is also elevated in patients who had a relapse [90]. Benyahia *et al.* reported that MRP1 was highly expressed in the vascular endothelial cells in 93% (14/15) of the glioma tumor samples examined. From this localization, it was proposed that MRP1 limits the uptake of drug into the brain tumor cells [91]. The protein was also found to be expressed along with MRP4 and MRP5 to the luminal side of brain capillary endothelial cells where again, it was proposed to influence drug penetration of the blood brain barrier [92]. The effect of MRP1 expression in human gliomas was also discussed in 2005 when a high level of expression was shown in 51% of the human glioma specimens and primary cell cultures examined. No difference between primary and recurrent glioma was observed, and it was subsequently concluded that MRP1-mediated drug resistance is intrinsic to glioma tumors [93].

5.4.3 ABCC2 (MRP2) and Cancer Drug Resistance

The effect of MRP2 on *in vivo* cancer drug resistance remains to be defined. However, it is clear that many agents used in the treatment of cancers are transported by the pump in *in vitro* studies. MRP2 was found to confer resistance to a large range of compounds including cisplatin and methotrexate. This resistance was highlighted after cisplatin resistance was successfully reversed using anti-MRP2 hammerhead ribozymes in the ovarian carcinoma cell line A2780RCIS [39]. MRP2-facilitated methotrexate resistance was also highlighted in an ovarian carcinoma cell line, with resistance observed following short-term exposure (1–4 h) of the drug [94]. Although overexpression of MRP2 has been shown to confer resistance to the tyrosine kinase inhibitor sorafenib in LLC-PK1 cells [95], earlier studies suggest that this is not common to all drugs of this class, with no active transport of erlotinib observed in MDCKII cells stably expressing MRP2 [96]. Other studies, using cell lines stably transfected with human MRP2, reveal that this protein confers resistance to etoposide, cisplatin, doxorubicin, and epirubicin. This resistance was up to 10-fold in the case of cisplatin and fourfold in the case of etoposide [35]. These results were supported in a study of drug resistance in esophageal squamous cell carcinoma (ESCC) treatment, which found that inhibition of MRP2 expression by siRNA reduced resistance to the 5-fluorouracil, doxorubicin, and cisplatin treatment regime [97]. Although *in vitro* studies had shown MRP2 to transport docetaxel, *in vivo* studies using MRP2 knockout mice revealed no difference in

drug pharmacokinetics compared to wild-type mice. When CYP3A and P-gp were also knocked out, however, (Cyp3a/Mdr1a/b/Mrp2^{-/-}) changes in the drug's pharmacokinetics were observed. These changes were evident when compared to both wild-type mice and Cyp3a/Mdr1a/b knockout mice proficient in MRP2, indicating MRP2 will reduce docetaxel exposure in the absence of both CYP3A and Mdr11/b [36].

5.4.4 ABCG2 (BCRP) and Cancer Drug Resistance

BCRP was originally identified by Doyle and colleagues [98] in 1998 in the MC7-AdrVp breast cancer cell line. Examination of BCRP and its role in drug resistance in leukemia patients has yielded a variety of results. In a study of 46 mixed lineage ALL samples, it was determined that BCRP was not only more highly expressed in the B-cell lineage compared to the T-cell lineage but also had a greater influence on MDR in these cells [99]. BCRP was also implicated in mitoxantrone efflux in 70% (21/30) of untreated ALL patients despite low mRNA levels being detected [100]. Furthermore, BCRP levels were found to be 10-fold higher in acute myeloid leukemia (AML) patients who did not achieve remission compared to those patients who responded well to therapy [101]. However, contradictory to these findings were reports that BCRP does not correlate to drug resistance in leukemia patients. One such report examining Ara-C resistance in 13 infants and 13 noninfants found that despite *ABCG2* mRNA expression being positively correlated to Ara-C resistance, the drug was not a substrate of the transporter and hence other mechanisms were responsible for this resistance [102]. Sauerbrey and colleagues also reported that although BCRP expression was lower in the T-cell lineage, there was no significant correlation between protein expression levels and response to therapy [103]. Similarly there are mixed reports on the influence of BCRP in drug resistance in solid tumors. In a recent study it was found that although there was no significant association between P-gp and MRP1 expression in SCLC patients' response to therapy and survival, there was a significant association between BCRP expression and tumor response to chemotherapy and progression-free survival [104].

5.4.5 Impact of Other ABC Transporters on MDR

Although there are few reports of the remaining ABC proteins influencing MDR *in vivo*, a substantial number of *in vitro* studies have provided evidence that implicates various ABC transport proteins in the onset of MDR. It was found that ABCA2 confers resistance to mitoxantrone in the BCRP1-negative GLC4-MITO cell line when compared to the GLC4 cell line [105], and it has also been established that the same protein confers resistance to the estrogen derivative, estramustine [106]. As previously mentioned, ABCA3 reportedly conferred resistance in leukemia cells to the anthracycline antibiotics, etoposide and vincristine, when localized to the lipid bilayer membranes of acidic vesicles [12]. This finding supported an earlier report that identified ABCA3 overexpression in pediatric AML using microarray technology. With a median expression level of threefold higher in patients who had failed to achieve remission following the first round of chemotherapy, it was suggested that ABCA3 conferred resistance to the anthracycline antibiotics in AML patients [107].

A greater number of ABCB members have been implicated in cancer drug resistance. Along with P-gp, both ABCB2 and ABCB3 have been shown to contribute

to mitoxantrone and etoposide/teniposide resistance [7,78], while both ABCB4 and ABCB11 have been implicated in resistance to paclitaxel [8]. ABCB5 has been shown to confer resistance to doxorubicin, camptothecin, and 5-fluorouracil [108]. One study that flagged ABCB5 as a contributor to doxorubicin resistance in malignant melanoma highlighted exclusive expression in a subpopulation of tumor stem cells. Blockade of the ABCB5 transporter using a specific inhibitory monoclonal antibody reversed doxorubicin resistance in G3361 melanoma cells, and the hypothesis was raised that targeting this transporter in an adjuvant therapy may enhance the efficacy of those agents used in the treatment of malignant melanoma [109]. The final member from the ABCB subfamily to be implicated in drug resistance is ABCB6 that has been shown *in vitro* to confer resistance to both cisplatin and camptothecin [78,110].

Like the ABCB subclass, a large number of ABCC subclass members have been shown to confer resistance to various anticancer agents. While MRP1 and MRP2 remain the most actively researched members of this subclass, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, and ABCC11 have all been shown to influence drug resistance phenotypes [78]. ABCC3 confers only a mild resistance to the vinca alkaloids, epipodophyllotoxins, methotrexate, and cisplatin [37]. Although the protein is able to transport organic compounds conjugated to GSH, experiments assessing GSH efflux suggested that ABCC3 cannot transport the tripeptide alone and does not require it for drug transport [37]. Evidence implicating ABCC4 in MDR phenotypes remains limited, although it has been suggested to be a possible target for cancer therapy. This is due to its role in the regulation of the intracellular concentrations of cAMP and the subsequent maturation of leukemic cells [111]. A report in 2005 concluded the transporter contributes to neuroblastoma prognosis through its activity in effluxing irinotecan and its active metabolite SN38 [112]. However, a later study assessing the systemic clearance of these agents in mice determined that ABCC4 did not influence their elimination, while P-gp did [113]. *In vitro* studies implicated ABCC4 in the resistance of cyclophosphamide and ifosfamide [114], topotecan and methotrexate [115], as well as camptothecin, SN-38, and other camptothecin derivatives [116] in HepG2 and HEK293. A larger number of substrates have been reported to be transported by ABCC5, yet it has also been highlighted that this transport is unlikely to contribute significantly to clinical MDR [117]. Various studies, each utilizing cells transfected with MRP5, have suggested a role for the protein in the transport of cisplatin, Oxaliplatin doxorubicin [118], etoposide, and teniposide [119]. Like many of the other members of the ABCC subclass, ABCC6, otherwise known as *MRP6*, has been shown to confer a low level of resistance to the anthracyclines, epipodophyllotoxins, and cisplatin [120]. Evidence to indicate this is, however, limited, and the suggestion has been made that like MRP5, MRP6 makes little contribution to clinical MDR [78]. After ruling out the influence of P-gp, BCRP, and various members of the ABCC family of drug transporters, it was recently shown that expression of ABCC10 (MRP7) confers resistance to vinorelbine in NSCLC cell lines [121]. This protein was also implicated in taxane resistance in transfected HEK293 cells where cellular accumulation of radiolabeled paclitaxel was reduced compared to cells transfected with a control plasmid [122]. The final member of the ABCC subclass that has been implicated in drug resistance is ABCC11. This protein was shown to confer resistance to 5-fluorouracil in a resistant subline of the PC-6 human SCLC cell line. In this case, resistance facilitated by MRP8 was reported at up to 25-fold [123].

5.5 INFLUENCE ON ADVERSE REACTIONS AND DRUG-DRUG INTERACTIONS

Drug-drug interactions (DDIs) manifest themselves clinically through altered pharmacokinetics, where one drug influences the absorption or disposition of another, and through altered pharmacodynamics, where drugs used in combination may produce additive, synergistic, or antagonistic effects [124]. Direct clinical evidence of P-gp inducing DDI is limited due to cross-specificity of P-gp substrates with the drug-metabolizing enzyme CYP3A4. While much of the current evidence indicates that CYP3A4 is a greater contributor to DDIs [124,125], it has been proposed that the ABC proteins contribute to DDI, and subsequent adverse side effects, following coadministration of antiretroviral agents used in the treatment of HIV with antifungals, antibiotics, cardiovascular agents, and recreational/illicit agents such as cocaine, lysergic acid diethylamide (LSD), marijuana, and 3,4-methylenedioxymethamphetamine (MDMA) [124].

While P-gp may not define drug disposition *in vivo* alone, its contribution to peak plasma concentrations and elimination rates of substrates with narrow therapeutic windows may be great enough to produce severe adverse reactions. This is the case with the cardiovascular drug digoxin, which is not metabolized by the cytochrome P450 family of enzymes but is a substrate of P-gp. Itraconazole, an antifungal agent that is known to modulate the function of P-gp, increases the plasma concentrations of digoxin while decreasing its renal clearance [126]. Similarly, the calcium channel blocker mibefradil was removed from the market as it increased the $C_{\max}$ and AUC of digoxin to levels that induced severe adverse reactions. [127]. P-gp was also implicated in DDIs between digoxin and rifampicin, an antibiotic used in the treatment of tuberculosis. In this case, the AUC of digoxin administered orally was significantly reduced when coadministered with rifampicin. This was attributed to a rifampicin-induced increase in the intestinal expression of P-gp, preventing the absorption of digoxin in the gut [128]. It should be noted, however, that although many studies have implicated digoxin in P-gp-mediated DDIs, a recent retrospective analysis of 123 such studies concluded that the majority of reported DDIs involving digoxin were not clinically significant [125].

The influence of P-gp on interactions between dopaminergic agonists used in the treatment of Parkinson's disease has also been assessed. While each of the agents examined were P-gp substrates, only bromocriptine inhibited substrate transport. The subsequent effect of this inhibition was evident on the intracellular transport of levodopa (L-dopa), which increased over twofold. With L-dopa, bromocriptine, and other dopaminergic agonists administered simultaneously in the treatment of Parkinson's disease, it was proposed that through its effect on P-gp function, bromocriptine could alter the disposition of L-dopa and subsequently induce an adverse effect as neurotoxicity [129].

5.6 MODULATION OF ABC PROTEIN FUNCTION

5.6.1 Molecular Mechanisms to Modulate ABC Function

Several molecular techniques have been adopted in an attempt to inhibit the function of the ABC drug transporters. P-gp targeting hammerhead ribozymes delivered via liposomes were utilized to achieve P-gp inhibition and reversal of vincristine resistance

in the breast cancer cell line MCF-7/R. This study, however, failed to achieve similar results in the MOLT-3/TMQ₈₀₀ cell line [130]. Successful inhibition of P-gp function in MOLT-3/TMQ₈₀₀ cell line was achieved, however, following transduction of MDR-1 hammerhead ribozymes into the cells. A retroviral vector containing Pol III was utilized to improve ribozyme activity and resulted in decreased levels of P-gp, along with an increase in vincristine sensitivity. On the basis of these results, it was suggested that the ribozyme targeting the translation initiation site was the most efficacious in reversing drug resistance [131].

Currently, gene silencing via the use of short siRNAs targeted toward the ABC transport proteins is proving successful in the reversal of drug resistance in *in vitro* studies. The efficacy of siRNAs targeted toward the *ABCB1* gene was revealed using the multidrug-resistant human pancreatic carcinoma and gastric carcinoma cell lines EPP85-181RDB and EPG85-257RDB. Expression of P-gp was reduced 91% at the mRNA and protein levels and this was correlated to significant improvements in the efficacy of daunorubicin. This reduction in drug resistance was reportedly at a level of 89% in EPP85-181RDB cells and 58% in EPG85-257RDB cells [132]. Successful inhibition of P-gp translation was also reported by Stege and colleagues in 2004. A complete reversal of resistance was achieved in the EPG85-257RDB cell line following exposure to an anti-P-gp short-hairpin RNA expression vector. This stable reversal of resistance was reportedly associated with a significant increase in intracellular accumulation of the anthracycline antibiotic daunorubicin [133].

5.6.2 ABC Modulators as Adjuvant Therapies

A large number of small molecules have been developed over the years, which modulate or inhibit the activity of the ABC transport proteins. Many of these molecules have entered clinical trials as adjuvant therapies, particularly in the treatment of cancer; however, the majority were not found to be clinically relevant, most often due to excessive adverse effects and toxicities at doses well below those required to inhibit the ABC proteins. These small molecules modulate the function of the ABC transport proteins through a number of mechanisms including competition at the drug substrate binding pocket, binding to high affinity sites in the NBD or through unique mechanisms.

Verapamil, cyclosporin A, and the phenothiazines, which act as competitors at the substrate binding site, are first-generation ABC inhibitors that were considered as adjuvant therapies for cancer treatment and they subsequently entered clinical trial. These compounds are associated with severe dose-limiting side effects that include cardiomyopathy, hypotension, and increased toxicity of the cytotoxic compounds. In the case of calcium channel blockers such as verapamil, adverse cardiovascular effects occur at dose concentrations lower than that required to inhibit the ABC transporters [134–136]. Clinical trials involving these first-generation inhibitors of ABC transporters indicated that although they may improve the efficacy of treatment in leukemia patients [137,138], the benefit in the treatment of solid tumors remains debatable. Early studies indicated that no advantage was gained in solid tumors, predominately due to the onset of cardiotoxicity at doses required to inhibit P-gp [139,140]. More recent studies, however, have reported that inclusion of verapamil in the treatment of doxorubicin-resistant metastatic breast cancer improved patient response without the onset of dose-limiting side effects [141].

The second-generation compounds dexverapamil and PSC833, which are analogs of verapamil and cyclosporin A, respectively, are associated with fewer and less intense

adverse effects. Their success in clinical trial, however, remains limited. Dexverapamil was assessed in combination with doxorubicin and epirubicin in patients with metastatic breast cancer, and although it was reported that inclusion of dexverapamil did not intensify anthracycline-associated cardiotoxicity, the number of patients achieving a complete response to therapy were limited [142,143]. Although the results obtained in these studies were considered promising, it could not be confirmed that this was a consequence of ABC transport inhibition. These conclusions were again reflected in another phase II trial in patients with advanced pancreatic cancer. The *in vivo* efficacy of PSC833 was assessed in combination with cytarabine, mitoxantrone, and etoposide in patients with AML. One study reported an improved efficacy and higher frequency of complete response (29%) compared to traditional response rates [144], and these results were supported by a separate study that reported a complete response rate of 26% in AML patients receiving the same combination of drugs [145]. Despite these positive results, it was also reported that excessive neurological side effects occur at doses higher than 10 mg/kg [146], and owing to a combination of poor response rates and excessive adverse reactions, one clinical trial was terminated prematurely [147].

Several newer “third-generation” compounds have been designed to specifically inhibit the function of P-gp. One agent known as *tariquidar* (XR9576) finds its mode of action in a noncompetitive mechanism, binding to P-gp at a site alternative to that of the transport substrate [148]. This compound is characterized by a greater selectivity toward P-gp and a slower dissociation rate from the protein. *In vitro* assessment of the agent suggested that P-gp inhibition may last up to 22 h after the drug is removed from cells [149]. Despite this high selectivity and potency, it was reported that tariquidar possessed limited clinical efficacy when used in combination with the anthracyclines and taxanes in patients with drug-resistant, advanced breast carcinoma [150].

Another third-generation P-gp inhibitor is LY335979. This agent shows no inhibition of BCRP or MRP1 and unlike tariquidar, competes with other P-gp substrates for binding at the substrate binding site [151,152]. Initial phase I clinical trials in patients with advanced malignancies reported several dose-limiting adverse reactions including neurotoxicity, cerebral dysfunction, hallucinations, and palinopsia [153]. A later study reported that a 640 mg/m² dose of LY335979 could be safely administered over 48 h intravenously. This study assessed the efficacy of the compound in combination with doxorubicin and reported that no dose-limiting side effects occurred [154]. Similarly, during a phase II clinical trial in patients with untreated nonHodgkin’s lymphoma, LY335979 was safely administered over a 24-h schedule without enhancement of cytotoxic related toxicities [155].

5.7 TRANSCRIPTIONAL REGULATION OF THE ABC GENES

In keeping with the focus of this chapter, the transcriptional regulation of *ABCB1*, *ABCC1*, and *ABCG2* in response to the presence of small molecules is discussed. It is important to remember, however, that with many of the ABC proteins having alternative physiological functions and cellular/organ locations, their transcriptional regulation may be induced and maintained by molecular mechanisms not discussed in this chapter.

The constitutive expression of each ABC drug transport protein is dictated by a complex network of transcription factors that interact with each other and response

elements within ABC gene promoter regions. Each of the ABC genes identified to date contains TATA-less promoter regions and possesses initiator elements to which various transcription factors bind before the recruitment of RNA polymerase II [156]. The *ABCB1* gene is known to possess an initiator sequence -6 to $+11$ nucleotides surrounding the transcription start site [157] as well as an inverted CCAAT box (-79 to -75) and a GC-rich element (-56 to -43). The latter two DNA sequence regions are among the most common polymerase II promoter elements and interact with the transcription factors NF-Y and Sp1/Sp3, respectively [158,159]. An additional GC element is known to reside upstream within the promoter region (-110 to -103) that does not interact with Sp1 but may interact with another member of the Sp transcription factor family.

Like *ABCB1*, GC-rich sequences play a vital role in the recruitment of the transcription machinery to the *ABCC1* and *ABCG2* genes. In the case of *ABCC1*, CG elements that lie -91 to $+103$ nucleotides adjacent to the transcription start site have been shown to interact with Sp1 [160], while in the case of *ABCG2*, several potential Sp1 sites were identified within sequences 300 bp upstream of the transcription start site [161]. There are several proteins that have been reported to interact with response elements within the ABC gene promoter regions to either induce or suppress transcription of the ABC drug transporters. These include p53, Ras/Raf, AP-1, and the Wilms' tumor suppressor protein [162]. Of greatest relevance to the pharmacology of small molecules, however, is the overexpression of the ABC drug transport proteins in response to drug exposure.

Several nuclear receptors, including steroid and xenobiotic receptor (SXR), retinoic acid receptor (RAR), farnesoid receptor (FXR), and constitutive androstane receptor (CAR), are known to influence transcription of the ABC genes in response to various xenobiotics [162]. SXR [also known as *pregnane X receptor* (PXR) in rodents, PAR and NR1I2] is activated by a range of small molecules. Xenobiotic detection and subsequent activation of the nuclear receptor involves a large and flexible hydrophobic pocket that resides in the N-terminal LBD. The PXR ligand binding cavity, unlike that of other nuclear receptors, senses a large range of molecules, serving a general protection mechanism against toxic substances [163]. Following activation, the nuclear receptor forms a heterodimer with the retinoid X receptor α (RXR α). This interaction, which is believed to be facilitated by two activation factor domains that lie within the LBD [164], is common among all nuclear receptors involved in the transcriptional regulation of the ABC drug transporters and precedes binding of the heterodimer to response elements within the promoter region of the ABC genes [164]. Binding of the heterodimer to DNA is mediated by a highly conserved region known as the *DNA-binding domain* (DBD). Both the PXR–RXR α and CAR–RXR α heterodimers can bind to the same response elements within the enhancer regions of the *ABCB1* promoter.

5.8 POLYMORPHISMS—EFFECT ON GENE EXPRESSION AND PROTEIN FUNCTION

5.8.1 Polymorphisms in ABC Transport Protein Genes

The growing desire for therapeutics tailored to the individual has, in recent years, stimulated much research into determining the effect of polymorphisms on the function and

transcriptional regulation of the ABC transport proteins. SNPs, which make up over 90% of all genetic mutations, are believed to contribute significantly to the interindividual differences of drug response [165]. This is often revealed through diminished drug efficacy and increased adverse reactions.

SNPs, which are defined as *single nucleotide variations* that exist in 0.1% of the population or greater, may be classified as synonymous or nonsynonymous. Synonymous SNPs are those that do not influence the tertiary structure of the protein but may play a role in the transcriptional regulation of the gene in which it resides. Nonsynonymous SNPs, on the other hand, produce an amino acid change in the protein sequence and hence may affect protein function, interaction with other proteins, or even folding of the tertiary structure. Predicting associations between SNPs and a particular disease, drug response, protein structure or function is made difficult by the number of SNPs believed to exist throughout the genome. This number is rapidly growing, and currently, over 6900 SNPs have been reported in 12 of the major ABC genes [166]. Owing to a phenomenon in which alleles in close proximity are often inherited together, strong correlations may be identified in the expression of minor alleles of different genomic regions. Termed *linkage disequilibrium* (LD), this redundancy of polymorphisms may be exploited in various ways to permit efficient detection of potentially functional SNPs and determine their association with disease and protein function [167].

One method that is commonly adopted in SNP association studies is the selection of a smaller proportion of SNPs, known as *tagging SNPs* (tSNPs), to represent most of the genetic variation within a particular region of interest [167,168]. These tSNPs must not only be in LD but it must also be assumed that no chromosomal recombination has occurred within the region to disrupt the SNP haplotype that would otherwise exist. tSNPs may be selected based on known haplotype distribution or pairwise r^2 LD, the latter being used primarily to select SNPs that best represent different LD blocks within a gene [167,169]. While each method has been shown to select tSNPs with similar predictive power, studies aimed at comparing the effectiveness of each technique suggest those based on haplotype information are most efficient, allowing fewer SNPs to be selected for the same predictive power [169]. Selection of tSNPs may not, however, represent other SNPs in regions of low LD. Similarly, an SNP in a genomic region that is under recent positive selection pressure is generally observed at a higher frequency and is rarely represented by other SNPs in the same region because of differences in ancestral history [166].

Signatures of recent positive selection have been implicated to permit the prediction of potentially functional SNPs. The SNP alleles e22/2677 T (rs2032582) and e27/3435 T (rs1045642) within the *ABCB1* (*MDR1*) gene were previously associated with various functional differences and differences in drug response/other phenotypes and were found to display signatures of recent positive selection in the Chinese population [170,171]. These two positively selected *ABCB1* SNP alleles were found to protect Chinese but not Caucasian men from late-onset (> 60 years) Parkinson's disease [172–174]. The signature of recent positive selection was later employed to demonstrate the feasibility of identifying a previously unknown potentially functionally significant SNP at the *ABCC1* gene locus [175]. The major G allele of SNP 5'UR/G-260C (rs504348) at the promoter region of the *ABCC1* gene was found to be under recent positive selection and to mediate lower *ABCC1* promoter activity compared to the C allele [175]. Before the identification of this potentially functionally significant SNP allele at the *ABCC1* gene that is under recent positive selection, researchers have

failed to detect associations between various SNPs at the *ABCC1* gene and functional differences [176–179]. One possible reason for their failure to detect an association between SNPs at the *ABCC* gene locus and functional difference is due to the general low LD observed at the *ABCC1* gene locus and that this potentially functionally significant promoter SNP under recent positive selection at the *ABCC1* gene locus was never examined in any of the studies. Signatures of recent positive selection were then employed to identify 32 SNPs of potential functional significance at 18 ABC gene loci that are known or implicated to transport drugs [171]. As discussed in this study, it may be assumed that positively selected genomic regions have benefitted the survival of the species in recent evolutionary times and hence play an important functional role. In the situation where an induced change to protein function aids survival, the SNP will be maintained within a population, while those SNPs that produce detrimental effects will reduce in frequency [171].

5.8.2 Effect of SNPs on the Function, Structure, and Expression of ABCB1 (P-gp)

The earliest SNPs believed to affect the expression of P-gp were identified using osteosarcoma cells in 1994. Two SNPs in the promoter region of *ABCB1* gene were identified and the 5'UR/T+103G point mutation was found to enhance the promoter activity of *ABCB1* in cells exposed to the anticancer drugs, doxorubicin and vincristine [180]. Since these early discoveries, advances in high throughput DNA sequencing techniques together with the popularity of this gene in the field of pharmacogenetics have seen a substantial number of additional polymorphisms identified. In the latest release of NCBI's SNP database (dbSNP build 130), 62 coding region SNPs were reported at the *ABCB1* locus. Of these SNPs 40 are nonsynonymous; and 22, synonymous. The majority of these SNPs have yet to be genotyped in any major population cohort, and many of the coding region SNPs associated with *ABCB1* are expressed at low frequency in each of the populations assessed HapMap (phase III) (<http://www.hapmap.org/>).

Nonetheless, three high frequency SNPs at the *ABCB1* gene locus, namely, e13/C1236T (rs1128503), e22/G2677T/A (rs2032582), and e27/C3435T (rs1045642), are often found to be in strong LD, having been identified as part of many haplotypes where the minor alleles may be expressed at highly variable frequencies in different populations [170,181–185]. Although a number of studies have drawn correlation between the presence of these SNPs and either drug response or disease progression, it remains difficult to determine which are causative and which are merely inherited with causative SNPs, which is a confounding issue of the haplotype phenomenon. Despite a general consensus that synonymous SNPs play insignificant roles in the function and expression of P-gp, a recent hypothesis has suggested that the minor T allele at the e27/C3435T (rs1045642) locus results in a codon that is rarely used in humans. This rare codon is hypothesized to result in ribosomal stalling during translation, which affects not only the rate of translation but also the chaperone-facilitated protein folding [186,187]. Early studies into the function of the synonymous SNP e27/C3435T (rs1045642) reported its association with decreased duodenal expression of P-gp and subsequent increase in the plasma levels of the cardiac glycoside digoxin [188]. However, these results could not be replicated by other studies that found either no influence on mRNA expression [189] or an increased expression of the protein in the

duodenum [190]. In a similar fashion, the influence this SNP has on drug activity is controversial. While expression of the minor e27/3435 C allele was correlated with increased efflux of the P-gp substrate, rhodamine 123 [191], it was associated with reduced efflux of nelfinavir [192] and no effect on fexofenadine efflux [193]. A few reports have implicated the wild-type e27/3435 C allele on drug response. Plasma levels of atazanavir, for example, were determined to be higher in HIV patients expressing the CC genotype [194], while the same genotype was linked to a greater incidence of steroid side effects in kidney transplant patients compared to the TT minor allele genotype [195].

Evidence regarding the influence of the nonsynonymous SNP e22/G2677T/A (rs2032582) on the function of P-gp is equally inconclusive. Again, many of the studies exploring the influence of this SNP on *in vivo* P-gp function have assessed this SNP in combination with other SNPs as a haplotype. Functional validation conducted *in vitro* has shown that expression of Ala893, which is produced by the minor 2677 T allele, was found to confer a reduced level of digoxin efflux compared to cells expressing the Ser893 variant [182]. This finding supported an *in vivo* assessment of fexofenadine disposition, where a reduced oral bioavailability of the drug was associated with expression of Ser893, suggesting an enhancement of P-gp activity. It was, however, noted that in most of these studies, the E22/G2677T/A (rs2032582) SNP was studied together with the E27/C3435T (rs1045642) SNP as a haplotype, making it difficult to determine which SNP exerts the greatest influence [182]. The 2677TT/3435CC genotype combination was also associated with lower plasma concentrations of fexofenadine compared to other genotype combinations involving these SNPs [196], while the 2677TT genotype was positively correlated with a higher risk of CsA failure in steroid-resistant ulcerative colitis [197]. e22/G2677T/A (rs2032582) SNP was also positively correlated to tacrolimus neurotoxicity [198], increased resistance to antiepileptic drugs [199], and increased response to cytarabine in AML patients [200]. Curiously, some studies have found no association between e22/G2677T/A (rs2032582), either individually or in haplotype with other SNPs, and drug response [185,201]. For example, e22/G2677T/A (rs2032582) did not influence plasma trough concentrations of ritonavir in HIV patients [202], rhodamine 123 efflux in peripheral blood lymphocytes [203], tacrolimus pharmacokinetics in renal transplant patients [204], or blood, semen and saliva concentrations of ritonavir or lopinavir in HIV patients [205].

The third coding SNP that occurs at highly variable frequencies in different populations and in strong LD with the e22/G2677T/A (rs2032582) and e27/C3435T (rs1045642) SNPs is the e13/C1236T (rs1128503) SNP. This SNP, being synonymous, does not change the amino acid Gly412. Nonetheless, some studies have indicated that this SNP may influence drug response. Significantly increased exposure to irinotecan was associated with the 1236TT genotype [177], whereas the 1236CC genotype was associated with increased response to temozolomide treatment in glioblastoma patients [206]. Positive correlation has also been drawn between this SNP and patient response to CsA [207] and nelfinavir [192]. Nonetheless, some studies also indicated that this SNP does not influence drug response. For example, response to both lopinavir and ritonavir was shown not to be affected by this SNP in HIV patients [205].

Aside from these three commonly reported SNPs, several other SNPs at the *ABCB1* gene locus have also been implicated in drug response. It has been reported that the SNP e27/T3421A (S1141T) (rs2229107) conferred decreased resistance to daunorubicin

in an *in vitro* setting, while the SNPs e5/C266T (M89T) (rs35810889), e17/T1985C (L662R) (rs35657960), and e17/C2005T (R669C) (rs35023033) all increased resistance to at least two of the drugs studied, which also included doxorubicin, valinomycin, and actinomycin D). In the same study e27/T3322C (W1108R) (rs35730308) reportedly decreased resistance to valinomycin when expressed in haplotype with e17/C2005T [208]. It should be noted, however, that opposing studies indicate that many of the SNPs do not influence P-gp function [209,210].

5.8.3 Effect of SNPs on the Function, Structure, and Expression of ABCC1 (MRP1)

SNPs at the *ABCC1* gene locus were found to be in low LD and high haplotype diversity when ~480 individuals from Chinese, Malay, Indian, European Caucasian, and African-American populations were screened [175]. Despite the large number of exonic SNPs identified in this gene, relatively few have been associated with drug response or disease progression. One SNP, e13/C1684T (rs35605), was reported to influence the pharmacokinetics of the irinotecan metabolites SN-38 and APC. However, it was highlighted that this may be due to alternative causative polymorphisms possibly in other membrane transporters [211]. Similarly, the SNPs e2/C218T (T73I) (rs41494447) and e23/G3173A (R1058Q) (rs41410450) were implicated in reduced resistance to at least one of the several drugs including daunorubicin, doxorubicin, etoposide, vinblastine, or vincristine in HEK293 and CHO-K1 cells. In the case of e17/G2168A (R723Q) (rs4148356), the SNP was found to confer reduced resistance to all drugs examined, while e2/G128C (C43S) (rs41395947) did not influence the efficacy of any agent [212]. Additional SNPs influencing the function of MRP1 include the SNP e28/G4009A (A1337T) (rs28364006) that was reported to influence the efficacy of methotrexate together with the intronic SNPs I22/G-1960A (rs2238476) and I9/T-176 C (rs35592) [213]. The e10/G1299T (rs60782127) polymorphism, which confers the amino acid exchange R443S, reportedly increased doxorubicin resistance while decreasing the transport of a number of organic anions [214]. Furthermore, the e2/G128C (C43S) (rs41395947) polymorphism, which was discovered not to influence protein expression or stability [215], was found to disrupt protein localization to the plasma membrane. Association between this SNP and reduced resistance to doxorubicin has also been highlighted [216]. In contrast to these positive associations, the results of a study that assessed the functional significance of 10 nonsynonymous SNPs in HEK293T cells reported that none altered the expression levels of MRP1 or have any impact on protein synthesis or stability [215].

5.8.4 Effect of SNPs on the Function, Structure and Expression of ABCG2

Although several SNPs have been identified in the *ABCG2* gene, few have been characterized and shown to influence the drug transport activity of the protein. One well-characterized nonsynonymous SNP, e5/C421A (Q141K) (rs2231142), is located between Walker A and signature motif C in the NBD. This SNP is associated with a less stable BCRP that is susceptible to proteasomal degradation and diminished localization to the plasma membrane [217]. This SNP was shown to reduce ATPase activity in HEK-293 cells, which was accompanied by a reduced mitoxantrone efflux [218]. An independent study also implicated e5/C421A (Q141K) (rs2231142) in drug

response after reporting an association between the heterozygous variant of the SNP and the onset of diarrhea following gefitinib treatment. Interestingly, however, there was no association in the same cohort with skin toxicity, another common side effect of gefitinib exposure [219]. The same SNP has been implicated in the pharmacokinetics of diflomotecan [220], imatinib [221], atorvastatin and rosuvastatin [222], and methotrexate [213]. SNP e5/C421A (Q141K) (rs2231142) was found not to affect the pharmacokinetics of the camptothecin analog, irinotecan [223] or the drug, lamivudine [200]. Two other nonsynonymous SNPs (V12M and Q126X) at the *ABCG2* gene locus also did not affect pharmacokinetics of the drug lamivudine [200].

5.8.5 Effect of SNPs in Other ABC Proteins

As previously mentioned, potentially functional SNPs have been reported in 12 ABC genes, with several of these associated with drug resistance or disease progression. A synonymous SNP, e15/C2127T (rs908832), found in the *ABCA2* gene was associated with early onset of Alzheimer's disease [224], while a nonsynonymous SNP in the *ABCB11* gene, e13/T1331C (rs2287622), was associated with altered canalicular expression of the protein leading to cholestasis [225,226]. Also, within the *ABCB11* gene, e28/G236A (rs473351), was found to be associated with intrahepatic cholestasis of pregnancy [227]. A few SNPs at the *ABCC2* gene locus have been associated with a defined phenotype including e10/G1249A (rs2273697), which was correlated to *ABCC2* mRNA expression in preterm babies but not in the human placenta [228], and e1/C-24T (rs717620), which was associated with *ABCC2* mRNA levels in normal kidney cortex [229] as well as alterations to the pharmacokinetics of methotrexate in pediatric ALL patients [230]. Similarly, the SNP e10/C1446G was associated with alterations to hepatic expression of MRP2 [231], while the SNPs e18/C2366T (rs56220353) and e31/G4348A (rs56296335) were correlated to both expression level and membrane localization of MRP2 in a kidney cell line [232]. The SNP -5'UR/C211T, which resides in the *ABCC3* gene, was found to be associated with *ABCC3* mRNA levels in human liver tissue [233], yet does not affect mRNA levels in ALL patients [234]. Similar associations were reported for two SNPs located in the *ABCC6* gene, with e24/C3421T (rs72653706) being correlated with a higher susceptibility to early cardiovascular disease [235] and the nonsynonymous SNP E27/G3803A (rs2238472) associated with plasma levels [236]. The final ABC gene to contain an SNP with reported associations to an established phenotypic property is *ABCC11*. SNP e5/G538A (rs17822931) at the *ABCC11* gene locus was found to be associated with earwax type [237].

5.8.6 Diseases Arising from ABC Gene Mutations

Several familial diseases are known to arise from loss-of-function mutations in ABC transporter genes (Table 5.3). Diseases have been associated with each subclass of ABC, with the exception of ABCE and ABCF, which are not associated with active substrate transport.

Familial high density lipoprotein (HDL) deficiency, a genetic disorder transmitted as a dominant trait, and the rarer autosomal recessive disease, but clinically similar, Tangier's disease are associated with defective *ABCA1* [238]. These diseases result

TABLE 5.3 Diseases Associated with Mutations in Genes Encoding Members of the ABC Superfamily

Disease	Associated ABC Transporter	References
Tangier disease, high density lipoprotein deficiency	ABCA1	238,239
Stargardt disease and age-related macular degeneration	ABCA4	4,240
PFIC	ABCB11 (PFIC-II) ABCB4 (PFIC-III)	241,242
Immune deficiency	ABCB2, ABCB3	2
Sideroblastic anemia with ataxia	ABCB7	243
Dubin–Johnson syndrome	ABCC2	244
Pseudoxanthoma elasticum	ABCC6	245
Cystic fibrosis	ABCC7	246,247
PHHI	ABCC8, ABCC9	248
X-linked adrenoleukodystrophy	ABCD1	9
Sitosterolemia	ABCG5, ABCG8	249

Abbreviations: PFIC, progressive familial intrahepatic cholestasis; PHHI, persistent hypoglycemia in infants.
Source: Adapted from Ref. 2.

from loss-of-function mutations, of which, over 50 different mutations have been identified within the *ABCA1* gene [239]. Both diseases are characterized by a complete absence of HDL in the blood plasma and subsequently an increased incidence of cardiovascular disease [238,239]. Similarly, various macular disorders, such as Stargardt's disease and age-related macular dystrophy, are linked to mutations in the *ABCA4* gene. This gene encodes the ABCR protein, which is exclusively expressed in photoreceptors and involved in the transport of vitamin A derivatives into the retinal pigment epithelium (RPE) [4]. Degeneration of rods and cones is the ultimate result of expressing loss-of-function mutations in *ABCA4* [240].

As previously highlighted, SNP mutations in *ABCB11* have been associated with the onset of cholestasis; however, it is important to note that mutations in *ABCB4* also contribute to the disease in a similar fashion. Mutations in *ABCB4* and *ABCB11* have been found to cause progressive familial intrahepatic cholestasis (PFIC) types III and II, respectively [241]. Cholestasis, which is characterized by a retention of bile salts in the liver, an absence of biliary phospholipids and elevated bile acid levels in blood plasma ultimately leads to liver failure and, without transplant, patient death [241]. In each disease, mutations in the responsible proteins produce a dysfunctional protein or a phenotype completely lacking detectable protein. This was observed in a screen of 50 PFIC3 patient samples, where ~30 different mutations were observed in the *ABCB4* gene. In this case, no ABCB4 protein was detected following immunostaining of the samples from liver failure patients and this lack of detectable protein was proposed to result from the presence of a premature stop codon and subsequent degradation of mRNA [242,250,251]. In other examples, missense mutations at the Walker A and Walker B motifs of the *ABCB4* gene locus were observed, which prevented ATP binding and hydrolysis [242,250].

One of the most prominent diseases associated with the ABC proteins is CF, which results from mutations in the gene known as *cystic fibrosis transmembrane regulator*

(*CFTR*) or *ABCC7*. Characterized by profuse mucosal secretions within the lungs and an increased vulnerability to bacterial infections, CF continues to be associated with an average life span in patients of just 34 years [246]. Although there have been over 1000 disease-associated mutations described in the *CFTR* gene, there are several that are consistently found in large proportions of CF patients [247]. The most common mutation, known as $\Delta F508$, is present in over 90% of CF patients in the United States and produces a protein lacking a phenylalanine residue at position 508 [247]. Three-dimensional modeling of the *CFTR* protein highlights the importance of this residue in facilitating an interaction in the tertiary structure between the surface of NBD1 and the cytoplasmic loop CS4 in the C-terminal TMD (TMD2) [252]. Despite the defective protein retaining its chloride channel function in cell-free *in vitro* studies, *in vivo* studies revealed that the $\Delta F508$ mutation resulted in a misfolded protein that is swiftly degraded before it localizes to the plasma membrane [247].

From the ABCG family, it has been established that deficiency in functional ABCG5 and ABCG8 proteins produces the autosomal recessively inherited disease, sitosterolemia. This is a rare disease with only 45 cases reported in the literature worldwide and produces significantly elevated levels of plant sterols in the blood plasma. While a healthy individual retains only 5% of the plant sterols consumed in a regular diet, sitosterolemia patients retain 10–25 times more, which ultimately produces coronary and aortic atherosclerosis leading to cardiac arrest [249].

5.9 CONCLUSIONS AND FUTURE PERSPECTIVES

MDR is a problem that diminishes the efficacy of chemotherapy in a range of diseases. It continues to hamper the development of new drugs and also prevents the optimization of new combinations of existing therapies. MDR may be intrinsic to some cell types or acquired following extended exposure to chemotherapy and most often results from a complex network of mechanisms that include drug metabolism, drug efflux, and alterations to the drug target. Despite often conflicting evidence on the role of the ABC transport proteins in clinical MDR, it is clear from decades of research that the ABC transport proteins contribute significantly to this phenomenon.

With the limited success in modulating or inhibiting the function of the ABC transport proteins, it can be argued that this family of proteins has failed as a therapeutic target. Although only meant as adjuvant therapies, the existing ABC transport protein modulators and inhibitors have proven ineffective in reversing MDR and continue to be associated with high toxicities at dose concentrations required to effectively inhibit the target protein. That said, to develop agents that improve existing chemotherapy regimens and diminish the effects of MDR, the ABC transport proteins must be targeted. Molecular mechanisms of inhibition such as RNA interference may prove more effective than small-molecule treatment. However, many ABC transporters are ubiquitously expressed and play an important physiological role. Hence, the specificity of any modulator or inhibitor must be improved in order to reduce the onset of adverse effects.

Attempts to develop novel strategies to counteract the effects of MDR will undoubtedly benefit from the recent interest in the pharmacogenomics of the ABC drug transporters. With growing evidence to suggest that interindividual differences in response to therapy are linked to the presence of SNPs in the coding and promoter regions of the

ABC genes and their protein regulators, it is clear that a better understanding in this field will permit chemotherapy protocols to be better tailored to individual populations.

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6 Solute Carrier (SLC) Family Transporters

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6.1	Introduction	1
6.2	Nomenclature and general structure of SLC transporters involved in xenobiotic transport	4
6.3	Distribution and pathophysiology of SLC transporters	8
6.4	Role of the major SLC transporters in different organs	14
6.5	In vitro models to assess SLC transporters	21
6.6	In vivo models of transport	23
6.7	Clinical extrapolations	26
6.8	Interplay between SLC transporters and drug-metabolizing enzymes	27
6.9	Transporter-mediated drug interactions due to inhibition	29
6.10	Nuclear receptor-mediated SLC transporter regulation	32
6.11	Genetic polymorphisms of SLC transporters and clinical implications	34
6.12	Regulatory Guidance for SLC transporters	36
6.13	Summary	37
	Abbreviations	38
	References	39

6.1 INTRODUCTION

Xenobiotic transporters are increasingly scrutinized for their contribution to the sub-optimal pharmacokinetics (PK) and safety of drug candidates that fail in clinical trials. Over the last decade, pharmaceutical industry has used structure–activity relationships (SARs) to develop compounds with half-lives conducive to no more than once-a-day dosing and a lower incidence of metabolism-based drug–drug interactions (DDIs), yet

unexpected drug interactions were encountered in the clinic. Several of these interactions have now been attributed to drug transporters. We now know that higher metabolic stability often results in the unpredictable involvement of drug transporters, leading to transporter-mediated drug interactions. Any significant changes in transporter expression and function can contribute to variable PK, unexpected toxicities, and drug interactions.

DDIs involving membrane transporters can be the result of competition for the same substrate-binding site, tight or allosteric binding leading to inhibition of transporter activity, or changes in transporter expression. These interactions could alter the blood-concentration-time profiles of drugs, leading to altered levels of a coadministered compound. Evaluating the substrate potential of a drug candidate for specific transporters *in vitro* is beneficial, especially when the organ is also the drug target. For example, the hepatitis C drugs, α -interferon and S-acyl-2-thioethyl esters, or the HMG-CoA inhibitors, such as the statins, must achieve adequate concentrations in the liver for pharmacological activity. Inhibition of transporter activity could result in changes to hepatic availability of xenobiotics. Transporters can also play a role in toxicities, as inhibition of transporter activity could lead to increased drug concentrations or inhibition clearance (CL) of endogenous substrates, for example, bile salts, leading to cholestasis.

The solute carrier (SLC) transporter family includes over 300 membrane-bound transporters that comprise 55 gene families and at least 362 putatively functional protein-coding genes. These transporters control the uptake of endogenous compounds and xenobiotics including sugars, amino acids, nucleotides, inorganic ions, and drugs, in all living cells. According to their mode of action, as depicted in Fig. 6.1, SLC transporters can be divided into passive (facilitated) and active transporters (symporters and antiporters). Passive transporters allow the passage of solutes across membranes down their electrochemical gradients, and active transporters create ion/solute gradients across membranes, utilizing diverse energy-coupling mechanisms. While symporters transport the two moieties across the membrane in the same direction, antiporters exchange these in opposite directions.

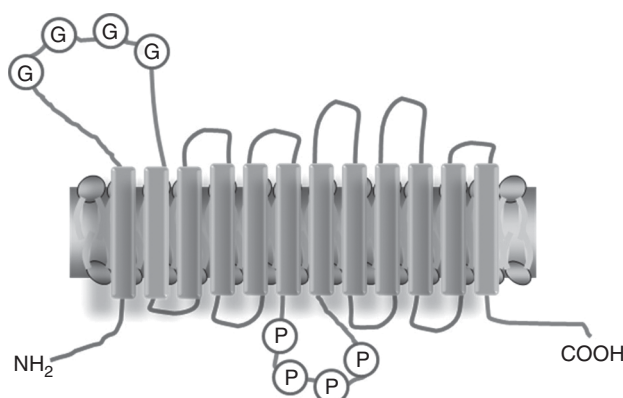


Figure 6.1 Schematic of membrane topology for SLC transporters, based on predicted structure of OAT.

SLC transporters are located in all cellular and organelle membranes, with the exception of the nuclear membrane. Substrates of SLC membrane proteins include endogenous substances such as amino acids, glucose, inorganic cations and anions, bile salts, carboxylate, essential metals, biogenic amines, neurotransmitters, vitamins, fatty acids, lipids, nucleosides, hormones, urea, and xenobiotics. These transporters are therefore essential for the normal functioning of cells and organ systems.

Less than 20 of the known membrane-bound transporters have been characterized as relevant to drug-related interactions. These participate in the absorption, disposition, and/or excretion of drugs, xenobiotics, and endogenous compounds in organs including the intestine, liver and/or kidney, and brain and perform homeostatic functions. The families of SLC transporters related to drug transport are SLCO, SLC1A10, SLC47, SLC1A22, and SLC1A7 and are discussed in details in this review. Of the six SLCO gene families, four transport organic anion-transporting polypeptides (OATPs) are most relevant to drug transport. These transporters form a superfamily of sodium-independent transport systems that facilitate the transmembrane transport of amphipathic, endogenous, and exogenous organic compounds. Among this class, SLCO1 family is the best characterized and includes OATP1B1, 1B3, and 2B1. SLCO5 and SLCO6 are also believed to be important in drug transport. The SLC10 family participates in bile salt transport and has two well-characterized members in humans: the Na^+ /taurocholate-cotransporting polypeptide (NTCP) and the apical Na^+ -dependent bile salt transporter (ASBT), which are critical to the enterohepatic recirculation of bile salts, along with efflux transporter bile salt export pump (BSEP) on the canalicular member of hepatocytes. NTCP and ASBT are cotransporters that mediate sodium-dependent, electrogenic uptake of mainly bile salts into hepatocytes (NTCP), biliary epithelial cells, ileal enterocytes, and renal proximal tubular cells (ASBT). SLC47 includes the multidrug and toxicant extrusion-1 and -2 genes (MATE1 and MATE2). SLC1A22 is the major facilitator superfamily and includes organic cation transporters (OCTs), zwitterion/cation transporters (OCTNs), and organic anion transporters (OATs). Transporters of the SLC22 family function as uniporters that mediate facilitated diffusion in either direction (OCTs), anion exchange (OAT1 and OAT3) and Na^+ /l-carnitine cotransport (OCTN2), and play an important role in the detoxification of exogenous compounds. SLC7, with 14 members, represents cationic amino acid and glycoprotein transporters. This family is divided into two subgroups, the cationic amino acid transporters and the glycoprotein-associated amino acid transporters, also called *light chains* or *catalytic chains* of the hetero(di)meric amino acid transporters.

The interplay between chemicals and transporters is evaluated using multiple *in vitro* and *in vivo* experimental approaches. For *in vitro* evaluations, the method of choice has been stably transfected cell lines. The cell lines most commonly used for transporter transfections are the MDCK, HEK-293, and LLC-PK1, and in most cases, singly transfected cell lines suffice. To evaluate hepatic transport, fresh or cryopreserved primary hepatocytes provide a good model of hepatic uptake, efflux, biliary excretion, transporter-mediated drug interactions, induction, and hepatotoxicity. *In vivo*, transgenic rodents have been used to study drug transport, for example, the oatp2 and oatp4 single-knockout mice and the oct1/oct2 double-knockout mice.

Since transporters are widely distributed and have a broad substrate spectrum, altered transport kinetics due to the functional consequences of genetic variations (polymorphisms) can contribute to the interindividual variability of drug PK and pharmacodynamic (PD) effects, as well as to idiosyncratic toxicities. Owing to this, evaluations

of the effects of polymorphisms on ADME (absorption, metabolism, distribution, and elimination) should also be evaluated.

6.2 NOMENCLATURE AND GENERAL STRUCTURE OF SLC TRANSPORTERS INVOLVED IN XENOBIOTIC TRANSPORT

The SLC superfamily comprises more than 360 putatively functional protein-coding genes organized into 55 SLC families on the basis of alignment similarities and differences in transport mechanisms [1]. A website maintained by the HUGO Gene Nomenclature Committee provides the latest updates of SLC transporter nomenclature (<http://www.genenames.org/genefamily/SLC.php>). Generally, the root symbol of SLC is used to name the genes followed by a number or letter and then another number denoting the isoform, for example, SLC22 represents SLC family 22. The letters, A–E are used to specify subfamilies (Table 6.1). Owing to the new isoforms discovered within a given species and the rapid evolution of this family, the initial naming system underwent modifications to accommodate a unique, species-independent classification. For example, in the subclass originally named SLC21 that encodes OATP, the numeral “21” and the letter “A” were replaced by the letter “O,” which stands for organic transporter [1]. It is also commonly accepted in transporter nomenclature that upper case letters denote the human protein, that is, OATP/SLCO and lower case letters indicate transporter proteins of preclinical species, for example, Oatp/Slco.

TABLE 6.1 Major Human SLC Transport Proteins Involved in ADME

Transporter Symbol	Transporter Name	Synonyms	Chromosome
SLC7A5	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	LAT1, E16, D16S469E, MPE16, CD98	16q24.3
SLC7A5P1	Solute carrier family 7 (amino acid transporter light chain, L system), member 5 pseudogene 1	LAT1-3TM, MLAS	16p11.2
SLC10A1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	NTCP	14q24
SLC10A2	Solute carrier family 10 (sodium/bile acid cotransporter family), member 2	ASBT, ISBT	13q33
SLC15A1	Solute carrier family 15 (oligopeptide transporter), member 1	PEPT1, HPECT1, HPEPT1	13q33-q34
SLC15A2	Solute carrier family 15 (H ⁺ /peptide transporter), member 2	PEPT2	3q21.1
SLC16A1	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	MCT, MCT1	1p12
SLC16A7	Solute carrier family 16, member 7 (monocarboxylic acid transporter 2)	MCT2	12q13
SLC22A1	Solute carrier family 22 (organic cation transporter), member 1	OCT1	6q25.3

TABLE 6.1 (*continued*)

Transporter Symbol	Transporter Name	Synonyms	Chromosome
SLC22A2	Solute carrier family 22 (organic cation transporter), member 2	OCT2	6q25.3
SLC22A3	Solute carrier family 22 (extraneuronal monoamine transporter), member 3	OCT3, EMT	6q25.3
SLC22A4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	OCTN1, MGC34546	5q23.3
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5	OCTN2, SCD	5q23.3
SLC22A6	Solute carrier family 22 (organic anion transporter), member 6	ROAT1, PAHT, OAT1	11q12.3
SLC22A7	Solute carrier family 22 (organic anion transporter), member 7	NLT, OAT2	6p21.1
SLC22A8	Solute carrier family 22 (organic anion transporter), member 8	OAT3	11q12.3
SLC22A9	Solute carrier family 22 (organic anion transporter), member 9	OAT4, FLJ23666, UST3, OAT7	11q12.3
SLC47A2	Solute carrier family 47, member 2	FLJ31196, MATE2, MATE2-K	17p11.2
SLC28A1	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 1	CNT1	15q25.3
SLC28A2	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	CNT2, SPNT1, HCNT2, HsT17153	15q15
SLC28A3	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	CNT3	9q21.33
SLC29A1	Solute carrier family 29 (nucleoside transporters), member 1	ENT1	6p21.1
SLC29A2	Solute carrier family 29 (nucleoside transporters), member 2	DER12	11q13
SLC29A3	Solute carrier family 29 (nucleoside transporters), member 3	ENT3, FLJ11160	10q22.2
SLCO1A2	Solute carrier organic anion transporter family, member 1A2	OATP, OATP1A2, OATP-A	12p12
SLCO1B1	Solute carrier organic anion transporter family, member 1B1	OATP-C, LST-1, OATP1B1	12p12
SLCO1B3	Solute carrier organic anion transporter family, member 1B3	OATP8, OATP1B3	12p12
SLCO2B1	Solute carrier organic anion transporter family, member 2B1	OATP-B, OATP2B1	11q13

Source: Data derived from website of HUGO Gene Nomenclature Committee (HGNC) (<http://www.genenames.org/genefamily/SLC.php>).

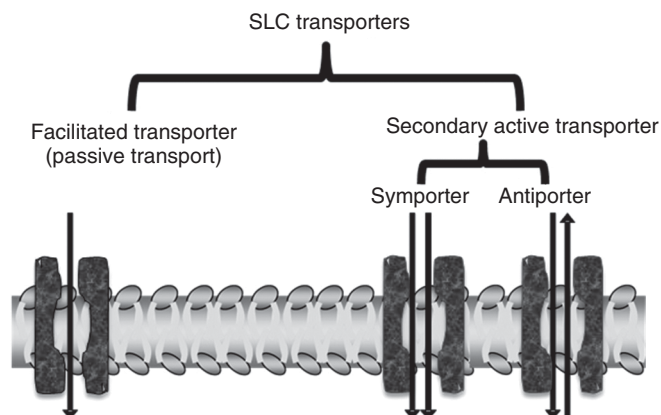


Figure 6.2 Schematic depicting passive and active transport processes of SLC transporters.

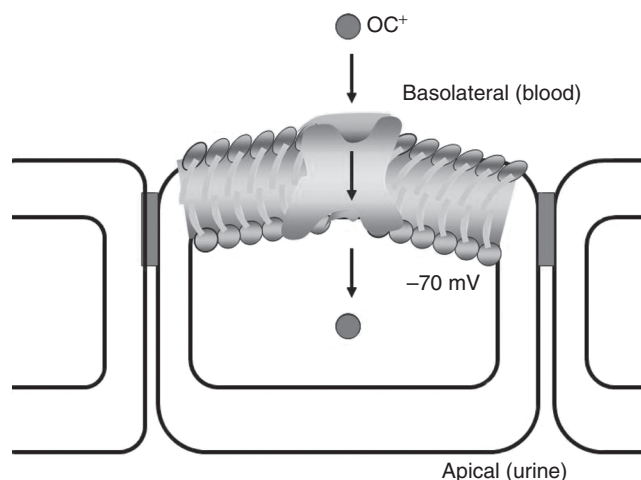


Figure 6.3 Depiction of the carrier-mediated facilitated transport processes for transporters such as OCT.

The SLC family consists of proteins containing multiple transmembrane domains (Fig. 6.1) and facilitates the movement of a substrate across cellular membranes, either along or against its concentration gradient. Based on the transporting mechanism, the SLC superfamily is categorized into passive (uniporters or facilitative transporters) and secondary active transporters (Fig. 6.2). Passive transporters, also known as *facilitated transporters*, utilize an electrochemical potential difference caused by the respective substrates to move substrates down a concentration gradient (Fig. 6.3). In contrast, secondary active transporters translocate the substrates uphill against their electrochemical gradient by coupling with another ion or molecule that flows downhill with its concentration gradient (Fig. 6.4). Basically, these processes are free-energy changes. The secondary active transporter families, including the OATPs (SLC21A or OATP), peptide transporters (SLC15A or PEPT), novel-type OCT (SLC22A or OCTN), and

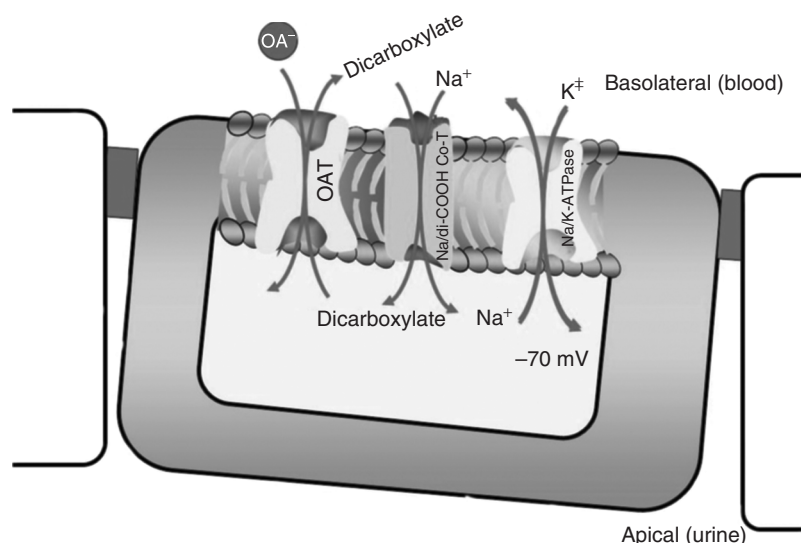


Figure 6.4 Depiction of carrier-mediated active transport processes for transporters such as OAT.

sodium-dependent bile acid transporter (SLC10A1 or NTCP), either utilize ion gradients across membranes or cotransport with intracellular and/extracellular ions [2,3] (Fig. 6.4). On the basis of the direction of the coupling ion or molecule, the secondary active transporters are further categorized as symporters and antiporters (Fig. 6.2). Symporters move the transported molecule and coupled ion toward the same direction, while antiporters move the molecules and coupled ion in opposite directions. In addition to localization at the cellular plasma membranes, the SLC proteins are also expressed on membranes of cellular organelles such as mitochondria, endoplasmic reticulum, Golgi, and other vesicles. These SLC transporters are termed *mitochondrial* and *vesicular transporters* [1] and are not discussed in this chapter.

Relatively few of the SLC transporters have been established to be actively involved in disposition of market drugs or drug candidates. From a pharmaceutical industry perspective, these SLC transporters are of great importance, in that they may rate-limit drug exposure to metabolizing enzymes in metabolic tissues, thus serving as gatekeepers for drug disposition and as drug targets, facilitating drug delivery and being responsible for drug interactions. For example, the glucose and neurotransmitter transporters have been exploited as drug targets for the past two decades, and more recently, the oligopeptide transporter (PEPT1) is being investigated as an endogenous delivery mechanism for drugs, where translocation across the intestinal mucosa or blood–brain barrier (BBB) is challenging [4]. Considerable progress has been made in understanding SLC transporter-mediated DDI and toxicities [5]. In 2010, the International Transporter Consortium summarized several SLC carriers including OATPs/SLCO, OCTs/SLC22, and OATs/SLC22, which are clinically relevant to DDI, and made recommendations for evaluating the substrate and inhibition potential of candidate drugs with these transporters [6]. These transporters are discussed in more details below.

6.3 DISTRIBUTION AND PATHOPHYSIOLOGY OF SLC TRANSPORTERS

The SLC family is distributed in the epithelia of various organs and transports a variety of endogenous and exogenous substrates (Table 6.2). For example, OAT3 is expressed on the basolateral membrane of the proximal tubule in the kidney and mediates the secretion of organic anions into urine via carrier-mediated active transport (Fig. 6.8) [7–9]. To move a substrate across the cell membrane, the SLC transporters need to collaborate with the ATP-binding cassette (ABC) transporters or other SLC transporters in the same or opposite membrane of polarized cells. Recently, the human MATE2-K transporter was identified as a luminal membrane transporter that transports organic cations out of renal proximal tubular cells in coordination with OAT3 [10]. Diverse organ distribution of the SLC transporters and coexistence of multiple transporter isoforms in tissues suggest that collaborative and compensatory functions of transporters might play an important role in physiological communication network between organs, as well as tissue distribution and elimination of xenobiotics. Transporter pharmacology is a rapidly emerging subject in drug discovery and development and therefore is of particular interest to the pharmaceutical industry. The SLC members discussed below are clinically relevant in drug absorption, drug disposition, and/or DDI.

6.3.1 SLCO Superfamily: Organic Anion-Transporting Polypeptides (OATPs)

The SLCO subfamily proteins are predicted to have a 12-transmembrane-domain structure, similar to that depicted in Fig. 6.1. These transporters are expressed in organs such as the intestine, liver, muscle, and BBB. Members of the SLCO family include several OATP transporter isoforms that play an important role in uptake of structurally diverse endogenous compounds and xenobiotics, including clinical therapeutics. OATP transporters are involved in the entry of diverse drugs into organs. The inhibition or downregulation of OATP expression and/or function by a drug, or gene polymorphisms, may lead to PK alterations and an increase in the propensity for DDI [11].

OATP1A2/SLCO1A2 (formerly OATP-A) has been identified in the liver, brain, kidney, and testes [12] and transports diverse compounds, such as steroid sulfates, thyroid hormones, and opioid peptides [13]. In contrast to the distribution of OATP1A2, OATP1B3/SLCO1B3 and OATP1B1/SLCO1B1 are believed to be liver specific (Fig. 6.6) [14]. Immunohistochemical analysis indicates that OATP1B1, also referred to as *liver-specific transporter 1* (LST-1), OATP2, or OATP-C is localized to the basolateral (sinusoidal) membrane of hepatocytes [15]. Generally, OATP transporters exhibit a broad substrate selectivity that overlaps across isoforms. For example, the substrates for OATP1B1 or 1B3 include anionic (e.g., statins such as pravastatin, pitavastatin, and rosuvastatin), zwitterionic (e.g., rifampicin), and neutral lipophilic (e.g., paclitaxel) drugs. OATP1B1 and 1B3 also transport endogenous substances, such as bile acids, thyroid hormones, steroid sulfates, glucuronide conjugates, and peptides [12,16]. Comprehensive lists of human OATP substrates have been published and provide more details [17,18].

OATP2B1/SLCO2B1 (previously OATP-B) was initially cloned from the human brain and subsequently found in the liver, kidney, lung, heart, placenta, and gastrointestinal (GI) tract [19,20]. Functionally, OATP2B1 demonstrates higher transporter activity for estrone-3-sulfate and appears to have more limited substrate specificity relative to OATP1B1 or 1B3 [19].

TABLE 6.2 Major Human SLC Transport Proteins, with Some Known Substrates and Inhibitors

Transporter	Organ, Cell, Membrane	Substrates Used Experimentally	Substrate Drugs	Endogenous Substrates	Inhibitors and Inhibitor Drugs
OATP1B1	Liver, hepatocyte, basolateral membrane	BSP, E3S, E17 β -G	Statins, repaglinide, olmesartan, enalapril, temocaprilat, valsatan	Bile acids, bilirubin, steroid hormones	Rifampicin, rifamycin SV, saquinavir, ritonavir, cyclosporine
OATP1B3	Liver, hepatocyte, basolateral membrane	BSP, cholecystokinin 8, E17 β -G	Statins, fexofenadine, telmisartan, valsartan, paclitaxel, enalapril, erythromycin, phalioidin	Bile acids, steroid hormones	Rifampicin, cyclosporine, ritonavir
OATP2B1	Liver, hepatocyte, basolateral membrane. Endothelia	E3S, BSP	Statins, fexofenadine, glyburide, rifampicin	Bile acids, steroid hormones	Rifampicin, cyclosporine
OATP1A2	Brain capillaries, endothelia. Liver, cholangiocytes. Kidney, distal nephron	E3S, BSP, fexofenadine	Methotrexate ouabain, levofloxacin, statins	Bile acids	Naringin, ritonavir, lopinavir, saquinavir, rifampicin, verapamil, dexamethasone, ketoconazole, lovastatin
OAT1	Kidney, proximal tubule, basolateral membrane. Placenta	PAH	Adefovir, zidovudine, ciprofloxacin, methotrexate	Cyclic nucleotides, prostaglandin, uric acids	Probenecid, novobiocin

(continued overleaf)

TABLE 6.2 (*continued*)

Transporter	Organ, Cell, Membrane	Substrates Used Experimentally	Substrate Drugs	Endogenous Substrates	Inhibitors and Inhibitor Drugs
OAT3	Kidney, proximal tubule, basolateral membrane. Brain, choroid plexus, and blood–brain barrier	E3S, furosemide, bumetanide	NSAIDs, cefaclor, ceftizoxime,	Prostaglandin, uric acids, bile acids; conjugated hormones	Probenecid, novobiocin
OCT2	Brain, capillaries. Endothelia. Liver, cholangiocytes. Kidney, distal nephron	E3S, BSP, dehydroepiandrosterone sulfate	Fexofenadine, methotrexate, digoxin, statins	Bile acids, choline, acetylcholine, and monoamine neurotransmitters	Naringin, ritonavir, rifampicin
OCT1	Liver, hepatocytes, basolateral membrane. Intestine, enterocytes, apical membrane	TEA, MPP	Metformin, oxaliplatin	Choline, acetylcholine, and monoamine neurotransmitters	Quinine, quinidine, disopyramide

MATE1	Kidney, proximal tubular cells, apical membrane. Liver, hepatocytes, apical membrane. Skeletal muscle	TEA, MPP	Metformin, cephalixin, acyclovir, ganciclovir	Peptides and nucleosides	Quinidine, cimetidine, procainamide, verapamil
MATE2-K	Kidney, proximal tubular cells, apical membrane	Metformin, MPP	MPP, TEA	Creatine, guanidine, thiamine	Cimetidine, quinidine, pramipexole
NTCP	Liver, hepatocytes, apical membrane	Bile acids (e.g., taurocholic acid, glycocholic acid)	Rosuvastatin, pitavastatin, atorvastatin	Bile acids, iodothyronines	Troglitazone, bosentan

Abbreviations: BSP, bromosulfophthalein; E3S, estrone-3-sulfate; E17 β -G, estradiol-17 β -glucuronide; PAH, *para*-aminohippurate; TEA, tetraethylammonium; MPP, *N*-methylpyridinium.

Source: A significant part of this table is from the paper published by the International Transporter Consortium [6], with additions based on current literature cited elsewhere in the text.

6.3.2 SLC22 Superfamily: Organic Anion, Organic Cation, and Zwitterion Transporters (OATs/OCTs/OCTNs/MATEs)

Since Gründemann *et al.* discovered the rat Oct1 in 1994 [21], its orthologs were subsequently identified and found to be expressed in excretory organs such as the kidney. SLC22 transporters play a critical role in maintaining homeostasis of endogenous substances and urinary elimination of therapeutic agents by carrier-mediated facilitated transport (Fig. 6.3). The SLC22 transporters are also expressed in barrier organs such as the choroid plexus, retina, and placenta [22] and contribute to disposition and homeostasis of organic anions and cations, thereby modulating efficacy and toxicity of clinical therapeutics. SLC22 transporters interact with structurally diverse substrates. On the basis of the substrate specificity and mechanism of transport, SLC22 members are divided into several subgroups including OCTs, OATs, OCTNs, unknown soluble transporters (USTs), and fly-like putative transporters (FLIPTs) [23].

6.3.2.1 OAT Subtypes 1–5. The transporters in this group translocate organic anions including xenobiotics, hormones, and biogenic amines against electrical and chemical gradients. OAT1 (SLC22A6) is expressed mainly at the basolateral membrane of proximal tubule cells and mediates the uptake of organic anions such as *p*-aminohippurate (PAH) from blood into renal proximal tubular cells. In *in vitro* functional assays, an outwardly directed concentration gradient of α -ketoglutarate or glutarate can improve the OAT1-mediated PAH transport, suggesting that α -ketoglutarate gradient provides the driving force for the uptake of organic anions [24]. OAT1 is thus identified as an organic anion/dicarboxylate exchanger. OAT2 (SLC22A7) is expressed abundantly on the basolateral membrane of the liver (sinusoidal membrane) and weakly in the kidney, with marked gender-based differences in expression [25]. Unlike OAT1, OAT2 transports substrates without dependence on sodium, dicarboxylate, glutarate, and α -ketoglutarate gradients and is not considered an organic/dicarboxylate exchanger [26]. Oat3 (SLC22A8) was initially cloned from a rat brain cDNA library and subsequently detected in the kidney, brain, liver, and at low concentrations in the eyes [27]. OAT3 is located on the basolateral membrane of the proximal tubule in the kidney and on the apical membrane of the choroid plexus in the brain [28]. Largely overlapping with the substrates of OAT1 and 2, OAT3 is capable of transporting PAH, methotrexate, cimetidine, and estrone sulfate.

Within the OAT family, OAT4 (SLC22A11) is the only transporter highly expressed in both the placenta and the kidney [22]. Expression of the OAT4 protein was confirmed on the apical membrane of the proximal renal tubular cells, but thus far, there is no consensus on its function. Similar to OAT1 and OAT3, OAT4 is reported to be responsible for reabsorption of substrates from the lumen, in exchange for dicarboxylates. The substrate spectrum of OAT4 is relatively narrow as compared with the other OAT isoforms. OAT4 substrates include estrone sulfate, dehydroepiandrosterone sulfate, ochratoxin A, prostaglandin E₂ (PGE₂), and prostaglandin F (PGF) [29].

Through computational analysis of the genome database, Oat5 (Slc22a19) was discovered [30]. The expression of Oat5 in mouse and rat is confirmed, and a human OAT5 ortholog is yet to be identified. Human OAT6 (SLC22A20), OAT7 (SLC22A9), OAT8 (SLC22A25), OAT10 (SLC22A13), FLIPT1 (SLC22A15), and their rat orthologs have been identified by searching genome databases or via PCR homology screening approaches [31–33]. However, the functional characteristics of this so-called orphan

transporter group are yet to be determined. To date, there is no evidence that these transporters play a major role in xenobiotic disposition.

6.3.2.2 OCT Subtypes 1–3. Transporters in this class mainly translocate organic cations and weak bases and are electrogenic, sodium independent, and bidirectional [23]. OCT1 (SLC22A1) is predominantly expressed on the sinusoidal membrane of hepatocytes in human and rat liver and is also present in other preclinical species such as mouse and rabbit [34,35]. It is also found on the basolateral membrane of small intestinal enterocytes and the renal proximal tubular cells. OCT1 mediates Na^+ -independent transport of type I organic cations (protonated molecules), such as tetraethylammonium (TEA), 1-methyl-4-phenylpyridinium (MPP^+), *N*1-methylnicotinamide (NMN), dopamine, and choline [36], as well as type II cations (larger and bulkier cations) including methyl-quinine and quinidine. OCT1-mediated organic cations transport is potential, sensitive, and electrogenic.

OCT2 (SLC22A2) is expressed predominantly in kidney and also in placenta, lung, brain, and small intestine [23]. OCT2 is localized to the basolateral membrane of the distal tubule in the kidney and mediates uptake from the blood to the proximal tubular cells during the renal secretion of organic cations [37]. OCT2 transports many organic cations and plays an important role in the pharmacological, PK, and toxicological properties of therapeutics. In the brain, OCT2 is expressed on the apical (ventricular) membrane in epithelial cells of the choroid plexus. Since OCT2 transports monoamine neurotransmitters including dopamine, noradrenaline, adrenaline, and 5-hydroxytryptamine, tissue-dependent expression of OCT2 in tissues such as neurons, lung, and choroid plexus may participate in the regulation of interstitial and intracellular concentrations of monoamine neurotransmitters and cationic drugs [38].

OCT3 (SLC22A3) has a broader tissue distribution than OCT1 and OCT2 and is also detected in muscle, glial, cardiac, and cancer cell lines. OCT3 is localized on the basolateral membrane of the trophoblast in placenta, the epithelial cells of renal proximal tubules and hepatocytes, and the luminal membrane of bronchial epithelial cells and small intestinal enterocytes [39–41]. The broader distribution of OCT3 contributes to physiological functions such as regulation of the interstitial concentration of monoamine neurotransmitters and cationic drugs in the central nervous system and release of acetylcholine from placenta [42]. The substrate specificity of OCT3 is similar to that of OCT1 or OCT2, which is capable of transporting type I and II organic cations such as the neurotransmitters, namely, adrenaline, TEA, noradrenaline, and histamine, and the neurotoxin MPP^+ .

OCT6 (SLC22A16), also named CT2 or FLIPT2, is located predominantly in human testis but is also found in the embryonic liver, hematopoietic cells, and cancer cell lines. OCT6 transports carnitine with a high affinity and shows a greater selectivity for substrates. For example, OCT6 bidirectionally transports L-carnitine across cell membranes but does not interact with TEA and other organic cations [43].

6.3.2.3 OCTN1–3 (SLC22A4–6). These transporter proteins function as organic cation uniporter or H^+ /organic cation antiporters, or as uniporters for organic cations or Na^+ /carnitine cotransporters. OCTN1 (SLC22A4) is expressed in kidney, muscle, placenta, prostate, and heart [44]. OCTN1 is an electroneutral H^+ /organic cation antiporter and mediates the transport of monovalent organic cations including TEA, quinidine, pyrilamine, verapamil, and the zwitterion carnitine [45]. OCTN1 is expressed on the

luminal membrane of renal proximal tubular cells and mediates the cellular efflux of organic cations. The bidirectional function of this transporter indicates that it participates in reabsorption of organic cations [44]. There are significant species differences in the localization and transport mechanism of OCTN1 that is strongly expressed in the liver of rat, but not in humans [46].

OCTN2 (SLC22A5) is a Na^+ -dependent transporter for TEA, choline, verapamil, pyrilamine, L-carnitine, and the zwitterionic β -lactam antibiotic cephaloridine. It also serves as a Na^+ -independent cation transporter [41], mediating the Na^+ -independent cotransport of short-chain acyl esters of carnitine, zwitterionic β -lactam antibiotics, L-lysine, and L-methionine. In the absence of Na^+ , OCTN2 transports the organic cations and weak bases including quinidine, TEA, pyrilamine, verapamil, and choline [47]. Expression of OCTN2 is relatively ubiquitous and has been confirmed in kidney, muscle, heart, brain, lymphocyte, and sperm [23,46]. In kidney, luminal localization of OCTN2 in the proximal renal tubule provides the active process of reabsorption of L-carnitine, which can contribute to the secretion and reabsorption of organic cations. In the heart and muscles, OCTN2 mediates the uptake of L-carnitine into adipocytes and cardiac myocytes [23]. Polymorphic isoforms of OCTN1 and OCTN2 are linked to inflammatory bowel disease [48]. OCTN3 is found in the mouse testis and kidney and is believed to be less relevant for organic cation transport than OCTN1 and OCTN2.

6.3.2.4 MATE Transporters. MATEs are OCTs with multiple isoforms. MATE1 (SLC47A1) is a human and mouse ortholog of the multidrug and toxin extrusion family conferring multidrug resistance to bacteria [49]. MATE1 is primarily expressed in the liver, kidney, and skeletal muscles and weakly in the heart [50]. Human MATE1 is a Na^+ -independent transporter and transports TEA via a proton-cation antiport process. MATE1 interacts with MPP^+ , serotonin, cimetidine, quinidine, and verapamil, demonstrating functional similarity to the OCTs and OCTNs. MATE1 is localized to the luminal membranes of proximal tubular cells in the kidney and on the canalicular membrane of hepatocytes in the liver and is thought to play a key role in the excretion of organic cations through exchange of protons in the kidney. Recently, MATE1-coordinated transport of antibiotics with OAT3 was reported [10].

MATE2 comprises two splice variants, MATE2-K and MATE2-B. MATE2-K is predominantly expressed in the human kidney, whereas MATE2-B was identified in the human brain. MATE2-K is thought to be the kidney-specific H^+ /organic cation antiporter mediating the luminal efflux of a wide range of cationic compounds (endogenous substrates such as creatine, guanidine, and thiamine and drug substrates such as metformin and oxaliplatin) across the brush border membranes in the renal proximal tubular cells; however, the clear function of human MATE2-B is yet to be determined.

6.4 ROLE OF THE MAJOR SLC TRANSPORTERS IN DIFFERENT ORGANS

As described in the previous section, various drug transporters are expressed in the liver, kidney, intestine, placenta, and BBB. In most cases, individual SLC transporters are preferably expressed on either the apical or the basolateral membrane of polarized cells and work together to mediate the transport of xenobiotics. For example, in the liver, drugs are first taken up through the sinusoidal (basolateral or blood side)

membrane by hepatic uptake transporters. Once inside the cells, these drugs either are metabolized by P450 metabolism (Phase I) and/or conjugation (Phase II) and/or transported out of hepatocytes by transporter proteins located on the canalicular (apical) membrane and subsequently eliminated across bile canaliculi, or are transported back to the blood through sinusoidal (basolateral) transporters. The unidirectional transport process mediated by apical and basolateral membrane transporters guarantees the vectorial elimination of xenobiotics from the body. From a pharmaceutical perspective, organ distribution of SLC transporter proteins that is of the most interest with respect to absorption, disposition, and elimination is described below.

6.4.1 SLC Transporters in Intestine

Multiple parallel transport processes, such as passive transcellular diffusion, carrier-mediated absorption, and carrier-mediated efflux, exist in the intestinal barrier [51,52]. Absorption of therapeutics, nutrients, bile acids, and other compounds in the GI tract is often mediated by SLC transporters expressed in enterocytes (Fig. 6.5) [53]. Uptake transporters including PEPT1, ABST, concentrative or equilibrium nucleoside transporters (CNT1–3/SLC28A1–3 or ENT1–3/SLC29A1–3), OATP family, and organic cation/anion/zwitterion transporters (OAT/OCT/OCTN) facilitate drug absorption across the intestinal brush border membrane. These SLCs are critically involved in absorption, thereby contributing to the distribution and PK characteristics of many drugs. For example, β -lactam antibiotics and dipeptidyl-like anticancer drugs, such as bestatin, are absorbed by PEPT transporters [54,55].

Three subtypes of the SLC22 family have been identified in the GI tract: OCTs, OATs, and the OCTNs. OATPs of the SLCO family also have important functions in the intestine and transport structurally diverse substrates [56]. ASBT is critical for

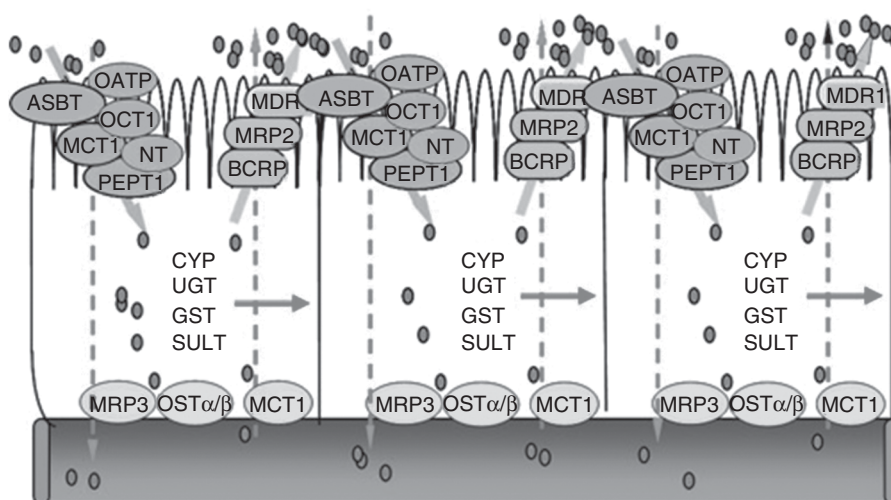


Figure 6.5 Schematic of apical and basolateral membrane transporters in human enterocytes [50].

enterohepatic circulation of bile acids by mediating their absorption at the apical membranes of enterocytes [57]. A defect in ASBT function could result in primary bile acid malabsorption characterized by congenital diarrhea and reduced plasma cholesterol levels [58]. Nucleoside transporters belonging to the SLC28 family are essential for *de novo* nucleic acid synthesis and for cancer therapy by mediating uptake of anti-cancer and antiviral nucleoside analogs into tumor tissues. CNT1–3 (concentrative) are localized to the apical membranes of epithelial cells and mediate nucleoside uptake against concentration gradients, whereas ENT1–3 (equilibrium) translocate nucleoside substrates following concentration gradients and are believed to be responsible for nucleoside efflux at the basolateral membranes of enterocytes. The oligopeptide transporter PEPT1 is present at the apical membrane of enterocytes and transports nutrient-derived peptides, as well as peptidomimetic drugs [59].

Recently, targeting uptake transporters in the design of poorly absorbed drugs has been proposed as a strategy for improving bioavailability and therapeutic efficacy [4,60]. Because active uptake and efflux transporters in the GI tract may contribute to the bioavailability of orally administered drugs, modulation of their expression levels or activities may lead to potential drug transporter interactions during absorption.

6.4.2 SLC Transporters in Liver

Hepatic drug elimination generally constitutes a series of events including (i) transport of drug into the hepatocyte via passive diffusion or hepatic uptake transporters (phase 0); (ii) intracellular metabolism, including cytochrome P450 metabolism (phase I) and/or conjugation (phase II); and (iii) flux into blood or bile via the hepatic transporters (phase III). Recently, the role of hepatic SLC uptake transporters in drug disposition has received considerable attention [6], as these proteins play an important role in translocating endogenous compounds and drugs from blood into the liver. This function represents a rate-limiting step in the entry of substrates into hepatocytes and plays a key role in drug elimination, even for the compounds that are primarily metabolized by hepatic enzymes. The distribution and elimination of drugs in the liver is dependent on the degree of expression of each transporter protein and substrate affinity for a transporter. Uptake transporters in liver may contribute to drug exposure, DDI, and toxicity of a therapeutic agent. Hepatic uptake transporters can also be targeted to selectively deliver therapeutic agents to the liver.

Hepatic uptake systems are divided into two classes, Na⁺-dependent and Na⁺-independent influx transporters (Fig. 6.6). The Na⁺-dependent bile acid uptake is mediated by the NTCP, while the OATP transporters, OATs, OCTs, and OCTNs transport a variety of organic anions through Na⁺-independent processes. Among the SLC superfamily, SLCO family, SLC22 family, and SLC10 gene family represent the predominant transport proteins that act as active carriers of organic anions across the sinusoidal membrane. Of the 11 functional human OATP transporters identified, only 3 isoforms (OATP1B1, OATP1B3, and OATP2B1) are currently believed to play an important role in hepatic uptake of exogenous therapeutics and endogenous compounds in hepatocytes [6]. OATP1B1, OATP2B1, and OATP1B3 are colocalized on the same membrane domain and have overlapping substrates, suggesting that inhibition of any one of the OATP would theoretically not cause a significant change in drug PK. While it is not easy to assess the relative contribution of each of these three transporters,

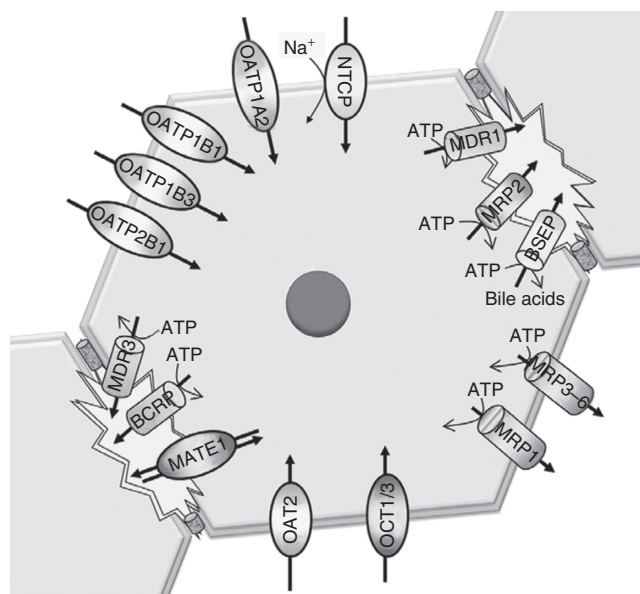


Figure 6.6 Schematic of apical and basolateral membrane transporters in human hepatocytes.

it is known that net inhibition of hepatic uptake can result in drug interactions and changes in systemic exposure and even unexpected toxicity. For example, in kidney transplant recipients receiving cyclosporine, a 3.8-fold increase in the area under the curve (AUC) of cerivastatin was observed [61] because of the inhibition of OATP1B1-mediated cerivastatin uptake into hepatocytes [62]. Recently, selective OATP inhibitors, such as estrone-3-sulfate for OATP1B1 and CCK-8 for OATP1B3, were used successfully to assess the relative roles of these isoforms in the hepatic uptake of pitavastatin, fexofenadine, and rosuvastatin [63].

Of the six distinct OAT members cloned from the human liver and kidney, OAT2 and OAT7 are predominately expressed on the sinusoidal membrane of hepatocytes. OAT7 shows 35–46% identity to other OAT family members and mediates an exchange of organic anions similar to other members from OAT family, but OAT7 has narrow substrate specificity, mainly encompassing sulfate conjugates in exchange for the four-carbon short-chain fatty acid butyrate [64,65]. The transport of cationic drugs is predominantly catalyzed by the OCTs. Of the three OCTs characterized, OCT1 is significantly expressed in the liver. The expression of OAT and OCT proteins is regulated by drugs and disease states. The activation of nuclear transcriptional factors by their respective ligands, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and nuclear factor erythroid 2-related factor 2 (Nrf2), could alter the expression of a number of transporters in the hepatocyte [66]. For example, phenobarbital (acting through CAR) effectively decreases Oat2 expression in liver [66]. On the other hand, hepatic mRNAs for Oct1 and Oct3, but not for Oat2, were decreased in rats following lipopolysaccharide treatment [67].

6.4.3 SLC Transporters in Blood–Brain Barrier

The BBB consists of brain capillary endothelial cells with complex tight junctions that separate circulating blood and cerebrospinal fluid in the central nervous system. The high density cell layers of the BBB serve to regulate the transport of nutrients, waste products, and clinical agents in and out of the brain. The passive diffusion of small molecules across the BBB has traditionally been dependent on lipophilicity, in which modifications of this physiochemical property is a strategy to increase BBB permeability. We now know that similar to other organs such as the liver and intestine, distinct transport systems expressed in the apical and/or basolateral membrane can influence the selectivity of compounds across the BBB. Physiologically, the mechanism of BBB transport is divided into three separate processes: (i) blood-to-brain uptake of drugs and nutrients (e.g., glucose, nucleotides, and amino acids) [68], (ii) efflux ABC transporters including P-glycoprotein (P-gp/ABCB2) and BCRP (ABCG2) that prevent entry of xenobiotics into the brain, and (iii) brain-to-blood transport systems for elimination of metabolites, neurotransmitters, and neurotoxins [69]. The interplay of transporters at the BBB makes it possible to selectively restrict the distribution of many drugs and therefore enhances our understanding of the molecular mechanism governing brain penetration [70].

While ABC efflux transporters with broad substrate specificity act as a functional barrier restricting drug entry into the brain, several SLC members in the BBB may play a key role on the uptake of neuroactive compounds (Fig. 6.7). Originally cloned from human liver, SLC21A3 (OATP-A), is highly expressed in the brain, followed by kidney, liver, lung, and testis [71]. Uptake assays with mRNA-injected *Xenopus laevis* oocytes indicate that OATP-A is involved in the transport of δ -opioid receptor agonists such as D-penicillamine (2,5)-enkephalin and deltorphin II, suggesting its involvement in the transport of centrally active opioids [72]. Other members of the SLCO family, such as SLC21A9 (OATP-B), SLCO3A1 (OATP-D), SLC21A10 (OATP-E), and SLCO4A1 (OATP-F), are also detected in human brain. However, the exact localization and functions of these transporters are yet to be determined [12]. Most OAT members of the SLC22 family are found in the brain. Among those, OAT3 is the most abundant

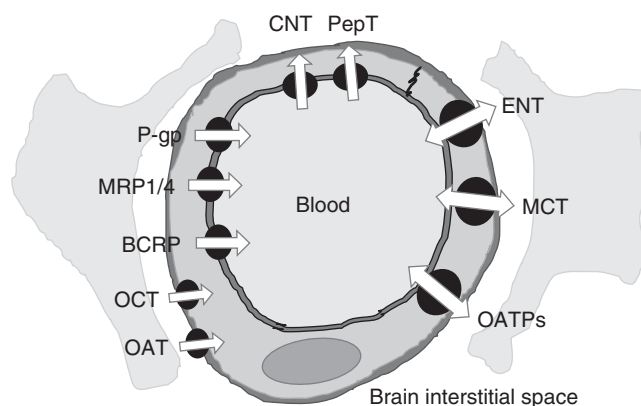


Figure 6.7 Schematic of apical and basolateral membrane transporters in human BBB endothelial cells.

OAT localized to the basolateral membrane of brain capillaries [73]. Although OAT3 is also expressed in the liver, kidney, lung, and muscles [74], it appears to play a vital role in the entry of substrates into choroid plexus and in the exclusion of anionic metabolites of neurotransmitters from the brain [28,75].

Three OCTs OCT1–3 are reported expressed in the brain at varying levels. While OCT1 mRNA expression is believed to be negligible, OCT2 mRNA found in neurons and choroid plexus is reported to be involved in neuron uptake and reabsorption of substrates such as dopamine, norepinephrine, serotonin, histamine, and choline from the cerebrospinal fluid [38]. In the cerebral cortex and cerebellar and hippocampal neurons in rodents, OCT3 mRNA expression is greater than that of OCT1 and 2 [76,77]. Although there is no direct evidence, the ability to interact with cationic neurotoxins and neurotransmitters suggests that OCT3 may play a significant role in handling neuron active compounds in the brain [78]. In addition to OCT and OAT, OCTN1 and 2 are detected in neurons in hippocampus, cerebellum, spinal cord, and superior cervical ganglion [77]. Systemic and brain concentrations of acetyl-L-carnitine are decreased in Octn-2 gene knockout mice, suggesting that luminal expressed Octn-2 could assist the brain uptake of carnitine [79].

6.4.4 SLC Transporter in Kidney

The kidney is a principal excretory organ for endogenous substances and clinical drugs and their metabolites through the processes of filtration, tubular secretion, and selective or passive reabsorption. The active secretion and reabsorption of organic anion occur mainly in the proximal tubule region of the kidney, particularly in the renal proximal tubular cells. SLC transporters in these cells play an important role in several processes: (i) uptake of chemicals into the cells; (ii) drug efflux into the glomerular filtrate; (iii) reabsorption of drug from the filtrate; and (iv) drug efflux back into the blood. The active renal secretion of drugs is accomplished through a vectorial transport process in the renal proximal tubules, which consists of uptake from systemic circulation via the basolateral membrane and subsequent efflux into urine across the luminal membrane [3,80,81]. Renal tubular epithelial cells express a variety of transporter proteins that handle diverse substrates to mediate proximal tubular secretion and, in some cases, reabsorption to maintain body homeostasis. Renal transporters have been recognized as important determinants in drug elimination for many clinically used drugs [3]. There are two primary groups of carrier-mediated transporters (SLC22 family) for organic anions and cations. OATs, OCTs, and OCTNs play key roles in active renal proximal tubular secretion (Fig. 6.8). Additional kidney transporters include several members of the SLC family, such as the OATPs, sodium-phosphate transporter (SLC17A1, NPT), PepTs, ENT and CNT, and MATE1 and MATE2-K.

Among the renal transporters detected, OAT1, OAT3, and OCT2 are located on the basolateral membrane of renal proximal tubule cells [37,82] and mediate the uptake of a variety of organic cations or organic anions from systemic circulation. The OATs and OCTs transport a broad range of substrates, including endogenous metabolites, anionic compounds such as nonsteroidal anti-inflammatory drugs, and the β -lactam antibiotics. Three OCT isoforms, OCT1–3, are identified as the primary transporters involved in renal secretion of several marketed drugs such as metformin, amantadine, and memantadine [3,83–85]. OATs and OCTs mediate the uptake of organic cationic and anionic compounds in concert with efflux carriers on the luminal membrane to

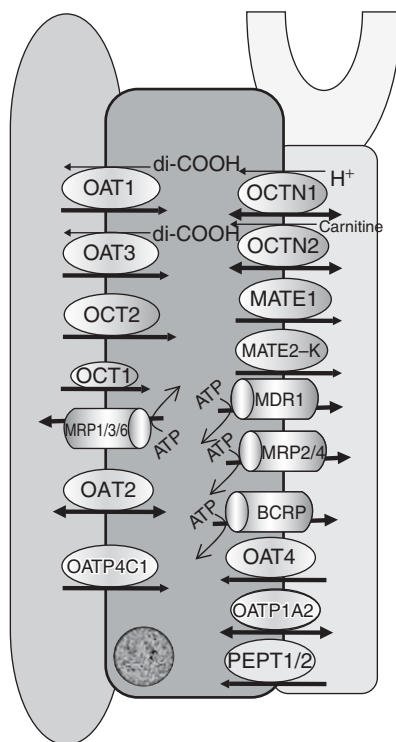


Figure 6.8 Schematic of apical and basolateral membrane transporters in human renal proximal tubular cells.

translocate xenobiotics into urine. Decrease in renal secretion and CL may produce an increase in systemic drug exposure, thereby resulting in clinically significant changes in the overall drug PK. Competitive inhibition of proximal tubular secretion is the most common type of renal DDI, for example, probenecid (an OAT substrate and inhibitor) prevents excessive accumulation of cidofovir or cephaloridine in proximal tubular cells, resulting in reduced nephrotoxicity [60,86]. Similar to renal OATs, DDI may also result from the modification of OCT1–3 functions. For example, cimetidine reduces the renal CL of metformin and thus increases exposure (increase in AUC) of metformin in patients via inhibition of OCT2 activity [87]. Substrates taken up from the systemic circulation may subsequently undergo efflux across the brush border membrane of the proximal tubule cells by various ABC efflux transporters such as P-gp and BCRP [88]. MATE1 and MATE2-K are located in the brush border membrane of proximal tubular cells in humans and preclinical species, and facilitate renal secretion of structurally diverse compounds [89]. MATE2-K works collaboratively with OAT3 for vectorial transport of substrates in proximal tubular cells [10]. OAT expression may be modulated by disease states, for example, increased Oat1 expression is found in rats with bilateral ureteral obstruction [90]. Chronic renal failure induced by nephrectomy can result in a decrease of OAT1 expression [91]. Modulation of transporter expression under disease conditions can potentially modify the renal excretion of substrate drugs.

6.5 IN VITRO MODELS TO ASSESS SLC TRANSPORTERS

Cell- and membrane-based systems are used *in vitro* to characterize transporter-mediated interactions. Whole-cell models use transformed/immortalized cell lines or primary cells, while membrane-based models include membrane fragments or vesicles.

6.5.1 Cell-Based Models

6.5.1.1 Immortalized Cell Lines. These cell lines are used to assess the absorption and permeability potential of compounds in drug discovery. The cells are referred to as *wild type* when used as a control for the cells overexpressing specific exogenous transporter genes. Caco-2 and MDCK cell lines are the most widely used system in ADME research for evaluating the substrate and inhibition potential of drug candidates. Although synonymous with efflux transporters, particularly MDR1 (P-gp), these cell lines are routinely used to assess the extent of transport across the epithelium because of the presence of well-developed and functional tight junctions. Caco-2 and MDCK cells can be cultured on Transwell® permeable supports to establish confluent monolayers with high transcellular resistance, allowing for measurement of uptake (apical to basolateral) and efflux (basolateral to apical) of test compounds through the monolayer. The advantage of the Caco-2 cells is that these are of human colonic origin and have many enterocytelike characteristics, while the MDCK cells are from the canine kidney. However, while the Caco-2 cells require three weeks to establish adequate tight junctions to form a resistant monolayer, the MDCK cells can do this within a week, making these more cost-effective, while essentially providing equivalent data [92]. Caco-2 and MDCK cells express multiple endogenous transporters such as ASBT; amino acid (e.g., LAT2), peptide (e.g., PEPT1), and monocarboxylate (MCT-1) transporters for uptake [93–95]; and MDR1, BCRP, and MRP1–3 for efflux [96]. Consequently, these cell lines (and others, e.g., LLC-PK1 and HK-2) are widely used for evaluation of oral bioavailability and predictions of BBB and tumor permeability. Although under appropriate culture conditions these wild-type cell lines produce polarized monolayers, with transporters correctly inserted in the cell plasma membrane, these are likely not present in the same quantities or ratios as those in the cells *in vivo*. Owing to the overlapping substrate specificities of many SLC transporters and the lack of transporter-specific inhibitors and substrates, an appropriate strategy is to use wild-type cell lines for the initial screening of test compounds, and if significant transport is observed, overexpressed/recombinant transporter systems are utilized to identify the specific transporters.

6.5.1.2 Stably Transfected Cell Lines. These are the most widely used system for drug-transporter-based studies. The cell lines most commonly used for drug transporter transfections are MDCK and HEK-293. For uptake transporters, singly transfected cell lines suffice, for example, OATP1B1, OATP1B3, OATP2B1, OAT1, OAT3, and OCT2 [97,98]; however, if evaluating efflux, in some cases, the coexpression of uptake transporter(s) along with the efflux transporter may be necessary to fully evaluate efflux transport. Since MDR1 transports a wide variety of lipophilic substrates that can diffuse through the cell membrane, an uptake transporter is generally not required. However, MRP2 transports organic anions and conjugates and an uptake transporter, for example, OATP1B1 may be required to facilitate the uptake of MRP2 substrate compounds into

the cell [99]. Similarly, to assess efflux of bile salts via BSEP, a vectorial coexpression system may be needed in which cells are transfected with bile salt uptake transporter, NTCP, and the efflux transporter, BSEP [97]. Studies have shown that multiple uptake transporters can be transfected simultaneously, for example, the coexpression of major hepatic uptake transporters OATP1B1 and OATP1B3 or the renal uptake transporters OAT1, OAT3, and OCT2 to evaluate substrate uptake involving several transporters [100].

6.5.1.3 Transiently Transfected Cell Systems. The advantage of the transiently transfected systems is that only one cell line is propagated in culture and the transporter of interest is transfected when needed, reducing the burden of maintaining multiple stable cell lines. Transient transfection systems are comparatively cost and resource sparing, especially when supporting large drug discovery programs. The vaccinia virus system is a widely used model [101] that yields high expression levels, as the virus shuts down the host cell protein synthesis by hijacking the host cell resources to generate recombinant viral proteins. Alternatively, *Xenopus* oocytes can be used as a recombinant system in which mRNA is injected into the cells to express the transporter of interest. In general, the overexpression of the transporter protein in this system is tedious and low throughput. This system has been used to express uptake transporters and has been applied to evaluate drug CL and address drug–transporter interactions [102].

6.5.1.4 Primary Cells. A majority of transporter studies in primary cells have been conducted with rat or human hepatocytes. Both suspension [103] and plated [104,105] primary hepatocytes are effective to evaluate drug uptake, biliary excretion, and drug–transporter interactions. In culture, when sandwiched between two layers of extracellular matrix, these cells polarize and establish functional bile canaliculi, in which transporters localize correctly to the apical and basolateral membranes [106]. Cryopreserved hepatocytes preserve both drug-metabolizing enzymes and the major uptake and biliary transporters [107,108], although levels of these change over time in culture. *In vitro*–*in vivo* correlations of uptake CL have been demonstrated using primary hepatocyte cultures utilizing the well-stirred model [109]. Cultured human and rat hepatocytes can be used to evaluate the hepatotoxic potential of drugs due to inhibition of bile acid excretion [104] and OATP-mediated transport [110], providing insight toward the prediction of drug disposition and DDI. On the other hand, limited success has been observed for primary human renal proximal tubular cells for transporter-mediated studies. While uptake can be evaluated effectively in fresh suspensions, these cells tend to lose membrane transporter activity when placed in primary culture on plastic culture plates, possibly because of internalization of the transporter proteins (authors' unpublished observations). However, when these cells are plated on permeable supports, they establish transepithelial resistance of 100–200 ohm², enabling their use to assess bidirectional transport across the monolayer [111]. Typically, the cells are cultured for 8–10 days as monolayers on permeable filter supports. To date, mRNA has been detected for OCT2, OCTN2, OAT1, OAT3, OAT4, MDR1, MRP2, and BCRP and protein for OAT1 and OAT3 [112]. Functional activity studies have been conducted demonstrating that PAH is transported (likely via uptake across the basolateral membrane by OAT1 and OAT3) and creatinine is secreted by OCT2-mediated uptake at the basolateral membrane

and efflux by MDR1 at the apical membrane. Similarly, there are a limited number of studies using primary brain endothelial cells and none to our knowledge that investigated the SLC transporters.

6.5.1.5 Hepatic Couplets. The hepatocyte couplet model allows for the evaluation of uptake and biliary elimination of test compounds. The advantage of the couplets is that these are polarized cells with a distinct basolateral membrane interacting with the media/buffer and an apical bile canaliculus, which is the sealed lumen between the two adjacent hepatocytes. Rat couplets have been used to evaluate bile transport and Bsep function by monitoring the uptake of taurocholate [113], canalicular transport of the organic cation daunorubicin [114], and electroneutral uptake and electrogenic secretion of the fluorescent bile salt 7 β -NBD-NCT [115]. Although the data obtained from these studies is valuable, isolating and working with hepatic couplets requires high technical skill and is low throughput, likely accounting for the limited ADME studies with this model.

6.5.2 Membranes and Vesicles

Both membranes and vesicles are made by overexpressing the transporter of interest by transfection of a cell line, or from organ tissues, for example, liver canalicular membranes. In the case of cell lines, once the transporter is expressed, the cell membranes are isolated and the transporter can be purified from the cell membranes [116,117]. The cell membranes can be used as is or made into vesicles to assess transporter-mediated uptake into the vesicular space. At present, only the ABC transporters have been successfully assessed in cell membranes or vesicles. It is more challenging to assess SLC activity in these systems, as the transporters are either cotransporters, antiporters, or work down a pH or concentration gradient. At present, these conditions have not been worked out in isolated membrane vesicles.

6.5.3 Small Interference RNA (siRNA/RNAi)

The mRNA of interest is specifically knocked out by the introduction of antisense RNA into the cells, resulting in degradation of the corresponding complementary mRNAs in a gene-dependent manner and loss of expression of the target protein [118]. In cell lines, RNAi has been used successfully to silence activities of specific transporters, to identify the transporters responsible for L-DOPA renal transport (rBAT and LAT2) [119], and to identify ABCA1 as the transporter mediating the secretion of the A β peptide [120]. Partial knockdown of Mrp2 (50% expression) in rat primary hepatocytes resulted in a 45% decrease in the biliary excretion index of carboxy-dichlorofluorescein without effecting Mrp3 expression [121]. In the absence of specific substrates and inhibitors for transporters, siRNA may be an appropriate system to assess the contribution of specific transporters to test compound disposition in primary cells and wild-type cell lines.

6.6 IN VIVO MODELS OF TRANSPORT

Transporter studies can also be conducted *in situ*, using isolated perfused organs or *in vivo* in wild-type, genetic knockout, genetic knockin, transgenic, or humanized/chimeric animals. Combining *in vivo* and *in vitro* data, the model provides additional insights toward potential transporter-related issues in humans.

6.6.1 Isolated Perfused Organ Systems

Compared to the *in vitro* assays, the isolated perfused intestine, liver, brain, and kidney provide a relatively more physiological determination of transporter functions in absorption, biliary elimination, brain penetration, and renal excretion, while also allowing for evaluation of the contribution of drug-metabolizing enzymes. For *in situ* studies, the organ (typically rodent) is directly infused with a physiological buffer, artificial blood, plasma, or saline via a major blood vessel entering the organ (e.g., hepatic artery for the liver) and the test compound is added to perfusate and infused for predetermined times. Concentrations of test compound and metabolites are determined in the organ and at the point of exit from the organ. The advantage of the isolated perfused organ system is that the concentration of a drug in the organ of interest can be controlled [122]. An example of such a study is using the rat-isolated liver perfusion model to determine the AUC of digoxin in the presence and absence of the Oatp2 inhibitor rifampicin and the Mdr1 inhibitor quinidine. Concentrations of digoxin in the perfusate were increased by rifampicin and decreased by quinidine, indicating that rifampicin limits enzyme exposure to digoxin by inhibiting Oatp2-mediated uptake, whereas quinidine increases enzyme exposure to digoxin by inhibiting the Mdr1-mediated efflux [123].

6.6.2 Knockout and Transgenic Models

Since transporters have broad substrate specificity, knocking out one or more related transporters at a time can theoretically result in a more clear understanding of their *in vivo* function. Care has to be taken when extrapolating these predictions to humans, as there are major differences in the rodent and human transporters in terms of substrate recognition as well as the lack of orthologs, for example, in the kidney, rodents express both Oct1 and Oct2, while humans express only OCT2.

6.6.2.1 Oatp Transporters. The hepatic transporter Oatp was first studied in a knockout mouse model of Oatp1b2 using the Oatp1b substrates pravastatin and rifampin. The liver-to-plasma ratios were lower in the knockouts as compared to wild type, indicating that Oatp1b2 is involved in the hepatic uptake and CL of these drugs [124]. The *Slco1b2*^{-/-} mice also exhibited lower liver-to-plasma ratios of the HMG-CoA reductase inhibitor lovastatin, but not of cerivastatin or simvastatin acid, suggesting that Oatp1b2 contribution in hepatic statin uptake is variable [125]. The *Slco1b2*^{-/-} mice were protected from hepatotoxicity induced by phalloidin and microcystin-LR, indicating a role for Oatp1b2 in transporting these toxins into the liver [126]. Transgenic mice expressing human OATP1B1 were created by feeding the mice a semisynthetic diet to downregulate endogenous *Slco* genes. Methotrexate PK revealed a 1.5-fold lower AUC in OATP1B1-expressing mice as compared to the wild types [127]. *Slco1a/1b*^{-/-} mice demonstrate significantly reduced hepatic uptake and elevated exposure to methotrexate and fexofenadine after IV or PO administration [128].

6.6.2.2 OATs. Oat1^{-/-} and Oat3^{-/-} mice have demonstrated the *in vivo* relevance of these transporters in renal elimination of overlapping substrates. In kidney slices from Oat3^{-/-} mice, taurocholate, estrone sulfate, and PAH concentrations were reduced

relative to those from wild-type, while there was no difference observed in the liver, which does not express Oat3 [28]. Penicillin G PK was reduced by one-half in Oat1^{-/-} and Oat3^{-/-} male mice and by two-thirds in female Oat3^{-/-} mice [129]. Gender-related differences were also observed in methotrexate CL [130].

6.6.2.3 OCTs. Knockout mouse models for all three OCTs have been developed. In the Oct1^{-/-} mice, TEA accumulation was reduced significantly as compared with wild-type mice, indicating that Oct1 is the main sinusoidal uptake system for TEA in the liver [131]. Small intestinal excretion of TEA was reduced by about 50%, indicating that Oct1 also mediates the basolateral uptake of TEA into enterocytes [132]. Knocking out Oct1 from the mouse liver resulted in a shift in the elimination of Oct1 substrate drugs from hepatic to renal elimination, and consequently, renal excretion of drugs was increased [132]. Since the Octs have overlapping substrates, knocking out all three Octs could result in more definitive data. To assess this, Oct2 single-knockout and Oct1/2 double-knockout mice were generated [131]. While removal of Oct2 did not have a significant effect on elimination of TEA, in the double-knockout mice, renal secretion of TEA was completely eliminated and higher plasma levels were observed. Oct3-deficient mice showed significant reduction in MPP⁺ accumulation in the heart as compared with wild-type mice [133]. This demonstrates that when extrapolating data with genetically manipulated preclinical species, it is advisable to consider the effects on all organs and not just on the one of interest to that particular study.

6.6.2.4 MATE Transporters. Knockout models show that as compared to the wild-type animals, Mate1^{-/-} mice exhibit a twofold increase in systemic exposure to metformin as a result of the reduced renal CL [134]. Mate1^{-/-} mice demonstrate increased cisplatin-induced nephrotoxicity and increased plasma and renal concentrations relative to wild-type mice [135]. Reduced renal CL of cephalixin was also observed in Mate1^{-/-} mice relative to that in the wild-type mice [136]. These studies showed that, in humans, the interplay between OCT2 and MATE1 may likely affect the net renal secretion of shared drug substrates.

6.6.2.5 Limitations of knockout and transgenic murine models. Although some of the murine transporter orthologs being studied are eliminated in transgenic models, the presence of other murine-specific transporters with overlapping substrate affinities are still present, and therefore, data obtained is the result of a combination of human and murine events. Another problem is that deletion of one transporter can cause alteration in expression of other transporters and/or enzymes. For example, Mdr1a/1b^{-/-} and Mdr1a^{-/-} knockout mice had significantly increased levels of CYP3A, 2B, and 1A proteins and activities [137]. Moreover, many of the SLC transporters, for example, OATPs do not have animal and human orthologs. Moreover, these models do not recapitulate the role of regulatory proteins such as PXR, liver X receptor α (LXR α), and farnesoid X receptor (FXR), which are involved in regulating the key nuclear receptors governing expression of these transporters. While these models are useful in studying a particular transport protein, the results should be extrapolated with caution since the SLC transporter being assessed may not have a human ortholog or other elimination pathways may be up- or downregulated.

6.6.3 Chimeric/Humanized Murine Models

Potential future models that could reduce deficiencies in the transgenic models because of the lack of animal and human orthologs are “humanized” mouse liver animal models. Typically, human hepatocytes are transplanted into immunodeficient mice with compromised livers (e.g., SCID mice) and between 70% and 90% replacement of murine hepatocytes with human hepatocytes is reported [138,139]. Hepatic mRNA expression of human drug-metabolizing enzymes and transporters in these chimeric mice revealed that 21 of the 23 transporters found in the wild-type mice were expressed. These mice have mainly been utilized for PK studies and have not gained widespread use because of the problems associated with using immunodeficient animals, high costs, and highly variable data, likely because of differences in hepatocyte repopulation between the mice. More recently, a humanized mouse model has been developed in immunocompetent mice with normal liver function, using facile and ectopic implantation of a tissue-engineered human liver [140]. The juxtacrine and paracrine signals in the polymeric scaffolds appear to stabilize the function of cryopreserved primary human hepatocytes. These mice exhibit humanized liver functions that persist for weeks, enabling the evaluation of drug metabolism, DDI, and drug-induced hepatotoxicity. It is likely that these models will continue to improve and that “humanized” mice will prove to be an effective *in vivo* model for transporter-based evaluations.

6.7 CLINICAL EXTRAPOLATIONS

Relatively limited work has been published thus far on successful quantitative correlations. One method published by Hirano *et al.* proposes correlation methodologies and predicts the degree of inhibition for OATP-transporter-mediated drug interactions [141]. If the compound inhibits a probe substrate with an IC_{50} value $<10 \times$ the unbound inhibitor concentration, then the compound has the potential to illicit a clinical drug–transporter interaction with the OATPs. These authors have furthered the drug interaction prediction by developing an algorithm based on *in vitro* and *in vivo* data, known as the *Degree of Interaction* (R) and estimated by the following equation:

$$R = 1 + \frac{f_u \times I_{in, max}}{K_i}$$

$I_{in, max}$ is the unbound inhibitor concentration (C_{max}) at the entry point of the organ (e.g., in the hepatic portal vein for the liver or the renal artery for the kidney), f_u is the blood unbound fraction, and K_i is the inhibition constant. However, an IC_{50} value is typically utilized instead of the K_i . The $I_{in, max}$ is determined as

$$I_{in, max} = I_{max} + \frac{F_a \times Dose \times k_a}{Q}$$

where I_{max} is the maximum circulating plasma concentration, F_a represents the absorbed fraction of inhibitor, k_a is the absorption rate constant in the intestine, and Q is the hepatic blood flow rate in humans (1500 mL/min) [142]. As the ratio of $(f_u \times I_{in, max})/IC_{50}$ increases, the likelihood of an interaction will increase as well. The potential for drug interaction is minimal if the calculated R -value is close to

one and drug interaction potential is likely with R values >2 . These extrapolations are confounded by the fact that many drugs that actively enter the liver are also metabolized, and thus far, there is no effective method to account for the interplay between transport and metabolism toward quantitative predictions of drug interactions.

In the case of the kidney, the quantitative extrapolations must also factor in the glomerular filtration rate (GFR) of the test article. When $CL > GFR$, it indicates active elimination via renal transporters, and when $CL < GFR$, it indicates active reabsorption is present. When $CL = GFR$, it may indicate no active transporter interaction or the potential for simultaneous active efflux and reabsorption, in which the two mechanisms negate each other but transporter-mediated drug interactions may still occur.

While for the CYP450 enzymes there are sufficient *in vitro* – *in vivo* correlations to classify *in vitro* IC_{50} and K_i data as low-, medium-, and high-drug interaction liability, this is not the case with the SLC transporters. For hepatic transporters, the K_m and K_i values that appear to be relevant to a clinical endpoint are quantitatively similar to the CYP enzymes, for example, K_i values in single digits for the OATP transporters [105]. However, in the case of the renal secretory transporters, in some cases, the K_m and K_i values that appear to be relevant to a clinical endpoint [143,144] can be relatively high compared to those reported for the hepatic transporters or CYP enzymes and are closer to what is reported for the UDP-glucuronosyltransferase (UGTs) [145]. For example, the CL for acyclovir in humans is higher than the GFR at 2.6 mL/min/kg and the affinity (K_m) for OAT1 is 242 μM [146]. Similarly, hydrochlorothiazide, which is actively transported predominantly via renal OATs, also has a $CL > GFR$ (4.9 mL/min/kg) and a high K_i value (150 μM) for the renal transporter OAT1 [147]. Until there are better methodologies developed to relate *in vitro* K_m and K_i values, the potential to predict *in vivo* transporter-mediated interactions will be weak. An effective strategy is to include benchmark marketed compounds, preferably similar chemotypes, as a comparator, to help guide predictions of potential interactions.

6.8 INTERPLAY BETWEEN SLC TRANSPORTERS AND DRUG-METABOLIZING ENZYMES

Transporters expressed in various tissues contribute to the distribution of physiological substances (e.g., bile acids and cholesterol) and nutrients and removal of metabolic waste [148]. The extent of drug movement across membranes is generally affected by physicochemical properties such as size, lipophilicity, charge, and degree of ionization. As transporters contribute to the ADME, they invariably influence the PK properties of drugs. The SLC/SLCO uptake transporters facilitate the distribution of hydrophilic drugs into tissues for greater access to drug-metabolizing enzymes. However, a majority of drugs are lipophilic and do not require an active transport process, as they can traverse the lipid bilayer of the cell membrane passively by diffusion.

Drug bioavailability and hepatic disposition can be greatly influenced by transporter–metabolism interplay. The interplay between drug-metabolizing enzymes and drug transporters was first identified in the 1990s from cyclosporine interaction studies with ketoconazole (CYP3A inhibitor) and rifampin (CYP3A4 inducer), in which changes in cyclosporine PK were not consistent with alterations of gut and hepatic metabolism by these two modulators [149–152]. The interplay became more

TABLE 6.3 Drugs Displaying Potential SLC Transporter and Drug-Metabolizing Enzyme Interplay

Object	Transporter(s)	Drug-Metabolizing Enzymes	References
Rosuvastatin	OATP1B1	CL _{hep} = 10%; metabolism by CYP2C9; metabolism by UGT1A1 and UGT1A3	155
Atorvastatin	P-gp, OATP1B1, BCRP	CL _{hep} ~ 100%; CYP3A4 extensive, metabolism by UGT1A3	156–158
Cerivastatin	P-gp, OATP1B1	CL _{hep} ~ 100%; CYP3A4 and CYP2C8	159
Fluvastatin	OATP1B1	CL _{hep} = 95%; metabolism by CYP2C9 (75%), but also by 3A4 and 2C8	160,161
Simvastatin	OATP1B1	CL _{hep} ~ 87%; CYP3A4 extensive first-pass metabolism, with only 5% available systemically	162
Bosentan	OATP1B1, OATP1B3	CL _{hep} ~ 97%; metabolized by CYP2C9 and CYP3A4	163
Bromocriptine	OATP1B1	CL _{hep} ~ 100%; CYP3A4, extensively metabolized	164
Repaglinide	OATP1B1	CL _{hep} ~ 98%; CYP2C8 and CYP3A approximately equal in contribution	165,166

lucid when overlap of substrate specificity for CYP3A4 and the ABC transporter MDR1 was identified. In addition to MDR1, enzyme–transporter interplay was also observed with SLC transporters, in which the transporter facilitated drug uptake into tissues (GI tract and liver) and exposed the drug to the metabolizing enzyme (Table 6.3). After metabolism, transporters, such as MDR1, BCRP, and MRP2, may contribute to the efflux of the metabolite to bile and urine [153]. Thus, transporters can be viewed as aiding the access of drugs to drug-metabolizing enzymes, thereby facilitating metabolism to protect the body from a buildup of endogenous and exogenous substances, whereas drug efflux decreases the load on detoxification enzymes [154].

6.8.1 OATP Transporters

The most widely evaluated anionic drugs are statins (e.g., fluvastatin, cerivastatin, simvastatin, and pravastatin) and diabetes drugs such as troglitazone and repaglinide. Many of the statins undergo significant phase I and phase II metabolism (fluvastatin, simvastatin, lovastatin), while pravastatin is excreted largely unchanged [167–169]. Clinical evidence based on genetic polymorphism studies for SLCO1B1 (encoding OATP1B1) of c.521 T>C (p.Val174Ala) reveal increases in the plasma concentrations

of simvastatin acid and pravastatin, but no significant effect on fluvastatin [160], consistent with hepatic uptake being rate limited. As shown in Table 6.3, there are several examples that highlight transporter–enzyme interplay in drug disposition: fluvastatin (OATP1B1 and CYP2C9, and to a minor extent CYP3A4 and CYP2C8), repaglinide (OATP1B1, MDR1, and CYP2C8 and CYP3A4), and bosentan (OATP1B1, OATP1B3, and CYP3A4 and CYP2C9) [163,165,170]. In addition to drugs with uptake and CYP metabolism, atorvastatin and rosuvastatin are also metabolized by the UGT enzymes [156–158]. The combination of transporter–drug metabolism interplay results in significant and complex drug interactions that are difficult to predict using *in vitro* systems.

6.8.2 OATs/OCTs

There are limited publications on drug transporter–enzyme interplay for OAT and OCT substrates. Many of the anti-infective drugs are predominately eliminated by renal OATs, for example, Tamiflu and olmesartan. These two prodrugs undergo metabolism that requires esterase cleavage for activity, and subsequently, their carboxylate metabolites are exclusively renally eliminated [171,172]. Ciprofloxacin and dicloxacillin undergo hepatic metabolism; however, renal CL is the predominant pathway. Methotrexate has some involvement with xanthine oxidase and aldehyde oxidase [174], and zidovudine is metabolized by UGT2B7 in the kidney [175], but there is no drug transporter–metabolizing enzyme interplay linkage reported clinically. For the OCTs, procainamide (an antiarrhythmic agent) undergoes CYP2D6 metabolism, and 65% of the dose is excreted unchanged in the urine [176], and theophylline is predominately metabolized by CYP1A2 [177]. Similarly, no reported transporter–metabolism interplay has been reported for the OCTs.

6.9 TRANSPORTER-MEDIATED DRUG INTERACTIONS DUE TO INHIBITION

6.9.1 OATP Transporters

The interplay between SLC/SLCO transporters and drug-metabolizing enzymes can be quite complex when multiple drugs are coadministered. The cotherapeutics may enhance or reduce active transport across membranes, as well as modulate the activity of metabolic enzymes, resulting in larger than expected drug interactions [178]. The uptake of HMG-CoA reductase inhibitors pravastatin, pitavastatin, atorvastatin, and fluvastatin is saturable and has been shown to be rate limiting in rats and human hepatocytes [179]. As shown in Table 6.3, statins are metabolized by CYP3A4 (simvastatin and lovastatin) and have up to 20-fold increases in plasma concentrations in the presence of strong CYP3A4 inhibitors such as itraconazole and ritonavir. Potent inducers of CYP3A can greatly decrease plasma concentrations of simvastatin and simvastatin acid, and probably those of lovastatin and lovastatin acid, while pravastatin plasma concentrations are not significantly affected by CYP inhibition and only slightly affected by CYP inducers [160]. Human PK interaction studies have shown that CYP3A4 inhibitors clarithromycin and itraconazole modestly increase the area under the plasma AUC of repaglinide to ~40% [166]. However, the AUC of repaglinide increased 8.1-fold when coadministered with gemfibrozil [166]. The large drug

interaction with gemfibrozil and its glucuronide metabolite is attributed to inhibition of OATP1B1 and CYP2C8 [166]. Further, OATP1B1-mediated hepatic uptake of repaglinide is important for its elimination by CYP-mediated reactions as evidenced by elevation of repaglinide plasma levels in subjects with the OATP1B1 (521CC) genotype.

In comparison to other statins, pravastatin has greater hydrophilicity that limits its penetration into the intracellular space of nonhepatic tissues, which is consistent with its poor penetration through the BBB [180]. Pravastatin CL is equivalent between renal and nonrenal routes (i.e., biliary excretion and biotransformation), and therefore, dose adjustments are generally not required during hepatic or renal impairment. Furthermore, as metabolism by CYPs is minimal, this reduces the extent of drug interactions for pravastatin when compared to other statins that undergo extensive metabolism. Despite pravastatin having minimal CYP metabolism, drug interactions can be pronounced, for example, a 10-fold change in pravastatin AUC is observed when it is coadministered with cyclosporine (Table 6.3).

6.9.2 OATs

It is well documented that renal tubular secretion is inhibited by the coadministration of probenecid, which increases the circulating levels of penicillin and cephalosporin antibiotics [181,182]. Probenecid inhibits active renal transport processes via inhibition of OAT1 and OAT3 [183]. As shown in Table 6.4, probenecid alters the exposure of several antibiotics, pravastatin, fexofenadine, and some antiviral drugs, with a change in AUC from 11% to 260%.

6.9.3 OCTs

The number of significant drug interactions associated with OCT is limited. Apricitabine is a deoxycytidine analog nucleoside reverse transcriptase inhibitor for the treatment of human immunodeficiency virus-1 infection. Increased apricitabine drug exposure (AUC) of 66% and 42% was observed with concomitant administration of cotrimoxazole (trimethoprim/sulfamethoxazole) and tipranavir/ritonavir combination, respectively [201,202]. The extent of drug interaction observed for cimetidine and metformin was modest as the AUC of metformin increased by 54% (Table 6.3). Genetic polymorphism of OCT2 was evaluated in the Chinese population, and the 808G>T polymorphism was attributed to a reduced metformin renal tubular CL. Furthermore, this mutation correlated with the extent of cimetidine-mediated inhibition of metformin renal tubular secretion [203].

6.9.4 NTCP Transporter

To date, there are no published clinically relevant drug–transporter interactions with human NTCP. However, *in vitro* studies utilizing HeLa-NTCP cell lines and human hepatocytes have demonstrated that rosuvastatin uptake is mediated by this transporter, accounting for ~35% of its uptake into the liver [204]. Moreover, cyclosporine and gemfibrozil can inhibit the uptake of rosuvastatin with IC₅₀ values of 0.4 and 23 μM, respectively. Polymorphisms related to NTCP have shown that the NTCP*2 variant,

TABLE 6.4 Summary of OAT-Mediated Clinical Drug Interactions

Precipitant	Object	Object Therapeutic Class	Percentage Change in AUC	References
Probenecid	Cephadrine	Antibiotics	263.2	182
	Furosemide	Diuretics	167.9	184
	Cephadrine	Antibiotics	141.6	185
	Cephadrine	Antibiotics	138.8	186
	Cefoxitin	Antibiotics	136.5	187
	Cefaclor	Antibiotics	114	185
	Cefonicid	Antibiotics	109.2	188
	Famotidine	H ₂ receptor antagonists	81.1	189
	Ciprofloxacin	Antibiotics	74.7	190
	Fexofenadine (terfenadine carboxylate)	H ₁ receptor antagonists	69.1	191
	Dicloxacillin	Antibiotics	66.8	192
	Cefmetazole	Antibiotics	58	193
	Cefoxitin	Antibiotics	45.3	187
	Acyclovir	Antivirals	40	194
	Ceftriaxone	Antibiotics	34	195
Rifampin	Pravastatin	HMG-CoA reductase inhibitors (statins)	126.6	196
Trimethoprim	Zidovudine	NRTIs	30.5	197
Disufenton sodium	Cefuroxime	Antibiotics	26.6	198
Pravastatin	Olmesartan	Angiotensin II inhibitors (ARBs)	21.1	199
Cotrimoxazole (trimethoprim/sulfamethoxazole)	Zidovudine	NRTIs	17.9	197
Furosemide	Lomefloxacin	Antibiotics	11.9	200

Abbreviations: ARBs, angiotensin receptor blockers; NRTIs, nucleoside reverse transcriptase inhibitors.

Source: The data for this table was obtained from the University of Washington Metabolism and Transporter Drug Interaction Database.

which is known to have a near-complete loss of function for bile acids, exhibits elevated activity toward rosuvastatin uptake *in vitro*. Therefore, *in vitro* data suggest that NTCP may contribute toward the disposition of rosuvastatin, and consequently, inhibitors of this transporter may have the potential to cause a clinical drug interaction; however, the extent of the interaction in humans is not known.

Circulating levels of bile acid are dependent on the interplay between several transporters such as NTCP, BSEP, and MRP2/3/4 [205,206]. Animal models have shown that reduced expression of Bsep and inhibition of this transporter may explain impaired bile excretion leading to cholestasis [207]. Caution must be applied when extrapolating animal data to humans, as bosentan is a more potent inhibitor of rat Ntcp than human NTCP [208]. While NTCP may contribute toward the regulation of bile acids, there is no clinical evidence directly linking this transporter to cholestasis.

6.10 NUCLEAR RECEPTOR-MEDIATED SLC TRANSPORTER REGULATION

Similar to drug-metabolizing enzymes, hepatic transcriptional factors mediate the regulation of drug transporters. The major transcriptional factors involved in transporter-mediated regulation are AhR; CAR; PXR; FXR; PPAR α (peroxisome proliferator-activated receptor α); Nrf2; hepatocyte nuclear factors HNF1 α , HNF3 β , and HNF4 α ; LXR α ; and retinoid X receptor/retinoic acid receptor dimer (RXR α /RAR α).

6.10.1 OATP Transporters

The regulation of these uptake transporters has been reported with AhR, PXR, CAR, and PPAR, on the basis of rodent induction studies [209]. Hepatic mRNA expression was evaluated after treatment with AhR ligands (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polychlorinated biphenyl 126, and α -naphthoflavone) and Oatp2b1 was increased, and PXR activators (pregnenolone-16 α -carbonitrile and spironolactone) increased Oatp1a4, while CAR activators (phenobarbital, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene and diallyl sulfide) decreased Oatp1a1 mRNA expression. PXR activators and PPAR α ligands (clofibrate, ciprofibrate, and diethylhexyl phthalate) decrease Oatp1a1, 1b2, 2a1, and 2b1 mRNA expression in liver, and Nrf2 activators (oltipraz, ethoxyquin, and butylated hydroxyanisole) downregulate Oatp1a1 and upregulate Oatp2b1 mRNA expression. In rodents, it appears that only a few transcriptional factors increase Oatp expression, while multiple factors decrease Oatp expression [209].

In humans, FXR, HNF1 α , HNF3 β , and HNF4 α transcriptionally regulate OATP1B1, OATP1B3, and OATP2B1 [210–214] by binding to the promoter region [212]. Site-directed mutagenesis revealed consensus binding sites in the 5'-flanking region from –38 to –24 bp for FXR, HNF1 α , and HNF3 β that are to be important for transcriptional activity of OATP1B3, as mutations in this region resulted in significantly decreased luciferase activity. Additionally, a vector containing the double mutant of HNF1 α and HNF3 β at the binding sites (–38 to –24 bp region) had significantly decreased luciferase activity as compared to the single transcriptional factor mutation. Using a different model, Kamiyama *et al.*, established an adenovirus vector expressing human HNF4 α -(hHNF4 α)-siRNA that selectively suppressed HNF4 α expression in human hepatocytes [212]. Data from this model suggests that OATP1B1 is directly regulated by hHNF4 α , unlike the CYP450 enzymes, which are indirectly regulated by HNF4 α via the upregulation of PXR and CAR expression in hepatocytes. Studies with the bile acid chenodeoxycholate (CDCA), the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA), endogenous ligands, and effective activators of FXR reveal a significant increase in OATP1B1 expression when FXR is activated [214].

6.10.2 OATs

Saji *et al.*, showed that deletion of Hnf1 α in mice (Hnf1 α null mice) resulted in a significant reduction of renal Oat1 mRNA levels. This was followed with the observation that promoter activity of Oat1 was stimulated by cotransfection of HNF1 α alone or the combination of HNF1 α and HNF1 β ; however, HNF1 β alone showed no effect [213].

For human OAT1, using the luciferase reporter gene assay, a series of 5'-truncated-promoter constructs of human OAT1 with or without the mutation in the HNF1 motif showed that HNF1 α alone, or HNF1 α and HNF1 β , bound to the promoter region within 111 bp upstream of the transcriptional start site significantly increased luciferase activity, confirming binding to the HNF1 motif [213]. HNF1 α /HNF1 α or HNF1 α /HNF1 β dimers were found to bind to the HNF1 motif in the OAT1 promoter [213]. Similarly, HNF4 α was shown to activate the OAT1 promoter in the regions spanning -1191 to -700 bp and -140 to -79 bp [215]. Kikuchi *et al.* showed that human OAT3 expression was transactivated by HNF1 α and HNF1 β . Similar to OAT1, there is a synergistic action of the HNF1 α /HNF1 α or HNF1 α /HNF1 β dimers toward transactivation and HNF1 β alone, and this does not activate expression of OAT3. Moreover, it was found that OAT3 promoter activity was repressed by DNA methylation [216].

6.10.3 OCTs

OCT1 is transcriptionally activated by HNF1 α , and two adjacent putative DNA-response elements interact with HNF1 α binding in the promoter region [212,217]. A luciferase reporter construct containing the OCT1 promoter was successfully activated by HNF4 α in transiently transfected Huh7 cells. Saborowski *et al.* showed that site-directed mutagenesis of either DR-2 element alone or in combination severely decreased the HNF4 α -mediated activation of the OCT1 promoter, indicating that both elements are functionally important [217]. In addition to HNF1/4 α , upstream stimulating factor (USF) 1 and 2 bind to the E-box regulatory element (CACGTG) of the proximal promoter, as deletion analysis suggested that the region spanning -141 to -69 bp was essential for the basal core transcriptional activity of OCT1 [218]. It appears there is coordination as the transactivation effect of USFs on the OCT1 promoter is further stimulated by HNF4 α binding.

In the case of OCT2, USF-1 binds to the E-box in the proximal promoter region, as a mutation of the E-box resulted in decreased OCT2 promoter activity, while the overexpression of USF-1 transcriptional factor enhanced OCT2 activity in a dose-dependent manner [219]. In addition, *in vitro* studies showed that methylation of the OCT2 proximal promoter resulted in significant reduction of transcriptional activity of USF-1 [220].

6.10.4 NTCP Transporter

The hepatocyte nuclear factors HNF1 α and 4 α and RXR α /RAR α bind and transactivate the rat but not the human or mouse NTCP/Ntcp promoters [221]. A 4-bp insertion in the HNF1 α recognition site located within the transcription start site of human and mouse NTCP/Ntcp gene resulted in a disrupted HNF1 α binding site and the transactivation of a consensus motif for the CCAAT/enhancer binding protein- β (CEBP- β) [221]. HNF3 β was found to be the only transcriptional factor with conserved binding sites in the 5'-regulatory region of the human, mouse, and rat NTCP/Ntcp genes. HNF3 β was found to repress NTCP activity by forming a specific DNA-protein complex, as assessed by electrophoretic mobility shift assays [221]. For RXR α /RAR α , Jung *et al.* suggested that a putative binding site for HNF4 α is localized with the RXR α /RAR α sequence based on coexpressed studies, in which rat Ntcp promoter activity increased two-fold [221].

On the basis of mutagenesis studies, a functional glucocorticoid receptor (GR) response element was identified in the promoter region of NTCP [222]. Dexamethasone increased reporter-linked NTCP promoter activity. Eloranta *et al.* showed that a DNA element arranged as a direct repeat of hexamers separated by nine nucleotides (DR-9) at nucleoside $-32/-12$ of the NTCP promoter mediates the glucocorticoid response and that GR directly interacts with this motif. The authors indicate that upstream and downstream elements are also required for full NTCP promoter transactivation activity by GR/dexamethasone. Adding more complexity, the induction of NTCP gene by GR in the presence of glucocorticoids can be enhanced by coexpression of the transcriptional coactivator, PPAR α (PGC-1 α) [222].

Bile acids may regulate NTCP/Ntcp indirectly by modulating the capacity of nuclear factors to activate gene expression [221]. When bile acid levels are elevated, the uptake of bile acids into hepatocytes from sinusoidal blood is reduced because of the reduced expression of NTCP/Ntcp in both rodents and humans [206]. In rats, bile acid-mediated suppression involves inhibition of the activity of the RXR α /RAR α heterodimer binding to the Ntcp promoter [221]. CDCA can cause reverse transcriptional activation of NTCP gene by GR/dexamethasone, and it has been proposed that GR is a target for negative feedback regulation of NTCP expression by bile acids in humans [222].

6.11 GENETIC POLYMORPHISMS OF SLC TRANSPORTERS AND CLINICAL IMPLICATIONS

Extensive human gene variation was reported after the near-complete sequencing of the human genome, with ~ 12 million single nucleotide polymorphisms (SNPs) identified [223]. These polymorphisms can occur in the regions coding for proteins (nonsynonymous polymorphisms) to give rise to amino acid changes or in noncoding regions of the genome (synonymous polymorphisms) to regulate the expression and function of proteins. Genetic polymorphisms could affect protein expression or functional activity via alteration of substrate affinity. Information from gene polymorphisms has advanced our understanding of the contribution of population PK variations to tolerability and safety profiles of therapeutics. Genetic variants may be associated with susceptibility to disease or important differences in PK/PD drug parameters. The PK and distribution of substrate drugs could be altered by the changes of transport capability caused by genetic variants. The variants might also affect the apparent activity of the transporter by lowering the affinity for substrates (K_m), and in some cases, they cause loss of function of the transporter.

Since membrane transporters handle many drugs that are prescribed clinically and regulate the ADME, these proteins play a critical role in pharmacological and toxicological processes. Genotypic and phenotypic polymorphisms in the SLC family contribute to interindividual, interethnic, and gender-based variability in PK and drug distribution. Transporter polymorphisms are associated with changes in efficacy, drug interactions, and safety concerns, including idiosyncratic hepatic toxicity. On the basis of the US Food and Drug Administration (FDA) *Guidance for Industry on Pharmacogenomics Data Submissions*, pharmacogenomics data is required for all known or probable valid biomarkers. The FDA also encourages submission of data for those genes (including

ADME genes) for which there is limited knowledge about the associated clinical variants. This pharmacogenetic information is needed to support scientific arguments and it could be included in the drug label.

6.11.1 OATP Transporters

Several genetic variants, including 388A>G (130Asn>Asp, rs2306283) and 521T>C (174Val>Ala, rs4149056), of the *SLCO1B1* gene that codes for OATP1B1 protein have been identified. The polymorphisms of OATP1B1 and 1B3 have been characterized to occur at different frequencies in various racial and ethnic populations. For example, the frequency of OATP1B1 c388G allele (*1b) in Caucasians, Asians, and African-Americans is about 40%, 60%, and 75%, respectively, and the 521T>C in codon 174 is ~15%, 15%, and 2%, respectively [6]. The polymorphisms have different activities that can affect PK and result in deleterious side effects. For example, individuals with the OATP1B1*5 or OATP1B1*15 haplotypes have increased exposure to the substrates including pravastatin, simvastatin acid, atorvastatin, and rosuvastatin, and this may result in myopathy or increased risk of hepatotoxicity. These variants also have a significant impact on the PK/PD profiles of substrate drugs. When OATP1B1 polymorphisms were mapped in individuals taking simvastatin, a significantly increased risk for myopathy correlated with increased tissue exposure and Val174Ala polymorphism [224]. Cerivastatin was withdrawn from the market because of a serious adverse effect rhabdomyolysis, which has been related to the variants in *SLCO1B1* with an odds ratio of 1.89, but not the variants of CYP2C8 or UGTs that metabolize cerivastatin [225]. When transfected with the variant *SLCO1B1**15 gene (rs2306283+rs4149056), HEK cells uptake activity with cerivastatin is reduced ~40% compared with the cells expressing the *SLCO1B1* wild-type gene [225]. The reduced transport capability is associated with decreased liver uptake of cerivastatin, leading to increased systemic exposure and risk of rhabdomyolysis. Pravastatin is an OATP1B1 substrate that is minimally metabolized and mainly excreted into bile unchanged. There is an ~10-fold variation in the AUC between individuals mainly because of the genetic polymorphisms in OATP1B1 that reduce elimination, thereby causing a significant increase in plasma concentrations of pravastatin. [226].

Genetic polymorphisms in regulatory regions of *SLCO* genes regulate the expression level of OATPs [227]. For example, OATP1A2 is expressed in brain capillary endothelial cells and contains one genetic variant located in a putative HNF1 α binding site [228].

6.11.2 OCTs

Western blotting and mRNA quantification in human liver samples revealed that expression of OCT1 and OCT3 was highly variable between individuals. A reduced expression associated with nonsynonymous coding variants of OCT1 (rs12208357) or OCT3 (rs2292344) was reported [229]. OCT transports metformin into hepatocytes, and the functional loss of variants of OCT1 is linked to the reduced hepatic uptake of metformin and subsequently, its PD effect [230]. Individuals carrying reduced function OCT1 alleles, including SNPs in positions 420, 401, and 465, appear to have reduced metformin CL based on the observed higher AUC and C_{max} [230].

Doors are opening to a new paradigm in drug development and therapy through pharmacogenetic research with human drug transporter proteins, which could lead to a clearer understanding of the individual susceptibility to drug toxicity and efficacy.

6.12 REGULATORY GUIDANCE FOR SLC TRANSPORTERS

The European Medicines Agency (EMA) issued a draft guideline on the investigation of drug interactions that included a section on drug transporters, in April 2010 and the Food and Drug Administration (FDA) issued a draft guidance for drug interaction studies in February 2012 that provides a more detailed information on drug interaction studies, including decision trees for the SLC (and ABC) transporters.

6.12.1 FDA Drug Interaction Guidance

As per the FDA guidance, all investigational drugs should be evaluated *in vitro*, using transfected cell lines and empty transfected cells to subtract out the background by endogenous transporters. Inhibition studies should be conducted *in vitro* for the hepatic transporters OATP1B1 and OATP1B3 and the renal transporters OAT1, OAT3 and OCT2, as well as the ABC transporters MDR1 and BCRP. Substrate assays for the hepatic transporters OATP1B1 and OATP1B3 are required when hepatic clearance is more than or equal to 25% of the total clearance. *In vitro* substrate assays for the three renal transporters are required when their renal active secretion is more than or equal to 25% of total clearance. The EMA includes the above transporters as well as OCT1 and BSEP, the latter being important for safety testing. An interaction is considered significant if uptake into the transfected cells is ≥ 2 fold of that in the empty vector transfected cells and can be inhibited by $\geq 50\%$ by a known inhibitor. The FDA guidance suggests that when evaluating drug candidates, selection of the interacting drugs should be based on established, relevant and strong inhibitors of the transporter pathways under investigation. Additional transporters: MRPs, MATE and BSEP are mentioned and it is suggested that these be considered when appropriate.

For clinical studies, the FDA advises that the choice of substrate for a particular transport pathway be based on the therapeutic area and most likely to be co-administered drugs, whose PK is markedly altered by known specific inhibitors of the transporter pathway, so as to enable the largest impact of the interacting investigational drug. The FDA acknowledges that the observed clinical interactions may be a result of inhibition of multiple pathways, if the investigational drug is also an inhibitor for multiple pathways.

6.12.2 EMA Drug Interaction Guidance

The EMA suggests that *in vitro* studies be conducted to assess substrate and inhibition potential of new drug candidates with OATP1B1, OATP1B3, OCT2, OCT1, OAT1, OAT3 and the efflux transporters P-gp and BCRP and also BSEP for pharmacodynamic and safety monitoring. *In vitro* inhibition assays are suggested for all the transporters. To assess the clinical substrate potential of an investigational drug for a particular transporter, the EMA suggests the transporter be evaluated if it contributes to the absorption, distribution or elimination of a compound. For distribution, evaluating *in*

vitro data along with tissue specific expression of the transporter, data on distribution in preclinical species, clinical safety data in patients with altered transport due to genetic polymorphism and expected clinical consequences of altered distribution should be considered.

The EMA guideline recommends that if renal and biliary secretion account for $\geq 25\%$ of systemic clearance, the transporter(s) involved in the active secretion should be identified. Potential OATP uptake should be investigated *in vitro* for non-cationic drugs with $\geq 25\%$ hepatic elimination. The contribution of biliary secretion should be based on available mass balance and interaction data, pharmacogenetic information and hepatic impairment, if any. Renal secretion should be estimated by comparing total renal clearance to renal filtration clearance of the unbound drug, similar to the FDA guideline.

The recommended time line for *in vitro* evaluations is before Phase II clinical trials; unless all concomitant drug treatments are not expected to have interactions and *in vivo* interaction studies should be conducted prior to Phase III. The EMA also suggests that the possible effects of transporter-enzyme interplay, such as between Pgp and CYP3A, should be discussed, and if required, clinical studies considered.

The major differences between the two guidelines are the inclusion of OCT1 and BSEP as major transporters suggested by the EMA, while this is not a part of the FDA guidelines. In addition, while the FDA guidance allow for IC_{50} values to be calculated, the EMA recommends K_i values.

6.12.3 Induction

The FDA and EMA guidance also discuss induction of transporters. Since expression levels of these transporters are regulated in coordination with metabolizing enzymes by activation of the same nuclear receptors, induction of CYP1A2, CYP2B6 and CYP3A4 can be used as the initial evaluation of the induction potential of an investigational drug on transporters. The definitive studies continue to be clinical induction studies. For example, induction of CYP3A4 by the drug candidate will serve as a trigger for a clinical P-gp induction study, using digoxin as the probe substrate.

6.13 SUMMARY

While earlier studies with transporters were conducted primarily with the ABC family, particularly MDR1, the SLC transporters are now considered equally important in the disposition of xenobiotics within the body and continue to be extensively evaluated. There is a significant amount of work being conducted that this review has not discussed. For example, we were not able to cover all transporters involved in drug disposition, for example, amino acid transporters such as System L (LAT) are not included. We have mentioned but not discussed the differences in transporter expression, regulation, and activity across ethnic groups and between different age groups mainly because there have been very few studies on this so far and we have limited knowledge of the effect of these on ADME. Another gap in this review and also in current knowledge is information on the number of binding domains in individual SLC transporters, similar to what has been reported for ABC transporters such as MRP2. Megaraj *et al.* developed transporter cells lines with MRP2 and various SNPs and

found that SNPs located in the MRP2 nucleotide-binding domains variably decreased the transport of all substrates studied; an SNP in the membrane spanning domain 1 selectively decreased the apparent affinity for glutathione and glucuronide conjugated substrates, and an SNP in the carboxyl terminus altered only bile acid transport [231]. The presence of multiple binding sites further complicates transporter evaluations, and these need to be identified for the SLC transporters as well. Most importantly, we still do not have adequate models to extrapolate *in vitro* transporter data to *in vivo*, and the different models currently available can result in very different outcomes. For example, fluvastatin uptake by OATP1B1, 1B3, and 2B1 is inhibited 60–90% by gemfibrozil in the transfected cell lines but only by 27% in the more intact primary hepatocytes, indicating that there is more to fluvastatin transport than uptake by these three transporters [161]. It is clear that we still have a long way to go in understanding the SLC transporters and their significance in ADME, and this story will evolve as the data unfolds.

ABBREVIATIONS

Transporters

ABC	ATP-Binding Cassette
ASBT	Sodium-Dependent Bile Salt Transporter
BSEP	Bile Salt Export Pump
CNT	Concentrative Nucleoside Transporter
CT	Na ⁺ /Carnitine Cotransporter
ENT	Equilibrative Nucleoside Transporter
LAT	System L Transporter
MATE	Multidrug and Toxic Compound Extrusion Transporter
MDR1	Multidrug Resistance Protein 1
NTCP	Sodium Taurocholate-Cotransporting Polypeptide
OAT	Organic Anion Transporter
OATP	Organic Anion-Transporting Polypeptide
OCT	Organic Cation Transporter
OCTN	Organic Cation Transporter Novel
SLC	Solute Carrier
UGT	UDP-Glucuronosyltransferase
URAT	Urate Transporter

Nuclear Receptors

AhR	Aryl Hydrocarbon Receptor
CAR	Constitutive Androstane Receptor
FXR	Farnesoid X Receptor
GR	Glucocorticoid Receptor
HNF	Hepatocyte Nuclear Factor
Nrf2	Nuclear Erythroid 2-Related Factor 2
PPAR	Peroxisome Proliferator-Activated Receptor

PXR	Pregnane X Receptor
RXR	Retinoid X Receptor
SNP	Single Nucleotide Polymorphism

ADME Terms

AUC	Area Under the Plasma Concentration Time Curve
BBB	Blood-Brain Barrier
CL	Clearance
DDI	Drug–Drug Interaction
GFR	Glomerular Filtration Rate
PK	Pharmacokinetics

Chemicals

6-CFL	6-Carboxyfluorescein
MPP ⁺	1-Methyl-4-Phenylpyridinium
PAH	<i>para</i> -Aminohippuric Acid
TEA	Tetraethylammonium

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7 Mechanisms of Drug Metabolism

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7.1	Introduction	1
7.2	Phase I drug metabolism reactions	10
7.3	Phase II drug metabolism reactions	32
7.4	Conclusion	47
	References	48

7.1 INTRODUCTION

Drug metabolism represents an integral contributor to the many physiological processes that govern the pharmacokinetic/dispositional fate of most therapeutic agents [1]. For the most part, drugs are taken orally and in order to be absorbed through the stomach and intestine, they need to possess a high degree of lipid solubility [2,3]. The requirement for drug lipid solubility is a physicochemical consequence to the fact that lipid-soluble (nonionized) molecules are able to pass through biological membranes by simple diffusion, while water-soluble (ionized) molecules cannot [4,5]. In this light, drug metabolism can be broadly described as the biological transformation of lipophilic, nonpolar molecules (drugs) to more hydrophilic, polar molecules (metabolites), which are in turn readily eliminated from the body. Restated, drug metabolism controls the systemic exposure of many drugs and without a metabolic pathway associated with drug clearance, these molecules would remain in the body for an extended period and possibly accumulate to toxic levels on repeated administration. In response to the role drug metabolism has in drug safety, regulatory agencies place a premium on gleaning information around the metabolic pathways and products associated for all new drug applications (NDAs) (e.g., Guidance for Industry: Safety Testing of Drug Metabolites).

In order to appreciate the role of drug metabolism in drug safety, it is important to consider how molecules are actually eliminated from the body. For the most part, molecules are removed from the body through two primary routes of elimination: the urinary and gastrointestinal systems [6]. The primary function of the kidney is the excretion of body wastes, and for the purpose of this chapter, drug metabolites. Renal elimination of drug metabolites is primarily dependent on the fraction of molecules that are susceptible to reabsorption in the distal renal tubule. In this region of the kidney,

nonionic diffusion is responsible for the reabsorption [7]. Reabsorption occurs primarily by passive transfer and is mediated by a large hydrogen ion gradient between plasma and acidic urine. In this environment, metabolites, which are typically ionized, remain in the urine and are excreted, while drugs that are generally present as nonionized molecules, readily diffuse from the urine back into circulation [8].

Extraction via biliary and intestinal excretion of drug metabolites is important for the elimination of many therapeutic classes of drugs and corresponding metabolites [9]. Typically, biliary excretion is usually the primary route of elimination for compounds with a molecular weight of more than 400 Da and generally involves active secretion rather than passive diffusion [10]. Specific transport systems appear to exist for certain classes of molecules (e.g., organic bases, organic acids, and neutral substances) [11]. However, given that the molecules most likely to be excreted via biliary elimination are large, ionized molecules, once a molecule has been excreted into the bile (and subsequently delivered into the intestinal tract), it is not likely to be reabsorbed by passive diffusion.

If we disregard the role of small molecule transporters in drug metabolite elimination, it is clear that the biliary elimination of drug metabolites, similar to renal excretion, is primarily mediated by the physical properties of the molecule. Those substances that are ionized typically leave the body, while lipid-soluble molecules can be reabsorbed and reenter the blood circulation, which lengthens their half-life in the body and potential for toxicity [12,13].

The process of drug reabsorption is conceptually similar to the manner in which any molecule distributes between the two phases of water and a lipid-like solvent. With respect to the drug metabolites, the two phases are fatty tissue (cell membrane) and water (plasma). In organic chemistry, this concept is described in terms of a quantitative feature, the partition coefficient P , or as it is usually expressed, $\log P$ [14]. Factors that influence $\log P$ can be further distilled into descriptors such as size and polarity or hydrogen bonding capability of a molecule [15]. Therefore, it is important to understand the chemical features that can influence the partition ratio of a molecule as these concepts are the primary areas of chemical modifications associated with drug metabolism and safety [16].

Generally, polar solute molecules will dissolve in polar solvents and nonpolar solute molecules will dissolve in nonpolar solvents. Polarity in organic chemistry refers to a separation of charge and can describe a bond or an entire molecule [17,18]. The polarity of the molecule is the sum of all the bond polarities in the molecule [19]. Since the dipole moment is a vector (a quantity with both magnitude and direction), the molecular dipole moment is the vector sum of the individual dipole moments [20]. Restated, polar solute molecules have a positive and a negative end to the molecule. If the solvent molecule (water in the case of biological systems) is also polar then positive ends of solvent molecules will attract negative ends of solute molecules (Fig. 7.1).

As described above, drug metabolites are typically described as either being weak acids (a substance that gives up a proton) or weak bases (a substance that accepts a proton) and because of this, the disposition of most drug metabolites are governed by passive diffusion [21]. The extent to which a given drug metabolite exists in an ionized and nonionized species varies at different pH values. Therefore, the pH, or more specifically the hydrogen ion concentration, influences the ratio of ionized to nonionized species for a particular drug metabolite (Fig. 7.2). This concept is expanded

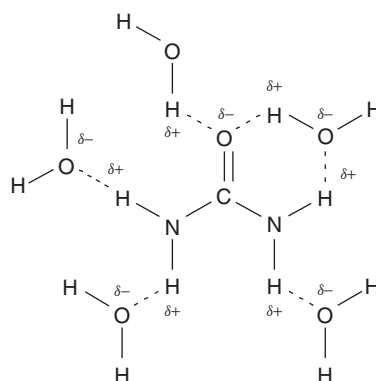


Figure 7.1 Hydrogen bonding of urea in water.

using the Brønsted–Lowry definition of acids and bases. The thermodynamic character that relates the charge in a molecule to the pH is the ionization constant, pK_a [22].

With respect to the elimination of drug metabolites, an understanding of the degree of ionization of a given molecule under physiological conditions is helpful to predict whether a metabolite will be long lived. At a particular pH, the relative concentration of the ionic and the molecular moieties of a given drug metabolite can be described by the Henderson–Hasselbach equation:

$$pH = pK_a + \log \frac{[\text{conjugate acid}]}{[\text{conjugate base}]}$$

Therefore, it follows, from the Henderson–Hasselbach equation, that when a substance is half ionized and half nonionized at a certain pH, its pK_a is equal to this pH. In other words, when the pH is equal to the pK_a value of the molecule, half of the molecules of a drug are in ionized and half are in nonionized form. It is important to consider that the relation between ionization and pH is not linear, but sigmoidal. Thus, small changes in pH will result in great changes in ionization, particularly if pH and pK_a values are close together.

With an appreciation of the physical chemical requirements that are required for the elimination of the drug metabolite, the next obvious question is what types of chemical modifications are biologically available to support drug metabolism enzyme reactions? The chemical routes by which drugs are susceptible to drug-metabolizing enzymes are large and varied. In fact, one particular feature that distinguishes drug-metabolizing enzymes from other “classical” enzymes is the wide variety of reactions that these enzymes can carry out. In 1959, Williams classified the drug metabolic reactions that are catalyzed by this group of enzymes into two general phases: phase I and II reactions [23]. Phase I reactions include oxidation [24], reduction [25], and hydrolysis [26], whereas phase II reactions are broadly defined as conjugation reactions and include glucuronidation [27], sulfation [28], acylation [29], methylation [30], and conjugation with glutathione (GSH) [31].

As with all enzyme-mediated reactions, drug-metabolizing enzymes are capable of accelerating a chemical reaction faster than reactions carried out on a laboratory bench top under the mild conditions of aqueous solution, room temperature, and neutral pH.

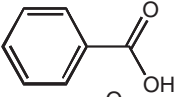
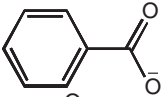
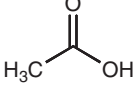
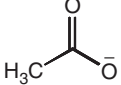
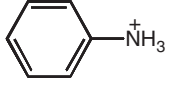
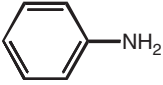
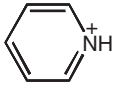
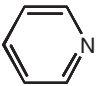
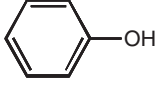
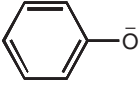
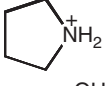
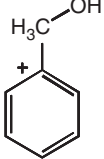
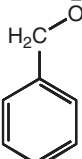
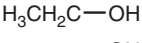
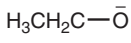
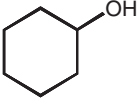
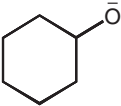
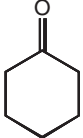
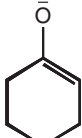
Conjugate acid	pK_a	Conjugate base
	4.2	
	4.8	
	4.6	
	5.3	
	9.6	
	11.2	
	15.4	
	16	
	17	
	20	

Figure 7.2 Examples of acids and acidity scale.

In terms of the concepts describing chemical thermodynamics, enzyme catalysis is essentially a lowering of the free energy function (G) associated with a given chemical reaction [32]. A change in free energy for a reaction, ΔG , determines if a reaction is spontaneous or not. Equilibrium conditions are governed by the standard free energy change ΔG° , and there are several means by which enzymes are able to decrease activation energy. The catalytic power and specificity of an enzyme is a reflection of the steric and electrostatic features associated with the enzyme's active site [33]. The most important of these involves the enzyme initially binding the substrate allows the molecule to exist in close proximity to the catalytic groups found within the active enzyme

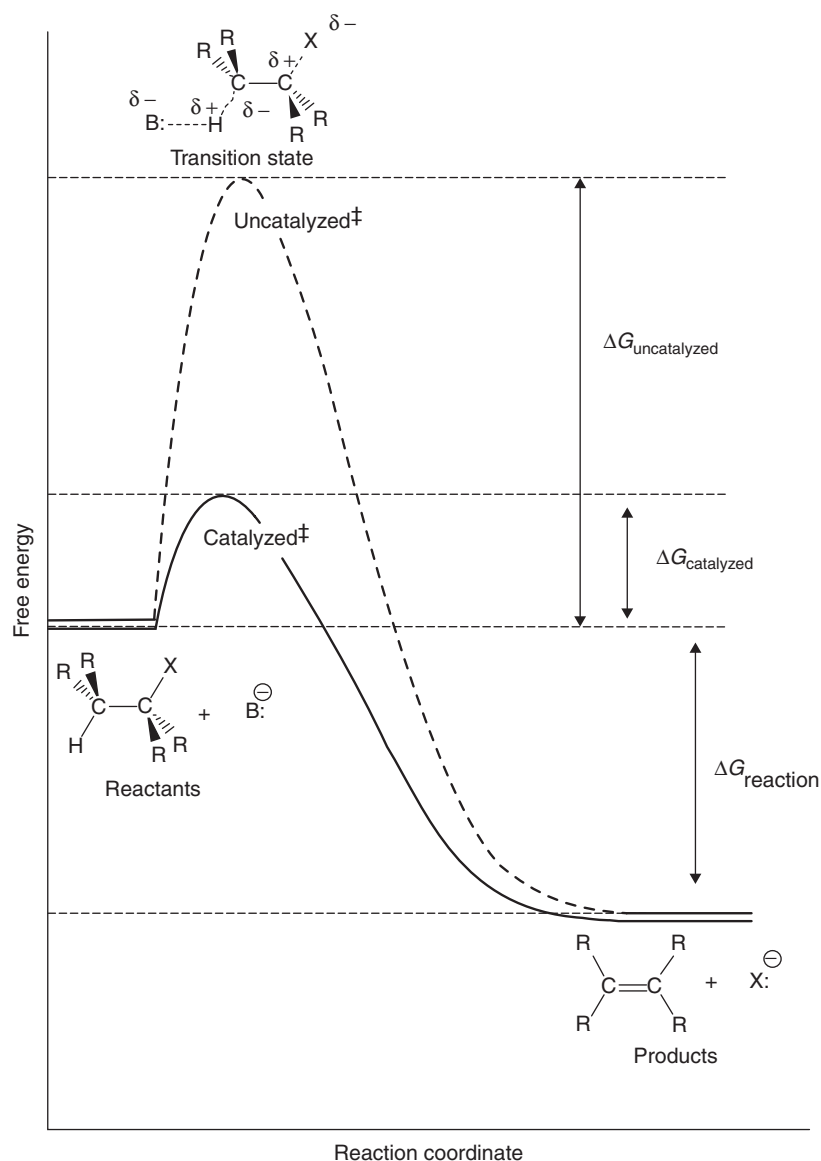


Figure 7.3 Free energy or potential energy diagram of a simple E2 elimination reaction of an alkyl halide to yield an alkene.

complex [34,35]. When the substrate is bound to the enzyme, the enzyme changes conformation and forces the substrate into a strained or distorted structure that mimics the reaction transition state [36], thus reducing the energy of activation (Fig. 7.3). In this conformation, the substrate is forced into a reactive state, due to the loss of the substrate's translational and rotational entropy, toward the total activation energy [37].

Although small in relative numbers, drug-metabolizing enzymes are able to catalyze a large variety of reactions because of their structural and chemical diversity. Some drug metabolism enzymatic reactions mimic organic chemistry reactions and proceed

via nucleophilic attack [38]. In this instance, enzyme's active residues can act as nucleophiles such as the sulfhydryl group of cysteine or the phenolic group of tyrosine. Alternatively, groups such as aspartic acid, glutamic acid, and histidine (as its conjugate acid) can act as general acids [39], while groups such as histidine, lysine, and arginine can act as general bases [40] in catalytic mechanisms. In contrast, while some drug-metabolizing enzymes can carry out their catalytic activity themselves, most others (phase I oxidative enzymes) require presence of cofactors and coenzymes to carry out reactions.

Coenzymes are organic molecules (often vitamins or vitamin derivatives) that are required by certain enzymes to carry out catalysis (Fig. 7.4). They bind to the active site of the enzyme and participate in catalysis but are not considered substrates of the reaction. Generally, coenzymes function as intermediate carriers of electrons, specific atoms, or functional groups that are transferred in the overall reaction [41]. An example of this would be the role of nicotinamide adenine dinucleotide phosphate (NADPH) in the transfer of electrons in certain coupled oxidation reactions [42]. In contrast, the term *cofactor* often refers to *inorganic substances* such as "metals" that are required for enzyme catalysis. In many instances, these metals are bound tightly by the enzyme or are complexed within prosthetic groups to form a permanent part of the protein structure. For example, cytochrome P450 enzymes derive catalytic function through a heme-complexed metal species (Fig. 7.4), through the formation of a reactive oxoiron(IV)-porphyrin π -radical cation, and are able to facilitate a wide range of reactions most notably the insertion of an oxygen atom between a carbon hydrogen bond [43].

While the notion of drug metabolism was appreciated before the 1950s, little was known about the oxidative source involved in these enzymatic reactions. However, in 1950s, several laboratories demonstrated the direct incorporation of molecular oxygen into small molecules [44–46]. In addition, during the same time period, other groups determined that the microsomal deamination of amphetamine [47] and dealkylation of aminopyrine [48] also required the presence of both the reduced form of NADPH and molecular oxygen (O_2) [24].

As illustrated in Fig. 7.5, the biological conversion of molecular oxygen to water involves four sequential one-electron reductions. The sequential nature of the reduction is necessitated by the fact that in the ground state, oxygen contains two unpaired electrons. Each stage of oxygen reduction generates a reactive intermediate, which can exist in a number of different energy levels or spin states and can generate new species, generally by the addition of a proton. This has special significance with regard to phase I drug-metabolizing enzymes that use oxene and hydrogen peroxide as the major oxygen species involved in oxidative drug metabolism reactions [49,50].

With respect to phase II drug-metabolizing enzymes, the chemistry that supports metabolite formation requires the presence of a cosubstrate. In these reactions, a foreign compound is covalently linked with a small endogenous molecule, derived from carbohydrate or amino acid sources, to yield a characteristic product known as a *conjugate* [23]. There are five major conjugation reactions that primarily make up phase II drug metabolism: glucuronidation, sulfation, acylation, methylation, and GSH conjugation. The corresponding cosubstrates involved in the listed reactions are listed in Fig. 7.6. Interestingly, while phase II reactions are collectively described as conjugation reactions, they differ greatly in terms of the nature of the substrates involved, their distribution through the body, and reaction mechanisms [51,52].

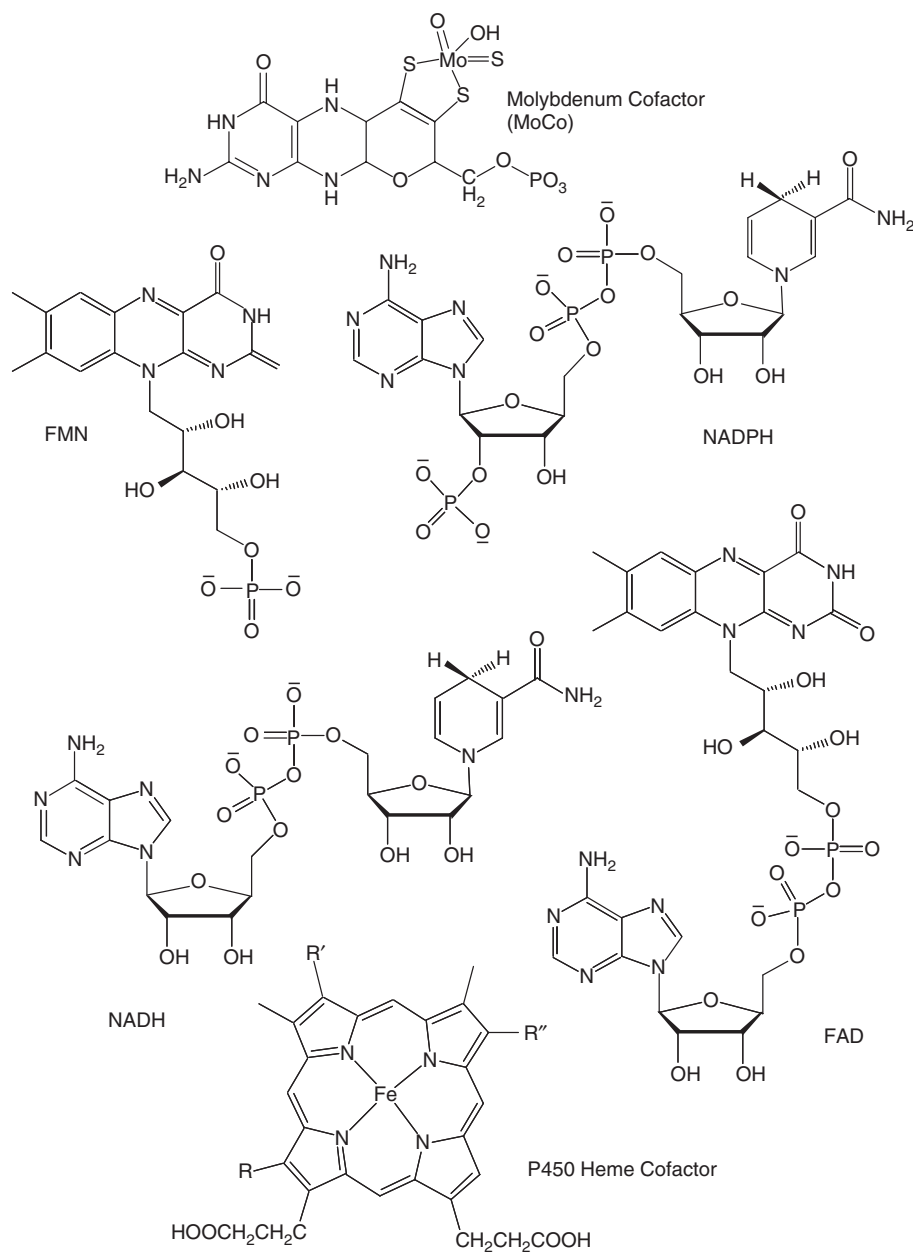


Figure 7.4 Structures of common phase I coenzymes and cofactors.

The biochemical tools used to understand the various mechanism of drug metabolism can be categorized into three primary groups: (i) experiments that are aimed to elucidate the factors that can affect the metabolite transition state; (ii) studies that characterize reaction pathways associated with product formation; and (iii) determinations of enzyme's active topography and influence on rates of metabolite formation.

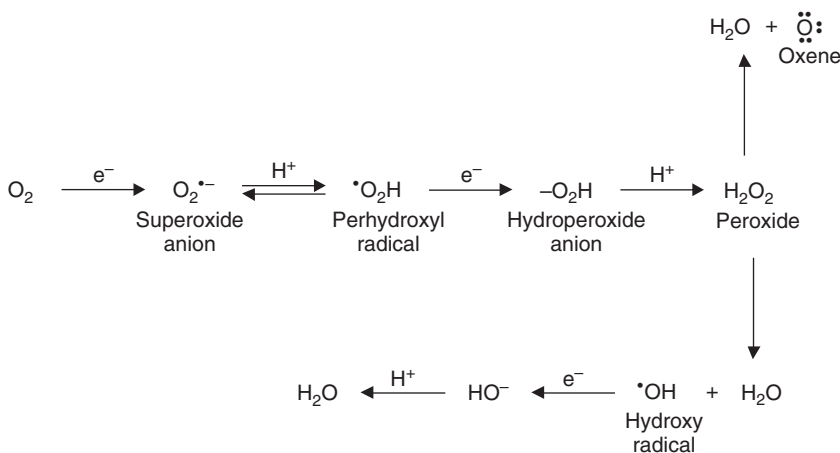


Figure 7.5 Reduced states of oxygen.

Kinetic deuterium isotope effect studies have been used extensively to understand the mechanism of drug metabolism reactions [53]. Primary kinetic isotope effects refer to processes in which the bond to deuterium (usually a C–D bond) is broken during the reaction and are expressed as the ratio of the specific rate constants, $k_{\text{H}}/k_{\text{D}}$, for the initial reaction of the protium- and deuterium-labeled compounds, respectively. The thermodynamic basis for the primary deuterium isotope effect lies in the large relative mass difference between H and D, which results in a difference (1.2–1.5 kcal mole) between the zero-point energy of a bond to deuterium versus hydrogen [54]. This difference results in a greater energy requirement for cleavage of a bond to deuterium and as a consequence, isotope effects on the rates of metabolic processes where rupture of a C–D bond is rate determining.

In addition to helping define the thermodynamics of an associated drug metabolism reaction transition state, selective use of isotopes (in particular, D and ^{18}O) has also been applied to dissect the nature of drug-metabolizing reactions [55]. For example, the mechanism by which most aromatic hydroxylations occur is via epoxidation of electron-rich π -bonds in the ring [56]. The resulting arene oxide is highly reactive for two main reasons: (i) three-membered rings are highly strained and open up readily and (ii) the ring on which the arene oxide forms is no longer aromatic (one double bond was used to make the epoxide). As a consequence, arene oxides can undergo two main types of reactions: rearrangement to yield a phenol or epoxide opening by reaction with a nucleophile. One of the first applications of selective deuterium labeling in drug metabolism was toward understanding the mechanism of reaction for the enzymatic conversion of naphthalene to 1-naphthol in rat microsomes [57]. The results of this experiment suggested that on enzyme-mediated oxidation of the aromatic ring to the corresponding *para*-phenolic metabolite, the deuterium was retained on the molecule (NIH shift), a result of rearrangement via a ring-opened intermediate [58].

In view of the widespread occurrence of oxidative transformations in drug metabolism, experiments with isotopically labeled oxygen ($^{18}\text{O}_2$ or H_2^{18}O) have been used extensively to characterize many drug metabolism reaction mechanisms [59,60]. A good example of the application of heavy oxygen to determine the mechanism of drug metabolism is through a series of *in vitro* experiments that were carried

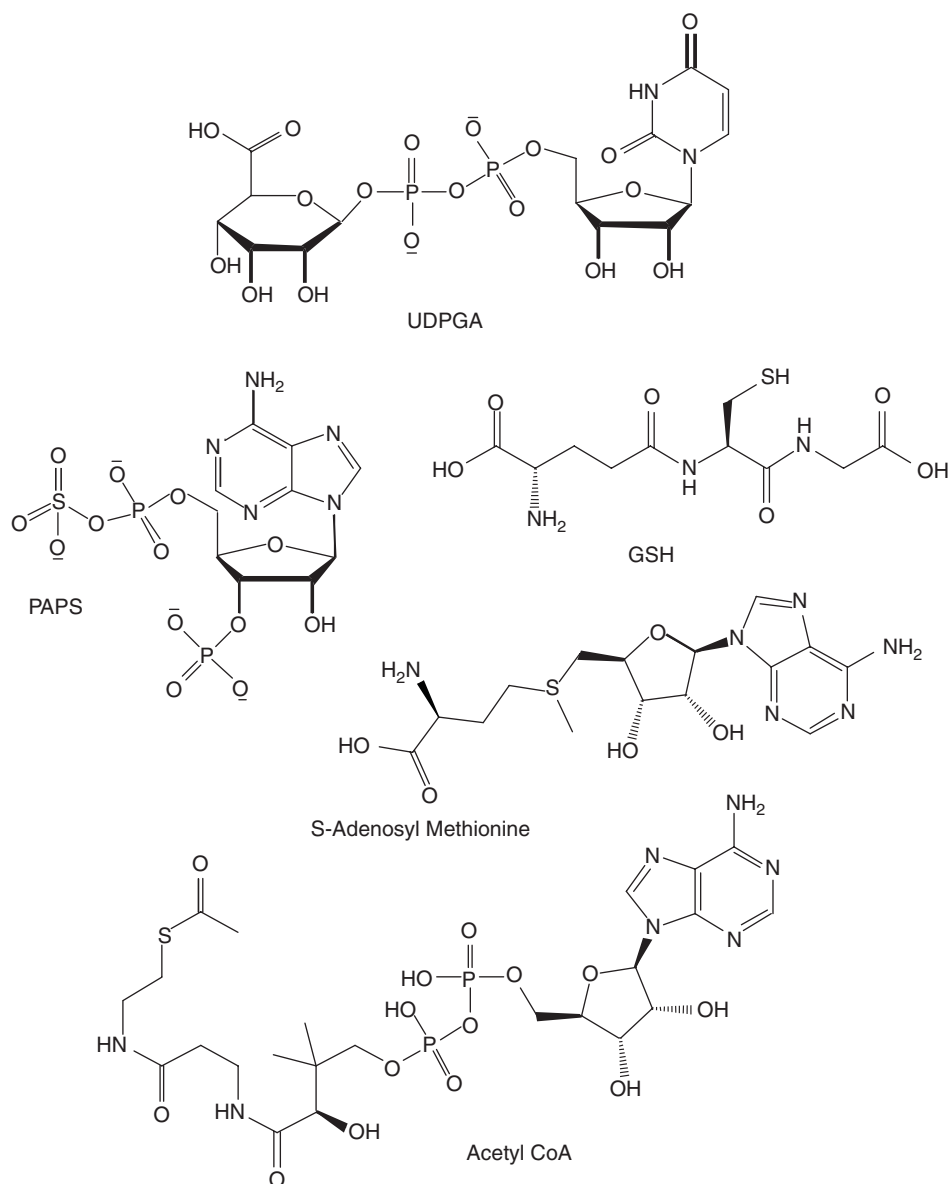


Figure 7.6 Structures of common phase II cosubstrates.

out under atmospheres enriched in $^{18}\text{O}_2$ and showed, by GC-MS analysis, that the dihydrodiol metabolite, 1,2-dihydro-1,2-dihydroxynaphthalene, contained only one atom of ^{18}O and that this labeled oxygen occupied the 1-position. In this study, the authors further inferred that the second oxygen atom was derived from water [61]. Subsequent experiments conducted by Jerina and others [62] verified this hypothesis by incubating the molecule in H_2^{18}O fortified rat liver microsomal preparations.

Site-directed mutagenesis of proteins has developed into a powerful tool for studies of structure–function relationships for many drug-metabolizing enzyme systems. The strategy of these types of studies is to alter enzyme’s amino acid residues that affect reaction features such as substrate specificity [63], catalytic competence [64,65], cooperativity [66], and product specificity [67]. In the past few years, advances in X-ray crystallography, homology modeling, and substrate docking techniques have allowed for more precise functions for drug-metabolizing enzyme residues, making it possible to utilize site-directed mutagenesis designed to test these predictions [68]. The primary aim of site-directed mutagenesis to investigate the catalytic mechanisms behind drug metabolism reactions is to replace essential residues (ionizable or nucleophilic amino acids) with unreactive ones [69,70]. In this fashion, general acids, general bases, and catalytic nucleophiles arguably represent the most “essential” residues in an active site as they directly participate in the formation and rupture of covalent bonds. For example, it has been proposed that the catalytic mechanism for uridine diphosphate glucuronosyltransferase (UGT) enzyme glucuronidation employs a serine proteaslike catalytic mechanism in which an Asp-His pair in the active-site deprotonates the hydroxyl on the substrate, which allows for nucleophilic attack on the sugar donor [71,72]. In an experiment carried out by Patana *et al.* [73], when the His37 residue of UGT1A9 was replaced with an alanine, drug metabolism studies conducted with the mutant enzyme revealed that His37 was not critical in UGT1A9 N-glucuronidation reactions, but was in O-glucuronidation reactions. The authors were able to conclude that for UGT1A9 reactions, *O*-nucleophiles require the active-site histidine residue to deprotonate them so that they become effective nucleophiles, while *N*-nucleophiles develop a formal positive charge during the reaction and thus requires a negatively charged active-site residue to stabilize the reaction transition state.

As described earlier, unlike most “traditional” enzyme systems, the group of enzymes that are loosely termed as *drug-metabolizing enzymes* exhibit considerable structural and catalytic diversity. For the most part, drug-metabolizing enzymes are capable of carrying out specific reactions. The multiplicity of various drug-metabolizing families was recognized more than 30 years ago [74,75]. Because most drug-metabolizing enzyme systems are actually a collection of isozymes, they have been broadly classified into families and subfamilies on a genetic basis [76,77]. For most drug metabolism enzyme families, there is some degree of substrate structural class preference within families and subfamilies, although some overlap in substrate recognition is observed [78,79]. However, independent of which isozyme is actually responsible for a drug metabolism reaction, it should be remembered that enzymes that make up a particular family, share the same chemical mechanism of catalysis. To this end, the scope of this chapter avoids any detailed description of any drug-metabolizing isozymes and will rather focus on the different reactions catalyzed within each family of drug-metabolizing enzymes and the chemistry involved in phase I (Section 7.2) and phase II (Section 7.3) reactions.

7.2 PHASE I DRUG METABOLISM REACTIONS

7.2.1 Cytochrome P450

Mammalian cytochrome P450 monooxygenases are heme-containing, membrane-bound enzymes that catalyze the oxidation and reduction of a variety of xenobiotics

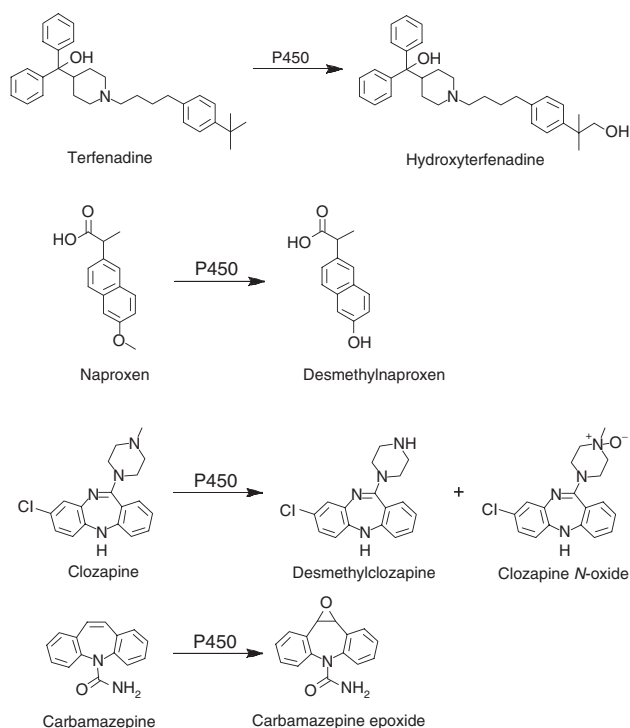


Figure 7.7 Examples of P450 metabolism reactions.

and endobiotics (Fig. 7.7) [80]. P450s require a redox partner protein to transfer electrons from NADPH for catalytic activity. Cytochrome P450 reductase serves as the redox partner for mammalian P450s; it accepts electrons from NADPH through its flavin adenine dinucleotide (FAD) moiety and donates electrons to P450 through its flavin mononucleotide (FMN) moiety [81–83]. Another redox partner protein, cytochrome *b5*, may enhance catalytic activity for certain mammalian P450s [84]. P450s frequently involved in human drug metabolism include CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5, which differ in tissue localization and substrate selectivity [85]. P450s are expressed in many mammalian tissues, with the highest expression in the liver, gastrointestinal, and respiratory tracts [86]. Major biological roles for the P450s include xenobiotic metabolism, steroid biosynthesis, vitamin metabolism, eicosanoid metabolism, and fatty acid oxidation [87].

The P450 catalytic cycle is a multistep process shown in Fig. 7.8 [80,88,89]. The cycle is initiated by substrate binding to the P450. Substrate binding displaces water as a heme ligand, changing the spin state of the heme iron, thereby allowing for easier reduction of the heme for many P450s [90,91]. An electron from NADPH is then introduced to the P450 through transfer via P450 reductase. Binding of molecular oxygen to the P450 heme is followed by introduction of a second electron to form a nucleophilic ferric peroxo intermediate [92]. This intermediate may continue through the catalytic cycle or lose superoxide in a shunt (nonproductive) pathway. Subsequent protonation of

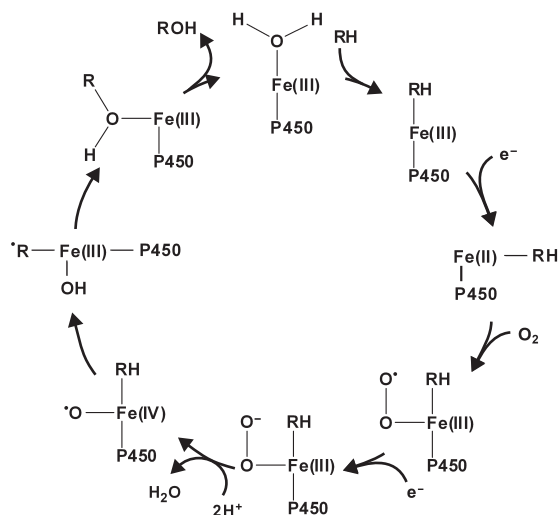


Figure 7.8 P450 catalytic cycle.

the distal oxygen results in an electrophilic ferric hydroperoxide intermediate [93–95]. The ferric hydroperoxide intermediate may continue through the catalytic cycle or lose hydrogen peroxide in a shunt pathway if the proximal oxygen is protonated. Protonation of the distal oxygen results in a species that rapidly undergoes heterolytic cleavage of the oxygen–oxygen bond to form a reactive iron–oxygen species, compound I [96]. Compound I may then react with substrate to form product or produce water via a shunt pathway through the introduction of two additional electrons and protons. While the general catalytic cycle applies to all P450-mediated reactions, specific reaction mechanisms are dependent on the functional groups present within the substrate.

7.2.1.1 Aliphatic Oxidation. P450s catalyze a variety of biotransformations (Fig. 7.9); common types of P450-mediated oxidations include hydrocarbon oxidation [97], olefin oxidation [98], aromatic hydroxylation [99], and heteroatom oxidation and dealkylation [100]. P450-mediated hydrocarbon oxidation is believed to proceed through a hydrogen atom abstraction (HAT) mechanism, where a hydrogen atom is abstracted from the substrate, followed by oxygen rebound to form a hydroxylation product (Fig. 7.10) [97]. Evidence for a HAT mechanism include a large isotope effect (typically >10) [101], partial stereochemical scrambling [102], and observed allylic rearrangement products [103].

Radical clocks have been used to assess the lifetime of radical intermediates and the rate of the oxygen rebound step [104]. Cyclopropyl groups are often used as radical clocks; radicals formed next to cyclopropyl rings may rearrange to homoallylic radicals that produce ring-opened products if the rate of rearrangement is faster than oxygen rebound or produce cyclopropyl carbinyl products if radical recombination is faster than rearrangement (Fig. 7.11). Adding ring strain to the cyclopropyl group increases the speed of rearrangement [105]. On the basis of experiments using radical clocks, rates for radical recombination during the oxygen rebound step of P450-mediated catalysis

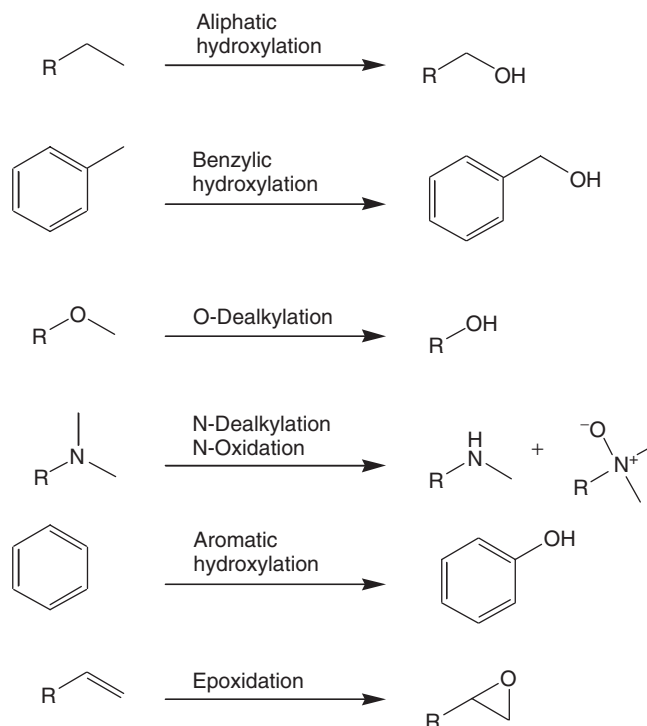


Figure 7.9 Generic examples of P450-mediated biotransformations.

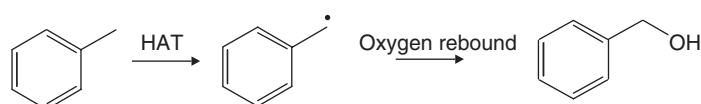


Figure 7.10 Example of the proposed hydrogen atom transfer mechanism.

vary from $1 \times 10^{10} \text{ s}^{-1}$ to those exceeding the theoretical rate constant of $2 \times 10^{12} \text{ s}^{-1}$ for highly strained radical clocks [106]. While higher ratios of rearranged products to carbonyl-derived products were expected from experiments using highly strained radical clocks, product formation ratios indicated that the rate of radical recombination generally increased in a compensatory fashion with radical rearrangement [107]. This finding suggests that P450-mediated hydrocarbon oxidation may proceed by a more complex mechanism than a simple radical recombination. Results observed from the radical clock experiments may be explained by a two-state reactivity model, with a low spin pathway that resembles concerted oxygen insertion and a high spin pathway that resembles radical recombination [108]. The influence of both substrate and enzyme environment influences which pathway predominates.

7.2.1.2 Olefin Oxidation. Olefins undergo oxidation by P450s to form epoxides, which may hydrolyze to form a diol product (Fig. 7.12). The olefin epoxidation mechanism may proceed through either a concerted or a nonconcerted mechanism. The

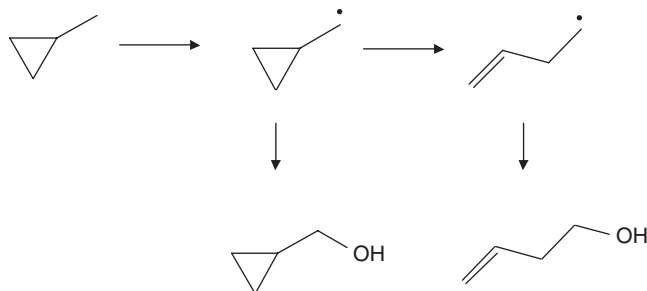


Figure 7.11 Example of a cyclopropyl radical clock.

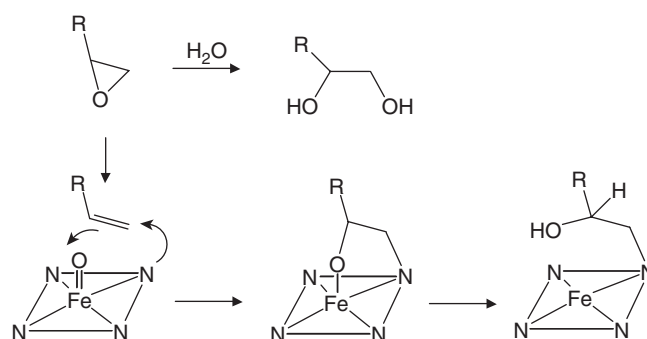


Figure 7.12 Proposed mechanism of P450-mediated olefin metabolism.

retention of stereochemistry on P450-mediated epoxidation suggests a concerted mechanism [109]. However, two pieces of evidence argue against a concerted mechanism. P450-mediated reactions with olefins form heme adducts in addition to epoxide products [110]. Direct addition of epoxides to the P450 did not alkylate the P450 prosthetic heme, suggesting that heme alkylation occurs before epoxidation [111]. Some olefins, such as trichloroethylene, form carbonyl compounds as a result of P450-mediated epoxidation [112]. The epoxides produced from these reactions do not form carbonyl products, suggesting that more than one oxidation pathway is needed to account for product formation. As with hydrocarbon oxidation, the observed results may be described using a two-state model, where closure of the epoxide ring in the low spin state proceeds with a small energy barrier such that the reaction is nearly concerted, while closure of the epoxide in the high spin state occurs with a larger energy barrier that allows for other mechanisms to compete with epoxidation [113].

Oxidation of acetylenes is believed to proceed through an oxirane intermediate (Fig. 7.13), which rearranges through shift of a terminal hydrogen to a ketene intermediate [114]. Evidence for this mechanism comes from the observed isotope effect, which suggests that deuterium migration occurs in the rate-limiting step as oxygen is transferred to the acetylene [115].

7.2.1.3 Aromatic Oxidation. Hydroxylation of aromatic rings proceeds through an arene oxide intermediate (Fig. 7.14) [99]. Formation of the arene oxide intermediate is

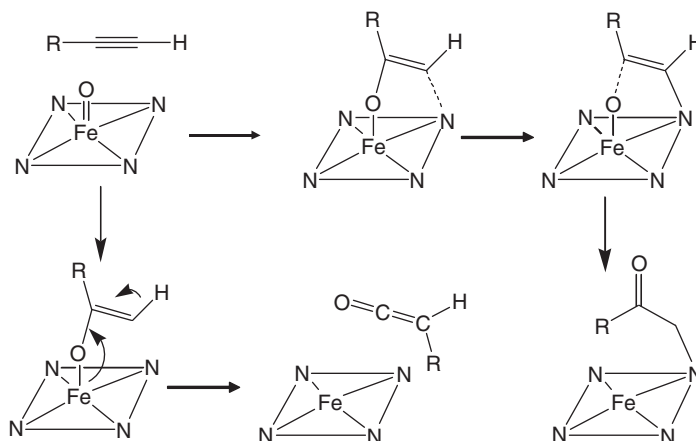


Figure 7.13 Proposed mechanism of P450-mediated acetylene metabolism.

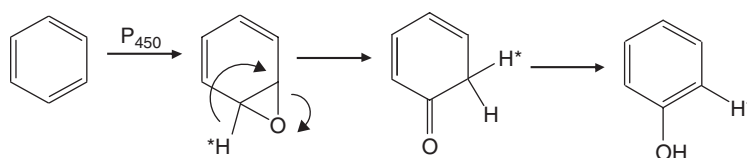


Figure 7.14 Proposed mechanism of P450-mediated aromatic oxidation.

followed by hydrogen migration in a process called an *NIH shift* [116]. Alkyl or halogen groups may also migrate, depending on the substrate [117]. The phenol product results from tautomerization. Evidence to support this mechanism includes partial retention of the shifted hydrogen and a small inverse secondary isotope effect for the reaction [118]. Since the NIH shift is not the rate-limiting step, the reaction is not sensitive to primary isotope effects. Hydroxylation of phenyl rings with adjacent cyclopropyl groups proceeds without ring opening, suggesting that a discrete radical mechanism is not favored for arene oxidation [119].

7.2.1.4 Heteroatom Dealkylation and Oxidation.

7.2.1.4.1 N-Dealkylation. Tertiary amines are metabolized predominantly through dealkylation, which can occur through various peroxidases, but the most common pathway for dealkylation is by the cytochrome P450 superfamily (Fig. 7.7). As such, a significant effort has been put forth to understand the mechanism of cytochrome P450 N-dealkylation reactions. The two predominant mechanisms in the literature are illustrated in Fig. 7.15. Mechanism A is that of single-electron transfer (SET) and mechanism B is that of HAT. For SET, the initial step is envisioned to occur by electron transfer from the amine nitrogen to the high valent iron oxene species forming a cation radical intermediate, which is then capable of catalyzing deprotonation of the ammonium radical followed by oxygen rebound to form the hydroxylamine [120]. Decomposition of the carbinolamine intermediate yields the N-dealkylated product.

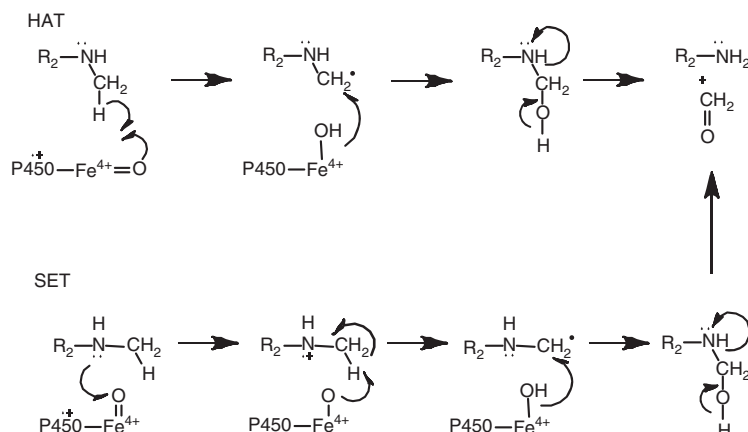


Figure 7.15 Proposed mechanisms for P450-mediated N-dealkylation: (i) single-electron transfer (SET) and (ii) hydrogen atom abstraction (HAT).

Alternatively, for HAT, a hydrogen atom is abstracted directly from the α -carbon followed directly by radical recombination to form the α -hydroxylamine. Differentiating between the two mechanisms has been explored extensively.

Kinetic isotope effects have been employed to differentiate between the two mechanisms. Initial interrogation of P450 N-dealkylation with selectively deuterated analogs such para-substituted *N,N*-dimethylanilines produces small kinetic isotope effects ($k_H/k_D < 4$). In contrast, alternate enzyme systems such as horseradish peroxidase (HRP) where SET is considered the mechanism of oxidation result in isotope effects that approximate the theoretical maximum. By comparison, HAT is the accepted mechanism for alkane hydroxylation which produces maximal kinetic deuterium isotope effect values ($k_H/k_D > 7$). The small isotope effects observed with *N,N*-dimethylaniline are consistent with hemeprotein catalyzed demethylation reactions that involve a deprotonation step [120]. However, small isotope effects have been demonstrated for demethylation of trimethylamine by *tert*-butoxy radical [121]. Dinnocenzo *et al.* [122] also observed a small isotope effect for hydrogen atom abstraction from *N,N*-dimethylaniline by *tert*-butoxy radical. The smaller observed isotope effects have been rationalized by the proposed weakening of the α -C–H bonds present in amines which facilitate a relatively early transition state. Nonsymmetrical transition states as described by the Westheimer kinetic postulate would produce an isotope effect below the theoretical maximum (Fig. 7.16). These contradicting results indicate isotope effect data alone may not be sufficient to distinguish the mechanism of P450 N-demethylation [123].

Synthetic heme mimetics and chemically designed metabolic probes have been employed to examine HAT (*tert*-butoxy radical) and SET (cation radical deprotonation) mechanisms in comparison to metabolites obtained directly from NADPH-supported P450 reactions. The combination of isotope effects with substituent effects has been used to generate isotope effect profiles for exploring reaction mechanisms. The increased number of compounds allows for trends to be explored in the resultant data to provide increased confidence in interpretation of reaction mechanism across different reaction systems. For example, P450 reactions revealed most similarity to *tert*-butoxy radical model supporting HAT as the mechanism for N-dealkylation (Fig. 7.17). The

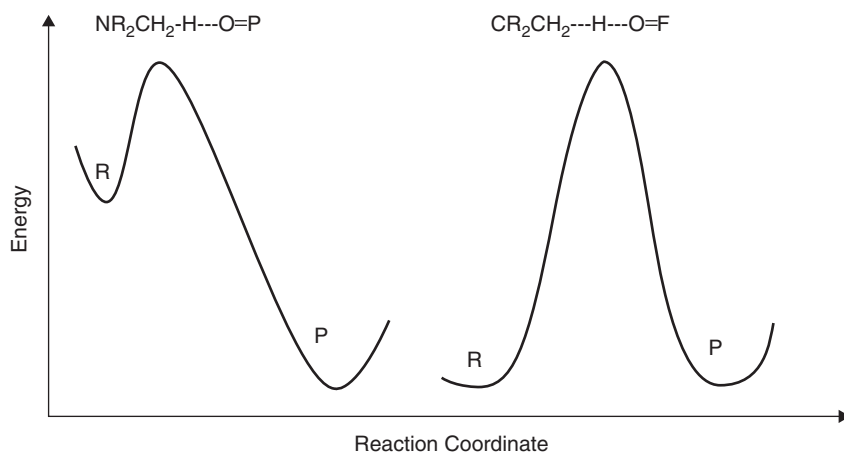


Figure 7.16 Example of asymmetrical and symmetrical transition state corresponding to theoretical difference in kinetic isotope effects: (a) early transition state $k_H/k_D > 4$ and (b) symmetrical transition state $k_H/k_D \sim 8$.

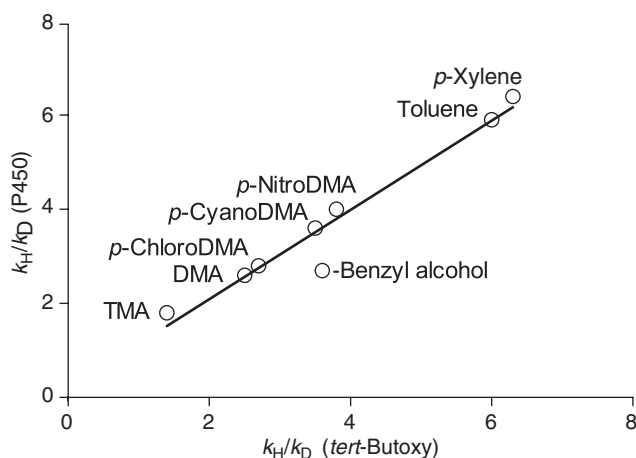


Figure 7.17 Isotope effects by *tert*-butoxy radical (HAT mediated) compared to P450-supported reactions. Plot includes accepted HAT pathways (xylene, toluene, and benzyl alcohol) in addition to several substituted *N,N*-dimethylanilines.

concordance between *tert*-butoxy radical and P450-supported reactions further support HAT mechanism for P450 reactions.

Chemical probes have been designed and synthesized to interrogate the mechanism of *N*-dealkylation. As described earlier in this section, radical clocks can differentiate oxidative mechanisms based on product ratios. A series of *N*-cyclopropyl anilines have been designed to evaluate different product ratios as a tool to rationalize HAT versus SET [105,124–126]. In principal, the rapid ring opening associated with *N*-cyclopropyl ring of the nitrogen cation radical would favor the *N*-decyclopropylation. However, the major pathway favored *N*-demethylation in support of HAT as the mechanism for oxidation (Fig. 7.18) [124]. Alternatively, Atkinson *et al.* [105] utilized a similar probe but in contrast yielded results supportive of the SET mechanism.

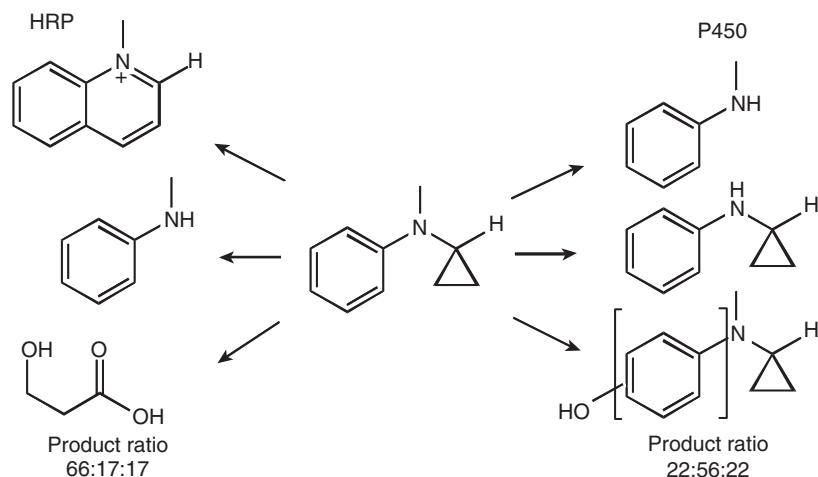


Figure 7.18 Example of product ratios from radical clock experiments with HRP and P450.

N-Oxides or *N*-hydroxyl products can also be catalyzed by P450s. The reaction is generally thought to proceed through the formation of a radical cation. The mechanism of partitioning between *N*-oxide and *N*-dealkylation remains unclear. Instability and potential for reduction of *N*-oxide may limit the extent of P450 *N*-oxide formation. The ability for *N*-oxides to back donate the oxygen to P450 was exploited in the form of synthesis of various deuterated *N*-oxide probes in order to specifically investigate the potential for high valent iron oxene to catalyze *N*-dealkylation reactions [127]. Specifically, the *N*-oxides for a series of para-substituted $^{13}\text{C}_2\text{H}_2$ -labeled *N,N*-dimethylanilines were synthesized with the intent to function as both substrates and surrogate oxygen atom donors. This particular substrate probe isolates the high valent iron oxene in the P450 catalytic cycle on reduction of the *N*-oxide to the iron–porphyrin complex. Coupled with deuterium isotope effect profiles obtained using the *N*-oxide system were found to closely match the profiles produced using NAD(P)H/NAD(P)-P450 reductase/ O_2 system.

7.2.1.4.2 O-Dealkylation. Oxidative O-dealkylation represents significant phase I oxidation pathway. O-Dealkylation reactions catalyzed by P450 enzymes have been studied using primary deuterium and tritium isotope effects to estimate the intrinsic isotope effect for this reaction using the technique of Northrop [128]. The large intrinsic isotope effect ($k_{\text{H}}/k_{\text{D}} > 7$) is consistent with a linear transition state on hemiacetal formation in which little to no bonding exists between the α -carbon and the hydrogen atom (Fig. 7.16) [128]. These results are consistent with hydrogen abstraction mechanism put forth by Groves and McClusky [97] for aliphatic oxidation. In addition, ^{18}O experiments illustrate that heavy water is incorporated into the metabolite byproduct [97]. Evidence for O-dealkylation to proceed through HAT is also provided by Dowers and Jones [129], wherein they designed and utilized quinoline analogs as evidence for HAT.

P450-mediated reactions follow the general reactivity pattern of *N*-dealkylation > benzylic hydroxylation > O-dealkylation > aliphatic hydroxylation > ω -hydroxylation [130]. These results correlate well with quantum mechanics-based AM1 calculations

of the stability and ionization potential of the radical resulting from HAT. These efforts have been extended to include aromatic oxidations through parameterization of the HAT and aromatic oxidation pathways based on experimentally determined regioselectivity data [131].

7.2.2 Flavin Monooxygenase

Mammalian flavin-containing monooxygenases (FMOs) are able to metabolize a variety of structurally diverse xenobiotics [132]. The structural requirements for FMO oxidation reactions (Fig. 7.19) are relatively straightforward as the substrate requires a soft nucleophile, usually a nitrogen or sulfur heteroatom but in rare cases may also include carbon, phosphorus, or selenium [133–138]. Interestingly, while nucleophilicity is the principal driver for FMO oxidations, structure–activity studies suggest that size and charge of potential substrates are important factors that mediate access to the enzyme’s active site [139,140]. As a consequence, the mechanism of FMO oxidation reactions are all similar and observed differences in the substrate specificities across the family of enzymes are all nearly due to differences in the active-site topography and access to the oxygenating species [141,142]. The influence of active-site topography on substrate oxidation is particularly evident in terms of the stereoselectivity observed with many

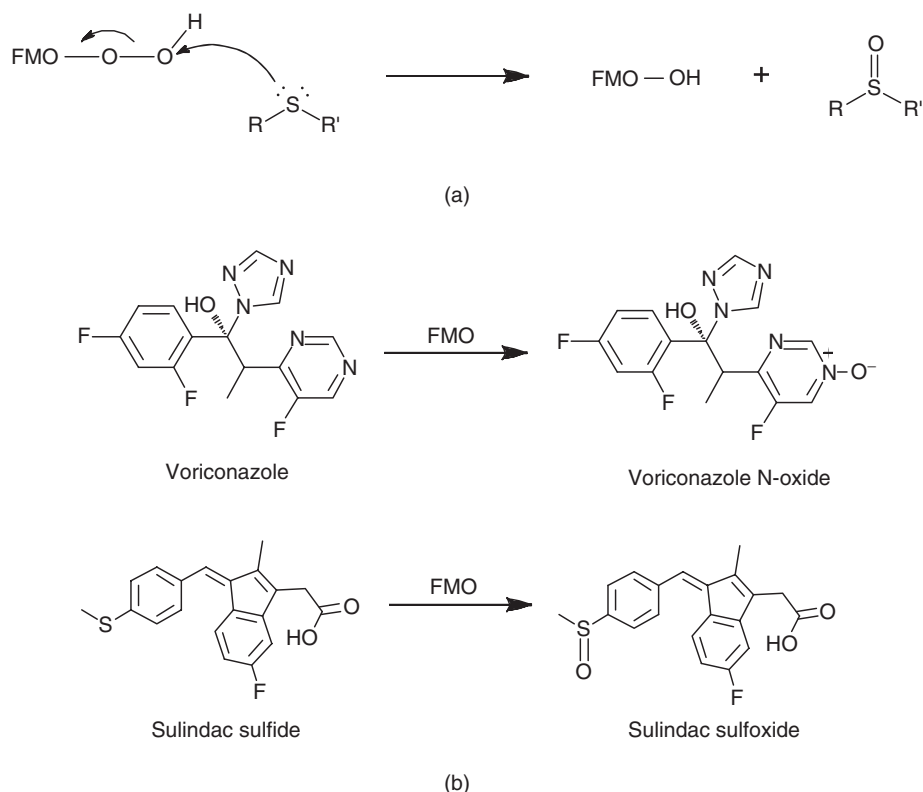


Figure 7.19 (a) Generic mechanism of FMO oxidation reaction. (b) Examples of FMO metabolism reactions with voriconazole and sulindac.

FMO oxidation reactions [143,144]. In the 1980s, Walsh and coworkers examined the stereochemistry of sulfoxidation of ethyl *p*-tolyl sulfide by hepatic microsomal FMO and found that the enzyme-catalyzed formation of the (R)-enantiomer of ethyl *p*-tolyl sulfoxide in great enantiomeric excess [145,146]. In this fashion, topography of the enzyme's active site not the substrate dictates the final chirality of the product as the enantiotopic electron pairs on the sulfur atom of an asymmetrically substituted sulfide are equivalent. Restated, chemical reactions that utilize peroxides or peracids as oxidants in solution proceed with no stereochemical preference and thus generate racemic sulfoxides [147]. However, in the chiral environment of the enzyme's active site, the electron pairs are not equivalent and stereoselective sulfoxidation can, and often times does, occur.

As with many biological oxidation reactions, the catalytic cycle for FMO enzymes is complex and involves several steps (Fig. 7.20). The underlying catalytic feature of this class of enzyme is their ability to stabilize a flavin-hydroperoxide intermediate [148,149]. Unlike the cytochromes P450 [150], the FMO peroxy intermediate has been found to be stable for minutes to hours at 4°C and can be observed spectrally [151].

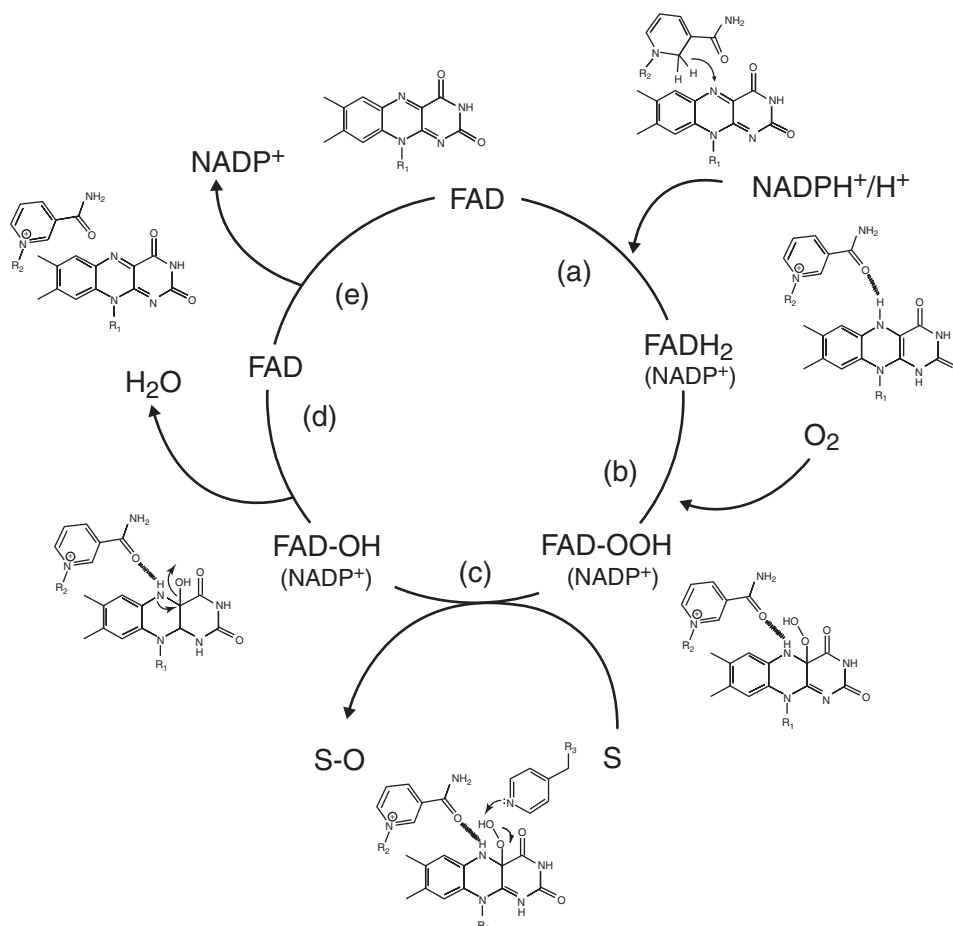


Figure 7.20 Proposed FMO catalytic cycle.

In the first step of the FMO catalytic cycle, FAD undergoes two-electron reduction by NADPH [152,153]. Chemistry of NADPH requires that reduction of the flavin cofactor is facilitated through a hydride transfer from the C4 atom of the nicotinamide ring to the flavin N5 atom (Fig. 7.20a) [154,155]. The reduced flavin reacts rapidly with molecular oxygen to form the stable peroxyflavin intermediate that is able to wait for a suitable nucleophile with which to react [156,157]. Stabilization of the intermediate requires the presence of NADP^+ [158], which remains tightly bound to the enzyme throughout the catalytic cycle [159]. It has been proposed through computer simulation of FMO active that NADP^+ is able to shield the enzyme's active site (Fig. 7.20b), and thus provide proper H-bonding environment, which in turn promotes a prolonged half-life to the peroxyflavin intermediate [160]. The binding of oxygen is followed by the nucleophilic attack by the substrate on the peroxyflavin, which results in one atom of molecular oxygen being transferred to the substrate (Fig. 7.20c). Following metabolite release, the resulting flavin hydroxy psuedobase is converted to FAD via the loss of water (Fig. 7.20d). It should be noted that the release of water and subsequent regeneration of FAD is a rate-limiting step in the FMO cycle [141]; thus, unlike the cytochrome P450 cycle [161], substrate binding has little influence on the rate of catalysis. The final step in the FMO catalytic cycle is the release of NADP^+ , which regenerates the FMO FAD moiety (Fig. 7.20e).

7.2.3 Aldehyde Oxidase

Aldehyde oxidase (AO) and xanthine oxidase (XO) are cytosolic members of the molybdenum hydrolase family. Both enzymes are capable of oxidizing aldehydes and nitrogen-containing aromatic compounds. While the scope of AO/XO involvement in xenobiotics remains somewhat limited relative to P450s, their role in the clearance of new drugs is likely to see an increase based on several factors [162,163]. The desire to reduce P450 clearance leads to increase number of heteroaromatic rings, a primary pharmacophore feature of AO/XO substrates. The awareness of AO/XO in drug metabolism has increased the availability of recombinant enzymes and selective chemical inhibitors for characterization of AO/XO metabolism. For example, planar nitrogen-containing heteroaromatic systems serve as the primary pharmacophore associated with competitive inhibitors of ATP-binding site for kinases that may coincide with AO/XO contributing to the clearance of these xenobiotics [164].

In accord with attempts to reduce P450 metabolism, the addition of nitrogens into aromatic ring systems is often attempted. The added nitrogens decrease the electron density of the carbon atoms in the heterocycle and tend to reduce P450 metabolism [127]. However, the reduced electron density of the carbon atoms in the heterocycle increases the potential for nucleophilic oxidation by AO/XO [165]. Both XO and AO enzymes contain molybdenum pyranopterin cofactors (MoCo) that facilitate nucleophilic oxidation of electron deficient substrates [166]. Most commonly, the oxidation occurs adjacent to the nitrogen in a heterocycle. The active sites are speculated to have similar structure capable of oxidizing a variety of dissimilar structures possessing significant overlap of substrates between AO/XO [165]. Catalysis involves cleavage of C–H bond and the oxygen incorporated into product arises from water and the byproduct of the oxidative step are superoxide and hydrogen peroxide [167]. A depiction of the catalytic mechanism for AO/XO metabolism is shown in Fig. 7.21. The oxidation has been demonstrated to occur through the formation of a tetrahedral transition state [168].

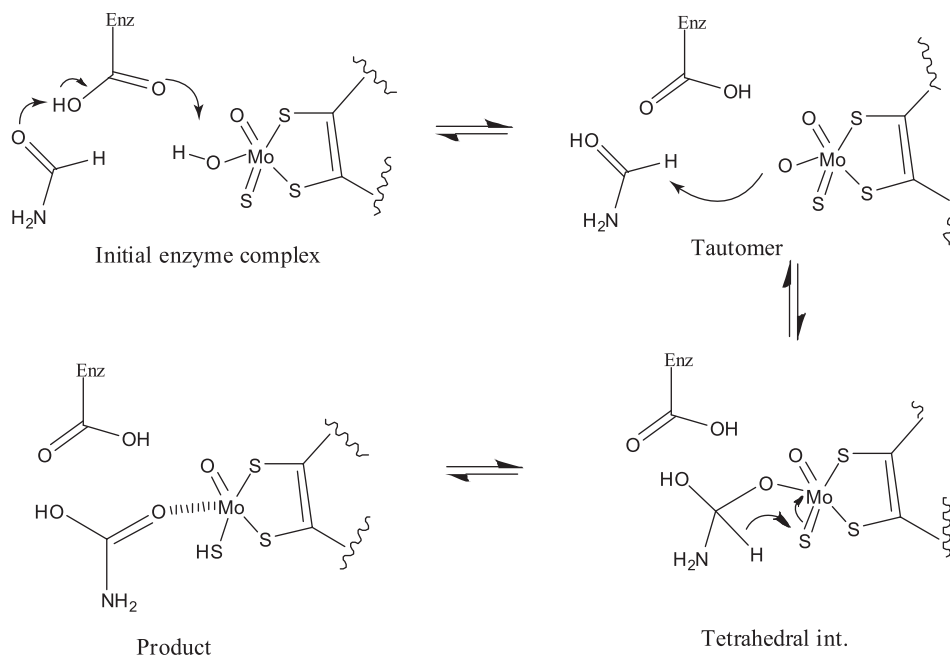


Figure 7.21 Proposed mechanism for AOX-mediated metabolism.

Several substrates and inhibitors exist for AO/XO, which can be helpful in distinguishing the enzymes involved in metabolism. XO is specific for the conversion of 1-methylxanthine to 1-methyluric acid, which are secondary metabolites of caffeine [169] (Fig. 7.22). Allopurinol is metabolized by XO to oxypurinol, which forms an inhibitory complex with the reduced form of the enzyme (Fig. 7.22) [170]. On reoxidation, the inhibition of oxypurinol is diminished [171]. FYX-051 also forms a tetrahedral complex and functions as structure-based inhibitor of XO [172]. In contrast, febuxostat does not complex to the molybdenum center but blocks substrate access directly [173,174]. Several compounds including hydralazine, chlorpromazine, and isovanillin were identified as reasonably selective inhibitors of AO after screening a panel of 239 drugs for AO inhibition [162].

AO/XO have also been exploited for the purpose of converting prodrugs to active drug species in order to overcome ADME-related issues. For example, 5-fluorouracil (5-FU) is a generic antineoplastic, which is rapidly metabolized in the gastrointestinal tract limiting the bioavailability of 5-FU [175]. In order to overcome this obstacle, a prodrug form, 5-fluoro-pyrimidinone, was employed, whereby the keto group is introduced by hepatic AO after absorption [176]. Another example is that of the antiviral penciclovir, which displays poor oral absorption [177]. The diacetyl 6-deoxy prodrug derivative of penciclovir, famciclovir, displays improved absorption and systemic availability [178].

Predictions of AO metabolism have been studied using density functional theory to qualitatively determine the site(s) of oxidation based on the analysis of the energetics of the proposed tetrahedral intermediate with the oxidized carbon [165]. In general, it has been suggested the AO metabolism will occur at the most electropositive carbon in a molecule with some influence on regioselectivity determined by steric bulk

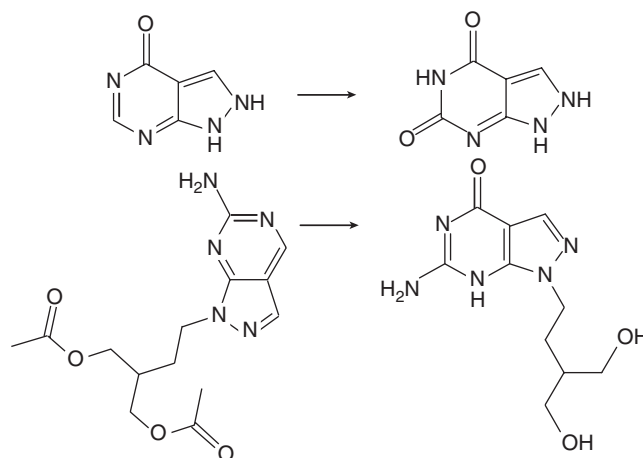


Figure 7.22 Example reactions catalyzed by AOX.

[179]. Additional factors must play a role in metabolite regioselectivity as illustrated by the fact that the most electropositive carbon is not always the site of metabolism. The DFT calculations from Torress *et al.* suggest that oxidation of substrates by AO appears reliant on the electronic effects of the substituents. The use of 6-substituted quinazolinones demonstrate the electronic effects and increase in reaction rates with increased electron-withdrawing groups at the 6-position [180,181]. The calculated results provide a rank ordering of potential products by evaluating the individual tetrahedral intermediates precluding metabolite formation. For the compounds examined, >90% of AO metabolites were correctly predicted. The calculations were also able to differentiate the metabolites generated by XO compared to AO for a series of closely related molecules.

7.2.4 Monoamine Oxidase

Mammalian monoamine oxidases (MAOs) are flavin-containing enzymes that catalyze the oxidation of amines to aldehydes (Fig. 7.23) [182,183]. Two isozymes are known, MAO-A and MAO-B, which differ in substrate and inhibitor selectivity [184]. MAOs are expressed in many mammalian tissues, with high expression in both the liver and placenta [185]. MAOs are also present in the brain; they are believed to have an important biological function for the inactivation of neurotransmitters [186].

The requirements for MAO oxidation are a basic nitrogen and an adjacent carbon with two α -hydrogens (Fig. 7.24). The substrate-binding region is a flat cavity lined with hydrophobic residues [187]. Structure–activity relationships (SARs) suggest that

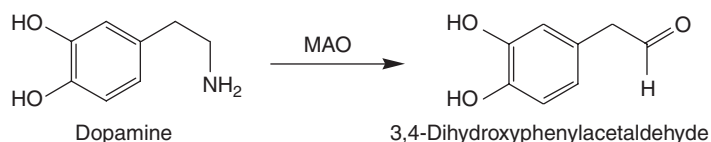


Figure 7.23 Example of MAO metabolism reactions with dopamine.

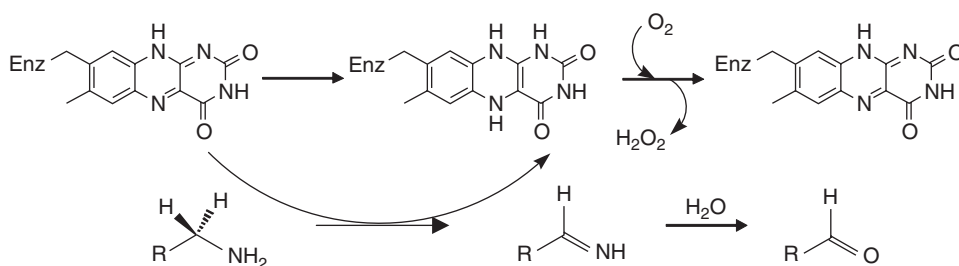


Figure 7.24 MAO catalytic cycle.

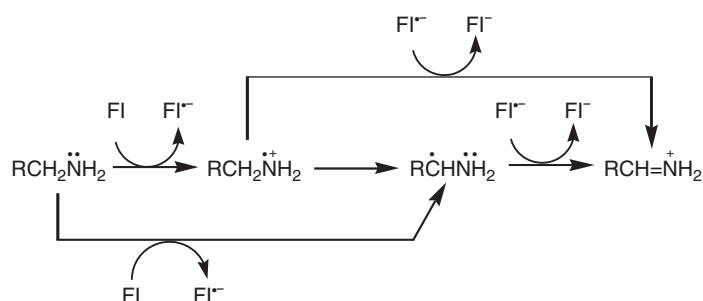


Figure 7.25 Proposed mechanisms of MAO-mediated reactions: single-electron transfer and hydrogen atom abstraction.

the size and shape of potential substrates determine access to the enzyme's active site [188]. The mechanisms of MAO-catalyzed reactions are generally similar; therefore, differences in observed kinetics are due to specific interactions between the enzyme and the substrate [189]. The influence of active-site topography is particularly important due to the stereochemical requirements for MAO oxidation. In the 1980s, Yu and coworkers found that the pro-(*R*)-hydrogen of MAO substrates was lost exclusively based on the reactivity of stereoselectively deuterated dopamine analogs [190,191]. In this sense, the active site dictates reaction stereochemistry. However, the aldehyde moiety resulting from substrate oxidation is not chiral, such that MAOs do not produce an inherently chiral product.

MAO-mediated oxidation has been proposed to proceed by three mechanisms: SET [192], HAT [193], and a polar/covalent mechanism (Fig. 7.25) [194]. The SET mechanism begins with transfer of an electron from the substrate nitrogen, forming an aminyl radical cation and a flavone semiquinone radical. Loss of an α -hydrogen creates a carbon-centered radical, which can then transfer an electron to form an iminium ion and a flavone semiquinone. On the basis of the results of SAR studies, the reactive carbon is positioned in front of the N5-C4a locus, with its α -hydrogens located ~ 3.6 Å from the flavin N5. The iminium ion is released from the active site and undergoes hydrolysis to form the aldehyde product. The HAT mechanism proceeds through a similar pathway, but begins with direct loss of an α -hydrogen. In the polar/covalent mechanism, the substrate forms a covalent bond with FAD (Fig. 7.26). Cleavage of the bond results in formation of an iminium ion and FADH₂.

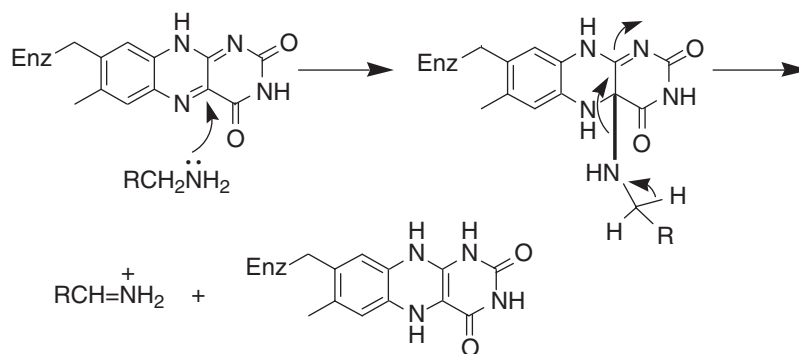


Figure 7.26 Proposed polar/covalent mechanism for MAO-mediated reactions.

MAO-A and MAO-B differ in both inhibitor and substrate selectivity. Substrates for MAO-A include 5-hydroxytryptamine and epinephrine; clorgyline is a selective inhibitor of MAO-A [195]. Substrates for MAO-B include benzyl amine and β -phenylethylamine; deprenyl is a selective inhibitor of MAO-B [196]. Differences in substrate selectivity may partially be explained by a smaller entrance cavity for MAO-B, although results from site-directed mutagenesis studies suggest that the Tyr326Ile MAO-B mutation conferred MAO-A activity and the Ile335Tyr MAO-A mutation conferred MAO-B activity [197].

7.2.5 Carboxylesterase

Carboxylesterases (CEs) metabolize and eliminate endogenous and xenobiotic esters. In humans, the highest hydrolase activity is found in the liver [198–200]. The mechanism for catalysis is similar to that of other α/β hydrolases that employ an active-site serine, histidine, and acidic (glutamic or aspartic acid) residue that are spatially oriented to form a catalytic triad (Fig. 7.27) [198,201–204]. The general mechanism occurs in two steps: first, the serine hydroxyl forms an acyl intermediate with the substrate. The acyl intermediate is then hydrolyzed to form a product. Like other drug-metabolizing enzymes, CEs display broad substrate specificity mediating the hydrolysis of esters, amides, and thioester substrates (Fig. 7.28) [205,206]. CEs have also demonstrated the ability to transesterify compounds such as cocaine, methylphenidate, and meperidine [207,208]. The inhibition of CEs has attracted attention as a mechanism of therapeutic intervention or modulation of coadministered prodrugs [209–211]. Furthermore, inhibition of CE is a primary off-target concern for therapeutics aimed toward inhibiting other esterase and hydrolase enzymes [212]. Differences in catalytic efficiencies have been observed when comparing human CE to CE found in preclinical species [199,213]. The mechanistic understanding of CE in drug metabolism has been established through a variety of techniques including ligand-based SAR, crystallography, and site-directed mutagenesis studies.

The strategically positioned amino acids for hydrolysis combined with the observed substrate promiscuity presents a unique dichotomy. One explanation is that CE displays significant induced fit of protein that is predicated on the proper spatial orientation of the substrate [214]. On substrate induced fit, the conformation of the catalytic triad

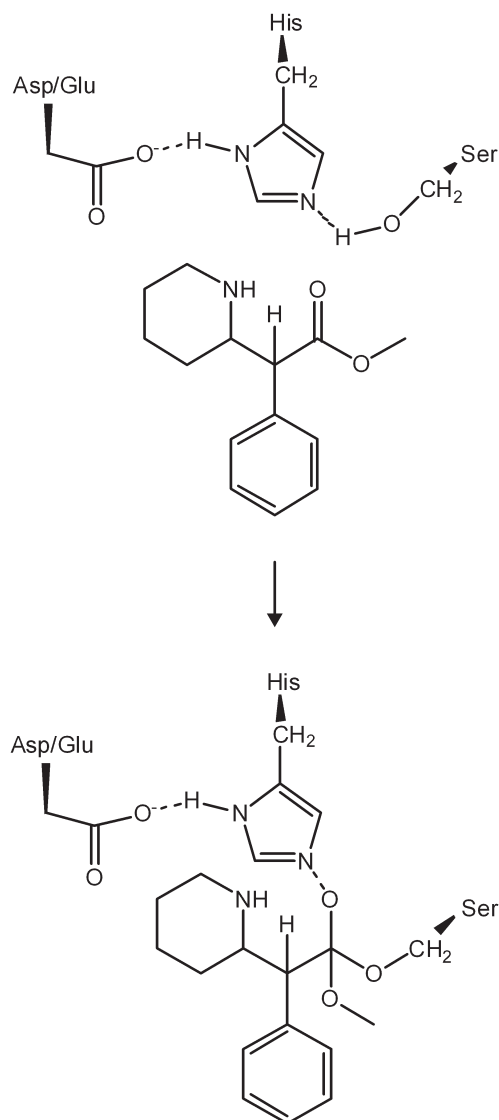


Figure 7.27 The catalytic triad binding methylphenidate to form tetrahedral transition state complex.

is postulated to form a strong hydrogen bond also known as a *low barrier hydrogen bond* between the catalytic His and acidic residue to yield an increased reactivity of His as a general base [215–217]. As might be expected, subtle changes in chemical structure of substrates and inhibitors can have a profound impact CE activity. For example, a series of cypermethrin analogs were examined to understand the role of stereochemistry on CE catalysis. Huang *et al.* [218] demonstrated a >20-fold difference in specific activity between *cis*- and *trans*-cypermethrin analogs. CE is the major first-pass enzymatic pathway for methylphenidate with higher activity toward the

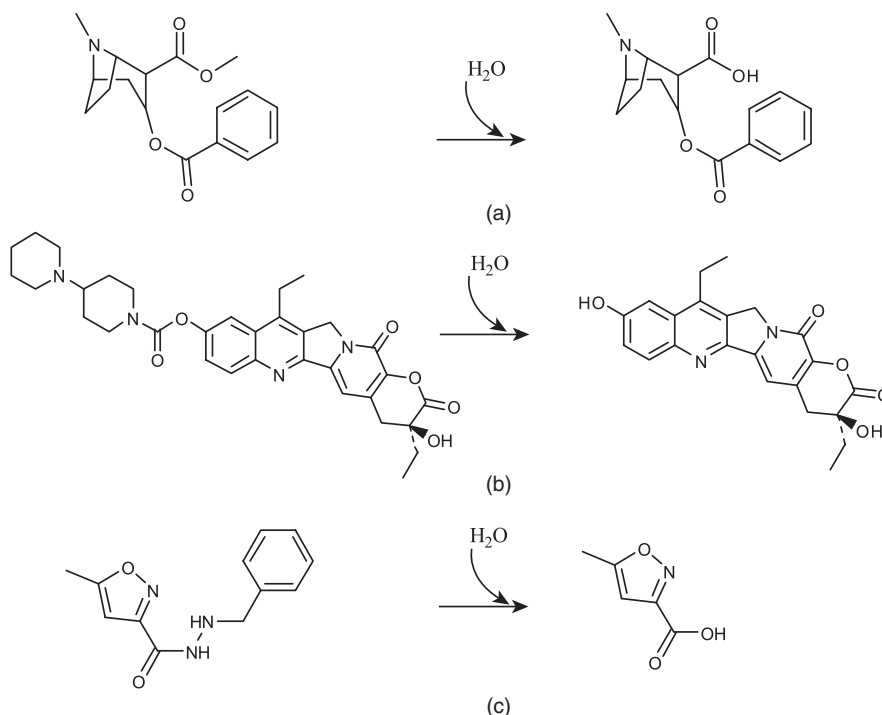


Figure 7.28 (a) Hydrolysis of cocaine ester, (b) hydrolysis of CPT-11 to bioactive SN-38, and (c) amide hydrolysis of isocarboxazid.

L-isomer compared to the D-isomer [219]. The stereochemistry leads to a subtle change in orientation of enzyme–substrate complex leading to profound changes in catalytic efficiency. This may be expected for CE based on the specific distance requirements for all three residues to act in concert with one another. The activity of the potent anti-cancer drug CPT-11 requires activation by hCE (Fig. 7.28). CPT-11 is the carbamate prodrug of the active SN-38 hCE-derived metabolite [206,220]. The conversion, however, is relatively inefficient where typically <5% of CPT-11 is converted to SN-38 [221]. Additional characterization of CE active site has been established with various competitive inhibitors. Trifluoromethyl ketones (TFKs) represent a class of compounds that display some of the most potent inhibitors of CE [222]. In general, the potency of TFK analogs is attributed to the enhanced electrophilicity of carbonyl carbon as a result of the electron-withdrawing nature of the CF_3 moiety (Fig. 7.29). In addition, TFK inhibitors also bind in the favored conformation of the transition state as a tetrahedral intermediate and thus are termed *transition state analog inhibitors*. Wheelock *et al.* [222] nicely demonstrated that TFK analogs were more amenable to mimicking the transition state through the formation of the gem diol (sp^3 hybridized conformation; tetrahedral intermediate common to CE transition state), which lead to improved inhibitory potencies (Fig. 7.29). Other inhibitors include organophosphorus compounds, which also exploit the nucleophilic behavior of the catalytic serine to form an irreversible covalent acyl enzyme intermediate with compounds such as soman and tabin

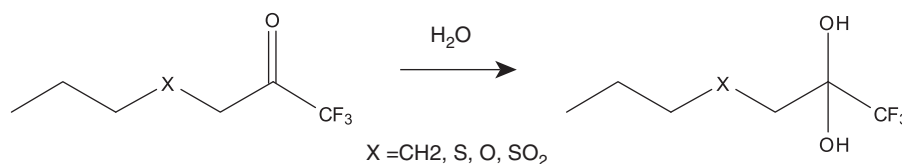


Figure 7.29 General structure of TFK inhibitor analogs where trifluoro substitution increase electrophilicity of carbonyl carbon favoring gem diol formation.

[223,224]. Interestingly, hCE does not seem to readily form the irreversible complex formed in rCE.

Site-directed mutagenesis of hCE identified key portions of the protein for substrate and metabolite entrance and exit channels from the active site [225,226]. Rational enzyme design was accomplished with site-directed mutagenesis studies in combination with crystal structure data to develop an hCE mutant with CPT-11 activity similar to that achievable with rCE. Modification of eight amino acids demonstrated improved CPT-11 hydrolysis, which was proposed to occur via an increased flexibility of the active-site cavity necessary to bind CPT-11.

The crystal structure of hCE in complex with the potent inhibitor benzil (45 nM) demonstrated the formation of covalent product [227]. Crystallization with modest inhibitor tamoxifen revealed binding at the α -site of the protein where in this may partially explain the partial inhibition properties of tamoxifen and provide evidence that the α -site may serve as an access channel for different substrates [227].

7.2.6 Epoxide Hydrolase

Mammalian epoxide hydrolases (EHs) catalyze the conversion of three-membered cyclic ethers, called *epoxides*, to diols (Fig. 7.30) [228]. EHs are expressed in many mammalian tissues with the highest concentrations found in the liver [229,230]. Several EHs are present in mammals; however, microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH) have been the focus of research efforts [231]. They differ in both subcellular localization and substrate specificity. mEH is found within the endoplasmic reticulum and metabolizes xenobiotic-derived epoxides [232]. sEH is found in the cytosol and metabolizes endogenous fatty acid epoxides [233]. The biological role of mEH is the detoxification of xenobiotics [234], while sEH appears to play a role in the hyperpolarization of vascular smooth muscle by controlling the levels of vasodilatory epoxyeicosatrienoic acids [235,236].

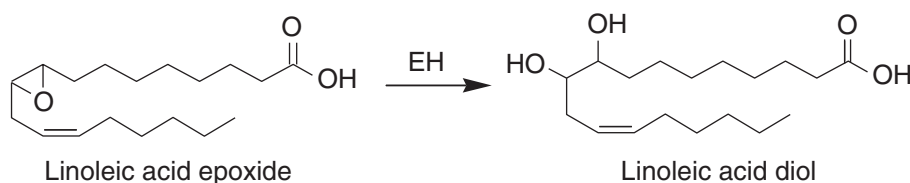


Figure 7.30 Example of EH metabolism reaction with linoleic acid epoxide.

Metabolism by EH requires the presence of an epoxide. The mechanisms of EH-mediated hydrolyses are similar; therefore, differences in observed kinetics are generally due to specific interactions between the enzyme and a potential substrate. SARs suggest that both the size and shape of a substrate are key factors in mediating access to the EH active site [237]. The active site of sEH contains a 25-Å long, L-shaped hydrophobic domain at the C-terminus where substrates bind for catalysis [231]. The influence of active-site topography on epoxide hydrolysis is particularly evident with regard to the stereoselectivity observed with many EH reactions. In the 1990s, Zeldin and coworkers [238] examined the stereochemistry of the conversion of *cis*-epoxyeicosatrienoic acids to diols by hepatic sEH and found that incubations containing the 8,9- or 14,15-epoxides formed diols stereoselectively, while incubations containing the 11,12-epoxide were not stereoselective. In this fashion, both the topography of the EH active site and the shape and orientation of the substrate within the EH active site influence the final stereochemistry of the product.

The catalytic cycle for EH is complex and involves several steps (Fig. 7.31). An underlying feature of this enzyme class is a catalytic triad containing a nucleophilic acid (usually Asp), an orienting acid (Asp or Glu), and a basic histidine [228]. In the first step of catalysis, polarization of the epoxide moiety by tyrosines located on the

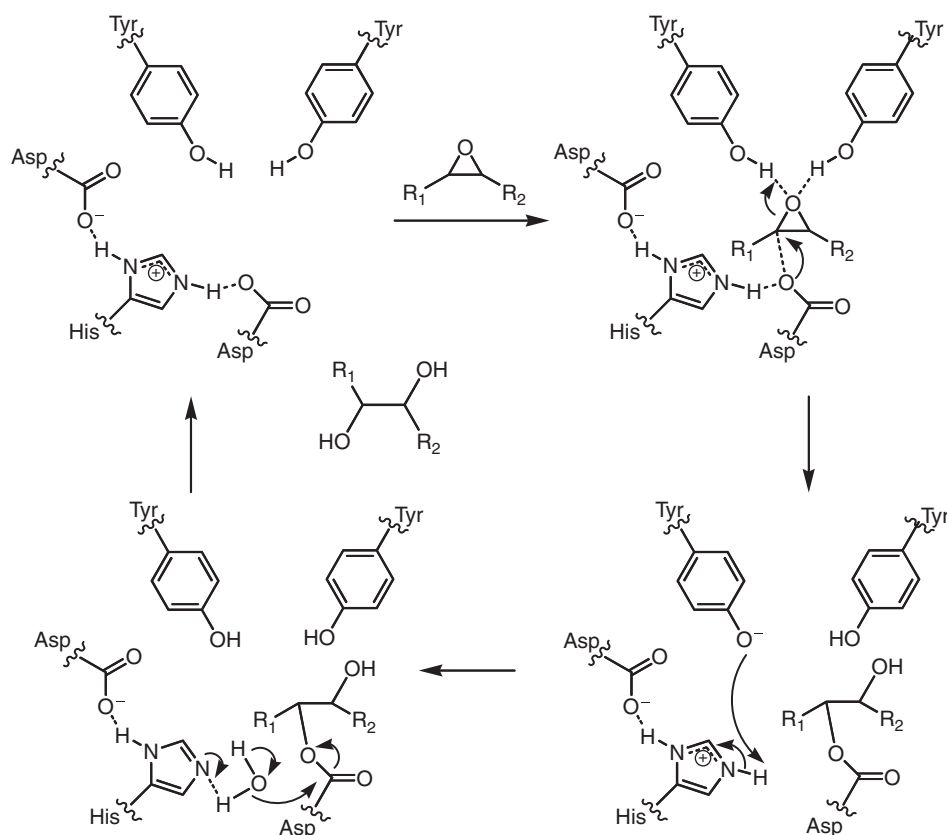


Figure 7.31 EH catalytic cycle.

top of the EH active site occurs concurrently with nucleophilic attack on an epoxide carbon by an acidic residue located on the opposite side of the active site [239]. This attack occurs preferentially at the least sterically hindered or most reactive carbon of the epoxide. Attack by the nucleophilic acid is aided and directed by a nearby histidine-orienting acid pair [240]. Ring opening of the epoxide results in formation of a covalent ester bond between the attacking acid and a carbon from the epoxide. This intermediate has been termed the *hydroxyl alkyl-enzyme intermediate* [228]. A water molecule, activated by the histidine-orienting acid pair, reacts with the hydroxyl alkyl-enzyme intermediate to release the enzyme and a diol as products.

Evidence for the mechanism of catalysis and the presence of a hydroxyl alkyl-enzyme intermediate comes from three sources. In a single turnover experiment using 1,10-phenanthrene-5,6-oxide as substrate, transfer of ^{18}O from hepatic mEH to the substrate was best explained through formation of a hydroxyl alkyl-enzyme intermediate [241]. Inhibition kinetics of EH-mediated reactions were well described using models based on formation of a covalent enzyme-inhibitor intermediate, whose half-life is inversely proportional to inhibition potency [242]. Additionally, a covalent adduct of hepatic mEH and $[1-^{14}\text{C}]$ epoxystearic acid was detected after denaturing SDS gel electrophoresis [243]. Formation of the adduct was inhibited in the presence of 1,1,1-trichloropropylene oxide, an inhibitor of mEH.

7.2.7 Ketoreductase

The aldo-keto reductases (AKRs) comprise a superfamily of monomeric (monomer molecular weight = 34–37 kDa), NADPH-dependent oxidoreductases with similar physical and chemical properties [244]. Much more is known about the physiological roles of carbonyl-reducing enzymes (e.g., steroid and prostaglandin metabolism, housekeeping, and stress response) than their roles in drug metabolism [245]. With respect to drug metabolism, typically the AKR enzymes catalyze the reduction of aldehydes and ketones to the corresponding alcohol products (Fig. 7.32) and the subsequent products often times undergo further conjugation and elimination [246]. For example, AKRs play an important role in the metabolism of the therapeutic agent such as haloperidol, ketotifen, and oracin [247–249]. In humans, an important pathway of warfarin metabolism is reduction of the ketone to a secondary alcohol [250]. This reaction is catalyzed by cytosolic ketone reductases and is stereospecific, producing only the (S)-enantiomer of warfarin enantiomers [251].

A nomenclature system to group AKRs has been adopted and currently 14 subfamilies have been identified [252]. However, only four AKR subfamilies, namely, 1A, 1B, 1C, and 7A, have documented roles in xenobiotic carbonyl metabolism [253]. To date, over 15 crystal structures of AKRs complexed with their ligands have been determined [254]. In each instance, the AKR structure reveal a $(\alpha/\beta)_8$ -folding pattern (triose isomerase or TIM barrel fold), where the central β -strands form the staves of a barrel, with large loops at the back that form the ligand-binding site [255]. The cofactor-binding site and the active site are highly conserved across the superfamily. The active site (Fig. 7.33) contains a conserved catalytic tetrad (Tyr, Asp, Lys, and His), which forms an oxyanion-binding site with the nicotinamide ring of the cofactor via a hydrogen bonding network [256–258]. In contrast, strong variation exists in the structure of the substrate-binding cavity, which is largely defined by the residues from the loops [259]. The reaction mechanism for this class of enzymes has been studied in

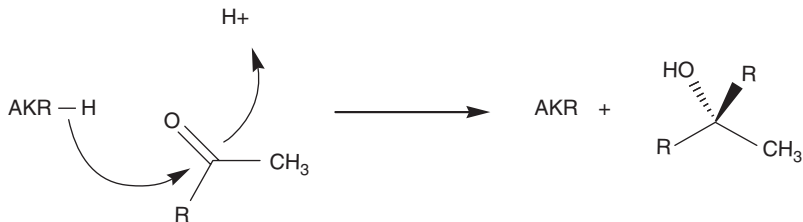


Figure 7.32 (a) Generic mechanism of ketone reduction. (b) Examples of AKR metabolism reactions with haloperidol and warfarin.

detail, revealing an ordered bi-bi mechanism [260], where the cofactor binds first and leaves last [261]. Interestingly, while the catalyzed reaction favors alcohol formation, it is possible for the reverse reaction to occur, albeit to a limited extent.

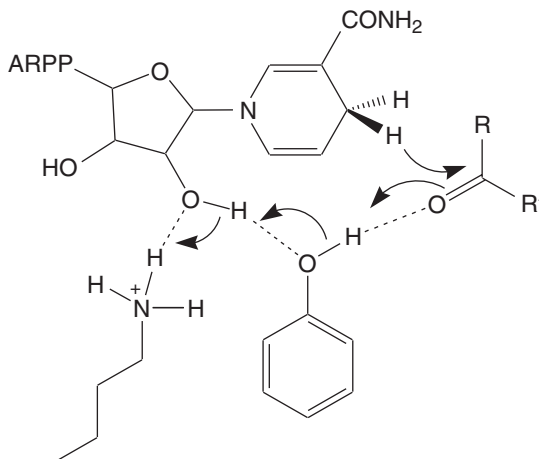


Figure 7.33 Proposed AKR active-site hydrogen bonding network to form the oxyanion-binding site with the nicotinamide ring in ketone reduction reaction.

A common feature of the carbonyl-reducing enzymes is their proposed two-electron reduction mechanism involving hydride transfer from the cofactor to the carbonyl carbon, which is facilitated by general acid [262]. In this fashion, unsymmetrical ketones generate chiral centers on reduction to alcohols. As a consequence, for many substrates, the stereoselectivity of carbonyl reductase-catalyzed ketone reduction is dependent on the steric situation of substrates and may be predicted with “Prelog’s rule” [263]. Prelog’s rule describes the reduction of ketone to yield optically active secondary alcohols. In this instance, the chirality of an alcohol arising from nucleophilic addition to a ketone depends on which prochiral face accepts the nucleophile and the sequence priority of the nucleophile relative to other substituents of the carbonyl function [264]. In hydride transfer reactions, the hydride nucleophile will always be the lowest priority group; thus, the reface addition of the hydride to a ketone will always generate an S-configuration alcohol [265].

7.3 PHASE II DRUG METABOLISM REACTIONS

7.3.1 Glucuronosyltransferase

The UGT family of enzymes catalyzes the conjugation of a glucuronic acid moiety from uridine diphosphoglucuronic acid (UDPGA) to a broad range of substrate molecules (Fig. 7.34) [266,267]. The glucuronidated compounds are more hydrophilic than their aglycone counterparts and thus more readily excreted from the body. Any functional group with sufficient nucleophilicity can be an acceptor for the glucuronic acid transfer, with the most common functional groups including phenols, aliphatic alcohols, carboxylic acids, amines, thiols, and nucleophilic carbon atoms [51]. Common substrates include endogenous compounds such as bilirubin, estrogens, and fatty acids as well as numerous xenobiotics.

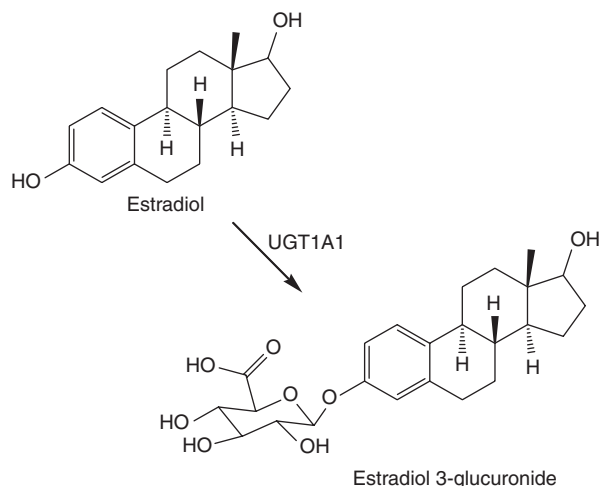


Figure 7.34 Example of UGT metabolism reaction with estradiol.

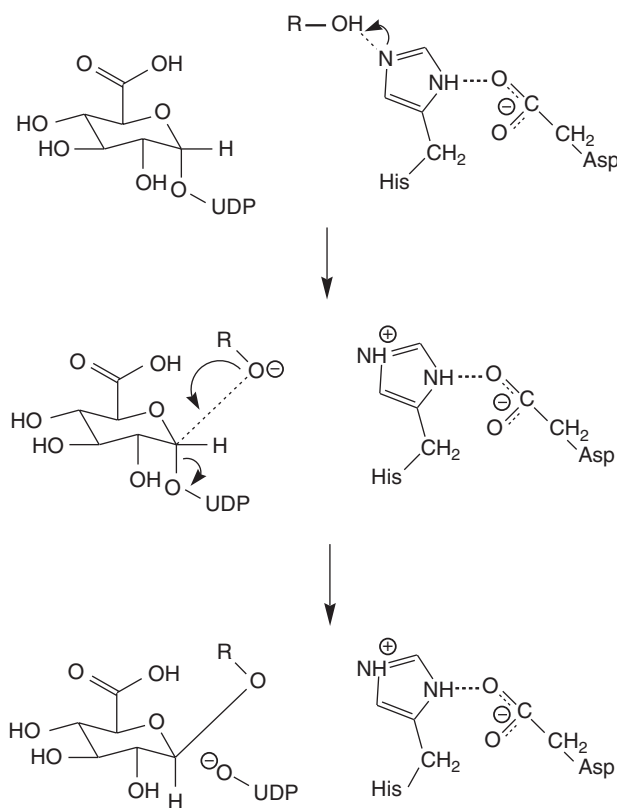


Figure 7.35 Proposed mechanism of UGT-catalyzed glucuronidation.

It is now generally accepted that UGT-catalyzed glucuronidation proceeds via an acid–base mechanism similar to that exhibited by serine hydrolases [268,269]. A conserved histidine residue serves as a catalytic base to deprotonate a nucleophilic site on the aglycone molecule, which then results in a nucleophilic substitution at the C1 atom of the glucuronic acid moiety. Consequently, the protonated histidine residue is stabilized through an interaction with a proximal aspartic acid residue that is also conserved across human UGTs (Fig. 7.35). The nucleophilic attack on the C1 carbon occurs through an S_N2 reaction mechanism with stereoinversion from the α -configuration at the C1 carbon of the glucuronic acid moiety in UDPGA to the β -configuration in the resulting glucuronide [270]. The reaction mechanism is supported by data showing a correlation between reaction rates and nucleophilicity for a series of phenol-containing compounds [271]. The order in which the aglycone and UDPGA bind during the UGT-catalyzed S_N2 reaction remains ambiguous and may be isoform dependent, with proposed mechanisms including a compulsory-ordered bi-bi mechanism, a random-ordered bi-bi mechanism, or a Theorell–Chance reaction mechanism [271–278].

A unique scenario exists following a nucleophilic attack on the C1 carbon by a carboxylic acid moiety. The resulting acyl glucuronides are more reactive than ether-linked glucuronides and present the possibility for additional reaction steps, where the aglycone migrates via intramolecular transesterification from the C1-hydroxyl group

of the glucuronic acid moiety to the C2-, C3-, or C4-hydroxyl groups [279,280]. All the steps in the migration are reversible, except for the first migration step from the C1 to the C2 position. On migration, acyl glucuronides can covalently adduct proteins and other macromolecules through one of two mechanisms. First, a direct nucleophilic attack on the glucuronic acid moiety by an amino acid residue results in binding of the aglycone to protein through a carbonyl linkage and liberation of D-glucuronic acid [281–285]. The second proposed mechanism for the irreversible binding of acyl glucuronides to protein involves the interaction of a protein nucleophile, such as an internal lysine residue or the N-terminus of a protein, and the free aldehyde of the open-chain glucuronic acid moiety to form an imine bond between the two functional groups. The initial interaction is reversible; however, it can subsequently be followed by an irreversible imine reduction or Amadori rearrangement, which occurs when the aglycone moiety has migrated to the C3 or C4 position, and is converted to the more stable 1-amino-2-keto product [286,287].

In addition to the observed stereochemistry observed at the C1 carbon of UDPGA during glucuronidation, the UGT family of enzymes also has also been shown to exhibit a high degree of stereoselectivity regarding the aglycone acceptor molecules. A recent study aimed at measuring the eudismic ratios of a series of chiral secondary alcohols for UGT2B7 and UGT2B17 demonstrated that the UGTs can display a degree of stereoselectivity that is similar to that generally reserved for highly specific receptor proteins [288]. Similarly, both isoforms have shown substrate specificities that are dependent on the configuration (α vs β) of the 17-hydroxyl group of steroids such as estradiol and testosterone [289,290]. Interestingly, the stereochemistry of the aglycone had a negligible effect on the affinity of the enzyme–substrate complex and thus may reflect the importance of substrate orientation in the transfer of the glucuronic acid moiety. Additional studies have also demonstrated the importance of the orientation of the glucuronic acid, uridine diphosphate, and aglycone groups in forming the ternary UGT-UDPGA-aglycone complex [291].

While substrate selectivities vary across the different UGT isoforms, it is generally accepted that the N-terminal domain plays an important role in substrate recognition [67,268, 292–302]. It has been suggested that a conserved histidine residue is generally required for glucuronidation through proton abstraction, while a proline residue is more likely involved in the glucuronidation of tertiary amines [303]. From the standpoint of the acceptor molecule, compounds that are hydrophobic in nature and contain a nucleophilic site generally make good substrates for glucuronidation. More recently, the effect of chemical substituents within close proximity to the nucleophile on the rate of glucuronidation has been examined. In general, an aromatic ring adjacent to the nucleophilic site appears to greatly increase the likelihood of glucuronidation at the nucleophilic site, possibly through electronic stabilization of the reaction intermediate or through π -stacking interactions within the active site of the enzyme, which serve to decrease the entropy of bond formation between the acceptor molecule and UDPGA [304,305]. It has also been noted that nucleophiles with a more negative partial charge combined with a higher Fukui function (describing the likelihood of electrophilic or nucleophilic attack at a given atom) are more prone to glucuronidation [305]. In addition to the nucleophilicity of the acceptor molecule (aglycone), glucuronidation by hepatic UGTs is also highly dependent on the hydrophobicity of the aglycone, with an optimal octanol–water partition coefficient ($\log P$) of ~ 2 having been reported [306]. Finally, steric interactions can also play a role in substrate selectivity as well as overall

rates of glucuronidation, as has been observed for a series of substituted phenols, where bulky substituents in the ortho-position to the hydroxyl group generally decreased the susceptibility of the nucleophile to glucuronidation [307,308].

7.3.2 Glutathione Transferase

Cytosolic and membrane-bound forms of glutathione-*S*-transferase (GST) are encoded by two distinct superfamilies [309]. The soluble protein form exists as two polypeptide subunits creating dimer with a molecular mass of 50 kDa. GSTs catalyze the transfer of the endogenous tripeptide, GSH to the electrophilic center of a diverse set of substrates to form a polar *S*-glutathionylated reaction product (R-SG). GST subunits contain a GSH-binding site (G-site) and a hydrophobic substrate site (H-site) (Fig. 7.36) [310,311]. On proper orientation of substrate and GSH, the reaction proceeds through the formation of a Meisenheimer complex (Fig. 7.37).

The G-site binds GSH extending the tripeptide in a conformation that points the sulfur toward the subunit in which it is bound. Electrostatic interactions with the glycine of GSH are favorable with the subunit it binds ($K_d = 20 \mu\text{M}$). The spatial orientation of GSH in the active site is conserved across soluble forms of GST. However, the interactions stabilizing the GSH-GST subunit complex are unique to the isoform and isoform-specific interactions may contribute to differences in conjugate regioselectivity and catalytic activity of the different enzyme forms [312]. Ultimately, GSH binding at the G-site lowers the activation energy for the nucleophilic attack by reducing the thiol pK_a of GSH. The thiol pK_a of unbound GSH is 9.0 ($\text{GSH} \rightarrow \text{GS}^-$ in water) compared to GSH in complex with GST subunit where the pK_a is 6.0–7.5 [313–315]. A shift

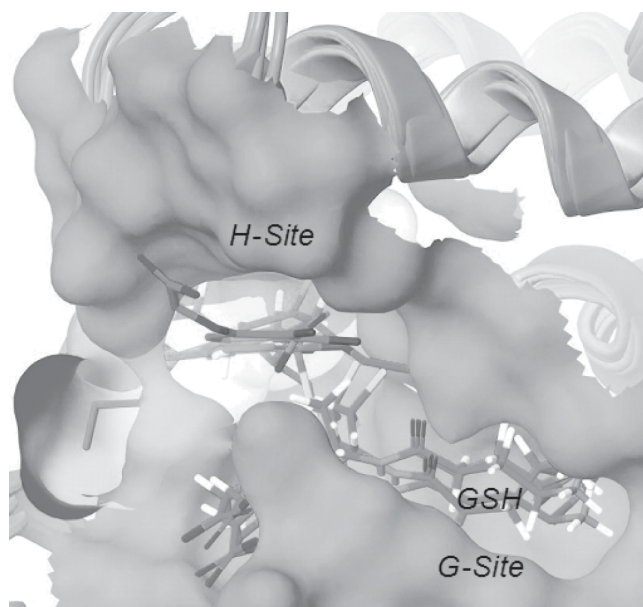


Figure 7.36 General binding site for GSH (G-site) and electrophilic substrate (H-site). (See color insert.)

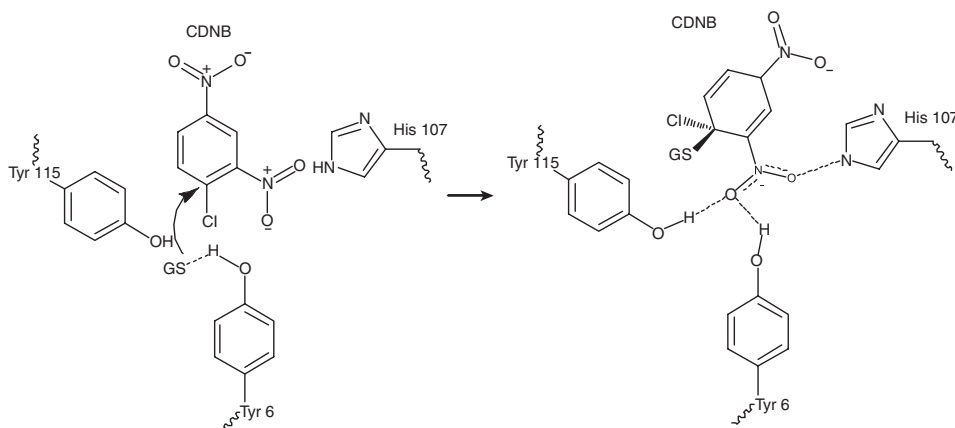


Figure 7.37 Meisenheimer complex formation with CDNB.

in pK_a of that magnitude requires ~ 3 kcal/mol of energy, which is generated through the binding of GSH at the G-site.

The H-site is composed of the C-terminal domain and displays greater primary sequence diversity providing the substrate diversity observed across the different enzyme forms. For example, α -class GSTs show substrate specificity for cumene hydroperoxide (CuOOH) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-C1), due to unique structural component proximal to the H-site, there is a short three-residue β -strand near the C-terminal segment and a longer α -7 helix (due to insertions at the N-terminus and near to the middle of this helix); domain I is formed from two separate segments of the sequence. This occurs because an extra helix (α -11) formed via folding of the C-terminal region of the polypeptide chain is also part of this domain. This helix covers the substrate bound in the H-site, which is thought to explain the preference of α -class GSTs for more hydrophobic compounds. In fact, different GSTs have unique linear free energy plots relative to hydrophobicity in the form of alkyl chain length (Fig. 7.38) [316].

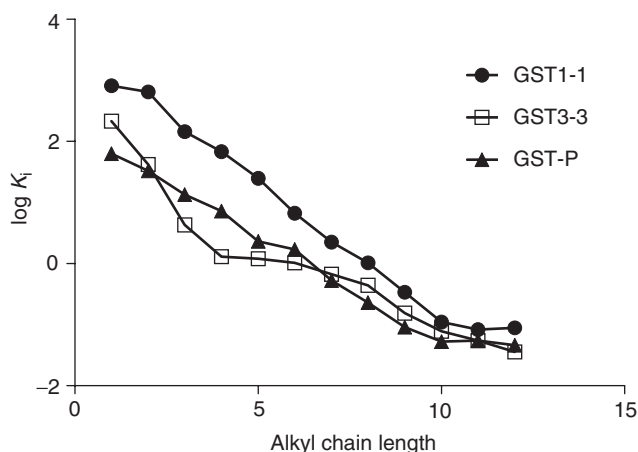


Figure 7.38 Linear free energy relationship between inhibitory potency and hydrophobicity.

Product inhibition and transition state analogs of GST metabolites provide information regarding structure function of GST enzymatic activity. Nucleophilic substitution between GSH and chlorodinitrobenzene leads to formation of Meisenheimer complex (σ -complex) (Fig. 7.37) [317]. The complex presents as a competitive inhibitor of rat isoform 3-3 with a $K_i = 20 \mu\text{M}$. The generation of anionic tripeptide analogs of GSH where the thiol group was replaced with carboxylic acid functional group yielded competitive inhibitors to GSH [313]. Most notably the substitution of the cysteine residue for 2-aminomalonyl derivative yielded a K_i of $0.74 \mu\text{M}$. Chen *et al.* [318] employed the use of GSH in comparison to three GSH analogs in concert with several 4-substituted L-halo-2-nitrobenzenes to investigate the catalytic mechanism of rat liver GSH transferase (isozyme 4-4). The observed substituent effects were consistent with a catalytic mechanism where the Meisenheimer complex formation is rate-limiting or partially rate-limiting step of conjugation (Fig. 7.37). Structural variants of GSH peptides affected the effective pK_a of the thiol and altered the orientation of the thiol toward the nucleophile [318]. The design of novel bivalent inhibitors have been used to realize the in-solution distance of the two H-sites from different dimerized forms of GST [319]. Endogenous ligands have also proven useful as tools to study structure function of GST. S-Nitroso glutathione serves as a carrier for NO and has been demonstrated to be a competitive reversible inhibitor of GSH with a K_i of $180 \mu\text{M}$ [320].

In comparing the structural differences across GST classes, the helix $\alpha 2$ region displays significant variance and not surprisingly comprises part of the H-site. The helix $\alpha 2$ region has been established as a flexible region in the protein that influences catalysis in human GSTP1-1 [321,322]. For instance, S-nitrosoglutathione (GSNO) was shown to modify Cys47 in a temperature dependant fashion. Below physiological temperature, GSNO does not readily label the buried Cys residue, but under physiological conditions, the modification leads to enzyme inactivation. Mobility of the GST has also been identified and characterized by Atkins *et al.* [64].

Single-residue substitutions have also revealed significant information regarding active-site structure activity. Ile104 in GSTP1-1 is a critical residue affecting the binding at the H-site as demonstrated by unique binding of 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol in the wild-type and mutant enzyme [323]. The significance of the histidine residue depicted in the catalytic mechanism of Fig. 7.37 was explored with hGSTM1a-1a and mutagenesis studies to demonstrate the critical role for His107 in catalysis [324]. Numerous other mutagenesis studies have highlighted important active-site residues in G- and H-sites [64,325,326].

7.3.3 Sulfotransferase

The cytosolic sulfotransferases catalyze the conjugation of a sulfate moiety to a nucleophilic acceptor molecule (Fig. 7.39). Sulfotransferases utilize 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the universal sulfuryl donor to increase the water solubility and decrease the biological activity of the acceptor substrate, though examples of bioactivation do exist [327–330]. Known acceptor moieties for sulfotransferase reactions include phenolic and aliphatic hydroxyl groups as well as N-substituted hydroxylamines [331]. Unprotonated amines can also undergo sulfation, resulting in the formation of a sulfamate product [332].

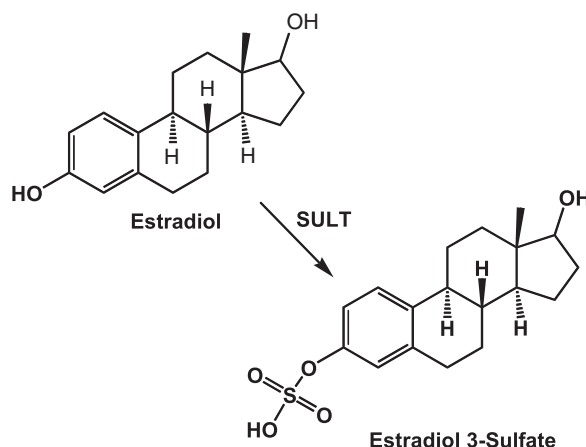


Figure 7.39 Example of sulfotransferase metabolism reaction with estradiol.

The proposed reaction mechanism for sulfotransferases involves an S_N2 nucleophilic attack on the sulfate moiety of PAPS by the acceptor substrate. As many of these substrates have pK_a values of ~ 6.4 – 9.0 and thus insufficient nucleophilicity at physiological pH, it has been suggested that a conserved histidine residue acts as a catalytic base to deprotonate the acceptor molecule [333], while active-site serine and lysine residues serve to orient PAPS in the sulfotransferase active site (Fig. 7.40) [334]. The overall basicity of the catalytic histidine residue is increased through hydrogen bonding with neighboring threonine residues, which serve to distribute the resulting charge on the histidine. Following the formation of a ternary sulfotransferase-PAPS-deprotonated acceptor molecule complex, a trigonal bi-pyramidal transition state intermediate has been proposed [335]. This transition state geometry may be stabilized by highly conserved serine and lysine residues that interact with the bridging oxygen of the 5'-phosphate group of PAP [333,334,336]. As the lysine residues may also directly catalyze the cleavage of the sulfate moiety from PAPS, an in-line displacement reaction mechanism has been proposed for sulfation. The ordering of the sulfation reaction appears to differ between species and sulfotransferase isoforms, as kinetic studies with rat aryl sulfotransferase suggest either a random bi-bi mechanism or an ordered Theorell–Chance mechanism [337], while a random bi-bi mechanism has been proposed for mouse and human SULT1E1 [338] and multiple studies with additional human sulfotransferases have suggested an ordered bi-bi mechanism in which PAPS binds before the acceptor molecule [339–341]. Interestingly, studies with bacterial sulfotransferases have implicated a random two-site ping-pong bi-bi mechanism, whereby PAPS and the sulfate acceptor molecule bind randomly and independently of each other [342].

Sulfotransferase enzymes can exhibit stereoselectivity among chiral substrates, as is observed with the increased sulfation rate of (*R*)-4'-hydroxypropranolol as compared to (*S*)-4'-hydroxypropranolol [343]. Additional examples include salbutamol, albuterol, isoprenaline, and various hydroxysteroid analogs [343–349]. While active-site characteristics may play a role in the preferred sulfation of one enantiomer over the other, steric interactions within the substrate molecule must also be considered. Studies with

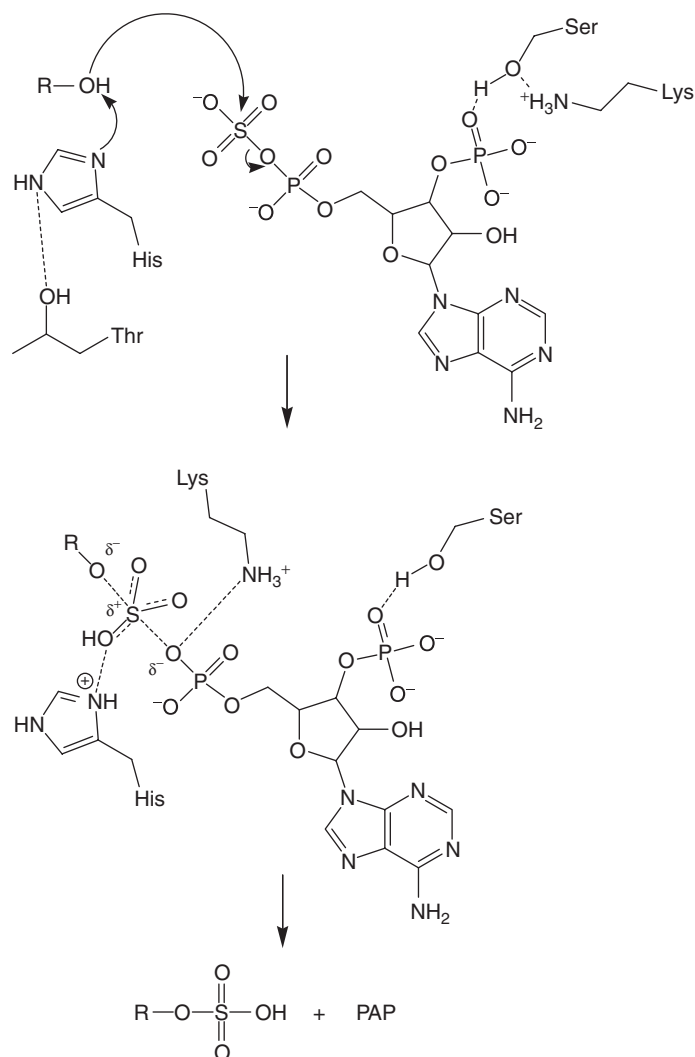


Figure 7.40 Proposed mechanism of SULT-catalyzed sulfation.

chiral aryl alcohols such as 1-(1-naphthyl)ethanol have suggested that spatial orientation may either sterically inhibit the interaction of the PAPS sulfate group with the nucleophilic site on the acceptor molecule or conversely, may orient the nucleophilic site in too distal a position to allow an S_N2 attack to occur on the sulfate group [344]. The resulting conformational rotation needed to overcome the stereoselectivity imposed by the spatial orientation of the substrate molecule would require a significant amount of energy and as such does not readily occur (Fig. 7.41).

From the standpoint of the acceptor molecule, substrate specificity appears to be linked to a number of molecular descriptors, including the size of the acceptor molecule, local structural features, lipophilicity, and electronic characteristics [350–352]. For example, both chain length as well as substituent positions (ortho-, meta-, and para-)

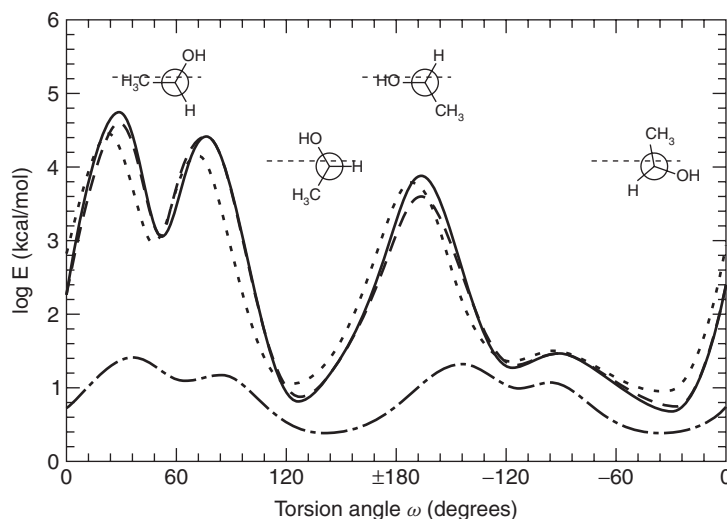


Figure 7.41 Conformational energies of chiral aryl alcohols. *Source:* Reproduced with permission from Banoglu E, Duffel MW. *Chem Res Toxicol* 1999;12(3):278–285.

have been shown to play a role in the substrate profiles of SULT1A1 and SULT1A2 [108,353]. Meanwhile, relationships have been developed between the K_m for a series of phenol sulfotransferase ligands and their respective $\log P$ values, molar refractivities, and Hammett constants [350]. Finally, the presence of a formal charge at physiological pH may also affect the substrate specificity for a given sulfotransferase, as observed for SULT1A3. Tyramine, which contains a primary amine that is positively charged at physiological pH, binds to SULT1A3 with an affinity that is 200-fold greater than that of *p*-ethylphenol, a structurally similar compound to tyramine that differs in only the lack of the primary amine [354].

7.3.4 *N*-Acetyltransferase

Mammalian *N*-acetyltransferases (NATs) are cytosolic proteins that transfer an acetyl group from acetyl coenzyme A to xenobiotics (Fig. 7.42) [355]. Two isozymes are known, NAT1 and NAT2, which differ in tissue localization and substrate selectivity. NAT1 is found in a variety of tissues including liver, colon, blood, and skeletal muscle [356]. NAT2 is present mainly in the liver and gut [357]. The biological function of NATs outside of xenobiotic metabolism is unclear; the only other known role of NATs is involvement in folate catabolism [358].

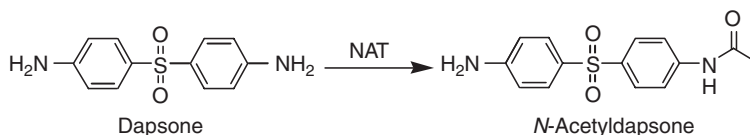


Figure 7.42 Example of NAT metabolism with dapsone.

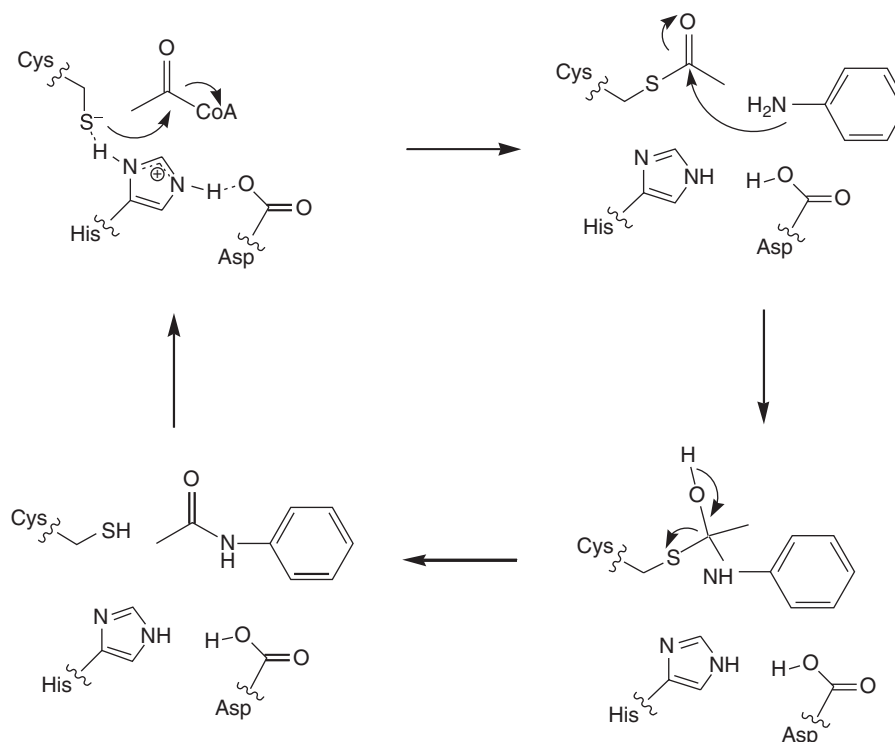


Figure 7.43 NAT catalytic cycle.

The general requirement for NAT conjugation is the presence of an aromatic amine. The catalytic cycle of NAT conjugation is complex and proceeds through a ping-pong bi-bi mechanism [355]. The reaction is initiated through acetylation of an active-site cysteine by acetyl-CoA (Fig. 7.43). Activation of the active-site cysteine is aided by adjacent histidine and acidic residues [359,360]. The positioning of the C-terminal region of human NATs may also facilitate interactions with acetyl-CoA, as a serine residue is within hydrogen bonding distance of the adenine ring (N6) of acetyl-CoA [361]. The acetyl group is then transferred from the enzyme to the substrate, forming an acetylated product and releasing the enzyme.

The hydrophobic nature of the active-site pocket may contribute to the stability of the acetylated enzyme intermediate [356]. While the lifetimes of these intermediates is NAT-dependent, the estimated half-life in hamster is 88 s [359]. Variable access of water to the active site, resulting in competing hydrolysis, may explain lifetime differences. Further evidence to support this hypothesis includes kinetic models, which are most consistent with reaction of unionized anilines with the reactive species. Ionized alkylamines, which tend to remain unionized at physiological pH, generally do not react with NATs [356].

NAT1 and NAT2 exhibit different substrate selectivity. Substrates for NAT1 include *p*-aminobenzoic acid and *p*-aminophenol [362]. Substrates for NAT2 include sulfmet-hazine, dapsone, and procainamide [363]. Substrate selectivity may be explained by differences in active-site topography. The active site of NAT1 is ~40% smaller than

NAT2 [364]. The NAT1 active site contains an arginine residue (Arg127) that is available for hydrogen bonding to acidic moieties and a phenylalanine residue (Phe125) that is closely located to orient linear, aromatic substrates toward the acetylated cysteine through π -stacking [365]. In contrast, docking studies based on the NAT2 crystal structure suggest that a phenylalanine residue (Phe93) is positioned to interact with hinged-aromatic substrates such as sulfmethazine [361]. The Phe125Ser mutation conferred NAT1 activity to NAT2 [366].

7.3.5 Acyl-CoA Synthetase

The acyl-CoA synthetases (also referred to as *acyl-CoA ligases*) catalyze the formation of thioester conjugates from carboxylic acids (Fig. 7.44). The activation of endogenous and xenobiotic acids to their thioester conjugates is often the first step in detoxification processes that end in amino acid conjugation and subsequent elimination [367]. The acyl-CoA synthetases are classified by their substrate specificity

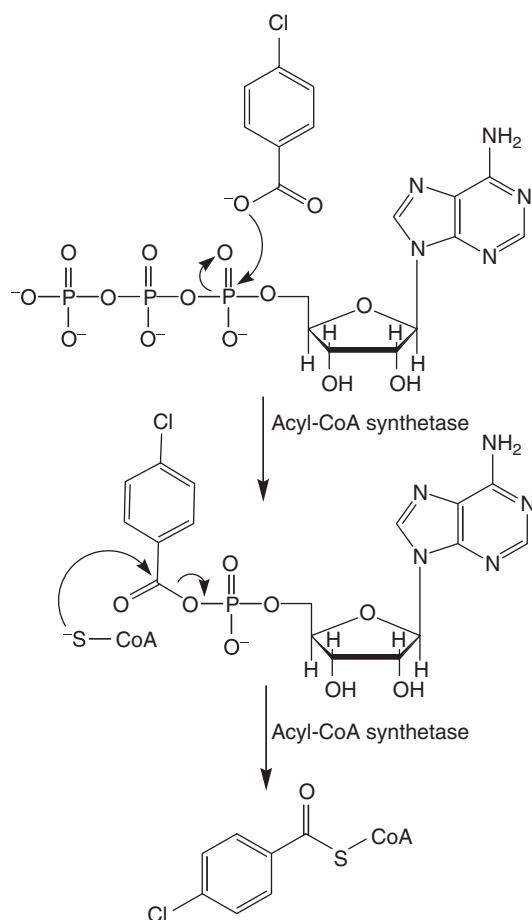


Figure 7.44 Example of acyl-CoA synthetase metabolism reaction with *p*-chlorobenzoic acid.

(short chain, medium chain, and long chain) and primary pharmacophores include aromatic carboxylic acids as well as some aliphatic and arylacetic acids [368]. Well-characterized substrates include nonsteroidal anti-inflammatory drugs such as ibuprofen and flunoxaprofen, hypolipidemic drugs such as clofibrate, and the environmental pollutant 4-chlorobenzoate [369–378].

The initial step in the acyl-CoA synthetase catalyzed reaction involves a nucleophilic attack of a carboxylate anion on the α -phosphate group of adenosine triphosphate (ATP) resulting in the formation of an acyl-adenylate intermediate, which does not dissociate from the acyl-CoA synthetase [379]. The acyl-adenylate intermediate then reacts with a deprotonated coenzyme A molecule resulting in the activated thioester conjugate and free adenosine monophosphate [380]. It has been proposed that the acetyl-CoA synthetase may adopt a different conformation for each step in the reaction [381]. The esterification of the carboxylate moiety has been proposed to proceed via a unidirectional bi-uni-uni-bi ping-pong mechanism [382]. Proper orientation of the reactants in the active site of acyl-CoA synthetase is achieved through hydrogen bonding with active-site residues as well as through coordination with a divalent magnesium ion [378,381–383].

A unique attribute of acyl-CoA synthetases is their involvement in the chiral inversion of nonsteroidal anti-inflammatory drugs such as ibuprofen, flunoxaprofen, and 2-phenylpropionic acid [373,375,377,384], resulting in a unidirectional inversion of the (*R*)-enantiomer to the (*S*)-enantiomer. In each case, the acyl-CoA enzyme displays a stereoselective preference for the formation of a thioester conjugate of the (*R*)-enantiomer, with a minimal amount of acyl-CoA transfer to the (*S*)-enantiomer. Using ibuprofen as an example, the first step in the chiral inversion process is the formation of an (*R*)-ibuprofen-CoA thioester intermediate (Fig. 7.45), as demonstrated through studies utilizing deuterium-labeled ibuprofen [385]. The next step involves the abstraction of the C2 proton of the ibuprofen-CoA conjugate by epimerase/carnitine dehydratase. Though the inherent acidity of this proton is increased by the presence of the thioester conjugate, the deprotonation step is not spontaneous at physiological pH and requires the presence of a catalytic base provided by epimerase [372,375,386]. The resulting carbanion tautomerizes to the planar and more stable enolate, allowing for a proton attack from either side of the double bond to form both enantiomers of the ibuprofen thioester conjugate. Finally, hydrolysis of the thioester bond by acyl-CoA hydrolase results in the formation of unconjugated (*S*)-ibuprofen [375,377,387].

While substrate specificities may vary among the different acyl-CoA synthetase homologs, the enzymes generally catalyze the thioester conjugation of aliphatic and aromatic carboxylic acids. For the aliphatic acids, the acyl chain length is often the determining factor in the specificity of individual acyl-CoA isoforms. In addition, both the pK_a of the carboxylic acid as well as the $\log P$ of the compound have been shown to be important determinants in the formation of thioester conjugates [368,388,389], though electronic and steric factors may also play a role. For example, examination of the rates of thioester formation for a series of cinnamic acid analogs with various electron-donating or electron-withdrawing groups in the para-position resulted in a biphasic Hammett plot (Fig. 7.46), implicating the importance of a negative charge in formation of the conjugate as well as a potential change in the rate-determining step in the reaction for electron-donating versus withdrawing groups [390]. Sterically, compounds with a flat hydrophobic region that is coplanar to the carboxylic acid moiety appear to be much better substrates for acyl-CoA synthetases [391]. It has been

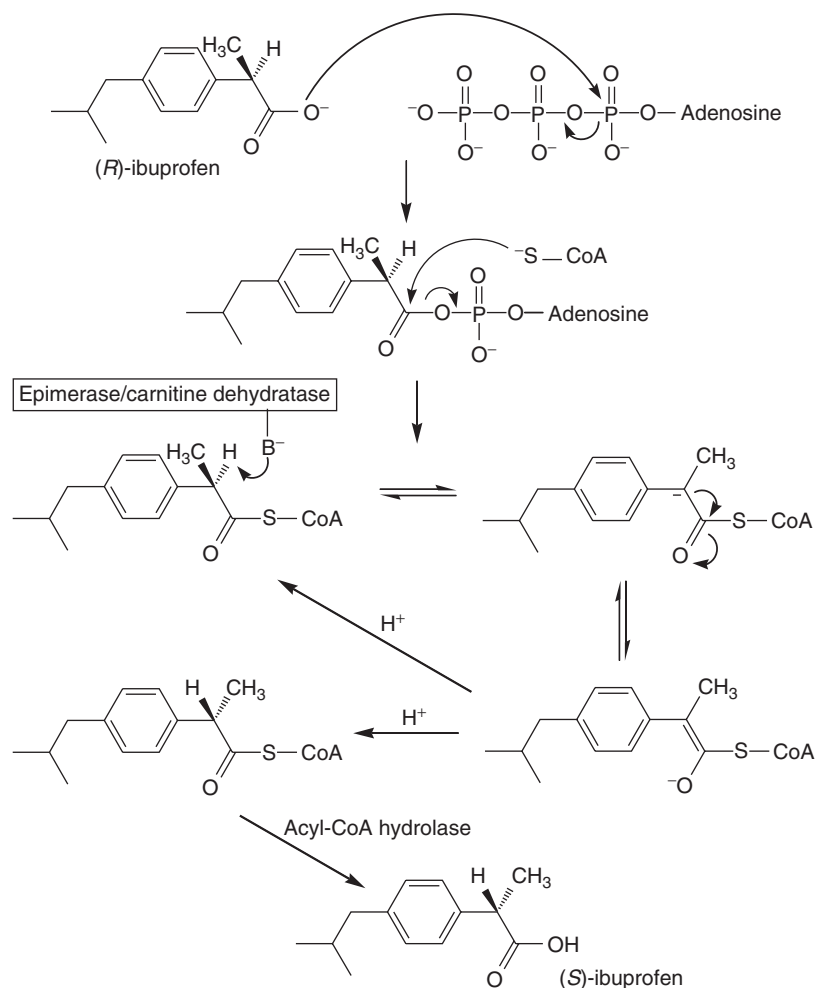


Figure 7.45 Proposed acyl-CoA synthetase reaction mechanism resulting in stereoinversion of (*R*)-ibuprofen to (*S*)-ibuprofen.

suggested that compounds that do not meet this coplanar criteria may have difficulty accessing the active site of the enzyme.

7.3.6 Methyltransferase

The methyltransferase family of enzymes (including thiopurine-*S*-methyl transferase, thiol methyltransferase, catechol-*O*-methyltransferase, phenethanolamine-*N*-methyltransferase, and multiple other forms involved in biological homeostasis) catalyze the transfer of a methyl group from *S*-adenosyl methionine to nucleophilic substrate [392–394]. Drugs that are metabolized by methyltransferases include 6-mercaptopurine, 6-thioguanine, and azathioprine (thiopurine-*S*-methyl

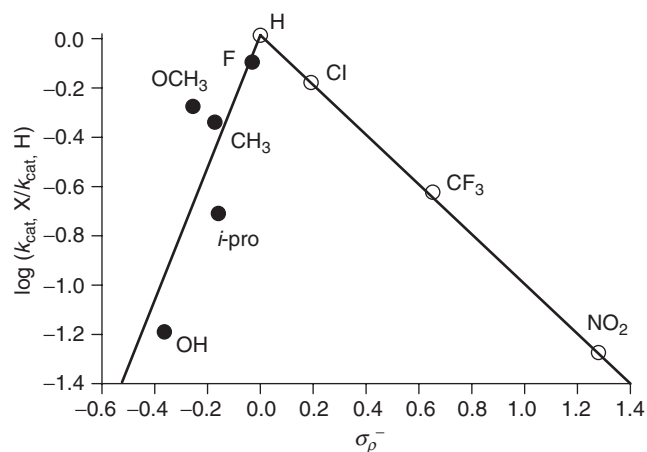


Figure 7.46 A Hammett plot for acyl-CoA synthetase reactions with para-substituted cinnamic acids. *Source:* Reproduced with permission from Koetsier MJ, *et al. Biochem J* 2009;417:467–476. © The Biochemical Society, <http://www.biochemj.org>.

transferase), dopamine, L-3,4-dihydroxyphenylalanine and isoprenaline (catechol-*O*-methyltransferase), captopril and D-penicillamine (thiol methyltransferase), and norepinephrine (phenethanolamine-*N*-methyltransferase) [395–404].

The mechanism by which methyltransferases catalyze the addition of a methyl group to a substrate proceeds via an S_N2 nucleophilic attack on the methyl carbon of *S*-adenosyl methionine by the substrate (Fig. 7.47). The reaction may require the presence of Mg^{2+} (catechol-*O*-methyltransferase) and has strict geometrical requirements for the orientation of substrate and cofactor [396,405,406]. An ordered mechanism has been suggested for the transfer of the methyl group, with the order of binding

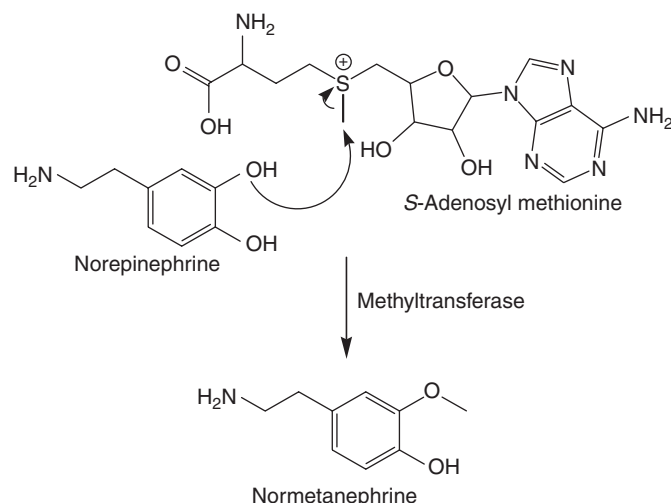


Figure 7.47 Example of methyltransferase metabolism reaction with norepinephrine.

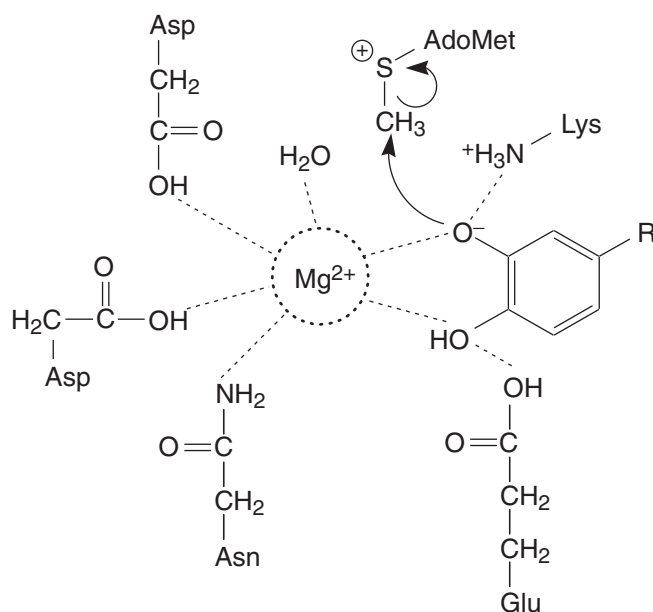


Figure 7.48 Proposed methyltransferase reaction mechanism.

being (i) *S*-adenosyl methionine; (ii) Mg^{2+} ; and (iii) substrate [407–409]. Following transfer of the methyl group, *S*-adenosyl homocysteine (the demethylated byproduct of *S*-adenosyl methionine) is the last ligand to dissociate from the binding pocket of the methyltransferase [410,411]. Kinetic isotope effect studies and hybrid density functional theory models have been used to identify the transfer of the methyl group as the rate-limiting step in the reaction [393,412,413]. In a similar manner to other enzyme-catalyzed S_N2 reactions (i.e., glucuronidation and sulfation), a conserved active-site lysine residue (catechol-*O*-methyltransferase) or arginine residue (thiopurine-*S*-methyltransferase) acts as a catalytic base to deprotonate the nucleophile before the S_N2 attack (Fig. 7.48) [398,414].

Substrate selectivity varies widely across the range of methyltransferases. For catechol-*O*-methyltransferases, which catalyze the transfer of a methyl group to catechols and catecholestrogens, it appears that the presence of vicinal aromatic hydroxyl groups is a requirement for catalysis, as the enzyme does not bind the nonaromatic dihydroxyl cyclohexane analogs [415,416]. In addition, the presence of a strong electron-withdrawing group on the catechol has been shown to greatly decrease the rate of *O*-methylation, though the compounds can still bind and inhibit the enzyme [417–419]. Orientation of the substrate within the active site is controlled by the bound magnesium ion, which is coordinated to active-site aspartate and asparagine residues, a bound water molecule, and both hydroxyl groups of the catechol, while overall substrate selectivity may be conferred by hydrophobic “gatekeeper” residues (Fig. 7.48). For thiol methyltransferases, the preferred substrates are aliphatic sulfhydryl-containing compounds, such as 2-mercaptoethanol [420]. Potent inhibitors of thiol methyltransferase include the calcium channel blocker SKF 525A [421]. Lastly, while thiopurine methyltransferases catalyze the methylation of

thiopurines, thiopyrimidines and aromatic sulfhydryl compounds, a natural substrate has not yet been identified [402,414,422]. Though compounds with a purine moiety are understandably the primary substrates for thiopurine methyltransferase, the presence of a heterocyclic aromatic functional group is not a requirement for substrate recognition, as demonstrated by studies with thiophenol-containing compounds [402,423]. Substrate recognition by thiopurine methyltransferases also appears to be dependent on the ability of the acceptor molecule to achieve proper orientation within the substrate-binding site of thiopurine methyltransferase through hydrophobic and van der Waals interactions [414].

Methyltransferases are also known to display both stereoselectivity and regioselectivity in the transfer of a methyl group to an acceptor molecule. Regioselectivity by methyltransferases has been observed for the methylation of catechols by catechol-O-methyltransferase, with 3-O-methylation (meta) generally preferred over 4-O-methylation (para), especially *in vivo* [398,407]. Furthermore, dimethylation is not readily observed. This observed phenomenon may be due in part to the unfavorable interactions required between the substrate and hydrophobic active-site residues to allow the 4-hydroxy position to gain proper alignment for an S_N2 attack on *S*-adenosyl methionine. The catechol-O-methyltransferases also exhibit stereoselectivity in the methylation reaction, as noted with a series of β -adrenoceptor agents, where the enzyme preferentially catalyzed the methylation of (–)-isoprenaline and (–)-noradrenaline over their respective (+) counterparts [424].

7.4 CONCLUSION

Understanding the biochemistry of drug metabolism is one of the most critical aspects of new drug development to assure safe and efficacious medicines of the future. As described in this chapter, drug metabolism is heavily dependent on the molecular properties of the substrate and the topography of the enzyme's active site. Considerable advances have been made in recent years, such that basic rules can be applied to determine the fate of a compound in man based on physicochemistry character and structure of a drug metabolite. In this light, the impact of the identification of drug metabolites allows the drug metabolism scientist to predict drug clearance pathways and factors affecting drug/metabolite exposures early in the drug development process. Moreover, it is anticipated that a strong understanding of the chemical basis for metabolite formation will greatly enhance our ability to elucidate the mechanisms involved in idiosyncratic drug reactions and off-target toxicity.

In addition to gaining greater insights into the biochemical factors that influence the chemistry of drug metabolism, other technological and analytical advances will continue to be made and their application will continue to grow, which should enable scientists to have even greater impact in the area of drug metabolism. Of course, in order to have the greatest benefits from these advances, drug metabolism scientists need to embrace and incorporate emerging trends in science and technology, select the appropriate tools, and accurately interpret data generated from drug metabolism studies with a link toward the fundamental chemistry associated with the observed biotransformation. We hope the present work provides a reasonable chemical foundation for the biochemistry of drug metabolism on which both experienced and newly initiated drug metabolism scientists may rely on.

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8 Extrahepatic Drug-Metabolizing Enzymes and Their Significance

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8.1	Summary	1
8.2	Introduction	2
8.3	Distribution and expression of DMEs in extrahepatic tissues	2
8.4	Factors influencing expression of extrahepatic DMEs	30
8.5	Role of extrahepatic DMEs in bioactivation and deactivation of xenobiotics	40
8.6	Significance of extrahepatic metabolism	42
8.7	Conclusions	63
	References	67

8.1 SUMMARY

Drug-metabolizing enzymes (DMEs) are primarily expressed in the liver but their role in the extrahepatic tissues such as gastrointestinal tract (GIT), pulmonary, excretory, nervous, cardiovascular system, and skin cannot be neglected. Generally, the expression of DMEs in extrahepatic tissues is quantitatively lower than that in the liver, but there are a few enzymes such as CYP1A1, CYP1B1, CYP2F1, and CYP2U1 that are more abundant in extrahepatic organs. As many extrahepatic organs are portals for administered drugs, DMEs expressed in these organs can be responsible for significant metabolism, leading to first-pass effects and lower bioavailability. Extrahepatic DMEs are also involved in bioactivation of prodrugs and formation of reactive metabolites that may interact with cellular components, resulting in organ-specific toxicity. Activity and expression of extrahepatic DMEs is often altered by coadministered drugs, leading to drug–drug interactions. Expression of DMEs in living beings affected by a host of

environmental and genetic factors such as genetic polymorphism, age, gender, pathophysiological conditions, inborn errors in metabolism, food habits, and environmental pollutants, contributing to varied drug effects and idiosyncratic toxicities.

8.2 INTRODUCTION

There was a stage when it was believed that DMEs in the liver were mainly responsible for the protection of the body against exogenous challenges (environment, diet, drugs, chemicals, etc.), while adrenal glands were involved in the metabolism of endogenous steroids. With time, DMEs have been found in extrahepatic organs and tissues including GIT, pulmonary, urinary, central nervous system (CNS), and cardiovascular system (CVS), as well as in skin, placenta, adrenal glands, reproductive organs, plasma, eyes, pancreas, and mammary glands. However, overall quantitative expression of extrahepatic DMEs represents just 10–20% of total hepatic metabolism. Nevertheless, some DMEs are found expressed solely in extrahepatic organs and tissues. Extensive studies have shown that extrahepatic enzymes play a significant role in drug metabolism and detoxification of environmental/food contaminants and provide protection to specific organs and the body as a whole.

A number of factors such as genetic makeup, age, gender, pathophysiological conditions, and environmental factors contribute to differential expression of DMEs in extrahepatic organs. The expression of DMEs, especially of cytochrome P450s (CYPs), is greatly influenced by both age and gender, and varies from fetal to geriatric age because of environmental factors as well as changes in levels of sex hormones. These DMEs contribute to bioactivation and deactivation of xenobiotics and to the development of diseases, especially cancer. The Human Genome Project has identified a correlation between extrahepatic CYPs and development of both solid and hematological malignancies.

DMEs in the GIT influence bioavailability of drugs significantly and drug clearance is dependent upon the rates of metabolism from hepatic and extrahepatic organs, in addition to the rate of excretion of the unchanged drug. These changes in turn affect the efficacy and safety of drugs by influencing their concentration and that of their metabolite(s) at the site(s) of action, which can result in severe drug–drug interactions in case of polypharmacy.

This chapter provides a comprehensive review of the type and nature of DMEs expressed in organs and tissues of the body, excluding liver. It also encompasses factors affecting the expression of extrahepatic DMEs, such as genetic polymorphism, age, gender, pathophysiological conditions, and food habits. Additionally discussed are observations on bioactivation and deactivation of xenobiotics, bioavailability and toxicity of drugs, and drug–drug interactions.

8.3 DISTRIBUTION AND EXPRESSION OF DMEs IN EXTRAHEPATIC TISSUES

Liver is quantitatively and qualitatively the most important site for metabolism of xenobiotics, including therapeutic drugs that enter the body after absorption from the GIT. A majority of the DMEs are expressed in the liver, although not evenly distributed

in the organ. DMEs such as CYPs are highly concentrated in the periportal region of the liver, while other enzymes such as glutathione *S*-transferases (GST) are expressed in the centrilobular of liver. This uneven distribution provides significant protection to this vital organ, for example, if toxic electrophilic intermediates are generated then only pericentral necrosis will occur, rather than massive necrosis [1].

DMEs similar to those expressed in the liver are also expressed in the extrahepatic organs. The distribution of DMEs in extrahepatic organs and tissues is depicted in Fig. 8.1 and detailed information, based on current literature, is described in Table 8.1. The expression of extrahepatic DMEs varies from organ to organ, depending on exposure to endogenous substances, routinely used drugs and the challenges faced because of food habits and the consequence of exposure to environmental contaminants [2–5]. There are some instances where DMEs show higher expression in extrahepatic tissues relative to the liver because of exposure to certain environmental components or because of life style choices (e.g., smoking or type of food intake).

8.3.1 CYPs and Subtypes

CYPs are monooxygenases and consist of an apoprotein and a heme moiety that is common to all isoforms. CYPs are involved in reactions that include hydroxylation, sulfoxidation, epoxidation, deamination, desulfuration, dehalogenation, peroxidation, *N*-oxide reduction, *N*-dealkylation, *O*-dealkylation, and *S*-dealkylation. CYPs constitute the largest family of DMEs localized in every organ of the body and are categorized based on amino acid sequence similarities. Among CYPs, CYP1, CYP2, and CYP3 are the major types responsible for the metabolism of xenobiotics [125]. The distribution of CYPs varies across the body. Pie charts depicting relative distribution of CYPs in different human organs are shown in Fig. 8.2 [126]. The total specific contents of CYPs in small intestine, lungs, kidneys, and brain have been observed to be 0.02–0.21 [127], ~0.01 [128], 0.1–0.2 [129], and ~0.1 nmol/mg [15], respectively.

8.3.1.1 Gastrointestinal Tract. Orally administered drugs first come in contact with CYPs located in epithelial cells of GIT. Major activity of CYPs at the cellular

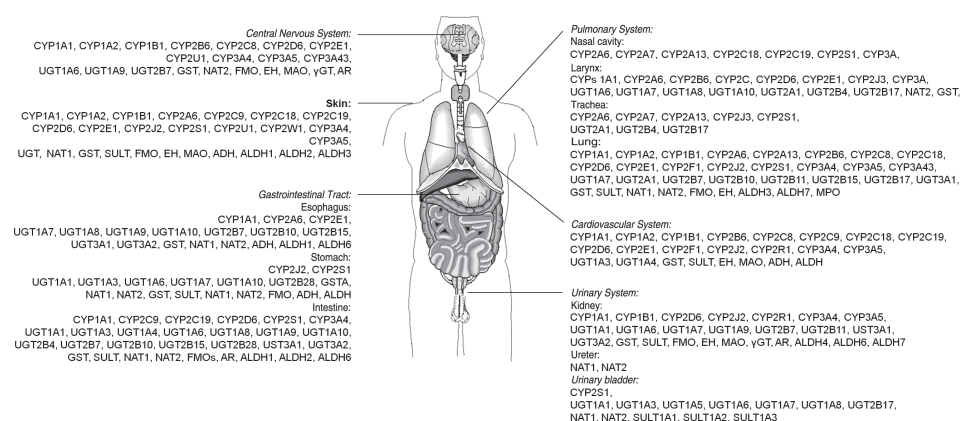


Figure 8.1 Distribution of drug-metabolizing enzymes in extrahepatic organs and tissues in humans.

TABLE 8.1 Location of Drug-Metabolizing Enzymes in Extrahepatic Organs and Tissues

Enzyme	Gastrointestinal Tract	Pulmonary System	Urinary System	Central Nervous System	CVS	Skin
CYP1A1	Enterocytes, esophagus 6	Bronchial epithelial cells, capillary endothelium, alveolar epithelium, alveolar type I and II cells, ciliated columnar epithelial cell lining, bronchoalveolar airways, alveolar macrophages 7	PCT 8	Cerebellum, cortex, basal ganglion, hippocampus, substantia nigra, pons, medulla oblongata 9,10	Left and right ventricles 11,12, coronary smooth muscle cells 13	Epidermis 14
CYP1A2	ND	Lung peripheral tissue 7	ND	Cerebellum, cortex, basal ganglion, hippocampus, substantia nigra, pons, medulla oblongata 15	Endothelium of endocardium, coronary vessels 16	Epidermis 17
CYP1B1	ND	Alveolar macrophage, bronchial epithelium 7	PCT 8	Putamen, spinal cord, medulla oblongata, frontal, temporal cortex 15	Heart 18, coronary smooth muscle cells 13	Epidermis 14
CYP2A6	Esophageal mucosa 19	Nasal mucosa, trachea, alveolar epithelium 7,20	NS	Striatum, thalamus, pons, medulla oblongata 21	Left ventricle 12	NS
CYP2A13	ND	Nasal mucosa, bronchial mucosa, alveolar epithelium 7,20	ND	Brain 20	—	NS

CYP2B6	ND	Clara cells, bronchial and peripheral tissue, epithelial cells, larynx, bronchial mucosa 7, bronchial epithelial cells 22	NS	NS	Endothelial cells 18	Foreskin epidermis, sebaceous glands, hair follicles 23
CYP2C8	Enterocytes 24	Serous cells of bronchial glands, bronchial tissue, peripheral tissue 7	NS	Cerebellum, cortex, basal ganglion 15	Right ventricle, left ventricle 11,12	NS
CYP2C9	Enterocytes 6	ND	NS	ND	Right ventricle, left ventricle 11,12	Epidermal keratinocytes 25
CYP2C18	Enterocytes 26	Trachea, serous cells of bronchial glands, bronchial tissue, peripheral tissue 7,27	NS	ND	Right ventricle 11	NS
CYP2C19	Enterocytes 6	Trachea 27	NS	ND	Right ventricle 11	Epidermal keratinocytes 25
CYP2D6	Detected at mRNA level but no activity reported 6,24	Bronchial mucosa 7	PCT 8	Putamen, globus pallidus substantia nigra 15	Right ventricle 11	Dermal fibroblasts 17
CYP2E1	Esophageal mucosa 19, colon mucosa 28	Nasopharynx, bronchial, bronchiolar epithelium, alveolar epithelium, endothelial cells 7	NS	Cerebellum, cortex, basal ganglion, hippocampus, substantia nigra, pons, medulla oblongata 15,29	Right ventricle 11, endothelium of endocardium, coronary vessels 16	Suprabasal cell layers of foreskin epidermis, keratinocytes 17

(continued overleaf)

TABLE 8.1 *(continued)*

Enzyme	Gastrointestinal Tract	Pulmonary System	Urinary System	Central Nervous System	CVS	Skin
CYP2F1	ND	Alveolar macrophages, epithelial cells, endothelial cells 7	ND	ND	Endocardium, coronary vessels 16, left ventricle 12, vascular endothelium 16	NS
CYP2J2	Epithelium lining of gastric pits, parietal cells of the gastric glands, intestinal smooth muscle cells 30	Bronchiolial and vascular smooth muscle cells, vascular endothelium, alveolar macrophages 7	PCT, CD, DCT 31	Autonomic ganglia cells 30	Heart 32	Keratinocytes 17
CYP2R1	NS	ND	Kidney 33	ND	Left ventricle 12,18	Keratinocytes 17
CYP2S1	Stomach, small intestine 33	Epithelial cells 7	Urinary bladder 34	White matter 34	ND	Cutaneous 35
CYP2U1	ND	ND	Kidney 36	Cerebellum 15,33	NS	NS
CYP3A4	Enterocytes 6, gastric parietal cells 37	Bronchial, bronchiolial and alveolar epithelium, alveolar endothelium, alveolar macrophages 7,38, nasal cavity, pharynx, larynx, trachea 39	CD 40	Cortex, basal ganglion 15	Endothelium of endocardium, coronary vessels 16	Keratinocytes 41

CYP3A5	ND	Bronchial, bronchiolial and alveolar epithelium, alveolar endothelium, alveolar macrophages 7,38, nasal cavity, pharynx, larynx, trachea 39	PCT 40	Midbrain, basal ganglion, frontal cortex 42	Endothelium, endocardium, coronary vessels, vascular endothelium 16	Keratinocytes 41
UGT	Complete GIT 43,44	Lung 43	Complete kidney 45,46	Brain 47	Heart 12	Stratum corneum 48
UGT1A1	Stomach, colon 43	NS	PCT, DCT 49, urinary bladder 50	NS	NS	NS
UGT1A3	Stomach, colon 43	NS	Urinary bladder 50	NS	Left ventricle 12	NS
UGT1A4	Colon 43	NS	NS	NS	Right ventricle 11	NS
UGT1A5	Complete GIT 50	NS	Kidney, urinary bladder 50	NS	NS	NS
UGT1A6	Stomach, colon 43	NS	PCT, DCT 49, urinary bladder 50	Brain 43	NS	NS
UGT1A7	Esophagus, stomach 43, small intestine, colon 50	Lung 51	PCT, DCT 49, urinary bladder 50	NS	NS	NS
UGT1A8	Esophagus, jejunum, ileum, colon 43	NS	Urinary bladder 50	NS	NS	NS

(continued overleaf)

TABLE 8.1 *(continued)*

Enzyme	Gastrointestinal Tract	Pulmonary System	Urinary System	Central Nervous System	CVS	Skin
UGT1A9	Colon 43	NS	PCT, DCT 43,49	Brain 43	NS	NS
UGT1A10	Esophagus, stomach, intestine, colon 43	NS	NS	NS	NS	NS
UGT2A1	NS	Lung 7	NS	Brain 52	NS	NS
UGT2B7	Esophagus, intestine, colon 43	Lung 7	PCT, DCT 43,49	Brain 43	NS	NS
UGT2B10	Esophagus 43	Lung 7	NS	NS	NS	NS
UGT2B11	NS	Lung 43	PCT, DCT 43,49	NS	NS	NS
UGT2B15	Esophagus 43	Lung 7	NS	NS	NS	NS
UGT2B17	NS	Lung 7	Urinary bladder 50	NS	NS	NS
UGT3A1	Complete GIT 53	Lung 53	Kidney 53	ND	NS	NS
UGT3A2	Complete GIT 54	NS	Kidney 54	NS	NS	NS
NAT1	Esophagus, stomach, small intestine, colon 55	Bronchial epithelial cells, alveolar lining cells 7	Ureter, urinary bladder 55	NS	NS	Epidermal keratinocytes 56

NAT2	Esophagus, stomach, small intestine, colon 55	Bronchial epithelial cells, alveolar lining cells, laryngeal mucosa 7,55	Ureter, urinary bladder 55	Neural tube, hind brain 57	NS	ND
GST	Complete GIT 58	Lung 59	Kidney 60	Brain 61	Heart 62,63	Skin cytosol 64,65
GSTA	Duodenum, stomach, colon 58	Bronchial epithelium, bronchiolar epithelium 7	PCT, loop of henle, CD 66	NS	NS	Skin cytosol 64,65
GSTP	Duodenum, stomach, colon 58	Bronchial epithelium, bronchiolar epithelium 7	DCT, Bowmen's capsules, CD 31,67	Choroid plexus, vascular endothelium, ventricular lining cells, pia-arachnoid, astrocytes 61	NS	Skin cytosol 64,65
GSTM	NS	Lung 7	DCT, Bowmen's capsules, CD 31,67	Brain 61	NS	Skin cytosol 64,65
SULT	Duodenum, jejunum, ileum, stomach 68	Bronchial epithelial cells 7	CD medulla 31	Brain 69–72	NS	Skin 73,74
COMT	NS	NS	NS	Right inferior frontal gyrus, intraparietal sulcus 75	NS	NS

(continued overleaf)

TABLE 8.1 (continued)

Enzyme	Gastrointestinal Tract	Pulmonary System	Urinary System	Central Nervous System	CVS	Skin
γ GT	NS	NS	Cortex 76	Brain 77	NS	NS
EH	NS	Bronchial epithelial cells 7	Cortex, medulla 78	Cerebral blood vessels, brain parenchyma 79	Heart 80	Epidermis, dermis, epithelial cells, fibroblasts 81–84
FMO1	Small intestine 85	Lung 85,86	Kidney 87	Magnocellular reticular nuclei, colliculi, substantia nigra 88	NS	Epidermis, dermis 89,90
FMO2	Small intestine 85	Lung 85,86,91	NS	Magnocellular reticular nuclei, colliculi, substantia nigra 88	Heart muscle 92	NS
FMO3	ND	Lung 85,86	Kidney 93	Magnocellular reticular nuclei, colliculi, substantia nigra 88	NS	Dermis 89
FMO4	Small intestine 85	Lung 85,86	Kidney 87	Magnocellular reticular nuclei, colliculi, substantia nigra 88	NS	Dermis, epidermis 94,95
FMO5	Small intestine 85	Lung 85,86	Kidney 96	Magnocellular reticular nuclei, colliculi, substantia nigra 88	Heart muscle 92	Dermis, epidermis 94,95
MAO	Small intestine 97	Lung 97	Kidney 98	Brain 99,100	Heart 97	Hair follicles, cutaneous nerve fibers beneath epidermis 101, fibroblasts 102–104

MAO-A	Small intestine 97	Lung 97	Cortex, medulla 98	Catecholaminergic neurons (cells of the substantia nigra, locus coeruleus, periventricular regions of hypothalamus) 99,100	Heart 97	NS
MAO-B	Small intestine 97	Lung 97	Cortex, medulla 98	Serotonergic neurons (dorsal raphe nucleus), astrocytes, posterior hypothalamic regions 99,100	NS	NS
β -Gluc	Brush border epithelium, microflora 105	Alveolar macrophages 106	NS	NS	NS	ND
ADH	Intestine 107	Lung 107	Kidney 107	Brain 108	Heart 109	Epidermis, sebaceous glands and hair follicles 110
ADH Class I	NS	NS	NS	Neurons of cerebral cortex, hypothalamus, infundibular stalk of the pituitary, purkinje cells of the brain cerebellum 108,111,112	NS	NS

(continued overleaf)

TABLE 8.1 *(continued)*

Enzyme	Gastrointestinal Tract	Pulmonary System	Urinary System	Central Nervous System	CVS	Skin
ADH Class II	Esophagus, stomach 112	NS	NS	NS	NS	NS
ADH Class III	Whole body 112	Whole body 112	Kidney 112	Whole brain 112,113	NS	NS
ADH Class IV	Stomach 112	NS	NS	NS	NS	NS
ADH Class V	Stomach 112	NS	Fetal kidney 112	NS	NS	NS
ALDH	Esophagus, stomach, intestine 114	Lung 114	Kidney 115	Brain 116	Heart 109	Skin 117
ALDH1	Esophagus, stomach 118	Lung 119	ND	NS	NS	Epidermis, dermal appendages 117

ALDH2	Esophagus, stomach 118	ND	ND	NS	NS	Epidermis, hair follicles 120
ALDH3	ND	Lung 115	ND	NS	NS	Epidermis, dermal appendages 117
ALDH4	ND	ND	Kidney 115	NS	NS	NS
ALDH5	ND	ND	ND	NS	NS	NS
ALDH6	Esophagus, stomach 118	ND	Kidney 115	NS	NS	NS
ALDH7	ND	Lung 115	Kidney 115	NS	NS	NS
AR	Intestine 121	NS	Papilla of epithelial cells, endothelial cells, interstitial cells, CD 122	Brain 123	NS	NS
MPO	NS	Lung 124	NS	NS	NS	NS

Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AR, aldose reductase; β -Gluc, β -glucuronidase; CD, collecting duct; COMT, catechol-*O*-methyltransferase; CT, carboxyltransferase; DCT, distal convoluted tubule; EH, epoxide hydrolase; FMO, flavin-containing monooxygenase; GST, glutathione *S*-transferase; γ -GT, γ -glutamyltransferase; MAO, monoamine oxidase; MPO, myeloperoxidase; NAT, *N*-acetyltransferase; ND, not detected; NS, not specified; PCT, proximal convoluted tubule; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; TPMT, thiopurine *S*-methyltransferase.

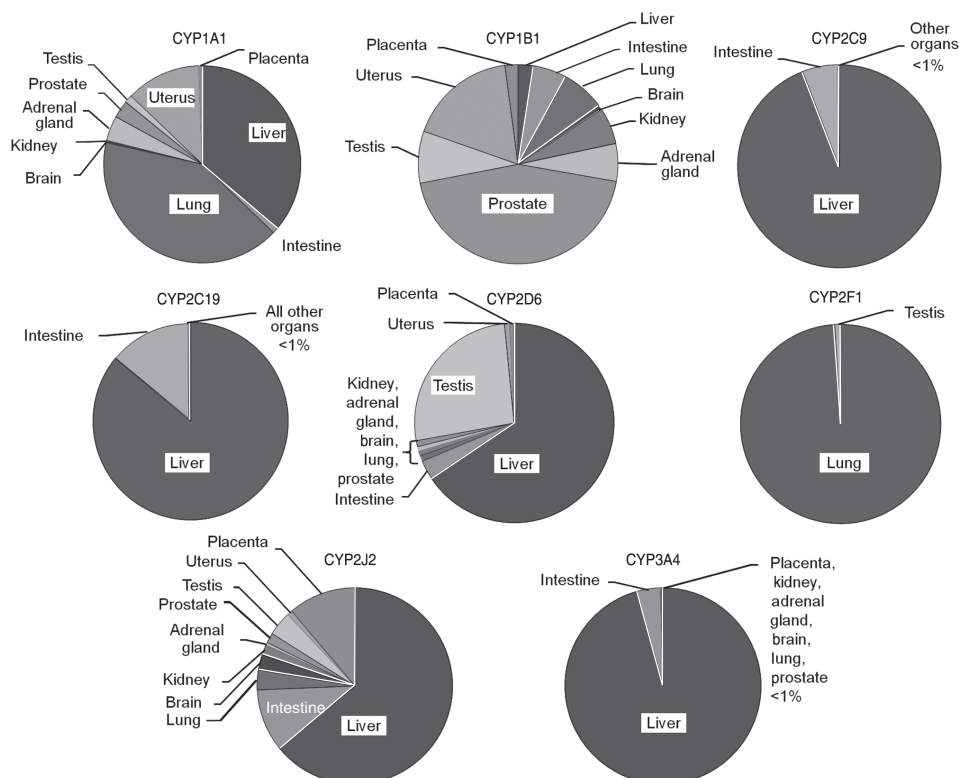


Figure 8.2 Percentage of expression of CYP mRNA in different organs. *Source:* The figure is based on data from Nishimura *et al.* [126].

level is in the villous tips and decreases in the direction of crypts. At the tissue level, CYP activity gradually decreases from the duodenum to the colon [127]. CYP1A1, CYP2C9, CYP2C19, CYP2D6, CYP2J2, CYP3A4, and CYP3A5 are the primary enzymes expressed in the GIT. In addition, CYP1A2, CYP2A6, CYP2A13, CYP2E1, CYP2F1, CYP2B6, and CYP2S1 are expressed at lower levels. Recently, mRNAs for CYP2S1, CYP2R1, CYP2U1, and CYP2W1 have been reported in the GIT but their contribution to the drug metabolism is not yet known [33,127].

The specific content of CYP1A1 in the small intestine varies from 3.6 to 7.7 pmol/mg of protein [127]. CYP1A1 expression is regulated by aryl hydrocarbon receptor (AhR) [130]. The ligands for the receptor are aromatic hydrocarbons, which are toxic to the body [131]. Zhang *et al.* correlated the expression of AhR with CYP1A1 along the length of the intestine from duodenum to ileum and found expression diminished markedly along the oral direction of intestine [130,132]. The extent of induction of CYP1A1 expression and activity could be related to the challenges faced, for example, exposure to polyaromatic hydrocarbons (PAHs) [24]. This enzyme can be induced in GIT by smoking, omeprazole, β -naphthoflavone (BNF), 3-methylcholanthrene, isosafrole, cyclohexanol, albendazole, phenobarbital, imazalil (food contaminant), benzo(α) pyrene, green tea extract and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [133–139]. Although like CYP1A1, CYP1B1 is also regulated by AhR, no expression of this enzyme has been detected in the intestine [24].

Expression of CYP2B1/2 is reported to be 137 pmol/mg microsomal protein in the upper villi and it is not detected in the crypt cells of the human intestine except in the induced state, for example, by phenobarbital and imazalil [135,140]. CYP2B6 mRNA has also been reported in the human intestine, although its activity is not established [141].

CYP2C9 constitutes 15% of the total CYP content, with an average specific content of ~ 8 pmol/mg, as compared to 89 pmol/mg microsomal protein in the human liver [142]. The presence of this enzyme in the intestine is responsible for poor bioavailability of fluvastatin [127]. CYP2C19 constitutes 2% of the total CYP content in the human intestine, with a specific content of 2 pmol/mg microsomal protein [142]. mRNA expression of CYP2C isoforms in the human intestine is in the order of CYP2C9 = CYP2C18 > CYP2C19 > CYP2C8, while protein expression is in the order of CYP2C9 > CYP2C19 > CYP2C8 (below limit of quantification) > CYP2C18 (not detected) [143]. In human jejunum, both CYP2C9 and CYP2C19 exhibit polymorphisms [26] with a 19-fold interindividual variation in CYP2C19 activity [143].

The expression of CYP2D6 in the human intestine is reported to be 0.9 pmol/mg microsomal protein, which is ~ 14 times lower than the liver. Its concentration decreases in the order of jejunum > duodenum > ileum [144]. Although CYP2D6 shows 100-fold interindividual content variation in the intestine, while clinically, its contribution to first-pass metabolism is not significant [144]. CYP2E1 mRNA is detected in the intestine, but its activity has not been established [127]. CYP2J2 (arachidonic acid epoxygenase) constitutes about 1.4% of the total human intestinal CYPs [142], with a specific content of 2.1 ± 0.6 pmol/mg protein [145]. Its protein expression is relatively constant across the GIT [30]. CYP2J2 contributes to the first-pass metabolism of nonsedative antihistamines, astemizole, and ebastine [145]. CYP2S1 is highly expressed in epithelial cells of the GIT, with its expression being higher in the basal cell layer of the stratified squamous epithelium of esophagus. In stomach, CYP2S1 is located in the mucus secreting epithelial cells of the glandular mucosa covering the fundus and body. Its expression in the duodenum is strong in the columnar epithelial lining of the villi, in crypts, as well as in the cells surrounding the glands in the lamina propria. It is also located in the glandular epithelium of both colon and rectum [34].

CYP3A is the major enzyme expressed in the gut and constitutes up to 82% of the total CYPs [142]. CYP3A4 expression is highest in the small intestine and lowest in the stomach [146]. The specific content of CYP3A4 protein in human duodenum, distal jejunum, and distal ileum is in the range of 3–90, 2–98, and 2–38 pmol/mg, respectively [147]. CYP3A4 expression is regulated by the pregnane X nuclear receptor (PXR) [148] and induced by drugs such as rifampicin [149], phenobarbital [150], sodium arsenite [151], nafcillin, and dicloxacillin [152]. Intestinal CYP3A can also be inhibited by drugs including maslinic acid, corosolic acid, ursolic acid [153], ketoconazole [154], and imazalil [155]. CYP3A inhibitors and inducers affect the bioavailability of orally administered CYP3A substrates.

8.3.1.2 Pulmonary System. DMEs in the pulmonary system are responsible for the metabolism of drugs and also of environmental pollutants such as organic solvents and PAHs that are present in inhaled air [156]. Despite relatively low expression, these enzymes provide protection to the brain from inhaled chemicals and are important for homeostasis and metabolism of odorant molecules [157]. Apart from inhaled xenobiotics, drugs given by parenteral routes have the potential to accumulate in the lungs

as 100% of the cardiac output reaches the pulmonary system before passing through the liver and undergoes metabolism. Examples include amiodarone, imipramine, lidocaine, propranolol, pethidine, amphetamine, chlorphentermine, chlorpromazine, local anesthetics, and fentanyl [158–160].

The CYPs expressed in the pulmonary system are CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C18, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2S1, CYP3A4, CYP3A5, CYP3A43, and so on [7,156]. Of these CYP1A1, CYP1B1, CYP2A13, CYP2E1, CYP2F1, CYP2S1, and CYP3A5 are abundantly expressed in the pulmonary system at concentrations higher than that in the liver [7].

CYP1A1 is inductively expressed in bronchial epithelial cells, capillary endothelium, and alveolar epithelium of the human lungs in response to inhaled chemicals such as TCDD, BNF, pyridine, acetone, benzo(α)pyrene (cigarette smoke constituent), 3-methylcholanthrene, TCDD, nicotine, omeprazole, 2-hydroxypyridine, 7,12-dimethylbenzo(α)anthracene, and diesel exhaust [7,161–166]. Its presence in the epithelium of bronchi is restricted to airways with a diameter greater than 1 mm [7]. A study to estimate the influence of smoking on the expression of CYP1A1 revealed that specific content of this isoform in nonsmokers was 6.0 pmol/mg in comparison to $\sim$ 15.5 and 19.0 pmol/mg in smokers and ex-smokers, respectively [167]. In contrast to CYP1A1, CYP1A2 is expressed constitutively [132], although inhaled benzo(α)pyrene and diesel exhaust can lead to its induction [166].

CYP1B1 is expressed in alveolar type I and II, ciliated columnar epithelial, alveolar macrophage epithelial, and bronchial epithelial cells of the human lungs [7,167]. It is responsible for 2- and 4-hydroxylation of 17-estradiol and metabolic activation of PAHs and aromatic amines, similar to CYP1A1 [166]. CYP1B1 has five-fold higher expression in the smokers' lungs [167]. Its expression is reported to be 1.8, 1.0, and 4.4 pmol/mg microsomal protein in smokers, nonsmokers, and ex-smokers, respectively [167].

In the human lungs, CYP2A6 and CYP2A7 are present in the trachea and alveolar epithelium, respectively [7]. CYP2A6 is prominently expressed in the liver and also found in the pulmonary system with mRNA expression of 9 ± 1 , 76, and 160 ± 140 amol/mg RNA in lungs, trachea, and nasal mucosa, respectively [20]. CYP2A7 is virtually quiescent or absent in the human nasal mucosa and lungs [168]. CYP2A13 is present in pulmonary epithelial cells and the bronchial mucosa, with highest expression in the nasal mucosa, while hepatic expression is below the limit of quantitation [7]. mRNA expression in the lungs, trachea, and nasal mucosa is 78 ± 32 , 130, and 750 ± 210 amol/mg RNA, respectively. The specific content of CYP2A13 in microsomes is in the range of 0.26–0.56 nmol/mg proteins [20]. It is associated with metabolism of 2'-methoxyacetophenone, 2,6-dichlorobenzonitrile, hexamethylphosphoramide (HMPA), *N,N*-dimethylaniline, *N*-nitrosodiethylamine (NDEA), *N*-nitrosomethylphenylamine, aflatoxin B, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK) [7]. CYP2B6/7 isoforms are located in bronchial epithelial cells and alveolar type II pneumocytes. CYP2B6 is associated with oxidation of NKK in the human lungs and bioactivation of the prodrug, cyclophosphamide [7].

CYP2C8 and CYP2C18 are predominantly expressed in serous cells of bronchial glands, bronchial mucosa, and peripheral tissues of the human lungs, while CYP2C9 shows very low expression in the bronchial mucosa [7,27]. CYP2C8 is believed to have a role in regulation of the pulmonary vascular and bronchial tone [159]. This

subfamily is involved in metabolic activation of drugs as well as carcinogens [27]. CYP2C18 has more abundance in the trachea, as compared to CYP2C19, which is predominant in the human lungs [27].

Guidice *et al.* [169] examined gene expression of CYP2D6 in the human bronchial mucosa and lung parenchyma and reported that the mean levels of CYP2D6 mRNA in the bronchial mucosa and lung parenchyma were three- and six-folds, respectively, lower than the liver and that the gene was nonuniformly distributed within the lung. CYP2E1 is present in the bronchial, bronchiolar and alveolar epithelium, endothelial cells, and the nasopharynx, and reported to metabolize tobacco-specific nitrosamines, such as *N*-nitrosodimethylamine (NDMA), *N*-nitrosodibutylamine, and *N*-nitrosodiethylamine [159,170]. CYP2E1 expression is significantly higher in lung adenocarcinomas and is induced by acetone and ethanol [162]. CYP2F1 is specifically expressed in the human lungs with no or minimal expression in hepatic and other organs [171]. It is responsible for the metabolism of environmental toxicants, such as components of cigarette smoke, gasoline, and industrial by-products such as benzene, styrene, naphthalene, and 3-methylindole [172,173].

The CYP2J subfamily is expressed in bronchial and vascular smooth muscle cells as well as endothelium. The enzymes are involved in epoxidation of arachidonic acid (AA), leading to epoxide AA metabolite, which modulates bronchial smooth muscle tone and airway transepithelial ion transport [7,159,174]. CYP2J2 and CYP2J3 subtypes are primarily expressed in ciliated epithelial cells lining of the airway [174]. CYP2S1 is expressed in the upper and lower respiratory systems, in particular, in the nasal mucosal epithelium of the upper respiratory tract, and at high levels in the epithelium of bronchus and cuboidal and columnar epithelia of bronchioles in the lung. The enzyme is induced by TCDD and smoking [34,175].

CYP3A is expressed in bronchial epithelium, bronchial glands, alveolar epithelium, vascular epithelium, capillary epithelium, and alveolar macrophages of the human lungs. It is also expressed in the nasal cavity, pharynx, larynx, and trachea [39]. CYP3A4 and CYP3A5 are expressed at a significant level in human lungs, whereas CYP3A7 and CYP3A43 have low expression [39,176]. CYP3A4 is located in epithelium cells of bronchial glands, bronchiolar ciliated and terminal cuboidal, epithelial cells, and type II alveolar epithelial cells [176]. CYP3A5 is present in bronchial and alveolar epithelial cells, bronchial glands, and alveolar macrophages [39]. Its levels are decreased in alveolar macrophages on cigarette smoking [177].

8.3.1.3 Urinary System. CYPs expressed in the human kidneys are CYP1A1, CYP2D6, CYP2E1, CYP2J2, CYP2R1, CYP3A4, CYP3A5, and so on [8,129]. These enzymes are present throughout the kidney, with highest concentrations in the microsomal fraction of the cortex and proximal convoluted tubule (PCT). The specific content of CYP1A1 is <0.005 pmol/mg proteins in each kidney [8]. CYP2E1 is located in the PCT and is induced by pyridine, ethanol, acetone, and pyrazole [129]. It is responsible for nephrotoxicity, as it generates reactive metabolites of chloroform, ipomeanol, 1,1-dichloroethene, and paracetamol [129]. CYP2J2 plays an important role in carcinogenesis and is located in PCT, collecting duct (CD), and distal convoluted tubule (DCT) cells, while it is absent in glomerulus and thin loop of Henle [31]. CYP2S1 is highly expressed in urothelial cell of the urinary bladder and in PCT and DCT of the kidney [34]. CYP3A4 is predominantly expressed in PCT, along with CYP3A5 [129,178].

8.3.1.4 Central Nervous System. The CYPs in the brain are distributed unevenly in different cells and regions. For example, CYP content in mitochondrial fraction of the brain cells is nine times higher than that in the microsomal fraction [179]. CYPs play a diverse role in the brain, including metabolism of xenobiotics, aromatization of androgens to estrogens, formation of catechols, and metabolism of neurotransmitters [180]. Centrally acting drugs, such as analgesics, antidementia agents, beta-blockers, tricyclic antidepressants, antipsychotics, monoamine oxidase inhibitors, and vasodilators, are metabolized in the brain [15]. CYPs are also present in the microvessels and choroid plexus of the brain, which indicates that regulation of the penetration of xenobiotics in the brain compartment might be due to their metabolism in endothelial cells of the blood–brain barrier [180].

The CYPs expressed in the human brain include CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C8, CYP2D6, CYP2E1, CYP2U1, CYP3A4, CYP3A5, and CYP3A43 [15]. The subtypes, CYP1A1, CYP1A2, CYP2B6, CYP2D6, and CYP2E1, are expressed in the cortex, cerebellum, basal ganglia, hippocampus, substantia nigra, medulla oblongata, and pons. Of these, CYP1A1, CYP1A2, and CYP2E1 along with CYP2A6 are expressed in the mitochondria of the striatum, thalamus, pons, and medulla oblongata. CYP1A1 bioactivates PAHs that form adducts with DNA and thus it has the potential to induce carcinogenesis [15]. Interestingly, the structure of human brain CYP1A1 is different than that present in the liver [9]. The CYP2A6 isozyme is mainly responsible for the metabolism of nicotine to cotinine through C-oxidation and also to *trans*-3'-hydroxycotinine by 3'-hydroxylation [181,182]. Some studies have shown that its activity is inhibited by neurotransmitters and steroids, such as tryptamine, serotonin, dopamine, histamine, noradrenaline, adrenaline, estrogen, androgen, and corticosterone [183]. CYP1B1 is predominantly located in the putamen, spinal cord, medulla oblongata, frontal, and temporal cortex and to a lesser extent in cerebellum, hippocampus, thalamus, and amygdale. It is predominantly expressed in the nuclei of a majority of astrocytes and neurons in human brain cortex [15].

CYP2B6 is located in the caudate nucleus, putamen, hippocampus, cerebellum, brain stem, thalamus, and frontal cortex [15]. Induction of CYP2B6 isoform has been reported in monkeys after chronic treatment with phenobarbital [184]. CYP2B6 and CYP2C19 are the major enzymes responsible for metabolism of selegiline, a drug used in the treatment of Parkinson's disease [185,186]. The activity of CYP2B6 toward metabolism of nicotine is reported to be relatively higher than CYP2A6 [15].

CYP2C8 is located in the frontal cortex, putamen, and hippocampus of the brain [187]. Typical CYP2C8 substrates are tricyclic antidepressants, diazepam, and verapamil [15]. CYP2D6 is highly expressed in the cerebral cortex, purkinje and granule cell layers of cerebellum, reticular neurons of midbrain, and pyramidal neurons and its expression in brain fractions, viz putamen, globus pallidus, and substantia nigra, is higher in alcoholics than in nonalcoholics [188–190]. The isoform is involved in metabolism of neurotransmitters, tryptamine, and tyramine [15]. CYP2E1 plays a significant role in fetal alcohol syndrome, as it is induced by ethanol. Increased expression of this subtype was observed in human neuroblast cultured cells treated with nicotine [191]. CYP2S1 is expressed in the white matter of the brain [34]. CYP2U1 is specific to the cerebellum [15] and is involved in the metabolism of arachidonic acid, docosahexaenoic acid (DHA), and other long-chain fatty acids [36].

CYP3A is present in the cortex and basal ganglia where it metabolizes psychoactive drugs, such as codeine [192,193]. The subtypes CYP3A4 and CYP3A43 are expressed

in equal amounts in the brain. CYP3A43 is highly expressed in the brain as compared to the liver and is involved in metabolism of drugs such as alprazolam [194]. CYP3A5 mRNA is reported in the midbrain, basal ganglia, and frontal cortex [42].

8.3.1.5 Cardiovascular System. CYPs play a vital role in modulation of cardiac Ca^{+} current and cell contraction and in maintaining vascular tone by generating epoxides such as 5,6-epoxyeicosatrienoic acids (EETs), 8,9-EETs, 11,12-EETs, and 14,15-EETs from AA [195]. The CYPs expressed in the human heart are CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A7, CYP2C8, CYP2D6, CYP2E1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, and CYP3A4 [196,197]. Of these, CYP1B1 and CYP2J2 are prominently expressed, while CYP1A1, CYP1A2, CYP2B6, CYP2B7, and CYP2C9 are induced [198]. Cardiomyocytes are efficiently involved in metabolism and clearance of drugs from the heart [199].

Healthy human heart shows no expression of CYP1A2, while there is limited expression of CYP1A1 in the left ventricle. In diseased conditions, like dilated cardiomyopathy, CYP1A1 is also detected in the right ventricle, ascending aorta, pulmonary aorta, and right atrium of the heart. CYP1A1 and CYP1B1 are expressed in coronary artery smooth muscle cells and are involved in the conversion of estradiol to hydroxyestradiol, which has cardiovascular protective effects [13].

CYP2C8, CYP2C9, and CYP2J2 show epoxigenase activity in the heart. CYP2C8 and CYP2C9 are present in the right and left ventricle cardiomyocytes [11,12,199] and are induced during ischemic heart injury [198]. CYP2J2 is abundantly expressed in coronary artery endothelial cells, smooth muscle cells, and cardiac myocytes of the normal human heart [200]. Expression of CYP2J2 is aggrandized on exposure with cocaine [201]. CYP2D6 shows very low expression in heart and is involved in the metabolism of verapamil [198]. CYP2E1 is expressed in the endothelium of the endocardium and coronary vessels [16], while CYP2S1 is absent in the heart [34,202]. CYP3A4 is expressed in the endothelium of the endocardium and coronary vessels. The CYP3A4-NADPH-CYP reductase system is involved in formation of nitric oxide from isosorbide dinitrate [16].

8.3.1.6 Skin. CYPs are present in keratinocytes, monocytes, lymphocytes, and fibroblasts of the human skin. CYPs are relatively highly expressed in keratinocytes [203] and also found within the differentiated cells of hair follicles and sebaceous glands, as well as in epidermis [204]. The role of CYPs is to provide protection to exposed skin from ingredients in cosmetics, toiletries, and healthcare products, as well as from many allergens, environment toxicants, and carcinogens. CYP isoforms expressed in human skin are CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP2S1, CYP2U1, CYP2W1, CYP3A4, and CYP3A5. Among these, CYP2C9, CYP2C18, CYP2C19, CYP2W1, CYP3A4, and CYP4B1 are upregulated by cellular differentiation [17]. CYP1A1 and CYP1B1 are expressed in the differentiated keratinocytes [14] and CYP2U1 is expressed at highest levels in undifferentiated keratinocytes [17].

CYP1A1 is expressed inductively in the human skin and hair follicles [205] and can be induced by ultraviolet (UV) irradiation, PAHs, BNF, polyhalogenated dibenzo-*p*-dioxins, polyhalogenated dibenzofurans and coplanar polyhalogenated biphenyls [14,17,206]. It is involved in the metabolism of benzo(α)pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz(α)anthracene in the skin [203]. CYP1A2 is expressed at mRNA

level in epidermis of the human skin [17]. CYP1B1 is also expressed in the hair follicles and plays a major role in the metabolism of PAHs (7,12-dimethylbenz(α)anthracene). It is induced in the skin on exposure to UV radiation, especially in keratinocytes, basal cell layer, and dermis [14,207].

CYP2A7 mRNA is detected in human skin fibroblasts, although no catalytic activity has been observed [208]. CYP2B6 is reported in foreskin epidermis, epidermis, sebaceous glands, and hair follicles of adult skin [23]. Weak CYP2C mRNA expression is shown in epidermal keratinocytes and dermal fibroblasts of the human skin. This subfamily may play a role in the pathogenesis of drug-induced skin allergy, as this group of enzymes is known to metabolically activate drugs, including phenytoin [209]. CYP2C9 and CYP2C19 are unregulated in epidermal keratinocytes during cellular differentiation, and CYP2C18 is present in the human skin [17,25]. CYP2D6 is detected in dermal fibroblasts, while CYP2E1 is present in the suprabasal cell layers of human foreskin epidermis and keratinocytes [17]. The latter is induced by ethanol, dexamethasone, and salicylic acid [210]. CYP2J, CYP2W, and CYP2R mRNA has been detected in human keratinocytes [17]. CYP2S1 is constitutively expressed in the human skin and can be induced on UV exposure or topical drug treatment. Other factors responsible for CYP2S1 expression in the cutaneous tissue are PUVA (psoralen + UV-A treatment for eczema, psoriasis, graft-versus-host disease, and vitiligo), coal tar, and all-*trans* retinoic acid [35].

CYP3A4 is not expressed constitutively in the proliferating human skin keratinocyte but is induced by dexamethasone. In contrast, CYP3A5 mRNA is expressed constitutively [41].

8.3.2 Non-CYP Enzymes

This category includes phase I (e.g., aldehyde oxidase) and phase II DMEs that are involved in metabolic reactions including oxidation, reduction, hydrolysis, hydration, and group transfer. Extrahepatic non-CYPs DMEs include transferases, hydrolases, oxidases, dehydrogenases, reductases, peroxidases, and glucuronidase. These enzymes convert drugs into active/reactive/inactive metabolites in extrahepatic tissues and are essential for clearance and pharmacological activity of xenobiotics and are also responsible for the drug-induced toxicity.

8.3.2.1 Transferases. Transferases catalyze the transfer of functional groups from a donor to an acceptor molecule. These enzymes play a crucial role in converting electrophilic metabolites produced by phase I enzymes (e.g., CYPs) into less or nontoxic products [211–214]. Metabolic reactions catalyzed by transferase enzymes are shown in Fig. 8.3.

8.3.2.1.1 UDP-Glucuronosyltransferases. UDP-glucuronosyltransferases (UGTs) are located in the endoplasmic reticulum of mammalian cells and detoxify xenobiotics (e.g., therapeutic drugs, carcinogens and environmental pollutants) as well as endogenous compounds (e.g., bilirubin, bile acids, thyroid hormones and steroid hormones) [50,215]. About 40–70% of clinically used drugs are subjected to glucuronidation, resulting in hydrophilic products with low pharmacologically activity [216]. In humans, families of UGTs reported are UGT1, UGT2, UGT3,

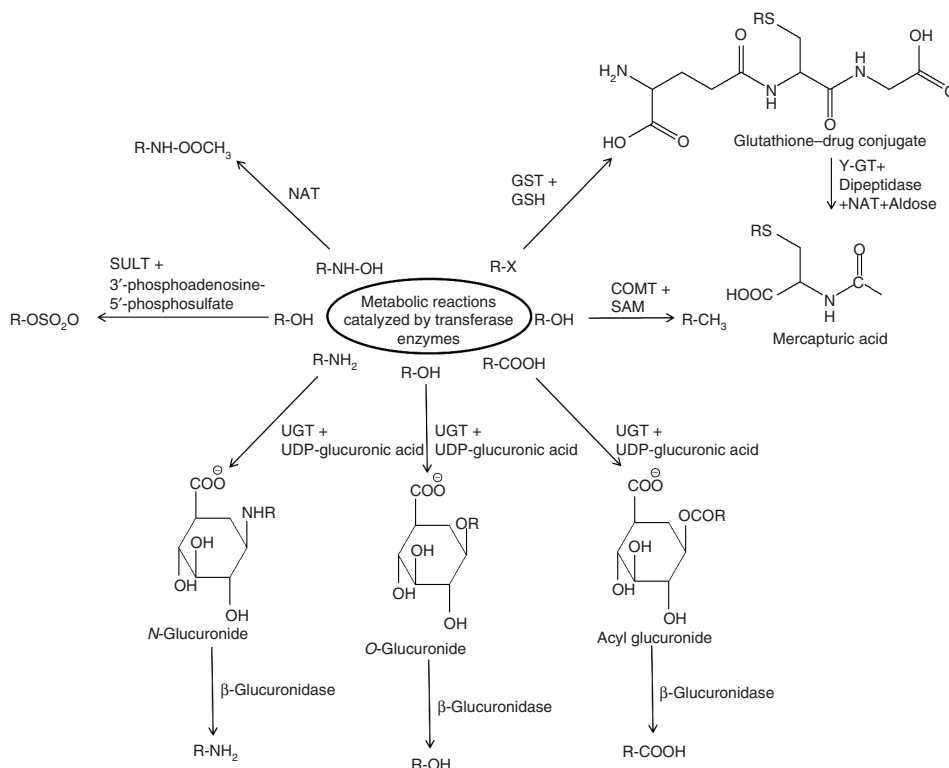


Figure 8.3 Metabolic reactions catalyzed by transferase enzymes.

and UGT8 [50,216]. UGT1A is involved in glucuronidation of NSAIDs, anticonvulsants, chemotherapeutics, steroid hormones, bile acids, and bilirubin. UGT2B isoforms, UGT2B7 and UGT2B15 metabolize endogenous substrates, including hydroxylated steroids and bile acids, and xenobiotics [50,217]. UGT3A1 uses UDP-*N*-acetylglucosamine as a substrate to glycosidate xenobiotics, while UGT3A2 uses both UDP-glucose and UDP-xylose to glycosidate a broad range of substrates, including 4-methylumbelliferone, 1-hydroxypyrene, bioflavones, and estrogens [54]. UGT3A1 contributes in the metabolism and elimination of ursodeoxycholic acid for ameliorating the symptoms of cholestasis or dissolving gallstones [53]. UGT3A2 has a more selective role in protecting organs from toxins [54]. UGT8 converts UDP-galactose to galactosidate ceramide, an important step in the biosynthesis of glycosphingolipids and cerebroside [53,218]. These enzymes are distributed in the liver, GIT, lungs, kidneys, brain, placenta, reproductive organs, and so on [43,219]. The highest activity is reported in kidneys, followed by GIT and lungs [220].

The isoforms of UGTs expressed in human GIT are UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2A1, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, and UGT2B28. UGT1A7 is prominently expressed in upper GIT, while UGT1A8 exists in the lower GIT [219]. The UGTs present in human esophagus are UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B10, and UGT2B15. In stomach, the isozymes reported are UGT1A7, UGT1A10,

and UGT2B28. The subtypes, UGT1A1, UGT1A3, and UGT1A6, exhibit polymorphisms in stomach. Most of the UGTs present in the duodenum exhibit polymorphism (viz UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT2B4, UGT2B7, and UGT2B15), except UGT1A10 and UGT2B28. In both jejunum and ileum, UGT1A10, UGT2B15, and UGT2B28 are the predominant DMEs, while UGT1A3 is additionally expressed in the jejunum. Expression of UGT isoforms UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B10, and UGT2B15 are highest in the colon [43,221,222].

Gregory *et al.* [219] reviewed literature and report that glucuronidation of bilirubin is catalyzed by UGT1A1 in the GIT and this isoform is involved in the conjugation of endogenous compounds (e.g., retinoic acid and estrogens) and xenobiotics. The isozymes, UGT1A5, UGT1A6, and UGT1A7, are involved in conjugation of xenobiotics, although their role in conjugating endogenous substrates is not yet established. The subtypes, UGT1A8, UGT1A9, and UGT1A10, are responsible for conjugation of mycophenolic acid and environmental pollutants such as PAHs. Tertiary amine drugs are conjugated by UGT1A4, oestrone by UGT1A3 and UGT1A10, while UGT2B4 and UGT2B7 are primarily involved in catalysis of hyodeoxycholic acid glucuronidation [223,224].

In human pulmonary system, the only UGT1A enzyme reported is UGT1A5 [43,225,226]. UGT1A6 and UGT1A8 are expressed in the larynx; UGT1A7 and UGT1A10 in the tongue, tonsils, floor of mouth, larynx, and esophagus; while UGT2B4 and UGT2B17 exhibit significant levels of expression through the aerodigestive tract [227]. UGT2A1 is prevalent in the aerodigestive tract, with highest expression in the lungs followed by trachea > tonsil > larynx > colon > olfactory tissue [43,225,226]. It provides protection against cancer by neutralizing tobacco carcinogens (PAHs, including 1-hydroxy benzo(α)pyrene, benzo(α)pyrene-7,8-diol, and 5-methylchrysene-1,2-diol) [226]. In other studies, UGT2B4, UGT2B11, and UGT2B17 have been shown to be present in the human bronchial epithelial cells [225,228].

The activity of UGT enzymes in the human kidneys is highest in PCT and DCT cells. For example, the propofol glucuronidation rate is higher in kidney than liver because of relative higher expression of UGT1A8 and UGT1A9 [43,45]. The human kidneys have low levels of UGT1A1 and UGT1A6 [49]. UGT1A9 and UGT2B7 are involved in the metabolism of endogenous and exogenous substances, which can result in renal drug–endobiotic interactions [229].

In the human brain, UGT1 is expressed in cerebellum and involved in glucuronidation of serotonin [230]. UGT1A6 is responsible for glucuronidation of propofol in the brain [231]; UGT2A1 is involved in glucuronidation of odorant compounds such as monoterpenoids, aliphatic alcohols, phenols, and coumarins and UGT2B7 metabolizes opioids and their metabolites (e.g., 3- and 6-hydroxyl morphine) [232,233]. UGT activity has been observed in the stratum corneum of the human skin [48].

8.3.2.1.2 *N*-Acetyltransferases. *N*-Acetyltransferases (NATs) are directly involved in *N*-acetylation of xenobiotics, such as aromatic and heterocyclic amines as well as drugs such as mesalamine (5-aminosalicylic acid, 5-ASA), isoniazid, sulfamethazine, and sulphasalazine [234–237]. Arylhydroxylamine metabolites generated by CYPs also undergo acetylation by NATs [234,235]. These enzymes have a role in the biotransformation of carcinogens and are inhibited by anticancer drugs, such as cisplatin and

tamoxifen [235]. NAT1 activity is increased in human cancer cells and can be induced by trichostatin A and inhibited by caffeic acid, ferulic acid, and gallic acid [238,239]. A well-known nonselective CYP inhibitor, 1-aminobenzotriazole, acts both as a substrate and an inhibitor of NAT1 [240]. The second human isoform, NAT2, is involved in *N*-acetylation of carbocyclic arylamines, such as 4-aminobiphenyl and β -naphthylamine [241]. Paracetamol, scopuletin, and curcumin [239,242] are reported to be inhibitors of NAT2.

NAT1 is widely distributed in the human body, whereas NAT2 is expressed predominantly in the intestine and liver [243]. In the intestine, both NATs are expressed in the GIT from esophagus to colon [55]. NAT2 is predominantly present in epithelial cells of the human intestine and involved in activation of compounds passing through this organ. The lung and urothelium of the urinary bladder express NAT1 and NAT2, while these are not expressed in the human kidneys [55,243]. In the human brain, the homologous enzymes NAT8L and NAT14 are involved in *N*-acetylaspartate synthesis [244]. NAT1 is expressed in human skin epidermal keratinocytes [56].

8.3.2.1.3 Glutathione *S*-Transferases. Glutathione *S*-transferases (GSTs) utilize glutathione (GSH) to form conjugates with a wide range of structurally different electrophilic substrates. This conjugation provides protection against cellular/organ toxicity [245]. GSTs are induced by diverse chemical compounds, such as isothiocyanates (such as benzylisothiocyanate, allylisothiocyanate, and sulforaphane), phenobarbital, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, pregnenolone-16 α -carbonitrile, 3-methylcholanthrene, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin, BNF, butylated hydroxyanisole, ethoxyquin, oltipraz, fumaric acid, coumarin, dexamethasone, thiazolidinediones 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole, 12-*O*-tetradecanoylphorbol-13-acetate, glyphosate, citrus triterpenoids (nomilin, isoobacunoic acid, and deacetyl nomilin), rambo (0.6% permethrin), cinnamon, cardamom, protocatechuic acid, myristicin, and so on [246–255]. The gene expression of GSTs is mediated by multiple receptors, viz, constitutive androstane receptor (CAR), PXR, AhR, nuclear factor E2 related factor (Nrf2), peroxisome proliferator-activated receptor- α/γ (PPAR α/γ), and CAATT/enhancer-binding protein (C/EBP) β [249,256,257]. Caffeic acid phenethyl ester selectively inhibits GSTs in the presence of tyrosine [258].

The mammalian GSTs have been very well characterized and classified as GST- α (GSTA), GST- μ (GSTM), GST- π (GSTP), and GST- θ (GSTT) subtypes based on structural conformation of protein, immunological identity and their susceptibility to inducers and inhibitors [245,259]. The GST isozymes reported in humans are GSTA1-4, GSTM1-5, GSTP1, GSTT1, and GSTT2 [260]. GSTA1, GSTA2, GSTM1, GSTM2, and GSTP1 are inhibited by polyphenolic compounds, such as ellagic acid and curcumin. GSTM1 and GSTM2 are also inhibited by genistein, kaempferol and quercetin [261].

GSTAs, GSTMs, and GSTPs are mainly expressed in the human GIT [262,263]. Overall, the activity of GSTs and expression of GSTA in the GIT increases in the following order: duodenum > stomach > sigmoid colon > transverse colon [58]. GSTA4, GSTM3, and GSTT1 are organ specific and located in the small intestine [148]. Duodenum shows higher GST activity than the colon [264]. GSTPs are highly expressed in the stomach, and their expression decreases from the proximal to distal direction [58]. The contents of GSTA and GSTP in the stomach are 4.5 ± 0.5 and $16.5 \pm 0.7 \mu\text{g/mg}$ and in duodenum are 20.0 ± 0.7 and $11.2 \pm 0.5 \mu\text{g/mg}$, respectively [58]. The main role of

GSTs in GIT is to detoxify electrophilic compounds ingested with foods (e.g., quinones and 4-hydroxyalk-2-enals), dietary supplements, and orally administered medicaments [265]. There is a report where 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) induced GST by 2- and 15-folds in the mice stomach and small intestine, respectively [266]. PAHs and phenobarbital also induce GST in the intestine [267].

Multiple GSTs are expressed in the human lungs [268]. The GSTM isoforms, GSTM1 and GSTM3, are collectively expressed at 89.3 ± 27.8 ng/mg and GSTP1 at 762 ± 27.9 ng/mg. The combined expression of GSTA1 and GSTA2 in the lung is 80.8 ± 17.6 ng/mg [269]. Similar to GIT, GST expression in the lungs is induced by BHA, providing protection against lung cancer induced by PAHs [266]. GSTA, GSTM, and GSTP are expressed in the human kidneys and GSTA isoforms are highly expressed (50–70%) in the PCT, loop of Henle, and CD. GSTM and GSTP isoforms are expressed in DCT, Bowman's capsule, and CD [31,67,270]. GST isozymes in kidney are inhibited competitively by bromosulphophthalein and noncompetitively by bilirubin and haematin [60]. GSTM3, GSTM5, and GSTP are expressed in the human brain. GSTP is mainly localized in choroid plexus, pia-arachnoid, tanycytes, astrocytes, and ependyma of the brain, while GSTM3 is localized in astrocytes [61,271,272].

The reported activity of GSTs in epidermis, dermis, and whole skin is 27.5 ± 2.8 , 44.2 ± 3.0 , and 48.3 ± 3.05 nmol conjugate/min/mg protein, respectively [64]. GSTA, GSTM, and GSTP are expressed in the human skin cytosol, where GSTM is involved in detoxification of environmental toxicants that lead to skin allergies [64,65,273]. GSTP mRNA is expressed in cultured keratinocytes and fibroblasts of the human skin [274].

8.3.2.1.4 Sulfotransferases. Sulfotransferases (SULTs) are responsible for *O*- and *N*-sulfation of xenobiotics. Sulfation of molecules usually increases water solubility, often accompanied by a decrease in biological activity. SULTs are present either as membrane-bound or cytosolic enzymes. Membrane-bound SULTs are responsible for the sulfonation of peptides, proteins, lipids, and glycosaminoglycans, while cytosolic SULTs metabolize endogenous steroids, bile acids, neurotransmitters, and xenobiotics [275]. Three families of SULTs, that is, SULT1 (SULT1A1, SULT1A3, SULT1B1, SULT1C1, SULT1C2, SULT1C3, and SULT1E1), SULT2 (SULT2A1 and SULT2B), and SULT4 (SULT4A1) have been identified in humans, and SULT3 has primarily been reported in zebra fish [276–279].

SULT1 and its subtypes are responsible for the sulfonation of planar phenolic molecules such as estradiol, thyroid hormones, and xenobiotics [280]. SULT1A1, SULT1A3, SULT1C1, and SULT1E1 are involved in the sulfation of small neutral phenols, monoamine neurotransmitters, *N*-hydroxy-2-acetylaminofluorene, and 17 β -estradiol [280–283], respectively. SULT1C2 catalyzes sulfonation of thyroid hormone [284]. SULT2s are responsible for the sulfonation of hydroxyl groups of steroids, such as androsterone, allopregnanolone, and dehydroepiandrosterone [280]. For example, SULT2A1 metabolizes 3 α - and 3 β -hydroxysteroids as well as aliphatic hydroxyls of xenobiotics, whereas SULT2B1 is involved in selective sulfonation of 3 β -hydroxysteroids [281].

After the liver, human intestine has been reported to have highest SULTs activity, followed by the lungs and the kidneys [220,285]. The SULT subtypes reported in the human intestine are SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1, with the order of expression being: SULT1B1 (2400 ± 1300 ng/mg) > SULT1A3

(2000 ± 1100 ng/mg) > SULT1A1 (1300 ± 650 ng/mg) > SULT1E1 (490 ± 470 ng/mg) > SULT2A1 (390 ± 130 ng/mg) [286]. SULT1C2 is identified at mRNA level in the adult stomach [278]. The total expression of SULTs in the human intestine cytosol has been reported to be 7800 ± 4600 ng/mg, higher than their expression in the liver (5960 ± 1400 ng/mg) [286]. SULT1A1, SULT1A3, and SULT1B1 are expressed throughout the GIT; SULT1C1 is found in the stomach, while SULT1E1 and SULT2A1 are detected in the jejunum, ileum, and colon [68]. SULTs exhibit intersubject variability and do not exhibit any consistent trend across the intestine. In some subjects, SULT expression is higher in the central part, while in others it is higher at either end of the intestine [287,288]. SULTs are induced by methotrexate in Caco-2 cells and by tamoxifen in the rat intestine [289,290]. Isoetharine, isoprenaline, ethinyloestradiol, and paracetamol undergo sulfate conjugation in GIT [236].

SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1 have been detected in the human lungs. While total content of SULTs in the lung is reported to be 290 ± 130 ng/mg, the individual contents of SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1 subtypes are 60 ± 50 , 60 ± 80 , 40 ± 20 , 110 ± 110 , and 30 ± 20 ng/mg, respectively [286]. SULT1C3 is present in the fetal lungs [278]. In the human kidneys, high concentration of SULTs is reported in the cytoplasm of PCT and specific subtypes are expressed in CD and medulla [31]. Total SULT content in kidney is 430 ± 270 ng/mg. The expression of SULT1A1, SULT1A3, SULT1B1, and SULT2A1 is 170 ± 90 , 120 ± 70 , 130 ± 110 , and 5 ± 10 ng/mg, respectively, whereas expression of SULT1E1 in kidney is below the limit of quantitation [286]. SULT1C2 and SULT1C3 are expressed in the fetal kidney and SULT1C2 in the adults' kidneys [278].

Human brain expresses SULT1A1, SULT1A3, SULT1C2, SULT1E1, SULT2A1, SULT2B1, and SULT4A1 [69–72]. SULT1A1 is located in cytosol of the cerebellum, occipital, and frontal lobes, whereas SULT1A3 is present in cytosol of superior temporal gyrus, hippocampus, and temporal lobe. SULT1A1 is involved in the metabolism of small phenols and SULT1A3 metabolizes catecholamine neurotransmitters [70]. The location of SULT1E1 and SULT2B1 in the human brain is not specified [71,291]. SULT4A1 is located in the cortex, substantia nigra, cerebellum, pituitary, and brainstem; hence, it regulates functions and neurochemistry of different regions of the brain [72].

SULTs are present in the human scalp skin and skin fibroblasts [292,293]. The subtypes include SULT1A1, SULT1A3, SULT1E1, and SULT2B1 [73,74]. SULT2B1b is localized in the granular layer of skin epidermis and involved in synthesis of cholesterol sulfate, which is a critical regulator of keratinocyte differentiation and desquamation, and is a well-known mediator of barrier homeostasis [294,295]. Parabens inhibit SULTs, leading to prolonged estrogenic effects in skin, thus providing antiaging benefit [296].

8.3.2.1.5 Other Transferases. Carboxyltransferase (CT), thiopurine *S*-methyltransferase (TPMT), catechol-*O*-methyltransferase (COMT), and γ -glutamyltransferase (γ -GT) have been detected in extrahepatic sites. CT is expressed in PCT of the kidney and is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) [31]. TPMT, a cytosolic enzyme, is expressed in RBCs, kidneys, lungs, and brain, apart from the liver and is responsible for the *S*-methylation of thiopurines such as 6-mercaptopurine, azathioprine, and 6-thioguanine [297,298]. Naproxen, mefenamic acid, and tolfemic acid are noncompetitive inhibitors of this DME [216].

COMT is a phase II enzyme that is highly expressed in liver, kidneys, intestine, and brain (postsynaptic neuron) in both cytoplasmic-soluble and membrane-bound forms [216] and is mainly expressed in the right inferior frontal gyrus and intraparietal sulcus of the brain [75,299]. Compounds with a catechol moiety, for example, catecholestrogens and catechol-containing flavonoids, are substrates of COMT. It introduces the methyl group in catecholamine, which is donated by *S*-adenosyl-L-methionine (SAM) [299]. COMT is involved in inactivation of catecholamine neurotransmitters, viz, dopamine, epinephrine, and norepinephrine, which can ultimately lead to behavioral problems, psychiatric disorders, chronic pain, and cancer [216,300,301]. The enzyme also plays a key role in metabolism of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) [302]. Other specific reactions catalyzed by COMT include conversion of dihydroxyphenylethylene glycol (DOPEG) to methoxyhydroxyphenylglycol (MOPEG), and 3,4-dihydroxymandelic acid (DOMA) to vanillylmandelic acid (VMA) [303]. Entacapone increases bioavailability of L-DOPA by inhibiting COMT both peripherally and centrally and also by blocking conversion of dopamine into 3-methoxytyramine [216,304].

γ -GT is present in lungs, kidneys, brain, spleen, heart, and seminal vesicles [77,305–309]. This enzyme is primarily expressed in the PCT of kidney, which plays an important role in the transport of amino acids through a sequence of reactions forming a “gamma-glutamyl cycle” [310]. This enzyme also releases glutamate from drug-GSH adducts, leaving behind conjugates, which cause nephrotoxicity. It is irreversibly inhibited by acivicin [305].

8.3.2.2 Epoxide Hydrolases. Epoxide hydrolases (EHs) are phase I enzymes that metabolize exogenous and endogenous epoxides to vicinal diols, which are subsequently eliminated in a conjugated form [311,312]. EH enzymes have broad substrate specificity, including epoxide derivative of PAHs, anticonvulsant drugs, and steroids. On the basis of their molecular weights, subcellular localization, and substrate specificity, these are classified into five subgroups, viz, hepoxilin EH, leukotriene A4 hydrolase, cholesterol EH, microsomal EH, and soluble epoxide hydrolase (sEH) [312]. These have been subject of intensive investigations because their inhibition protects heart, brain, and kidneys against injury [80,313–316]. EHs are expressed in bronchial epithelial cells of lung and provide protection against development of lung cancer by hydrolyzing carcinogenic products of PAHs [317]. Lung EHs metabolize epoxytetraenoic acid (EET), which is responsible for blood pressure regulation [318]. Low levels of this enzyme have been reported in cytosolic fractions of the kidney [78]. EHs are differentially expressed in cerebral blood vessels and brain parenchyma, demonstrating their involvement in metabolism of vasodilator EETs into inactive diol metabolite in the brain [79,319]. EHs are found in epidermis, dermis, epithelial cells, and fibroblasts in the human skin [81–84]. These are reported to metabolize benzo(α)pyrene-4,5-oxide and *trans*-stilbene oxide in rat skin microsomes [320].

8.3.2.3 Flavin-Containing Monooxygenases. CYPs and flavin-containing monooxygenases (FMOs) have partially overlapping substrate specificities, although they may or may not generate the same metabolites. For example, FMOs exhibit highest catalytic activity toward pesticides, such as carbophenothion, methiocarb, and aldicarb and result in the generation of a sulfone metabolite [321]. In comparison, these pesticides are metabolized by CYPs into sulfoxide and sulfone [322]. Apart from qualitative and quantitative differences in the generation of metabolites, FMOs

and CYPs also exhibit differential stereoselectivity [323,324]. These enzymes are mainly involved in the metabolism of nucleophilic xenobiotics that contain nitrogen, sulfur, and phosphorous heteroatoms, for example, trimethylamine, sulindac, and methionine [88,325–328]. FMOs are of five subtypes, viz FMO1, FMO2, FMO3, FMO4, and FMO5 [329]. Their expression varies across extrahepatic tissues.

The relative expression of FMO subtypes in the human intestine is in the following order: FMO5 > FMO2 > FMO1 > FMO4 > FMO3 [86]. FMO1 is expressed in the human small intestine to a level of 2.9 ± 1.9 pmol/mg microsomal protein [329]. In the human lungs, FMOs are located in nonciliated bronchiolar epithelial (Clara) cells [330]. Among these, FMO2 is specifically expressed in lung, and primary amines and alkyl sulfides are important substrates for it [331,332]. The relative expression of FMO3, FMO4, and FMO5 in the human lungs as compared to the liver is ~4.5%, ~7% and ~4%, respectively. The overall order of expression of FMO subtypes in lung is FMO2 > FMO5 > FMO3 > FMO4 > FMO1 [86].

The primary site of the expression of FMO1 and FMO4 is the human kidneys [87]. Reported expression levels of FMO1, FMO3, and FMO5 in the human kidneys are 5.8 ± 2.3 , 0.5 ± 0.4 , and 2.4 ± 1.4 pmol/mg, respectively [96]. The expression of FMO1 varies from 5.0 ± 1.7 to 7.7 ± 2.4 pmol/mg of protein. It is involved in the metabolism of nitrogen- and sulfur-containing nucleophiles [96]. In human kidneys, FMO3 has very low expression (~3.7% of liver), while FMO4 is expressed moderately [93]. Renal FMOs are involved in the biotransformation of 5-HT₃ antagonists, viz, tropisetron and meperidine, into *N*-oxide metabolite [86,333]. In brain, FMOs are localized predominantly in neuronal cell bodies in magnocellular reticular nuclei, colliculi, and substantia nigra with relatively constant expression [88]. These enzymes are responsible for the metabolism of CNS drugs, for example, imipramine, fluoxetine, and chlorpromazine [334]. Relative expression of FMO subtypes in the human brain is in the order of FMO2 > FMO5 > FMO4 > FMO3 > FMO1 [86]. In the rat brain, FMOs metabolize *N,N*-dimethylaniline, methimazole, thiabenzamide, and antidepressant drugs such as imipramine and fluoxetine [88,335]. FMO1 and its polymorphs have potential influence on nicotine metabolism and hence, modulate its concentration in the brain [87,336].

In the skin, FMOs are localized in the epidermis, sebaceous glands, and hair follicles, with their basic role being detoxification of xenobiotics to which the skin is exposed [94]. FMO1 is expressed in epidermis and dermis and FMO3 in dermis, while FMO4 and FMO5 are expressed almost equally in total skin, dermis, and epidermis [89,94,95]. Another study has also reported the presence of FMO1, FMO3, FMO4, and FMO5 in the human skin, and these subtypes were found to play an important role in the metabolism of phorate [337].

8.3.2.4 Monoamine Oxidases. Monoamine oxidases (MAOs) are involved in the oxidative deamination of a range of monoamines, including 5-hydroxytryptamine (serotonin), histamine, and catecholamines (viz, dopamine, noradrenaline, and adrenaline), to yield respective aldehydes by liberating ammonia and hydrogen peroxide [338]. These enzymes are located predominantly in the outer membrane of mitochondria, with low concentrations associated with microsomal fractions [99]. MAOs are abundantly expressed in organs with direct exposure to the environment, for example, lungs, GIT, and liver [338]. In humans, MAO-A and MAO-B are expressed, with the order of expression of MAO-A being small intestine $\geq$ placenta $\geq$ lungs $\geq$ muscle > kidneys

> brain $\geq$ spinal cord $\geq$ meninges $\geq$ liver > spleen > adrenal glands, and MAO-B: small intestine > kidneys $\geq$ liver > adrenal glands > heart > spinal cord > lungs. The combined MAO activity in different parts of the brain is in the order: frontal cortex > locus coeruleus > temporal cortex $\geq$ posterior pensylvian cortex-supramarginal gyri > anterior pensylvian cortex-opercular gyri $\gg$ hippocampus and thalamus [97]. MAO-A is present in catecholaminergic neurons (cells of the substantia nigra, the locus coeruleus, and the periventricular regions of the hypothalamus); while predominant expression of MAO-B is in serotonergic neurons (dorsal raphe nucleus), astrocytes, lateral and posterior hypothalamic regions, and platelets [99,100]. These MAOs are nonselectively inhibited by stilbene-like derivatives [339]. In different organs, MAO-A is selectively inhibited by clorgyline, toloxatone, and cimoxatone, while MAO-B is inhibited by selegiline (L-deprenyl) [340–342]. The MAO enzymes are expressed in nerves around hair follicles, cutaneous nerve fibers beneath the epidermis, and human skin fibroblasts [101–104].

8.3.2.5 Additional Non-CYP Enzymes. β -Glucuronidase (β -Gluc), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), aldose reductase (AR), myeloperoxidase (MPO), and GSH reductase are additional non-CYP enzymes present in extrahepatic sites. β -Gluc is located in the brush border epithelium and microflora of human GIT, lung alveolar macrophages, spleen, and placenta [105,106,343,344]. It is responsible for the cleavage of glucuronide complexes and for the liberation of the active moiety for reabsorption, in cases, leading to enhanced drug action [49]. In gut, β -Gluc is responsible for the hydrolysis of exogenous and endogenous β -glucuronides produced in the liver by UGT (e.g., morphine glucuronide conjugates), aiding in enterohepatic reabsorption [105].

ADHs are cytosolic dimeric metalloenzymes involved in the metabolism of ethanol to aldehydes in the presence of nicotinamide adenine dinucleotide [118]. ADHs are located in the gingiva, tongue, esophagus, stomach, and colon in GIT. These do not show any specific pattern of expression from distal to proximal end of the human GIT but are highly expressed in esophagus and stomach [118]. They have also been reported to be expressed in the human lungs [107]. In the human kidneys, ADHs are mainly located in epithelial tubuli, glomeruli, and collecting tubules. Low content of ADHs is also reported in adrenal glands, neurons of the cerebral cortex, hypothalamus, infundibular stalk of the pituitary, and purkinje cells of the brain cerebellum [108,111]. In the human skin, ADH is localized in the epidermis, sebaceous glands, and hair follicles and is involved in detoxication and toxication of compounds that can cause allergic contact dermatitis, such as *trans*-cinnamaldehyde and *trans*-cinnamic alcohol [110,345].

Human ADHs have been divided into following five classes based on structural and catalytic features: Class I (α -ADH, β -ADH, and γ -ADHs); Class II (π -ADH), Class III (χ -ADH), Class IV (μ -ADH or σ -ADH), and Class V (resembles Class I, named as $\alpha\alpha$ -isozyme) [112,118,346]. Class I ADH mRNA is widely distributed in most tissues, including brain and placenta, although it is abundantly expressed in hepatic region and plays major role in the first-step metabolism of ethanol. The expression of Class II and Class V mRNA is mainly limited to hepatic tissues, with low mRNA concentrations of Class II detected in stomach, pancreas, and small intestine. Class V enzymes demonstrate similar distribution in fetal kidneys and small intestine. The distribution pattern of Class III mRNA is consistent with those of housekeeping enzymes, while expression of Class IV mRNA is restricted to the stomach [112].

ALDHs are responsible for the oxidation of aldehydes generated during retinoic acid biosynthesis and alcohol metabolism and also for the metabolism of amino acids, biogenic amines, steroids, lipids, carbohydrates, and drugs into their respective carboxylic acids [347]. Ten types of ALDHs (ALDH1-10) are known [115,118]. ALDH1, ALDH2, and ALDH6 are expressed in the gut; ALDH3 and ALDH7 in the lung; and ALDH4, ALDH6, ALDH7, and ALDH9 in the kidney [115]. In the GIT, ALDHs are located in gingiva, tongue, esophagus, and stomach. Like ADHs, these enzymes are also highly expressed in esophagus and stomach [118]. Expression of ALDHs in the lung is increased by carcinogenic aldehydes found in cigarette smoke [348]. In the kidney, ALDH enzymes are located in cortex tubules and provide protection against toxicity of aldehydic metabolites, for example, chloroacetaldehyde, a metabolic product of ifosfamide [349]. In the human brain, expression of ALDHs is in the order of spinal cord > corpus callosum > subthalamic nucleus > medulla > thalamus > amygdale > cerebral cortex > substantia nigra > hippocampus > caudate nucleus > temporal lobe > cerebellum > putamen > frontal lobe > occipital pole [350]. These are reported to be involved in metabolism of γ -aminobutyraldehyde [350]. ALDH enzymes are located in the epidermis, sebaceous glands, and hair follicles of the human skin and play an important role in the metabolism of exogenous aldehydes. ALDH1, ALDH2, and ALDH3 are detected in different regions of the human skin. ALDH1 and ALDH3 are predominantly localized in the epidermis and dermal appendages [117], while ALDH2 is localized in the epidermis and hair follicles of the human skin [120].

AR is a NADPH-dependent oxidoreductase enzyme that is involved in the reduction of a variety of aldehydes and carbonyls, including monosaccharides. Substrates for AR include isocortisol, isocorticosterone, aromatic aldehydes, arylalkylaldehydes, and so on [351]. It is primarily known to catalyze reduction of glucose to sorbitol, the first step in the polyol pathway of glucose metabolism. The second and last step in the pathway is catalyzed by sorbitol dehydrogenase, which helps in the oxidation of sorbitol to fructose [352]. The aldose reductase reaction, responsible for sorbitol production, is important for the function of various organs in the body. For example, fructose produced from sorbitol is used by the sperm cells and is also used as an energy source for glycolysis and glycconeogenesis [353]. Incidentally, AR is also linked with toxicities, e.g. there is persistent increase in glucose levels, like in diabetes. Being hyperosmotic, the presence of sorbitol increases pressure inside various organs and hence leads to retinopathy, nephropathy, neuropathy, and other diabetic complications [354–356]. Retinopathy is linked to the presence of AR in the mural cell of retina and lens in the eyes [357,358]. AR in the human small intestine is coded HSI-AR and is reported to catalyze the reduction of carotene [121]. In the human kidneys, AR mRNA is expressed in the papilla of epithelial, endothelial, interstitial, and thin limb cells, connecting tubules, cortical CDs, and medullary CD. Weak expression is reported in the outer medulla, cortex, Bowman's capsule, and the glomerular tuft [122]. This enzyme is also reported in the human brain and involved in catalysis of NADPH-dependent reduction of physiological and xenobiotic aldehydes [123].

MPO is a phase I peroxidase enzyme present in the lysosome of neutrophils and exists in high concentration in the lung [124]. MPO is involved in the activation of benzo(α)pyrene and aromatic amines in tobacco smoke to generate carcinogenic free radicals [124,359]. 4-Aminobenzoic acid hydrazide is a specific irreversible inhibitor of MPO [360].

8.4 FACTORS INFLUENCING EXPRESSION OF EXTRAHEPATIC DMEs

Genetic polymorphism, age, gender, pathophysiological conditions, inborn errors in metabolism, environmental pollutants, and food contribute to the expression and activity of DMEs.

8.4.1 Genetic Polymorphism

Genetic polymorphism is defined as differential expression of genes, that is, having multiple alleles in different populations. Polymorphism in genes may be inherited and/or occur because of environmental factors. The study of alleles provides information on differences in levels of enzymes, which also helps to predict interpopulation variation in susceptibility to diseases. Genetic polymorphism of CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 alter their activities, resulting in diminished efficacy or increased toxicity of certain drugs [361,362]. For example, poor metabolizing phenotype of CYP2D6 results in dose-dependent toxicity of debrisoquine, perhexilene, and phenacetin, while the extensive metabolizing phenotype leads to therapeutic failure of codeine and selected antidepressant drugs in different populations [363]. Alterations in genes including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, GSTM1, GSTT1, GSTP1, NAT, and SULT have the potential to contribute to toxicities, including metabolic activation of procarcinogens, leading to development of carcinogenicity in extrahepatic organs [364–366]. Table 8.2 summarizes studies on genetic polymorphism of DMEs in extrahepatic tissues, mainly with relevance to the development of cancer.

8.4.2 Age and Gender

The expression of DMEs, especially CYPs, is greatly influenced by both age and gender. Usually, extrahepatic expression of CYPs is lower in neonatal and pediatric tissues, relative to the adults, for example, CYP1A1 is not found in kidneys of neonates [403]. In the fetal duodenum, CYP3A4 activity is absent, and a statistically significant increase in expression was observed from two weeks to 17 years of age [404]. The expression of duodenal CYP3A4 is maximum in young females and decreases by around 20% in postmenopausal women [405]. In the kidney, CYP3A4 and CYP3A5 are present at gestational age of eight weeks [406]. MAO-B activity is low at birth, remains constant in childhood, and then increases with age [407]. The level of EHs in fetal lungs is comparatively lower than the adult lungs [408]. Table 8.3 lists additional instances where DMEs are higher in fetus and neonates than adults.

Among the examples of the influence of gender, CYP2E1 is less active in women than men, with no gender differences in activities of CYP1A2, CYP2C19, CYP2D6, and CYP3A4 [412]. However, Paine *et al.* [405] reported higher CYP3A4 expression in the small intestine of men than women. In different studies on smokers, women had higher expression of CYP1A1, CYP1A2, and CYP3A4 in the lung as compared to men [413–415]. Higher CYP3A4 expression was associated with the metabolism of major lung procarcinogens/carcinogens to generate reactive oxygen species (ROS) or reactive metabolites, thus exacerbating the susceptibility to carcinogenicity [415].

Both age- and gender-dependent differences have also been reported in the expression and activity of GSTs, with women having higher expression in the distal colon, as

TABLE 8.2 Relationship of Genetic Polymorphism of DMEs with the Development of Cancer in Extrahepatic Organs

Enzyme	Organ with Expression of Polymorphic Gene	Genotype/Allele	Outcome	Population (State/Country)	References
CYP1A1	Esophagus	<i>CYP1A1</i> exon 7 polymorphism(Val/Val)	Slightly increased risk of cancer	Not specified (Taiwan)	367
		<i>CYP1A1</i> <i>MspI</i> polymorphism	No association with the development of cancer	Not specified (Taiwan)	367
		<i>CYP1A1</i> (Ile/Ile)	No association with the development of cancer	Japanese	368
		<i>CYP1A1</i> <i>MspI</i> , exon7	No association with the development of cancer	Caucasian	365
	Stomach	<i>CYP1A1</i> <i>MspI</i>	No significant influence on the development of cancer	Chinese (Dalian)	369
		<i>CYP1A1</i> Val	Increased probability of cancer on smoking	Chinese (Yangzhong)	370
	Colorectal	<i>CYP1A1</i> Ile/Val	Significantly associated with the development of cancer	Hungarian (Hungary)	371
		<i>CYP1A1</i> <i>MspI</i> , Ile462Val	Not associated with the development of cancer	Not specified (State of Utah)	372
		<i>CYP1A1</i> *2B	Increased frequency of cancer development	Caucasian	373
	Lung	<i>CYP1A1</i> w1/m1, <i>CYP1A1</i> w2/m2	Increased risk of squamous cell carcinoma	Caucasian	374
		<i>MspI</i>	Increased susceptibility to cancer development on the exposure to environmental pollutants	Chilean	375
		<i>CYP1A1</i> *2 C, <i>CYP1A1</i> <i>MspI</i>	Increased cancer susceptibility	North Indian	376

(continued overleaf)

TABLE 8.2 (continued)

Enzyme	Organ with Expression of Polymorphic Gene	Genotype/Allele	Outcome	Population (State/Country)	References
CYP2E1	Esophagus	<i>CYP1A1 Msp I</i>	Increased risk of cancer development	South Indian	377
		<i>CYP1A1*2B (MspI and Ile462Val)</i>	Increased risk of cancer development	Caucasian	378
		<i>c1/c2, c2/c2</i>	No association with the development of cancer	Japanese	368
	Colorectal	<i>RsaI</i>	Decreased risk of cancer development	Not specified (Hawaii)	379
		<i>96-bp</i> insertion	Increased risk of cancer development	Not specified (Hawaii)	379
		<i>c2</i>	Increased risk of cancer development	Hungarian	380
		<i>RsaI</i>	No association with the development of cancer	Australian	381
		<i>c2/c2</i>	Increased susceptibility of the development of rectal cancer on interaction with environment, and on smoking and drinking of alcohol	Chinese	382
	Lung	<i>c1/c2</i>	Significantly associated with the development of cancer	Hungarian	371
		<i>RsaI</i>	No association with the development of cancer	Caucasian	383

		<i>RsaI</i>	No association with the development of cancer	African-American	383
		<i>RsaI</i>	Decreased risk of cancer development	Not specified (Hawaii)	384
		<i>RsaI</i>	Decreased risk of cancer development	Chinese	385
		<i>c</i> and <i>c2</i>	No correlation with the development of cancer	Chilean	375
		<i>RsaI</i> and <i>DraI</i>	Can be protective to cancer development	Caucasian	386
GSTM1	Esophagus	Null	No association with development of cancer	Japanese	368
	Stomach	Null	Increased risk of cancer development	Chinese (Yangzhong)	370
		Null	Increased risk of cancer development	Chinese	387
	Colorectal	Null	Increased risk of cancer development	Spanish	388
		Null	Increased risk of cancer development	Caucasian	373
	Lung	Null	Increased susceptibility to cancer development on exposure to environmental pollutants	Chilean	375
		Null	Increased susceptibility of cancer development	French Caucasian	386
		Null	No association with cancer risk	Caucasian	389

(continued overleaf)

TABLE 8.2 (continued)

Enzyme	Organ with Expression of Polymorphic Gene	Genotype/Allele	Outcome	Population (State/Country)	References
GSTT1	Urinary bladder	Null	Increased susceptibility of cancer development	African-American	389
		Null	Increased susceptibility of cancer development	North Indian	390
		Null	Increased susceptibility of cancer development	South Indian	391
		Null	Increased susceptibility of cancer development	Caucasian (Germany)	392
	Ovarian	Null	No evidence of correlation	Australian	393
	Stomach	Null	Increased in risk of cancer development	Spanish	388
	Lung	Null	No association with the development of cancer	Caucasian	389
	Lung	<i>SULT1A1*2</i>	Increased risk of lung cancer development	Caucasian	394
SULT	Urinary bladder	<i>SULT1A1</i>	Reduced risk of urinary bladder cancer development	Not specified	395
NAT1	Stomach	<i>NAT1*10</i>	Increased risk of gastric cancer development	Japanese	396
	Colorectal	<i>NAT1*10</i>	Polymorphism not significant risk factor for colorectal adenocarcinoma	Japanese	396
		<i>NAT1*10</i>	Increased risk of cancer development	Caucasian (Staffordshire area of England).	397

NAT2	Lung	<i>NAT1*10, NAT1*11</i>	Increased risk of cancer development	French Caucasian	398
		<i>NAT1*3, NAT1*4</i>	Increased risk of cancer development	French Caucasian	398
		<i>NAT1*14, NAT1*15</i>	Increased risk of cancer development	French Caucasian	398
	Urinary bladder	<i>NAT1*10</i>	Increased risk of cancer development	Australian	399
		<i>NAT1*10</i>	Decreased risk of cancer development	Caucasian	400
	Colorectal	<i>NAT1*10</i>	No significant association found between polymorphism and gastric/colorectal cancer development	Japanese	396
	Lung	Not specified	No significant association found between polymorphism and lung cancer development	Not specified (France)	398
	Urinary bladder	<i>NAT2*4</i>	Decreased risk of cancer development	German	400
	EH	Combined <i>Exon 3</i> and <i>4</i> variant allele	Decreased risk of cancer development due to reduced EH activity	African-American	401
		Combined <i>Exon 3</i> and <i>4</i> variant allele	Not much promising results for reduced risk of cancer development	American Caucasian	401
		<i>Exon 3</i> variant allele	Provides protective effect against cancer development	Non-hispanic whites	402
		<i>Exon 4</i> variant allele	Increased risk of cancer development	Non-hispanic whites	402

TABLE 8.3 Examples of Higher Expression of DMEs in Early Years of Human Life as Compared to Adulthood

Enzyme	Age Group	Organ(s)	Anticipated Outcome	References
CYP1A1	Fetus	Brain	—	409
	8 and 21 weeks gestational age	Lung	—	403
CYP1B1	Fetus	Kidney, thymus, and lung	—	403,409,410
CYP2B6	Neonates	Kidney	Renders neonates more vulnerable to ifosfamide induced nephrotoxicity	404
CYP2R1	Fetus	Brain	—	409
CYP2W1	Fetus	Lung and kidney	—	409
CYP3A4	Neonates	Kidney	Renders neonates more vulnerable to ifosfamide-induced nephrotoxicity	404
GSTP1	Fetus and neonates	Lung	—	411
GSTA1/A2	20 Weeks gestational age	Kidney (nephron)	—	411
SULT1A3	Fetus (18–25 weeks)	Kidney	—	411
FMO1	Fetus	Brain	—	86

compared to men [264]. In this study, the GST activity increased with age in women, while it remained constant in men. Reversibly, expression of GSTM1 did not change in women with age but decreased in men on aging. Also, expression of GSTP1 in distal and proximal colon of women (50–70 years) was higher than men and women below 50 years.

The expression of ADHs in gastric mucosa of women has been reported to be less than that of men. ADH activity decreased in men with increasing age, while values in women between 41 and 60 years of age were higher than those between 20–40 and 61–80 years. In men of 40–50 years, gastric ADH level decreased significantly and became almost similar to that of women of the same age. There was reduced ADH activity in men between 20 and 40 years, who consumed large quantities of alcohol [416,417].

8.4.3 Pathophysiological Conditions and Inborn Errors of Metabolism

The expression of DMEs in extrahepatic organs can be altered by disease states, and certain diseases such as cancer are provoked because of alterations in the enzyme levels. Inborn errors of metabolism result in expression of inactive enzymes [418]. CYPs may be downregulated because of (i) inflammation in response to tissue damage, (ii) infection, (iii) burns, (iv) trauma, (v) tumors, and (vi) autoimmune diseases. Infectious agents, such as hepatitis A, influenza A/B, herpes simplex, HIV, helicobacter pylori, plasmodium falciparum, and so on, also modulate CYP-dependent biotransformation of drugs in humans [418]. Table 8.4 lists disease conditions and inborn errors

TABLE 8.4 Changes in the Expression of Enzymes in Extrahepatic Organs as a Result of Pathophysiological Conditions and Inborn Errors of Metabolism

Organ(s)	Disease/Infecting Agent/Inborn Error of Metabolism	Enzyme	Expression of mRNA/Proteins	References
Esophagus	Squamous cell carcinoma	CYP1A1, CYP1A2, CYP2E1, CYP1B1, CYP2C8, and CYP2C9	Expressed only in tumor cells	419–421
		CYP2B6 and CYP3A5	Increase	419–421
		CYP3A4	Decrease	419
	Adenocarcinoma	CYP2J2	Expressed only in tumor cells	419
Stomach	Foveolar epithelium with intestinal metaplasia of stomach	CYP3A4	Increase	422
	Gastric carcinoma	CYP1B1 and CYP2J2	Expressed only in tumor cells	419,420
		CYP2W1	Increase	419,420
	Chronic gastritis (caused by <i>Helicobacter pylori</i>)	ADH	Decrease	423,424
Small and large intestine	Celiac disease	CYP3A	Decrease	425–427
		CYP1A	Decrease	427
	Crohn's disease	CYP3A4 and CYP3A5	Increase	425
	Inflammatory bowel disease	CYP3A4 and CYP3A5	Increase	419
	Colon adenocarcinoma	She	Decrease	428
		CYP1B1	Expressed only in tumor cells	419,420
		CYP2J2 and CYP3A5	Increase	419,420
	Colorectal carcinoma	CYP2A6, CYP2B, CYP2F1, and CYP2W1	Decrease	419
		CYP2S1 and CYP2U1	Increase	419
	Colorectal adenomatous carcinoma	CYP2C8	Increase	419
	Ulcerative colitis	CYP3A4, CYP2C9, and CYP3A7	Dysregulation	425
	<i>Taenia taeniformis</i> infection	CYP1A1 and CYP2B1	Increase	418

(continued overleaf)

TABLE 8.4 (*continued*)

Organ(s)	Disease/Infecting Agent/Inborn Error of Metabolism	Enzyme	Expression of mRNA/Proteins	References
Nasopharynx	Nasopharyngeal carcinoma	CYP1B1	Increase	419
Lung	Non-small-cell lung cancer	CYP1A1 and CYP2B7	Decrease	419
	A549 adenocarcinoma cell lines	CYP1A1	Decrease	419
	Bronchioloalveolar carcinoma	CYP1A1	Decrease	419
	Pulmonary squamous cell carcinoma	CYP1B1 and CYP2J2	Expressed only in tumor cells	419,420
	Lung carcinoma	CTP2W1	Expressed only in tumor cells	419
		CYP3A7 and CYP4B1	Decrease	419
	Respiratory viral infection	CYP1A	Decrease	429
	Squamous cell lung cancer	ALDH1A1 and ALDH3A1	Increase	348
Kidney	Renal cell carcinoma	CYP1A1, CYP3A1, CYP3A2, and CYP3A5	Increase	31
		CYP1B1	Increase	420
		γ -GT	Increase	31
		Quinone oxidoreductase	Decrease	31
		sEH, CYP2C9, CYP2C8, and CYP2J2	Decrease	428
	Clear cell carcinoma, transitional cell carcinoma	CYP1B1	Expressed only in tumor cells	419
	Uremia	CYP2D6	Decrease	31
	Adenocarcinoma	CYP4A2	Decrease	31
	Acute renal failure	γ -GT	Increase	31
	Diabetes mellitus	UGT2B7	Decrease	430
Bladder	Bladder carcinoma	CYP2D6 and CYP4B1	Increase	419
		CYP2W1	Decrease	419
	Transitional cell carcinoma	CYP1B1	Expressed only in tumor cells	419,420
Brain	Glial cell tumor	CYP1B1	Expressed only in tumor cells	419
	Alzheimer's disease	CYP27 and CYP46	Redistribution	15
	Parkinson's disease	CYP2D6	Decrease	15

Heart	Inflammation followed by ischemic brain injury (astrocytes of hippocampus)	CYP2E1	Increase	429,431
	Inflammation due to trauma-evoked head injury	CYP1A1	Decrease	429,431
		CYP4F4 and CYP4F5	First decrease and then increase after two weeks	418
	Dilated cardiomyopathy	CYP1A1	Detected in right ventricle, ascending aorta, pulmonary aorta, and right atrium. In normal patients, it is detected only in left ventricle	11,12
		CYP2B6 and CYP2B7	Detected in right ventricle and aorta	11
		CYP4B1	Detected in right ventricle	11
		CYP2D6	Detected only in right ventricle	11
		CYP2C8 and CYP2C9	Induced following ischemic injury	432
	Different organs	CYP2D6	Decrease	418
	Inborn errors of metabolism with effect on different organs	CYP11A1, CYP11B1, CYP11B2, CYP17, and CYP21	Autosomal recessive disorder	3
	Vitamin-D-dependent rickets (bones)	CYP27B1 and 25-hydroxy D ₃ 1 α -hydroxylase	Autosomal recessive disorder	3
	Primary congenital glaucoma (eyes)	CYP1B1	Autosomal recessive eye disorder (deletions, insertions)	3
	Sjögren–Larsson syndrome (skin and nervous system)	ALDH10	Autosomal recessive disorder	3

of metabolism, which can alter expression of DMEs and defects in enzyme genes, respectively.

8.4.4 Food

Another category that influences expression of CYPs in various extrahepatic organs is food. For example, chronic intake of char-grilled meat induces CYP1A1 in the intestine, and this is linked with potentiating the risk of intestinal cancer [433]. The nature of food also affects GST expression, for example, GSTA and GSTP were induced in duodenum on intake of vegetables and GSTT1 in gastric mucosa was induced on the consumption of certain fruits. Citrus fruits induced GST activity in the rectal mucosa, and a brassica vegetable also leads to induction of GSTM1 [434]. However, no reports exist on food-dependent changes in colonic GST activity. A majority of commonly used food products, such as potato, onion, garlic, spices, rice, and bread, do not change DME activities significantly [58].

8.5 ROLE OF EXTRAHEPATIC DMEs IN BIOACTIVATION AND DEACTIVATION OF XENOBIOTICS

DMEs in extrahepatic organs play an important role in bioactivation of drugs/prodrugs/precancerogens and/or deactivation of drugs/metabolites/carcinogens. For example, terfenadine (a nonsedating antihistaminic prodrug) is metabolized by CYP3A4 in the intestine, to the active moiety, fexofenadine (Fig. 8.4). The active metabolite acts on H₁ receptors at different sites in the body, resulting in pharmacological activity [198]. Similarly, metabolism of cyclophosphamide is directly responsible for its therapeutic activity and also for toxicity. The initial activation step involves oxidation of the heterocyclic ring to produce 4-hydroxycyclophosphamide, which is in spontaneous equilibrium with its cyclic tautomer aldophosphamide. Acrolein is spontaneously eliminated from aldophosphamide to yield phosphoramidate mustard, an active alkylating agent. 4-Hydroxycyclophosphamide, acrolein, and phosphoramidate mustard in the presence of CYPs and other oxidative enzymes are responsible for the pharmacological activity as well as toxicity of the drug (Fig. 8.5) [435]. Another example is of sulindac, a sulfoxide prodrug, which is metabolized in kidney into inactive sulfone and bioactive sulfide (Fig. 8.6). The formation of

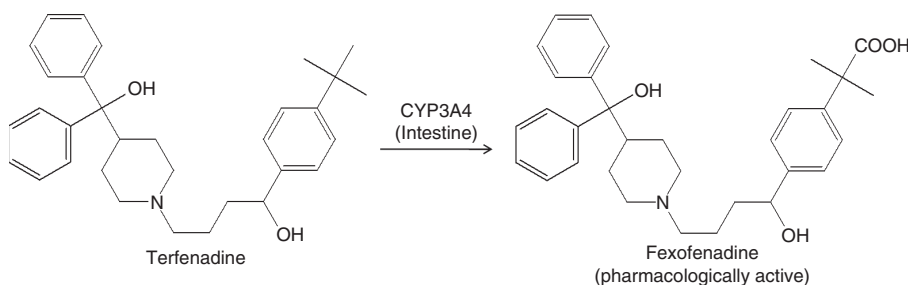


Figure 8.4 Bioactivation of terfenadine in the GIT.

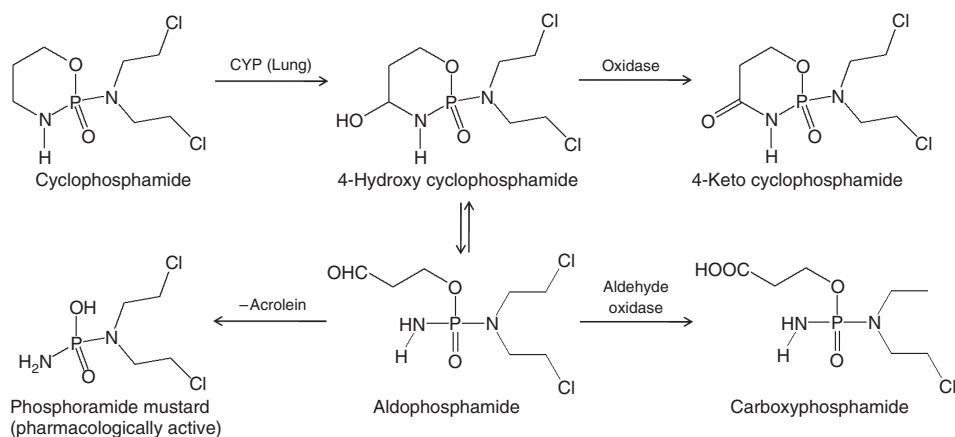


Figure 8.5 Metabolism of cyclophosphamide in the lung.

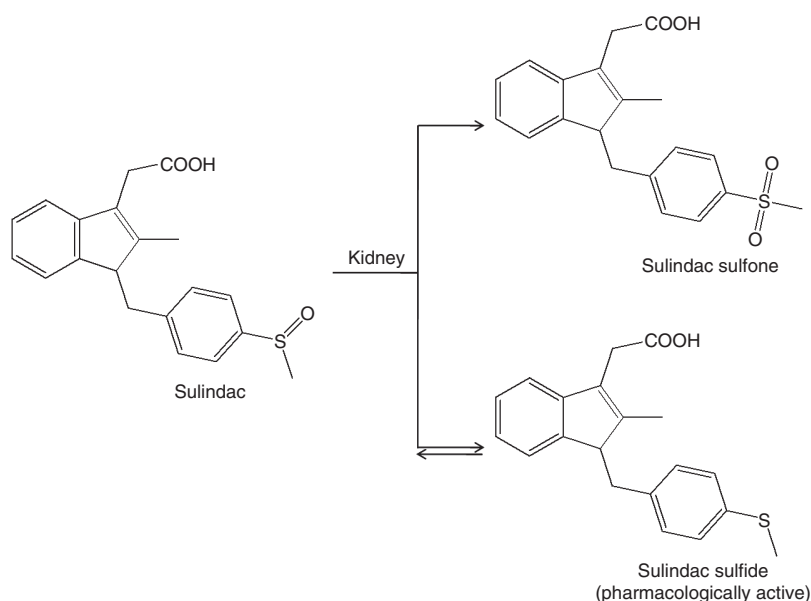


Figure 8.6 Metabolism of sulindac in the kidneys [436].

the latter is reversible, therefore, the kidney rapidly interconverts active sulfide back to inactive sulfoxide [436]. The reaction is reported to be mediated in humans by kidney microsomal FMOs [437]. Similarly, codeine is metabolized into morphine by CYP2D6 in the brain (Fig. 8.7), which is responsible for the analgesic effect of the former [438]. Additional examples of bioactivation and deactivation of drugs in GIT, lungs, kidneys, and brain, along with their metabolites, are listed in Table 8.5.

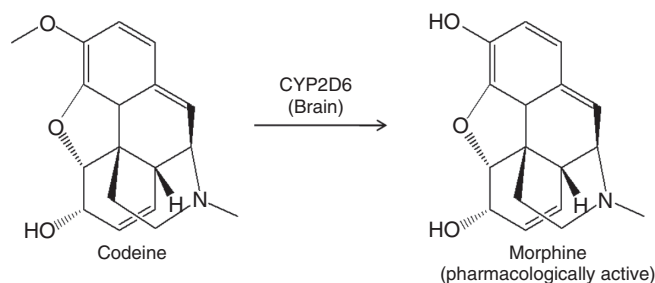


Figure 8.7 Metabolic bioactivation of codeine in the brain.

8.6 SIGNIFICANCE OF EXTRAHEPATIC METABOLISM

The metabolism of drugs in extrahepatic organs results in alteration of systemic bioavailability and toxicity. Inhibition/induction of DMEs in extrahepatic tissues and organs by the drugs can alter pharmacokinetic/pharmacodynamics of other concomitantly administered drugs. These effects have significant impact on the overall clinical outcome of the therapy.

8.6.1 Bioavailability

The therapeutic action of a drug is determined by the dose achieved at the target site in the body. The latter in turn is determined by the amount of the substance absorbed, which further depends on its bioavailability. The bioavailability of drugs can be limited by their absorption from the GIT epithelium and/or through first-pass metabolism. Although the major site for the first-pass metabolism is liver, GIT is a significant contributor, as it presents a very large area for the metabolism, with numerous DMEs being expressed along its length. For example, Marier *et al.* [543] explored the contribution of rabbit liver and intestine on the elimination rate of different enantiomers of propranolol. At a concentration of $5.8 \mu\text{M}$, *R*(+) propranolol was metabolized faster in the intestine in comparison to the liver. Likewise, the bioavailability of inhaled drugs is significantly affected by their metabolism in the lung. In addition, factors such as age, sex, food, disease state, and interindividual variation were found to influence the bioavailability of drugs and xenobiotics, owing to increase or decrease in the expression of DMEs [544,545].

8.6.2 Toxicity

Drug-induced organ toxicity is not only dose-dependent but also a result of the generation of reactive metabolites, which include electrophiles and/or free radicals. The latter have the potential to interact with nucleophiles, such as DNA and proteins. The mechanism of reactive metabolite-induced toxicity is shown in Fig. 8.8 [546]. Toxicity can be significantly alleviated as a result of the binding of reactive metabolites to GSH, but once most of the GSH gets consumed, reactive metabolites exceed a limit, leading to significant toxic effects.

The wide distribution of DMEs in different organs provides the possibility for generation of reactive metabolites across the body and hence, the widespread toxicity.

TABLE 8.5 Examples of Drugs that Undergo Bioactivation and Deactivation in Extrahepatic Tissues

Organ	Substrate	DME	Metabolic Product(s)	Outcome/Significance	References
Gastrointestinal tract	Chlorpromazine	CYP	<i>S</i> -oxide, <i>N</i> -oxide, 7-hydroxyl, desmethyl, and didesmethyl chlorpromazine	Deactivation and first-pass metabolism	439,440
	Carbamazepine	CYP	10,11-Epoxy and 10,11- <i>trans</i> dihydrodiol carbamazepine	Deactivation and first-pass metabolism	441,442
	Astemizole	CYP	<i>O</i> -Desmethyl astemizole	Deactivation and first-pass metabolism	145
	Benzo(α)pyrene	CYP1A1	Benzo(α)pyrene-7,8-dihydrodiol	DNA adduct formation	443
	Astemizole	CYP2J2	Astemizole <i>O</i> -demethylation	First-pass metabolism	145
	Alprazolam	CYP3A4	Alprazolam-4-hydroxylation	Deactivation and first-pass metabolism	444,445
	Terfenadine	CYP3A4	Fexofenadine	Bioactivation	198
	Indinavir	CYP3A4	Oxidative metabolite	Deactivation	446
	Simvastatin	CYP3A4	—	Deactivation and first-pass metabolism	442
	Buspirone	CYP3A4	—	Deactivation and first-pass metabolism	442
	Carvedilol	CYP3A4	Oxidative metabolite	Biotransformation	447
	Diazepam	CYP3A4	—	Deactivation and first-pass metabolism	442
	Sirolimus	CYP3A4	Secorapamycin	Biotransformation	448
	Felodipine	CYP3A4	—	Deactivation and first-pass metabolism	442
	Clindamycin	CYP3A4	Clindamycin sulfoxide	Biotransformation	449
	Saquinavir	CYP3A4	—	Deactivation and first-pass metabolism	442
	Nicardipine	CYP3A4	—	Deactivation and first-pass metabolism	442
	Flurazepam	CYP3A4	Mono- and didesetyl flurazepam	Deactivation and first-pass metabolism	450
	Midazolam	CYP3A4 and CYP3A5	1'-Hydroxymidazolam	Deactivation and first-pass metabolism	444,451,452
	Verapamil	CYP3A4	—	Deactivation and first-pass metabolism	442
	Nifedipine	CYP3A4	Oxidized nifedipine	Deactivation and first-pass metabolism	444,453,454
	Nisoldipine	CYP3A4	BAY o 3199 and BAY r 9425	Biotransformation	455

(continued overleaf)

TABLE 8.5 (continued)

Organ	Substrate	DME	Metabolic Product(s)	Outcome/Significance	References
	Lovastatin	CYP3A4	6' β -Hydroxy and 6'-exomethylene lovastatin	Deactivation and first-pass metabolism	456,457
	Fentanyl	CYP3A4	<i>N</i> -Dealkylated fentanyl	Deactivation and first-pass metabolism	458
	Cyclosporine	CYP3A4	Hydroxylated and <i>N</i> -methylated cyclosporine	Deactivation and first-pass metabolism	459
	Tacrolimus	CYP3A4	13- <i>O</i> -Demethyl and 13,15- <i>O</i> -demethyl tacrolimus	Deactivation and first-pass metabolism	460
	Methadone	CYP3A4	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, 2-ethyl-5-methyl-3,3-diphenylpyraline	Deactivation	461
	L- α -Acetylmethadol	CYP3A4	L- α -Acetyl- <i>N</i> -normethadol, L- α -acetyl- <i>N,N</i> -dinormethadol	Bioactivation	461
	Ebastine	CYP3A4	Desalkylebastine	Deactivation	462
	Ritonavir	CYP3A4	<i>N</i> -Demethylation, oxidation of methylthiazolyl, hydroxylation of isopropyl side chain, and 2-(1-methylethyl)thiazolylmethyl group	Biotransformation	463
	Pravastatin	CYP3A4 and CYP3A5	3' α , 5' β , 6' β -Trihydroxypravastatin, sulphated pravastatin, 3' α -iso-pravastatin.	Deactivation and first-pass metabolism	456
	Oxycodone	CYP3A4 and CYP3A5	<i>N</i> -Demethyled oxycodone	Deactivation and first-pass metabolism	464
	Prasugrel	CYP3A and carboxylesterase 2	Thiol-containing active metabolites	Bioactivation	465

Quinidine	CYP3A62	3-Hydroxy quinidine	Deactivation and first-pass metabolism	466
Amiodarone	CYP3A1, CYP3A2, and CYP1A1	Desethylamiodarone	Deactivation and first-pass metabolism	467
Benzamidoxime	Reductase	Benzamidine	—	468
Guanoxaben	Reductase	Guanabenz	—	468
Ethanol	ADH	Acetaldehyde	First-pass metabolism	469
Polychlorinated biphenylol	UGTs	Glucuronide conjugate	Deactivation	470
Bisphenol A	UGTs	Glucuronide conjugate	Biotransformation	471
Galangin	UGTs	Galangin glucuronide	Deactivation	472
Kaempferol	UGTs	Kaempferol glucuronide	Deactivation	472
Indomethacin	UGTs	Indomethacin glucuronide	Deactivation	473
Paracetamol	UGTs	Glucuronide conjugate	Deactivation	474
Salbutamol	UGTs	Glucuronide conjugate	Deactivation	462
Salmeterol	UGTs	Glucuronide conjugate	Deactivation	462
Bambuterol	UGTs	Glucuronide conjugate	Deactivation	462
Formoterol	UGTs	Glucuronide conjugate	Deactivation	462
Fenoterol	UGTs	Glucuronide conjugate	Deactivation	475
Losartan	UGTs	Glucuronide conjugate	Deactivation	476
1-Naphthol	UGTs	Glucuronide conjugate	Deactivation	477
Biochanin A	UGT	Glucuronide conjugate	Deactivation	478
Raloxifene	UGT	Glucuronide conjugate	Deactivation	478
Scutellarin	UGTs and β -glucuronidase	Scutellarein	Bioactivation	479
Baicalein	UGT and SULT	Glucuronidation and sulfation of baicalein	Biotransformation	480
Clioquinol	SULT1A3	Sulphated clioquinol	Biotransformation	481

(continued overleaf)

TABLE 8.5 (continued)

Organ	Substrate	DME	Metabolic Product(s)	Outcome/Significance	References
	Fenthion	FMO1	Fenthion sulfoxide	Biotransformation	325
	Methiocarb	FMO1	Methiocarb sulfoxide	Biotransformation	325
	Cefpodoxime proxetil	Choline esterases	Cefpodoxime	Bioactivation	482,483
	<i>O</i> -Isovaleryl- propranolol	Esterase	Propranolol	Bioactivation	484
	L-751,164 (prodrug of potent fibrinogen receptor antagonist)	Esterase	L-742,998 (active drug)	Bioactivation	485
	Glycovir	Esterase	SC-48334 (active metabolite)	Bioactivation	486
	Timolol maleate	Esterase	Timolol	Bioactivation	487
	Levobunolol	Ketone reductase	Dihydrolevobunolol	—	487
	5-Aminosalicylic acid	NAT	<i>N</i> -Acetyled-5-aminosalicylic acid	Deactivation	488
	Ambamide	Amine oxidase	<i>p</i> -Carboxybenzene sulphonamide	Deactivation	489
	Isoprenaline	COMT	<i>O</i> -Methyl isoprenaline	Deactivation and first-pass metabolism	490,491
	Terbutaline	Not defined	Sulphate conjugate	Deactivation and first-pass metabolism	492
	3-Chloro-1,2- propanediol fatty acid esters	Not defined	Hydrolytic metabolites	Detoxification	493
	Halofantrine	Not defined	Desbutylhalofantrine	Bioactivation	494
	Daidzein	Not defined	Equol	Bioactivation	495
	Cycloastragenol	Not defined	Glucuronide conjugates and oxidative metabolites	Deactivation and first-pass metabolism	496
	Hesperetin	Not defined	Hesperidin	Deactivation and first-pass metabolism	497

Pulmonary system	Enalapril	Not defined	Enalaprilat	Bioactivation	498
	Cyadox	Not defined	Cyadox-1-monoxide	Biotransformation	499
	Phorate	CYP	(+) Phorate sulfoxide	Detoxification	324,500
	Nilutamide	CYP	Amine and hydroxylamine metabolite	Reactive metabolite generation	501
	Benzo(α)pyrene	CYP1A1	Benzo(α)pyrene-7,8-dihydroepoxide	Metabolic activation by epoxide hydroxylase and CYP1B1 followed by the formation of DNA adducts, leading to lung cancer	502,503
	NKK	CYP1A1	Keto aldehyde (major), keto alcohol (minor)	Carcinogenic product	20
	3-Methylindole	CYP1A1, CYP2F1, CYP2A13, and CYP4B1	3-Methyleneindolenine	Alkylates DNA and induces cell death through apoptosis	504
	Benzo(α)pyrene	CYP1B1	Benzo(α)pyrene-7,8-dihydroepoxide	Carcinogenic product leading to mutation in <i>p53</i> gene in lung	503
	Benzo(α)pyrene-7,8-dihydroepoxide	EH	($\pm$)-Benzo(α)pyrene- <i>trans</i> -7,8-dihydrodiol	—	503
	($\pm$)-Benzo(α)pyrene- <i>trans</i> -7,8-dihydrodiol	CYP1B1	Benzo(α)pyrene-7,8-dihydrodiol-9,10-epoxide	Carcinogenic product leading to mutation in <i>p53</i> gene in lung	503

(continued overleaf)

TABLE 8.5 (continued)

Organ	Substrate	DME	Metabolic Product(s)	Outcome/Significance	References
	AFB1	CYP2A13	AFB1-8,9-epoxide	Mutation of <i>p53</i> and then inactivation leading to cancer, DNA and protein adducts	10,505,506
	NKK	CYP2A6	Keto alcohol, keto aldehyde	Carcinogenic product	20
		CYP2A13	Keto alcohol (major), keto aldehyde (minor)	Carcinogenic product	20
		CYP2B6	NKK- <i>N</i> -oxide	Detoxification process of NKK in human lung	507
	1,1-Dichloro-ethylene	CYP2E1	2,2-Dichloro-acetaldehyde, 1,1-dichloroethylene-epoxide	Covalent binding with proteins/DNA, causes Clara cell toxicity	508,509
	Butadiene	CYP2E1	Butadiene oxide	Not defined	159
	Chlorzoxazone	CYP2E1	6-Hydroxylated chlorzoxazone	Not defined	159
	<i>N</i> -Nitrosodimethylamine	CYP2E1	Demethylated <i>N</i> -nitrosodimethylamine	Not defined	159
	<i>p</i> -Nitrophenol	CYP2E1	Hydroxylated <i>p</i> -nitrophenol	Not defined	159
	Trichloroethylene	CYP2E1 and CYP2F	Chloral hydrate	Clara cell toxicity	508
	Styrene	CYP2F	Epoxide styrene	Carcinogenic product	172
	Naphthalene	CYP2F	Naphthalene 5,6-epoxide, naphthalene 7,8-epoxide	Carcinogenic product	510
	Benzene	CYP2E1, CYP2B1, and CYP2F	Benzene oxide	May be responsible for the generation of tumors	511
	Nifedipine	CYP3A	Oxidative metabolite	Not defined	159
	4-Ipomenol	CYP4B1	Epoxide	DNA/protein covalent binding	512,513
	Benzamidoxime	Reductase	Benzamidine	—	468
	Guanoxaben	Reductase	Guanabenz	—	468

Urinary system	Phorate	FMO2	(–) Phorate sulfoxide	Detoxification	500
	Paracetamol	CYP2E1	NAPQI	Necrosis	514
	Cyclosporine A	CYP3A5	Hydroxylated cyclosporine (AM9)	Deactivation	178,515
	Benzamidoxime	Reductase	Benzamidine	—	468
	Guanoxaben	Reductase	Guanabenz	—	468
	Niflumic acid	UGT2B7	Glucuronide conjugate	Deactivation	516
	Paracetamol	UGT	Glucuronide conjugate	Deactivation	517
	Zidovudine	UGT	Glucuronide conjugate	Deactivation	518
	Almokalant	UGT	Glucuronide conjugate	Deactivation	519
	Diflunisal	UGT	Glucuronide conjugate	Deactivation	520
	Trichloroethylene	GST	<i>S</i> -(1,2-Dichlorovinyl)glutathione	Detoxification	521
	(<i>R</i>)- or (<i>S</i>)-2-bro- moisovalerylurea	GST	Glutathione conjugation	Detoxification	522
	<i>S</i> -(6-Purinyl) glutathione	GGT	<i>S</i> -(6-Purinyl)-L-cysteine	Bioactivation	523
	<i>S</i> -(6-Purinyl)-L- cysteine	β-Lyase	6-Mercaptopurine	Bioactivation	523
	6-Mercaptopurine	Xanthine oxidase	6-Thioxanthine	Deactivation	523
	MeDDC ^a	FMO1	MeDDC sulfine, MeDTC, MeDTC sulfoxide	Bioactivation	524
	Sulindac sulfide	FMO1	Sulindac sulfoxide	Deactivation	329,436
	Paracetamol	FMO1	Sulfate conjugate	Deactivation	517,525
	Fenthion	FMO1	Fenthion sulfoxide	Biotransformation	325
	Methiocarb	FMO1	Methiocarb sulfoxide	Biotransformation	325
	Methionine	FMO3	Methionine sulfoxide	—	328
	Tropisetron	FMO	<i>N</i> -Oxide metabolite	Biotransformation	86,333
	Meperidine	FMO	<i>N</i> -Oxide metabolite	Biotransformation	86,333

(continued overleaf)

TABLE 8.5 (continued)

Organ	Substrate	DME	Metabolic Product(s)	Outcome/Significance	References
Central nervous system	Nicotine	CYP2B1, CYP2A6, and CYP2B6	Cotinine	Deactivation	21,526,527
	Selegiline	CYP2B6 and CYP2C19	L-Methamphetamine, L-amphetamine, desmethylselegiline	Deactivation	185
	Imipramine	CYP2C18	N-Demethylated imipramine, imipramine-N-oxide	Deactivation	528
	Alprazolam	CYP3A43	α -Hydroxylated alprazolam	Biotransformation	194,529
	Imipramine	CYP4F6	10-Hydroxylated imipramine	Deactivation	528
	Codeine	CYP2D6	Morphine	Deactivation	438
	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine	CYP2D6	—	Detoxification	180
	Benzamidoxime	Reductase	Benzamidine	—	468
	Guanoxaben	Reductase	Guanabenz	—	468
	Propofol	UGT1A6	Propofolglucuronide	Deactivation	231
	Capsaicin	CYP	Vanillylamine, vanillic acid	Biotransformation	530
	Hesperetin	UGT	Hesperetin glucuronide	Biotransformation	531
	Fluroxypyr methyl ester	Esterase	Fluroxypyr	Acid metabolite	204
Skin	Fluroxypyr methylheptyl ester	Esterase	Fluroxypyr	Acid metabolite	204
	Amethocaine	Esterase	p-(Butyl)aminobenzoic acid	Biotransformation	532
	O-Acyl haloperidol ester	Esterase	Haloperidol	Bioactivation	533
	Acyclovir valerate	Esterase	Acyclovir	Bioactivation	534

	Sulfamethoxazole	NAT	<i>N</i> -Hydroxy and <i>N</i> -acetyl metabolites	Bioactivation	535
	Paraphenylene-diamine	NAT1	Monoacetyl paraphenylenediamine	Biotransformation	56
	Benzocaine	NAT	<i>N</i> -Acetylbenzocaine	Biotransformation	536
	Phorate	FMO	Phorate sulfoxidation	Biotransformation	337
	Triclosan	UGT	Glucuronide conjugate	Biotransformation	537
	Triclosan	SULT	Sulfate conjugate	Biotransformation	537
	Minoxidil	Phenol sulfotransferase	Minoxidil sulfate	Bioactivation	292,538
	Propranolol	Microsomes	4'-Hydroxypropranol	Biotransformation	539
	Theophylline	Microsomes	1,3,7-Trimethyluric acid and 1,3-dimethyl uric acid	Biotransformation	540
	Prednisolone farnesylate	Not specified	Prednisolone	Bioactivation	541
	TU-2100 [bis(<i>o</i> -carboxyphenyl ethyl ester) nonanedioate]	Not specified	—	Bioactivation	542
CVS	Glyceryl trinitrate	CYP3A4	NO	Biotransformation	16
	Isosorbide Dinitrate	CYP3A4	NO	Biotransformation	16
	Verapamil	CYP3A4,	Carbinolamine, <i>N</i> -formyl,	Biotransformation	92
		CYP3A5, CYP2C8, CYP2C18, CYP2D6 and CYP2E1	ahemiacetale, <i>N</i> -demethyl, <i>O</i> -demethylverapamil		

Abbreviations: AFB1, Aflatoxin B1; MeDDC, *S*-methyl *N,N*-diethyldithiocarbamate; MeDTC, *S*-methyl *N,N*-diethylthiocarbamate; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; NKK, 4-methylnitrosamino)-1-(3-pyridyl)-1-butanone.

^aDisulfiram metabolite generated in liver.

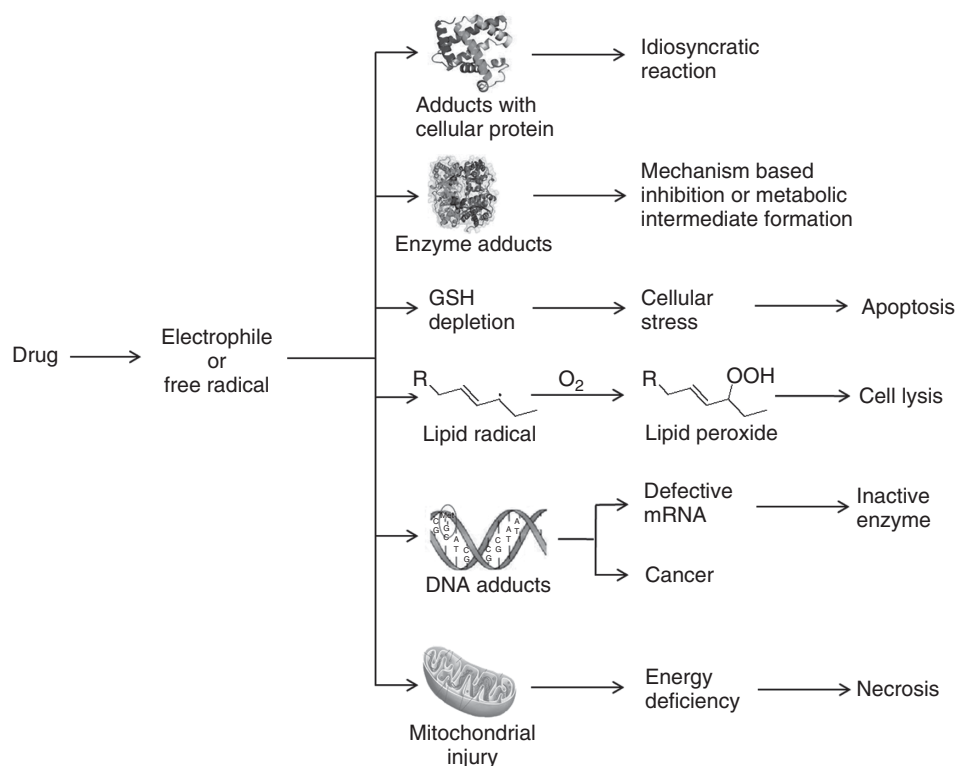


Figure 8.8 Mechanism of reactive metabolite-induced toxicity. (See color insert.)

Table 8.6 summarizes the nature of toxicity shown by different xenobiotics in extra-hepatic organs, with indications on the mechanisms involved.

Since the GIT is rich in different DMEs, orally administrated drugs can be metabolized, which may lead to the generation of reactive metabolites and subsequent toxicity. For example, acrylonitrile, a dietary carcinogen, is metabolized to form reactive epoxide in intestine by CYP2E1 and to a lesser extent by CYP2B1. This metabolite is responsible for the generation of toxicity in the intestine by three pathways: (i) direct binding with nucleophilic moieties, such as DNA and proteins, and the release of cyanide (CN^-); (ii) hydrolysis followed by the release of CN^- ; and (iii) interaction with sulfhydryl compounds (GSH and/or *N*-acetylcysteine) with associated release of CN^- (Fig. 8.9). This conversion of acrylonitrile to CN^- group is enhanced by phenobarbital, BNF, 4-methylpyrazole, or ethanol [547].

Like GIT, the lung is also susceptible to toxicity because of the formation of reactive metabolites. Lungs are directly exposed to air, so these come in direct contact with pollutants. Owing to abundant oxygen in lung, environmental contaminants have the potential to undergo redox reactions, resulting in the formation of oxygen superoxide radicals that can result in toxicity to the pulmonary system [580]. The lung is additionally exposed to drugs and xenobiotics through extensive cardiac output that circulates through them. Xenobiotics, such as 4-ipomeanol and 3-methylindole, are metabolized in different tissues and subsequently transported to lung through blood

TABLE 8.6 Toxic Events Attributed to Different Xenobiotics in Extrahepatic organs, Including the Mechanisms Involved

Organ	Xenobiotics	Enzyme	Reactive Metabolite	Mechanism	Outcome	References
Gastrointestinal tract	Acrylonitrile	CYP2E1 and CYP2B1	Epoxide intermediate	Can bind directly to nucleophilic moieties or release reactive CN ⁻	Carcinogenicity	547
	5-Fluorouracil	—	—	—	Apoptosis or cell cycle arrest in intestinal epithelial cells	548
Gastrointestinal tract + pulmonary system	Benzo(α)pyrene	CYP1A1, CYP1B1, CYP2C, CYP3A4, AKR1A1 AKR1C1-1 C4, and EH	Epoxide, quinone metabolites	Electrophile and free radical generation	Form covalent adducts with DNA, resulting in mutation of <i>p53</i> gene, leading to lung carcinogenicity	7,373,502,503, 549–551
	Aromatic and heterocyclic amines	CYP1A2, CYP1A1, CYP1B1, NAT1, NAT2, SULT1A1, SULT1A2, and SULT1A3	Nitrenium ion	N-Hydroxylation, sulfonation metabolism	DNA adduct formation, resulting into carcinogenicity	55,373,552
Pulmonary system	Naphthalene	CYP2F2	1 <i>R</i> , 2 <i>S</i> - to 1 <i>S</i> , 2 <i>R</i> -epoxide metabolite	Oxidative metabolism	GSH depletion, adduct formation cellular proteins, nonciliated airway epithelial cell injury	553–556

(continued overleaf)

TABLE 8.6 (continued)

Organ	Xenobiotics	Enzyme	Reactive Metabolite	Mechanism	Outcome	References
	4-Ipomeanol	CYP1A2, CYP2B7, CYP3A3, CYP3A4, and CYP2F1	Epoxide intermediate	Oxidative metabolism	Edema, congestion, and hemorrhage	513,557,558
	3-Methylfuran	CYP	—	—	Bronchiolar alkylation and necrosis of nonciliated bronchiolar cells (Clara cells)	559
	Bromobenzene	CYP	Bromobenzene-3,4-oxide	—	GSH depletion, cellular damage, and lipid peroxidation	560–562
	3-Methylindole	CYP1A1, CYP2A13, CYP2F1, and CYP4B1	Epoxides and free radical intermediates	Oxidative metabolism	Covalent binding to proteins and nucleic acids or stimulation of lipid peroxidation leads to alveolar type 1 and nonciliated bronchiolar epithelial cells injury	504,563,564
	Butylated hydroxy-toluene	CYP2B	6- <i>Tert</i> -butyl-2-[2'-(2'-hydroxy methyl)-propyl]-4-methylphenol, 4-hydroperoxy-4-methyl-2,6-di- <i>tert</i> -butyl-2,5-cyclohexadienone	Hydroxylation metabolism	Lung damage and elevated risk of lung tumor development	565–567
	Carbon tetrachloride	CYP	—	—	Clara and type II cell damage	561

1,1-Dichloro-ethylene	CYP2E1 and CYP2F2	2,2-Dichloroacetaldehyde, 1,1-dichloroethylene-epoxide	Oxidative metabolism	Clara cell toxicity	568
Styrene	CYP2E1 and CYP2F2	7,8-Styrene oxide	—	Tumor, Clara, and terminal bronchiole cell toxicity	569
<i>p</i> -Xylene	CYP and ADH	<i>p</i> -Methylbenzylalcohol, <i>p</i> -methylbenzaldehyde	—	Cell destruction	570,571
α -Naphthylthiourea	CYP	α -Naphthylurea, atomic sulfur	—	Atomic sulfur formed in this reaction covalently binds to macromolecules, pulmonary edema	572,573
Monocrotaline (pyrrolizidine alkaloids)	—	Ehrlich reactive (E+) metabolite	Activated in the liver to an Ehrlich reactive metabolite that is transported to the lung	Swelling of pulmonary capillary endothelial cells, thrombosis, and lesions of the arterial media	574,575
Bleomycin	Mixed-function oxidase	—	—	Increased lung collagen synthesis and deposition finally leads to pulmonary fibrosis	576

(continued overleaf)

TABLE 8.6 (continued)

Organ	Xenobiotics	Enzyme	Reactive Metabolite	Mechanism	Outcome	References
	Mitomycin C	NADPH:cytochrome <i>c</i> reductase and DT-diaphorase	Semiquinone radical and hydroquinone intermediate	Metabolism takes place under anaerobic conditions	Cellular toxicity	577
	Cyclophosphamide	—	4-Hydroxycyclophosphamide, acrolein and phosphoramidate mustard	—	Alterations in mixed-function oxidase activity, glutathione content, and lipid peroxidation	435,578
	Paraquat	NADPH cytochrome <i>c</i> reductase	Paraquat free radicals	Elevates superoxide levels by cyclic redox mechanism	Covalent adducts with DNA and proteins or form lipid peroxide and can lead to the damage of type II pneumocytes	549,579,580
	Nitrofurantoin	Not specified	Nitro free radical	Electron reduction	Covalent adducts with proteins or spontaneously reacts with oxygen molecules to yield reduced oxygen	579,580
	NKK	CYP2A6, CYP2A13, and CYP2B6	4-Hydroxy-1-(3-pyridyl)-1-butanone, 4-oxo-1-(3-pyridyl)-1-butanone, methyldiazohydroxide	α -Hydroxylation of methyl or methylene carbon	Forms adduct with DNA can lead to carcinogenicity	20,581,582

Urinary system	Aflatoxin B1	CYP2A13	Epoxide intermediate	Oxidative metabolism	Binds with DNA, RNA, proteins, and potentiates <i>p53</i> gene mutation and inactivation, resulting in lung cancer	7,505,583
	<i>N</i> -Nitrosamines	CYP2E1	—	—	Cancer of lungs, nasal cavity, liver, breast and esophagus	170
	Parathion	CYP reductase and amine oxidase	Paraoxon, diethyl hydrogen phosphorothionate, diethyl hydrogen phosphate	—	Respiratory edema, respiratory failure, and cancer development	561,584,585
	Skatole	CYP2F1	—	Oxidative metabolism	Necrosis	180
	Hexafluoropropene	GSH	<i>S</i> -(1,1,2,3,3,3-Hexafluoropropyl) glutathione	Conjugation	Undergoes further metabolism by γ -GT	586
	<i>S</i> -(1,1,2,3,3,3-hexafluoropropyl) glutathione	γ -GT	Cysteine <i>S</i> -conjugate	Glutamyl removal	Undergoes further metabolism by β -lyase	586
	Cysteine <i>S</i> -conjugate	β - Lyase	Thionoacyl fluoride	Lysis	Nephrotoxicity	586
	Chloroform	CYP2E1	Phosgene	Oxidative metabolism	Nephrotoxicity	587–589
	Cephaloridine	Mixed-function oxidase	Epoxide intermediate	Oxidative metabolism	Nephrotoxicity	590,591

(continued overleaf)

TABLE 8.6 (continued)

Organ	Xenobiotics	Enzyme	Reactive Metabolite	Mechanism	Outcome	References
Central nervous system	Aristocholic acid	CYP1A1	7-(Deoxyadenosin- <i>N</i> -6-yl) aristolactam I, 7-(deoxyguanosin- <i>N</i> -2-yl) aristolactam I, and 7-(deoxyadenosin- <i>N</i> -6-yl) aristolactam II	—	Urothelial cancer	8
	Bromobenzene	CYP	—	—	GSH depletion, cellular damage, and lipid peroxidation	562
	Parathion	CYP reductase	Paraoxon, diethyl hydrogen phosphorothionate, diethyl hydrogen phosphate	—	Nephrotoxicity	585
	Methoxyflurane	CYP2E1, CYP2A6, and CYP3A	—	Glutathione and cysteine conjugate	Nephrotoxicity	592
	Benzidine	CYP1A2	—	Oxidative metabolism	Urinary bladder cancer	180
	Cyclophosphamide	Multiple CYP	—	Oxidative metabolism	Urinary bladder cystitis	180
	<i>N</i> -Methyl-4-phenyl-1,2,3,6-tetrahydropyridine	MAO-B	<i>N</i> -Methyl-4-phenylpyridinium ion	—	Neuron degeneration resulting in Parkinson's disease	593

	Parathion	CYP reductase	Paraoxon, diethyl hydrogen phosphorothionate, diethyl hydrogen phosphate	—	Neurotoxicity	584,585,594
	Chlorpyrifos	—	<i>p</i> -Nitrophenol, trichloropyridinol	—	Neurotoxicity	594,595
	Amphetamine/ Methamphetamine	—	Peroxyxynitrite metabolite	Free radical formation	Failure of cellular energy metabolism followed by a secondary excitotoxicity, dopaminergic neurotoxicity	596
	Paclitaxel	CYP2C8 and CYP3A5	Hydroxylated paclitaxel	—	Neurotoxicity	597
Skin	Dapsone	FMO1, FMO3, and peroxidases	Arylhydroxylamine, arylnitroso metabolites	Oxidative metabolism	Protein adduct formed	598
	Sulfamethoxazole	FMO1, FMO3, and peroxidases	Arylhydroxylamine, arylnitroso metabolites	Oxidative metabolism	Protein adduct formed	598
Central nervous system	Daunomycin	Not specified	<i>N</i> -Acetyl daunomycin	Acetylation	Cardiac arrhythmias	599

Abbreviations: AKR, aldo-keto reductase; NKK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

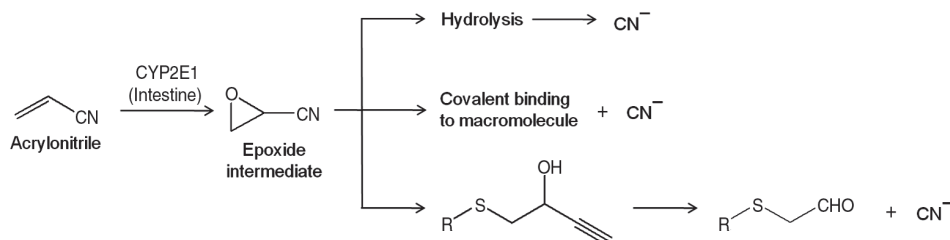


Figure 8.9 Conversion of acrylonitrile into reactive CN^- by CYP2E1 [547].

circulation, where these can result in toxicities due to covalent adduct formation [580]. Similarly, metabolic activation in lung and the formation of covalent adducts with nucleophilic species with resultant pulmonary toxicity is reported for many other xenobiotics [168]. Of special interest with respect to toxicity in both GIT and lungs are PAHs (food and air contaminants) that have the potential to result in carcinogenicity [7,373,502,503,549–551]. Inhaled air and cigarette smoke are major reasons for the exposure of lung to PAHs, while GIT is exposed to these as food contaminants. In both organs, CYP1A1, CYP1B1, EH, CYP2C, CYP3A4, aldo-keto reductase 1A1 (AKR1A1), AKR1C1, AKR1C2, AKR1C3, and AKR1C4 are responsible for the metabolic activation of PAHs, which actuate the formation of epoxide and quinone reactive metabolites, as shown in Fig. 8.10. These metabolites show genotoxicity by forming covalent adducts with DNA, resulting in mutation of *p53* gene and hence, the development of malignancy.

Similarly, the kidneys are also susceptible to drug toxicity. For example, kidneys receive 25% of the cardiac output, although these represent only 1% of the body mass, leading to high exposure to xenobiotics. The bioactivation of xenobiotics in kidney is mediated by local DMEs, and the ability of the kidneys to concentrate xenobiotics in the tubular fluid/tubular cells can result in renal toxicities with chronic administration. Examples of xenobiotics that induce nephrotoxicity are chloroform, cephaloridine, aristocholic acid (ARA), hexafluoropropene (HFP), and methoxyflurane [8,586–591,600,601].

Aromatic and heterocyclic amines present in cigarette smoke may cause tumors in GIT, lungs, kidneys, and urinary bladder after NAT-dependent modulation. NAT1 plays role in the activation of aromatic amines through O-acetylation of *N*-hydroxyl aromatic amines (generated after metabolized by CYP1A1, CYP1A2, and CYP1B1), whereas NAT2 is associated with N-acetylation, leading to deactivation of aromatic amines. These activation and deactivation reactions are responsible for the carcinogenic effects on GIT, lungs, and urinary bladder [55,373,552,602,603], as shown in Fig. 8.11.

N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an impurity present in a fast acting opioid analgesic, meperidine, has been reported to be neurotoxic. In brain, MPTP is metabolized by MAO-B to generate 1-methyl-4-phenyl 2,3-dihydropyridinium ion (MPDP⁺). This is finally converted into *N*-methyl-4-phenylpyridinium ion (MPP⁺), as shown in Fig. 8.12. MPP⁺ inhibits mitochondrial respiration in the nigrostriatal neurons and leads to an energy-deficient condition and degeneration of the latter, resulting in Parkinson's disease [593]. In this type of toxicity, MAO inhibitors and antioxidants have the potential to prevent oxidation of MPTP⁺ to MPDP⁺/MPP⁺ in mitochondria and thus act as neuroprotective agents [604].

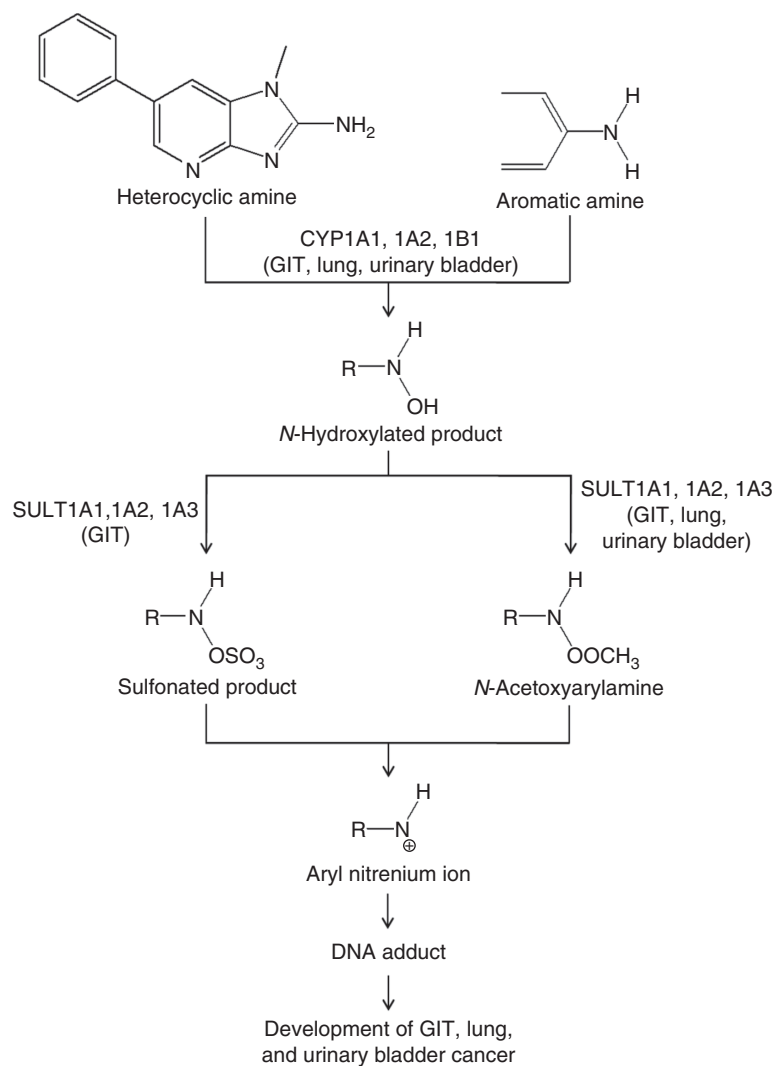


Figure 8.11 Bioactivation pathway of aromatic and heterocyclic amines depicting inception of lung/GIT/urinary bladder cancer [373].

8.6.3 Interactions in Extrahepatic Organs

Pharmacokinetic or pharmacodynamic properties of drugs may be altered in the presence of coadministered drugs, food, and environmental toxicants. While pharmacokinetic interactions are due to changes in absorption, distribution, metabolism, or excretion of drugs, the pharmacodynamic interactions are primarily attributed to changes at the target site (synergism or antagonism). A significant proportion of drug interactions occur owing to inhibition/induction of DMEs, not only in liver but also in extrahepatic organs. For example, oral administration of rifampicin results in significant induction of CYP3A4, CYP2C8, and CYP2C9 in GIT, which leads to enhancement of the metabolism and decrease in the bioavailability of

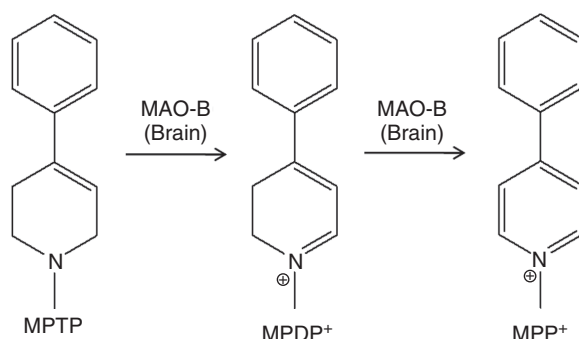


Figure 8.12 Generation of reactive *N*-methyl-4-phenylpyridinium ion (MPP⁺) in the brain from *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

the coadministered CYP substrate drugs [605]. Rifampicin increases apparent oral clearance of CYP3A4 substrates, for example, *S*-verapamil, cyclosporine, and midazolam, and accordingly decreases their bioavailability [605]. Symptoms of methadone withdrawal were observed in drug addicts taking rifampicin simultaneously for the treatment of tuberculosis [606]. Similarly, St. John's wort, a herb, has been reported to interact with a number of drugs by inducing CYP3A4 in the human intestine. CYP inhibitors, such as ketoconazole, erythromycin, clarithromycin, grape fruit juice, and troleandomycin, lead to dose-dependent toxicity of CYP3A4 substrates by increasing their bioavailability [154,458,459,607]. A summary of published drug–drug interactions in extrahepatic organs are described in Table 8.7.

8.7 CONCLUSIONS

Along with the liver, DMEs are expressed in extrahepatic organs, and their levels are significantly influenced by changes in food habits, pathophysiological conditions, exposure to environmental pollutants, smoking, and so on. These enzymes are responsible for the biotransformation of xenobiotics and convert them into relatively hydrophilic metabolites that can be cleared from the body. The high allelic variation in extrahepatic DMEs contributes to the generation of diseases observed in certain populations. Biotransformation reactions involving extrahepatic DMEs are involved in the generation of reactive metabolites, such as epoxide and quinone, which lead to organ toxicity by either directly binding with nucleophilic molecules in the cells or inducing cellular stress. The involvement of extrahepatic DMEs in the metabolism of drugs influence their bioavailability when administered by oral, inhalational, and transdermal route.

Although extensive work has already been done to explore DMEs in various extrahepatic organs and characterize their role, there are still some unanswered aspects. The total contribution of extrahepatic DMEs on the bioavailability of drugs given by non-parenteral route and in drug–drug interactions and organ toxicity are not completely understood. The relative ratio of contribution of extrahepatic xenobiotic transporters and DMEs to bioavailability and clearance is also not understood. Finally, it is not

TABLE 8.7 Drug Interactions in Extrahepatic Organs and Potential Risk Factors

Organ	Substrates	DME	Interacting Agent	Cause of DDI	Potential Risk	References
Gastrointestinal tract	Cyclosporine	CYP3A4	Grape fruit juice	Enzyme inhibition	Nephrotoxicity, hypertension, cerebral toxicity	607
			Erythromycin	Enzyme inhibition	Nephrotoxicity, hypertension, cerebral toxicity	459
			Ketoconazole	Enzyme inhibition	Nephrotoxicity, hypertension, cerebral toxicity	154
			St. John's wort	Enzyme induction	Nephrotoxicity, hypertension, cerebral toxicity	608
	Saquinavir	CYP3A4	Garlic	Enzyme induction	Reduced therapeutic efficacy due to the lowering of $C_{\max}$	609,610
	Ritonavir	CYP3A4	Garlic	Enzyme induction	Reduced therapeutic efficacy due to the lowering of $C_{\max}$	609
	Tacrolimus Midazolam	CYP3A4	Ketoconazole	Enzyme inhibition	Neurotoxic, nephrotoxicity	611,612
		CYP3A	Clarithromycin	Enzyme inhibition	Increased CNS depression	613
			Erythromycin	Enzyme inhibition	Increased CNS depression	614
			St. John's wort	Enzyme induction	Reduced therapeutic efficacy due to the lowering of $C_{\max}$	615
	Carvediol	CYP3A4	Grape fruit juice	Enzyme inhibition	Increased CNS depression	616
			Grape fruit juice	Enzyme inhibition	Lethal effect in congestive heart failure patient	610
	Felodipine	CYP3A	Grape fruit juice	Enzyme inhibition	Hypotension, ankle edema	617–619
	Indinavir	CYP3A	Ketoconazole	Enzyme inhibition	Nephrotoxicity	446,620
	Fentanyl	CYP3A	Troleandomycin	Enzyme inhibition	Reduced cerebral blood flow	458,621
	Alfentanil	CYP3A	Erythromycin	Enzyme inhibition	Vasodilation and hypotension	458
			Troleandomycin	Enzyme inhibition	Vasodilation and hypotension	458

	Sildenafil	CYP3A4	Grape fruit juice	Enzyme inhibition	Hypotension, decrease in arterial pressure	607,622
	Simvastatin	CYP3A	Grape fruit juice	Enzyme inhibition	Rhabdomyolysis, acute renal failure	623
	Verapamil	CYP3A4	Rifampicin	Enzyme induction	Reduce efficacy	605
			Grape fruit juice	Enzyme inhibition	Cardiovascular side effects	605,624
	Halofantrine	CYP3A4	Ketoconazole	Enzyme inhibition	Cardiac arrhythmia	625,626
	5-Fluorouracil	All DMEs	Nifedipine	Enzyme inhibition	Dose-dependent cardiac toxicity	627,628
	Finasteride	CYP3A4	Ketoconazole	Enzyme inhibition	—	629
	Tamoxifen	CYP2D, 3A1/2	Ondansetron	Enzyme inhibition	Pharmacokinetic interaction	630
	Hormone replacement therapy	CYP3A4	Licorice	Enzyme induction	Reduction in efficacy	610
Pulmonary system	Amiodarone	CYP1A1	PAHs	Enzyme induction	Pulmonary fibrosis	631
	Hormonal contraceptives	CYP1A1/2	PAHs	Enzyme induction	Increases chances of conceiving	632
	Inhaled corticosteroids	CYP1A1/2	PAHs	Enzyme induction	Aggravate of asthma	632
Urinary system	Diltiazem	CYP3A5	Tacrolimus	Enzyme inhibition	Nephrotoxicity	631

(continued overleaf)

TABLE 8.7 *(continued)*

Organ	Substrates	DME	Interacting Agent	Cause of DDI	Potential Risk	References
	Aldosterone	UGT2B7	Spironolactone, canrenone	Enzyme inhibition	—	633
	Valproic acid	UGT2B7	Spironolactone, canrenone	Enzyme inhibition	Bone marrow suppression	633,634
	5,6-dimethylxan- then-one-4- acetic acid, epirubicin	UGT2B7	Spironolactone, canrenone	Enzyme inhibition	—	633
	Diclofenac	UGT2B7	Spironolactone, canrenone	Enzyme inhibition	Risk of vascular events	633
	Naproxen	UGT2B7	Spironolactone, canrenone	Enzyme inhibition	—	633
	Morphine, codeine	UGT2B7	Spironolactone, canrenone	Enzyme inhibition	—	633
	β-blocker diuretics	Prostaglandin synthase	Sulindac	Enzyme inhibition	Kidney failure	635
Central nervous system	Vincristine	CYP	Nifedipine and itraconazole	Enzyme inhibition	Peripheral, autonomic, cranial nerve neuropathies and encephalopathy, neurotoxicity	636

clear whether extrahepatic metabolism provides beneficial effects by neutralizing carcinogens to which different organs of the body are exposed or if these are involved in detrimental effects by metabolic activation of procarcinogens. Continued research in this broad area will provide more answers and aid in the predictions of clinical end points.

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9 Mass Balance Studies in Animals and Humans

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9.1	Summary	1
9.2	Introduction	2
9.3	Purpose and specific aims	3
9.4	Experimental design of preclinical mass balance studies	4
9.5	Use, selection criteria, and limitations of radiolabel compounds	16
9.6	Determination and analysis of mass balance and pharmacokinetic parameters	17
9.7	Case example for mass balance study in preclinical species	21
9.8	Common issues during conduct of preclinical mass balance studies	24
9.9	Human mass balance studies	26
9.10	Human dosimetry calculations	27
9.11	Experimental design of human mass balance studies	33
9.12	Case example for human mass balance	34
9.13	Conclusions	36
	Acknowledgments	36
	References	36

9.1 SUMMARY

Mass balance studies play an important role in the development of new drugs. These studies give in-depth understanding of absorption, bioavailability, and routes (renal, biliary, hepatic, or gastrointestinal) and extent of excretion, metabolite profiling, metabolic pathways, and clearance mechanisms of a drug. In this chapter, fundamental scientific considerations for the successful design, conduct, and interpretation of conventional, basic mass balance and more comprehensive absorption, distribution, metabolism, and excretion (ADME) studies are reviewed and applied, practical case examples are provided.

9.2 INTRODUCTION

Before first-in-human (FIH) studies with a new drug candidate, preclinical studies need to be conducted to fulfill regulatory requirements or to facilitate regulatory approval [1]. In the United States, investigational new drug (IND) applications with the Food and Drug Administration (FDA) may include IND enabling studies, such as (i) *in vitro* and *in vivo* pharmacology studies for intended use; (ii) toxicology studies supporting the starting dose and duration of an FIH study (such as single or multiple-dose studies in one rodent and nonrodent species with toxicokinetics, genotoxicity battery, and safety pharmacology battery); (iii) single-dose pharmacokinetics (PKs) in one rodent and nonrodent species; (iv) *in vitro* plasma protein binding in relevant species; and (v) *in vitro* cross species metabolism study in relevant species [2]. While not mandatory for IND submissions, comprehensive preclinical ADME studies, including biotransformation or metabolite profiling, conventional mass balance, or tissue distribution by quantitative whole-body autoradiography (QWBA) [3] can make a submission package more compelling for progression toward phase I clinical studies. Typically, preclinical ADME together with mass balance studies are recommended before conducting human ADME and mass balance studies [4,5], which are generally initiated after the successful synthesis of the radiolabeled drug. An example of the typical timings for conducting mass balance studies during the drug development process is shown in Fig. 9.1. For pre-clinical ADME species, the selection of doses is often based on pre-existing toxicity information and targeted therapeutic doses. For human ADME studies, which include the evaluation of mass balance, a relevant dose in the anticipated therapeutic range is typically chosen. Conventional mass balance studies only allow the calculation of mass recovery and PK parameters of the radiolabeled drug by quantification of the radioactivity present in excreta such as urine, feces, or bile. Nowadays, these mass balance studies are often conducted as part of more comprehensive ADME studies, where additional information is obtained, including (i) PK properties of the drug and its metabolite(s) in blood or plasma; (ii) absorption estimates via plasma/blood data, urinary data, or fecal data; (iii) elimination pathways and drug metabolism; and (iv) total drug exposure and its metabolites in different organs and tissues at different time intervals.

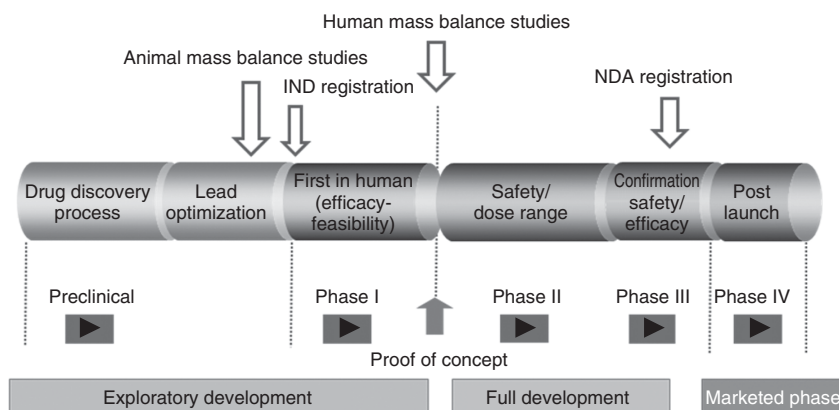


Figure 9.1 Representation of the timings for the conduct of animal and human mass balance studies parallelly in the drug development process.

9.3 PURPOSE AND SPECIFIC AIMS

The purpose of mass balance studies along with ADME studies [6,7] can be summarized as follows:

1. To determine absorption of drug after extravascular administration.
2. To identify excretory pathways and recovery of drug-related radioactivity (usually urine and feces) in early stages of drug development.
3. To characterize the PKs of the intact parent drug and its metabolites as well as their contribution toward pharmacology/toxicology.
4. To determine clearance mechanisms, including metabolic and excretory fates and understand potential contributors of intersubject variability associated with drug–drug interactions (DDIs), supported by the identities of drug-related material in excreta.
5. To determine the organs/tissues distribution in preclinical species, which can be a cause for incomplete recovery of radioactivity in excreta, caused by significant retention in specific organs or tissues.
6. To provide all necessary information from preclinical studies in terms of design and potential risks, including dosimetry of radiation to support mandatory human mass balance studies.
7. To guide the use and choice of preclinical species in the toxicity studies based on the metabolite profiles.

Conventional mass balance studies in their simplest form allow the determination of excretory pathways of drug and its metabolites by determining the cumulative radioactivity recovered in excreta after an extended collection period, relative to the dosed amount of radiolabeled drug, most commonly via the oral (p.o.) or intravenous (i.v.) routes [7]. To study the mass recovery, the radiolabeled drug is typically administered to facilitate the identification and quantification of parent drug as well as its metabolites in different biological matrices. The data obtained from these studies are multifarious, as when a drug is administered to animals or humans, it undergoes a series of events such as absorption, distribution, and metabolism, which govern the drugs systemic exposure, and its pharmacological response [8] or pharmacodynamic effect, as well as its excretion routes [7,9]. For investigational drug candidates, ADME and mass balance properties can be crucial determinants in the selection process, leading to optimized clinical outcomes for drug candidates [10]. Essential elements for design and conduct of mass balance study have been described previously by Tse *et al.* [11] and Beumer *et al.* [4]. This chapter summarizes detailed factors to be considered when designing and conducting various types of ADME and mass balance studies, including how to (i) choose of the best dose, dosing route, in a suitable preclinical species; (ii) clearly identify major excretory pathways; (iii) identify and troubleshoot causes of low drug recovery; (iv) determine PK and ADME properties using total radioactivity or intact parent drug data; and (v) conduct dosimetric calculation to extrapolate from animal to human. A case example in rats is given to provide details on the conduct of a mass balance studies. Moreover, the purpose and conduct of human mass balance studies are reviewed.

9.4 EXPERIMENTAL DESIGN OF PRECLINICAL MASS BALANCE STUDIES

To identify metabolic routes of a drug candidate in early development stages, *in vitro* studies provide a basic direction for the preclinical studies in animals [12,13]. A carefully designed mass balance study, which includes metabolite identification in plasma, urine, bile, and feces can further aid in the identification of excretory and metabolic routes. Common challenges related for the successful study conduct include proper dose selection, bioanalytical factors, suitable formulations, and potential toxicities. Details for experimental design, along with study result interpretation are outlined. To aid in clarity, a preclinical rodent study can be divided into six groups. Two groups, one each for p.o. and i.v. administration, are used for the interpretation of mass balance data, absorption, and PK parameters such as fraction absorbed (F_{abs}), area under the time–concentration curve (AUC), and clearance (CL). Two additional groups, one each for p.o. and i.v. administration, are dedicated for biotransformation studies and metabolic profiling. The metabolite profiles from the i.v. dosed group, when compare to the p.o. dosed group, can provide the information on whether the drug is subjected to the first-pass metabolism in gastrointestinal tract. The remaining two groups, each separately for p.o. and i.v. dose administration, are used for biliary excretion studies, in which one continuous loop nonvascular catheter is surgically implanted. The i.v. dose in bile-duct cannulated (BDC) rats can further distinguish biliary excretion from the secretion of drug from the gastrointestinal tract, if any. Blood, urine, feces, and bile samples are collected at the designated time following the dosing. The carcasses are also collected for potential analysis in cases where the total recovery in excreta is low.

From the group where the mass balance is studied, a mass balance recovery <80% can possibly lead to challenges from regulatory agencies, as the mass balance study might not provide a complete picture of ADME parameters. Some publications put the recovery cutoff at 90% [4] and 85–95% or above as the boundary for the success or failure of a mass balance study [14]. Understanding the causes for incomplete mass recovery is typically required but can be challenging at the early development stage. Sometimes, incorporation of QWBA data for the analysis of tissues and organs distribution of radioactivity in mass balance studies can be used to understand the incomplete mass balance recoveries.

9.4.1 Choice of Preclinical Species

For ADME studies as a part of nonclinical safety testing, two animal species, one rodent (rat or mouse) and one nonrodent (dog or monkey), are usually chosen [15,16]. Rodents are the most common and suitable choice of species for preclinical mass balance studies. For species selection, the difference in strain, age, sex, genetic modification, and body weight can have a significant impact on the studies [4] and must be considered. More importantly, the interpretations of any ADME studies often require consistency for results comparisons between studies; thus, it is recommended to use the same rodent strain with a close match of age and weight as used in the toxicity studies. Male animals are normally used for the ADME studies unless the drug is developed for use in female patients only. Female animals may be used if there are significant differences in toxicokinetics parameters between genders are observed and *in-vitro* metabolism cannot explain the results. If the drug is being developed for an

IND application with Japanese regulatory agencies, studies in both male and female animals may need to be conducted. Disposition studies may be conducted in other species such as rabbits, which is used for reproduction or teratogenicity studies, or in the mouse, which is used for carcinogenicity studies. Dogs and monkeys are normally used as nonnative animals. Special studies in female rats such as placenta and milk transfer studies are required and are usually conducted at late stage of the drug development. Before the actual dose administration, animals are usually housed for at least 14 days for quarantining or conditioning [2] with controlled conditions (in a room with controlled temperature ranges typically at $72 \pm 2^\circ\text{F}$), and a humidity range of $50 \pm 15\%$, and dietary conditions (fed or fasted) in accordance with Guidance for the Care and Use of Laboratory Animals. In this chapter, the focus is on the mass balance studies in rats in order to cover fundamental aspects of mass balance studies (including recovery from different matrices, e.g., excreta, tissue dissection, QWBA, carcass, and CO_2 exhalation) because rats are the most common and suitable choice of species. A schematic representation of the rat model used in a typical ADME study, including common routes of administration, absorption, metabolism, and excretion from different matrices, is illustrated in Fig. 9.2. A summary of animals commonly used for

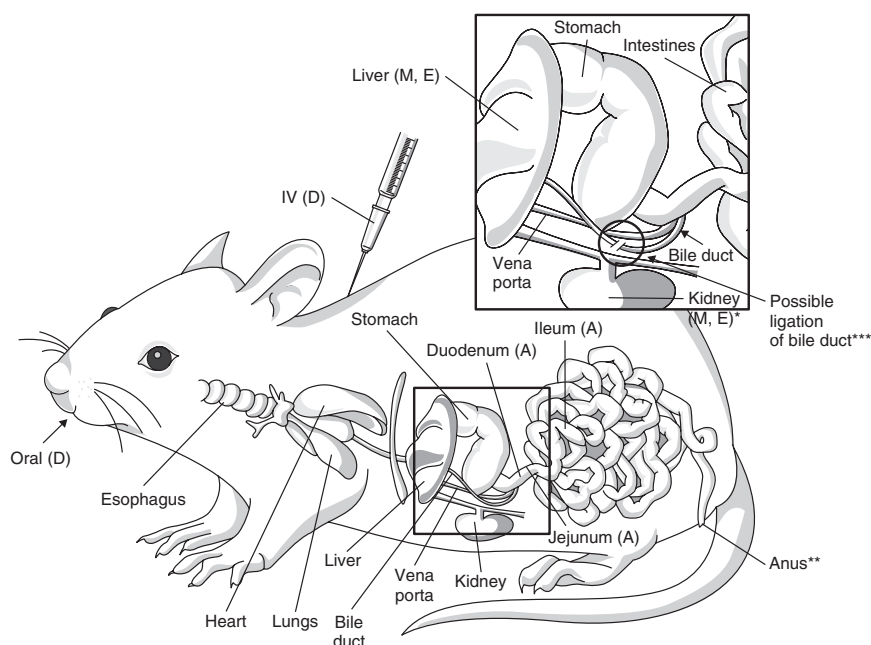


Figure 9.2 Schematic representations of various dosing, absorption, metabolism, and excretion routes. The most common dosing routes for mass balance studies are oral (p.o.) and intravenous (i.v.) administration. Urine and feces are separately collected after excretion. For biliary excretion studies, the bile duct that drains the bile from the liver to the upper part of intestine, usually duodenum, can be cannulated to collect bile. “D” represents p.o. or i.v. dosing routes, “A” represents various absorption sites, “M” represents metabolism sites, and “E” represents various excretion routes. *Urinary excretion site. **Fecal excretion site. ***Biliary excretion site. *Source:* The figure was drawn by Nicole Heimbach.

TABLE 9.1 Factors in the Choice of Animals, Dose, and Formulations for Mass Balance or ADME Studies with Rationale and Comments

Factors	Design	Rationale and Comments
Dose levels	<i>Intravenous dose:</i> usually 20–100% of oral dose (based on bioavailability and solubility) <i>Oral dose:</i> usually in the range of anticipated NOAEL dose studies	Ideally target similar exposure between oral and intravenous doses Support the toxicity study
Dose volume	<i>Intravenous:</i> ≤ 2 mL/kg <i>Oral:</i> ≤ 10 mL/kg for rodents and ≤ 5 mL/kg for large animals; ideally the same as planned for toxicity studies	Ethical reasons and animal welfare considerations
Formulation	Ideally same formulation as in toxicity studies	As in toxicity studies After administration, an aliquot of formulation is frozen as a reference check in metabolic studies
Dose regimen	Single dose	—
Species ^a /weight (kg)	Dose for [¹⁴ C]-labeled drug ^b (μ Ci/kg)	—
Mouse (0.025–0.040)	100–300	For higher species, avoid high radioactive doses as animals are returned to colony
Rat (0.15–0.30)	100–300	—
Dog (8–13)	20–30	—
Monkey (2.5–4)	20–30	—
Rabbit (2–4)	30–50	—
Subject number	$N = 3$ for each route ^c	—
Feeding	Should be same as in toxicity studies	
Housing	Individually in metabolism or housing cages	

Abbreviation: NOAEL, no observed adverse effect level.

^aMouse and rat studies support the carcinogenicity study; rat, dog, and monkey studies support the general toxicity study; rat and rabbit studies support the teratogenicity study.

^bFor [³H]-labeled drug, ≤ 500 μ Ci/kg for rodents and ≤ 100 μ Ci/kg for large animal.

^cIn mice, urine and feces may be pooled per time point. For PK and metabolite profiling in mouse plasma, due to small sample size, two to three animals per time point for each route may be used. In rats, additional three animals each will be needed for plasma metabolite identification and biliary excretion. In large animal, for intravenous dosing studies, often two animals are used due to more sample volume available and lower experimental variability compared to oral dosing.

the mass balance studies is listed in Table 9.1. All studies are designed to maximize quantitative aspects of the drug molecule, while minimizing radioactivity exposure.

9.4.2 Choice of Dose, Route of Administration, and Formulation

In mass balance studies, as most compounds undergo metabolic transformation before excretion, a radioisotopically labeled compound is commonly used so that both unchanged compound (parent drug) and its metabolites in different biological matrices can be accurately quantified. The total dose of the compound (radiolabeled and unlabeled) under investigation needs to be pharmacologically relevant and

comparable to toxicological doses [4]. Most of the time, the radiolabeled compound is diluted with nonradiolabeled compound to achieve the total targeted dose to be administered to the subjects for both preclinical (animal) and clinical (human) studies as the required radioactivity is typically only a trace amount. Moreover, the radiolabeled dose depends on the specific objectives of the study and sensitivity of the analytical method. The specific activity of the radioactivity in the dose must be chosen carefully, as the nonradiolabeled dose determines the PKs of the parent drug and mass balance studies for clinical practice, while the radiolabeled dose influences both the radioactive exposure in the animal/human and the analytical limits of quantification. For human studies, the route of administration preferred is the same as the intended route for the drug's clinical use, as the formation of metabolites and extent of metabolism may differ with the route of administration [7]. The selection of the radioactive dose amount is based on the balance between the radioactive exposure limits as outlined in 21 CFR 361.1 [1], other local radiation guidelines and regulations, and the safety/efficacy profiles of the drug molecule. The purity of the radioactive tracer used for the study plays an important role as impurities may result in different PK characteristics, metabolite profiles, and may result in toxicity concerns. The radioactivity purity must be >98% for mass balance studies unless there is a limit in purification technique. Various factors related to the choice of doses and formulations have been summarized in Table 9.1.

For p.o. administration, solutions, suspensions, or loose filled capsules prepared in certified labs are generally used for mass balance studies. A dosing vehicle also used in the toxicity studies is the preferred choice for preclinical mass balance studies. Solution formulations are a common choice with minimal difference in the physical properties (particle size) between radiolabeled and nonlabeled drug substance. For suspension or capsule formulations, care must be taken to ensure uniform distribution of radioactivity within the dose [7]. A premixed blend prepared by dissolving the radiolabeled and unlabeled drugs in appropriate solvent(s) followed by removal of solvent(s) can eliminate uniformity issues. Preparing the radiolabeled dosage form with commercially available large-scale manufacturing equipments will increase the risk of unnecessary radioactive contamination and is generally not preferred.

9.4.3 Choice of Matrices

The quantitative analysis of all mass balance studies is based on the cumulative amount of radioactivity collected in a given matrix at the given collection interval. Usually, blood, urine, and feces are routinely collected during a typical mass balance study. In order to understand the mechanism of clearance and potentially incomplete radioactivity recovery, more information can be obtained by sampling additional matrices, including bile, tissues, organs, carcass, and expired air [17]. The sample collection of blood and excreta begins before drug administration and continues until maximum recovery of radioactivity is achieved. The radioactivity of blood, plasma, urine, bile, feces, and cage wash samples is determined by liquid scintillation counting, while the parent drug is usually determined using the HPLC or liquid chromatography-tandem-mass spectrometry (LC-MS/MS) methods. The sample collection can be stopped when <1% of administered radioactivity is excreted for two consecutive days, as prolonged collection will not substantially increase recovery [4]. Moreover, the sample collection

time should also be practical for PK parameter calculations. The sample collection for different matrices is discussed in the following sections.

9.4.3.1 Blood and Plasma. Blood and plasma samples are generally used for the PK studies of both nonlabeled and radiolabeled compound. The choice of blood and plasma sample selection should be based on the blood-to-plasma distribution ratio, due to different PK behavior of unchanged drug and its corresponding metabolites in blood versus plasma. In the case of i.v. dosing, an early sample is imperative to determine the maximum plasma concentration (C_0). Thereafter, samples need to be collected at appropriate intervals in order to characterize the distribution and elimination phase separately. In the case of p.o. administered drugs, samples are frequently collected around the expected time of maximum plasma concentration ($C_{\max}$). When designing the time points to take a sample, the volume of samples collected should be sufficient for at least one bioanalytical measurement. In the example of the rat ($n = 3$), blood (200 μL) can typically be collected at 0.083 (i.v. only), 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 168 h post dose. The blood samples are added preheparinized tubes in cooled (4°C) sample collecting carousals using automatic sample collecting systems such as a Culex[®] system. Saline (200 μL) is automatically injected after each sample is collected to clear the cannula and replace the volume of blood samples. The blood samples are further spun to collect the plasma at 4°C . The total blood volume collected should not exceed 1% of the animal's body weight. For metabolite profiling, 250 μL of blood samples are collected from each of three rats at each time point up to 48 h using the same procedures mentioned above. The samples are pooled, and then spun to separate the plasma. The total radioactivity of radiolabeled drugs can be measured using the liquid scintillation counting (LSC) method. The concentration of nonlabeled or parent drug in blood or plasma can be measured using different chromatographic instruments (e.g., HPLC and GC), mass spectrometers (e.g., LC-MS/MS), or other suitable analytical methods (e.g., ELISA). Sample pretreatment methods for the determination of radioactivity differ for blood and plasma. The plasma samples are transparent and therefore can be directly measured after mixing with liquid scintillation fluid for the quantification of radioactivity using LSC. Generally, decolorization is required for blood samples to reduce color quenching. This is achieved using a tissue solubilizer and oxidizer (such as 30% hydrogen peroxide). EDTA (50 μL of 100 mM) as an antifoaming agent can be added to the solubilized sample before addition of hydrogen peroxide. Alternatively, the combusting method can be used to collect $^{14}\text{CO}_2$ or $^3\text{H}_2\text{O}$ derived from the samples. A detailed list of various parameters needed to be considered in designing and handling of blood and plasma matrices following with rationale and comments is provided in Table 9.2.

If a tritium radiolabeled compound is used, then the samples to be analyzed for the radioactivity should be dried before analysis, as $^3\text{H}_2\text{O}$ has a long half-life and can interfere with the radioactivity of drug-related substances. Since the dried sample is hard to redissolve in the liquid scintillation cocktail, it is recommended that the dried sample be dissolved in solubilizer first, if the solubilization method instead of the combusting method is used.

9.4.3.2 Excreta and Cage Wash. Urine and feces are typically collected from each PK study groups at defined time intervals: pre dose, 0–24, 24–48, 48–72, 72–96, and 96–168 h post dose. Urine is a liquid matrix that can be collected separately from the

TABLE 9.2 List of Various Parameters Needed to be Considered in Designing and Handling of Blood, Plasma, and Excreta Matrices, with Rationale and Comments

Sample Collection	Design	Rationale and Comments
<i>Blood samples</i>		
Blood/plasma collection time points	<i>Intravenous and oral dose:</i> pre dose, 6–8 points on day 1. Then sampling at desired time points up to seven days	Ideal for PK calculations even if terminal half-life is long
Volume of blood sample	≤0.25 mL from rodents; 2 mL from rabbits, dogs, and monkey; 10 mL from human	Allows analytical measurements for PK, including radioassay and metabolic profiling
Number of measurements	One per time point of each individual in ADME studies	Small volume Pooled samples per time point may be analyzed if limited sample available
<i>Excreta samples</i>		
Urine, feces, and cage wash collection	Separate collection of urine and feces on ice (no cooling after 96 h); modified metabolic cage preferred	Mass balance and metabolite profiling (avoid degradation) Pooled collection per time point for urine and feces may be needed for mice
Time intervals	Daily up to 168 h post dose; Fixed time interval (0–24, 24–48, 48–72, 72–96, 96–168 h) or pooled for mass balance studies	To determine mass balance, metabolic, and excretory pathways
Cage wash	Extensive cage wash after last day, separate workup	Increase recovery and safety aspects (clean cages to be returned to storage)
Number of radioactivity measurements	<i>Urine:</i> One per time interval and for each species under study; <i>Feces:</i> Two per time interval and for each species under study; <i>Cage wash:</i> One per animal	Repeat if mean difference is >10% and total content >1% of dose

feces with a modification of a metabolic cage as represented in Fig. 9.3 for rodents. The urine samples are collected in defined time intervals in order to calculate the total excretion of the compound and its metabolites. The urine collection containers should be placed on ice bath or frozen conditions to reduce possible chemical degradation. The total volume and pH of the urine sample should be accurately measured. After collection, urine samples are quantitatively analyzed for radiolabeled drug, unlabeled drug, and/or their metabolites. The percentage recovery of radioactivity in urine is calculated from the ratio of concentration and volume recovered in the urine to the total concentration and volume dosed, where concentration is given in disintegrations per minutes (DPM) per milliliter and is represented by Equation 9.1:

% Recovery in urine

$$= \frac{\text{Volume recovered in urine (mL)} \times \text{Urine concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{Dose concentration (DPM/mL)}} \times 100 \quad (9.1)$$

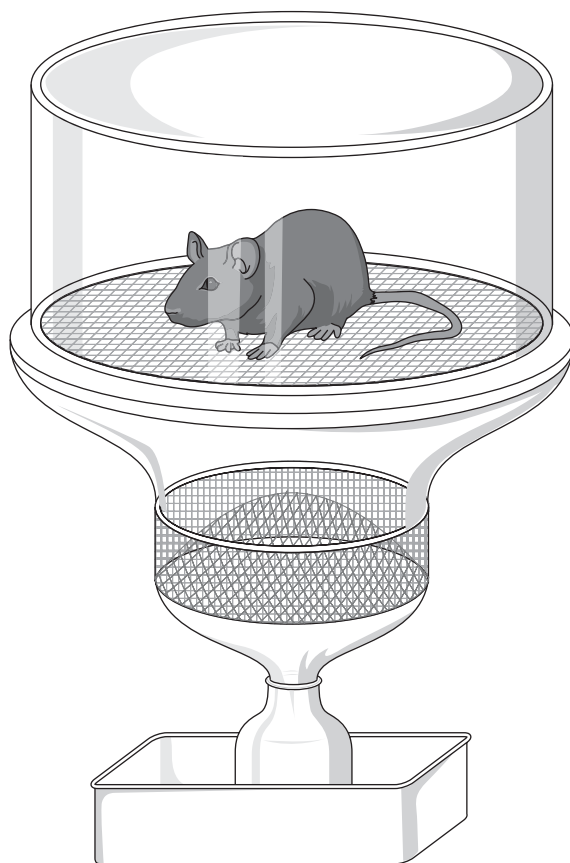


Figure 9.3 A representation of rat metabolic cage used for current mass balance studies for the separate collection of the urine (in bottle below) and feces (middle collection cone) for the rodents. For the metabolite profiling purpose, the samples (first 48 h) were collected over ice or frozen conditions.

Identification and quantification of metabolites of drugs in urine can be achieved using HPLC with off-line or on-line radioactivity detection and LC-MS/MS. For this purpose, urine samples can be pooled for analysis and may require to be concentrated in order to increase the detection sensitivity of the metabolites.

Feces are collected separately from urine with a modified metabolic cage for rodents, as represented in Fig. 9.3. Feces samples are subjected to homogenization, solubilization, and decolorization before radioactivity measurements. Homogenization can be done with water, buffer, bovine serum albumin solution [6], suspension agent solution, or water containing organic solvent(s). Similar to blood samples, after solubilization, decolorization is then performed with 30% hydrogen peroxide, if the solubilization method other than combusting method is used. As was the case for blood samples, EDTA (50 μ L of 100 mM) can be added as an antifoaming agent to the solubilized sample before addition of hydrogen peroxide. Finally, scintillation fluid is added to each vial. Overnight storage in a dark place is recommended before quantification of radioactivity with liquid scintillation counting to reduce chemiluminescence. The

percentage of the radioactive recovery from the feces is calculated from the weight of feces recovered, and the homogenization factor is given by expression given in Equation 9.2:

% Recovery in feces

$$= \frac{\text{Weight recovered (g)} \times \text{dilution factor (g/g)} \times \text{homogenate concentration (DPM/g)}}{\text{volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.2)$$

To determine the concentration of unchanged drug and/or metabolites in feces, the homogenates are processed and cleaned up using an appropriate method (such as solid-phase extraction or liquid–liquid extraction) and then analyzed by LC-MS/MS. After the final sample collection is completed, each cage is rinsed with water and 50% ethanol (or another appropriate solvent), and the resulting cage wash is quantitatively analyzed using LSC. The percentage of radioactivity recovery from the cage wash can be calculated from the ratio of volume recovered after cage wash and the concentration to the volume dosed and the concentrations recovered in cage wash, using Equation 9.3:

% Recovery from cage wash

$$= \frac{\text{Volume recovered in cage wash (mL)} \times \text{sample concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.3)$$

The detailed design of collection of samples in different matrices at different time intervals with applicable methods for the measurement of total radioactive drug, parent drug, and metabolites, with rationale and recommendations have been listed in Table 9.2.

If tritium-labeled compound is used, then tritiated water formation can be estimated to assess the metabolic stability of the radiolabel using the following equations [2]:

$$C_0 = C_{\text{mid}} \times \frac{0.693}{t_{1/2}} t$$

$$f = \frac{C_0 \times V_{\text{bw}}}{\text{dose}}$$

where C_0 is the concentration of tritiated water at time 0; C_{mid} is the concentration of tritiated water in urine at the midpoint of the collection interval; $t_{1/2}$ is the average tritiated water half-life in the rat, 85 h; t is the midpoint of the collection interval; f is the fraction of dose converted to tritiated water; and V_{bw} is the exchangeable body water volume in the relevant species.

9.4.3.3 Tissues and Organs. As part of preclinical mass balance studies employing a radiolabeled drug, tissue distribution studies are usually conducted when the disposition kinetics, distribution, and potential for accumulation of the drug and/or its metabolites in various parts of the animal body needs to be assessed [6,18]. The concepts of tissue/organ distribution studies, following administration of radiolabeled drugs in rodent, are briefly discussed in this section. The main objectives of these studies are as follows [19,20]:

1. To determine the distribution of drug-related material in various tissues/organs in the body.
2. To predict the exposure of drug-related materials at the target site of pharmacological response.
3. To investigate the potential sites involved in the metabolism.
4. To investigate the quantitative transfer across the blood–brain barrier, reproductive system, and placenta barriers.

These studies can aid in identifications of reasons for incomplete recovery of radioactivity, which may be due to reversible or irreversible tissue binding or incorporation into different organs/tissues. The use of radiolabeled compounds is extensive and very valuable in tissue distribution studies, and it is the basic requirement from regulatory agencies that preclinical animal tissue distribution data are submitted as a prerequisite for estimating the potential radioactivity exposure in clinical studies unless a trace radioactive dose with accelerator mass spectrometry (AMS) is used. Tissue distribution studies are performed to quantify the distribution of drug-related radioactivity (unchanged drugs or metabolites) in the target tissues of animal species. Typically, these studies are limited to single doses by the intended route of administration. After the administration of radiolabeled drugs, designated tissues and organs are promptly dissected after animals were sacrificed at various sampling time intervals [10], and the radioactivity was quantified using LSC. This quantification of the radioactivity by LSC in various tissues/organs can be achieved either by analyzing the homogenized tissue/organ or through combustion of tissue/organ to $[\text{CO}_2]$ that is further trapped in alkaline solution and quantified for ^{14}C radioactivity (for ^3H -labeled compound, $^3\text{H}_2\text{O}$ is trapped). The concentration of drug-related material (total radioactivity) in major tissues and organs reveals the degree of transient exposure of specific organs to the drug [20,21].

Another method to quantify tissue distribution of radioactivity into various organs/tissues is the QWBA technique [22]. QWBA is used to quantify the distribution of radioactivity in a larger extent to all body compartments, which helps target specific organs for further recovery of radioactivity as described before [3] or drug-related components, if needed. In QWBA studies, at least one animal per time interval is sacrificed at various intervals after dosing and then frozen in a “microtomb” at -20°C . Before whole-body section collection, standards fortified with ^{14}C radioactivity (as quality control) are placed into the frozen block for monitoring the uniformity of the section thickness. Then thin slides of the whole animal with a thickness of $\sim 40\ \mu\text{m}$, including representative of all major tissues, organs, and biological fluids, are prepared on adhesive tape. A section set from each animal is prepared by mounting a representative section from each level of interest. These mounted sections are exposed on phosphorimaging screens along with plastic embedded for subsequent calibration of the image analysis software. These screens are exposed for almost three days (or seven days for tritium) and then scanned using a phosphor imager to measure the radioactivity. On the basis of the body surface area and body weight, the exposure of radioactivity to each tissue and organ in rats can be scaled to human tissues for the calculation of human whole body exposure [2] and effective dose for human administration [23] (interested readers are advised to refer to Chapter 26, Volume V, for detailed explanation on quantitative whole-body

TABLE 9.3 Tissue Distribution Study Design: Quantitative Whole-Body Autoradiography with Various Rationale and Comments

Distribution (QWBA) ^a	Design	Rationale and Comments
Dose	Same as the mass balance study	Support toxicity studies Support human ADME study (dosimetry)
Time points	For example, 0.5, 1, 2, 4, 8, 24, 48, 72, and 168 h (or other time points according to pre-existing information)	Tissue distribution estimation in organs/tissues and spatial resolution
Number of animals (<i>N</i>)	<i>N</i> = 1 for each time point	Fewer animals necessary Pigmented rats with one albino at 168 h or v.v. (to investigate melanin binding in eyes or skin)

Abbreviation: v.v., vice versa.

^aUsually small animals are preferred, that is, rat and mouse, unless larger species are required. Selected organs of interest can also be collected for further analyses for the parent and/or metabolite(s).

autoradiography). The numbers of animal per study group, doses, and time intervals for both dissection and QWBA with rationale and comments have been compiled in Table 9.3.

9.4.3.4 Bile. Biliary excretion is an important elimination route and many compounds that are excreted in bile can be reabsorbed in the intestine, to complete an enterohepatic circulation cycle. The cycle can influence the absorption and elimination rate constants and alter the plasma concentration–time profile [24,25]. Bile-duct cannulation provides a way to examine the biliary excretion and potential enterohepatic circulation [26,27] and thus contributes useful information on the rate and extent of excretion of drug and/or metabolites via biliary pathways. Biliary excretion studies can be conducted by surgically ligating the common bile duct and inserting a cannula for bile collection [28] (see also Fig. 9.2). A recovery period of at least 24 h should be allowed before administering the test drug in order to avoid possible delays in gastrointestinal transit associated with postoperative adynamic ileus [29]. Tse and Ballard [26] developed a method using a pair of bile duct–duodenum cannula-linked rats, which permits a realistic approximation of biliary excretion and reabsorption in intact animals. After the acclimation period of the surgery, animals are lightly anesthetized and the continuous loop is then cut into two sections, for the bile collection and synthetic bile replacement to maintain the bile flow. A 50-cm extension tube is then attached to both sections of the continuous loop in order to pass through protective tether and spring. The animal is then placed back to metabolic cage and externalized tubing is attached to a dual channel swivel. The bile flow needs to be closely monitored for 1–2 h before the studies [30], and if possible, liver function tests can be performed by monitoring the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin [31]. Control bile is collected from the bile-duct cannula up to 0.5 h pre dose. After the administration of dose, synthetic bile is infused (via duodenal cannula in rodents) at a continuous rate of 1 mL/h for the duration of the study. Bile samples are collected at predefined time intervals (e.g., pre dose, 0–2, 2–4, 4–8, 8–12,

TABLE 9.4 List of Various Parameters Needed to be Considered in Designing and Handling of Bile-Duct Cannulated Animals with the Rationale and Comments

Bile-Duct Cannulation	Design	Rationale and Comments
Criteria	If >65% of radioactive dose is recovered in feces after intravenous dosing	If <65% of dose is recovered, study is conducted if there is strong interest in specific metabolites
Doses	Low for oral route (standard); <i>For intravenous route</i> : same as used in intact animals	Extent of biliary excretion/absorption/recycling
Bile substitution	Yes, method to be chosen	To avoid bile depletion
Time intervals	Bile in daily fractions up to 72 h (e.g., 0–2, 2–4, 4–8, 8–12, 12–24, 24–48, 48–72)	Maximum collection time feasible
Number of animals (<i>N</i>)	<i>N</i> = 3 for oral dosing; <i>N</i> = 2 for intravenous dosing	Means values are calculated
Volume of samples	Same as excretion studies	—
Number of measurements	Same as excretion studies	—

12–24, 24–48, and 48–72 h post dose). In addition, urine and feces are also collected in 24-h intervals for the duration of study. All excreta (urine, bile, and feces) are stored at approximately -20°C or lower until the time of analysis. After 72 h, the animals are sacrificed and the animal carcasses are retained at -20°C for further analysis as needed. To evaluate the enterohepatic circulation, the blood samples can be collected from BDC rats. The enterohepatic circulation can be estimated based on the blood or plasma exposures between BDC rats and intact rats. The enterohepatic circulation can also be accessed by introducing bile from the animal (donor) dosed with the drug to the intestine of another animal (receiver) without drug dosed. The design and handling of BDC animals are summarized in Table 9.4. The percentage of radioactivity recovered in bile is calculated from the ratio of the amount of radioactivity recovered in bile to the amount of radioactivity administered using Equation 9.4:

% Recovery from bile

$$= \frac{\text{Volume recovered in bile (mL)} \times \text{bile concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.4)$$

9.4.3.5 Carcass and CO_2 Exhalation. In some cases, the mass balance recovery is not complete, as determined from the total recovery in excreta, when compared with the administered dose. The most common reason is that the drug and/or its metabolites have a long half-life or are retained in certain tissue/organs, via, for example, irreversible binding to tissue/organ components or the incorporation of the radioisotope into endogenous components [6]. In such cases, as briefly discussed, the QWBA data may provide useful information. The radioactivity retained in the organs or carcass also can be analyzed to identify reasons for low recovery. In this method, animals are

sacrificed at the last time point, for example, 168 h, with an overdose of appropriate anesthesia and are stored at approximately -20°C pending potential analysis. Most organs can be easily homogenized and/or digested with a solubilizer. The carcass is often digested in organic alkaline solution followed by homogenization. For instance, the carcass may be digested in 30% methanolic potassium hydroxide solution (1-g sample weight per 1 mL) for approximately two days. Ethanol (two volumes of the sample weight) can be added to the solubilized sample and the net sample weight is measured for later calculation. The sample is further homogenized by a Polytron[®]. Owing to the high collagen content, a higher solvent volume to carcass weight ratio is generally required for homogenization on the large animal species, especially nonrodents, to prevent coalescing of the homogenate. An aliquot of homogenate (~ 200 mg) is taken in three to four replicates for the radioactivity determination using liquid scintillation counting to calculate the total remaining radioactivity. The percentage recovery in a carcass is calculated using Equation 9.5:

$$\begin{aligned} & \% \text{ of recovery in carcass} \\ &= \frac{\text{Total weight of solubilized material (g)} \times \text{sample concentration (DPM/g aliquot)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \\ & \quad \times 100 \end{aligned} \quad (9.5)$$

TABLE 9.5 List of Various Factors to be Considered, While Addressing Radioactivity Recovery Issues, Including Recovery From Carcass and CO₂ Exhalation with Rationale and Comments

	Design	Rationale and Comments
<i>Carcass</i>		
Criteria	If mass balance in excreta <90% (consider residual radioactivity in tissues)	Only in intact animals; Small animals preferred but mainly depend on drug under study
Method of analysis	Solubilization or homogenization and analysis using liquid scintillation counting or HPLC with radiochromagraphy detector	
Number of animals	Animals with recovery <90%	To add information about incomplete recovery
Time intervals	At the end of the study, for example, 168 h	—
<i>Expired CO₂</i>		
Criteria	If mean mass balance inclusive carcass <90%	To add information about incomplete recovery
Number of animals	$N = 2$ (only select one representative route)	—
Collection	0–12, 12–24 h <i>Optional:</i> 24–168 h in intervals metabolic cage	To avoid saturation of absorber To get mass balance recovery

Also see Fig. 9.6 for the basic considerations of troubleshooting.

If the mean mass balance including excreta and carcass is <90% for all routes and dosages and carbon dioxide is expelled as a metabolic product of the compound, one can monitor the radioactivity in the expired [^{14}C] CO_2 for more complete radioactivity recovery [32]. In a number of position of radioactive [^{14}C]-labeled compounds, the radioactivity of expired [^{14}C] CO_2 was successfully measured for the calculation of mass balance in the expired air [33]. Expired air can be collected by keeping the animals (rodents or nonrodents) in special metabolic cages in which air is drawn by vacuum pump with continuous air replacement. Exhaled air exiting the metabolic cage is passed through an appropriate trapping solution (usually alkaline solution), such as 2-methoxy-ethanol and 2-amino-ethanol (2:1) [34], and converted to nonvolatile carbonates. The trapping solution is replaced and assayed at designated times post dose so that the total radioactivity expired as labeled carbon dioxide can be determined [17,21,33]. The percentage recovery of expired radioactivity through exhalation is calculated from the concentration of radioactivity collected as carbonate using Equation 9.6:

$$\begin{aligned} & \% \text{ Recovery from } \text{CO}_2 \\ &= \frac{\text{Volume of trapping solution (mL)} \times \text{concentration (DPM/mL)}}{\text{Volume dosed (mL)} \times \text{dose concentration (DPM/mL)}} \times 100 \quad (9.6) \end{aligned}$$

Various parameters of designing and handling of radioactivity recovery in carcass and CO_2 are listed in Table 9.5.

9.5 USE, SELECTION CRITERIA, AND LIMITATIONS OF RADIOLABEL COMPOUNDS

Radiolabeled drugs have been widely used in mass balance studies since the radioactivity can be easily detected and quantified using scintillation techniques, and the disposition fate of drugs can be simultaneously characterized. The radioisotope needs to be carefully selected or designed for a successful mass balance study, along with the position of radiolabel in the compound, radiochemical purity, and the specific activity, for they can have a significant impact on the formation of metabolites and detection and thus affect the recovery of total radioactivity. For example, radiolabel isotope should be at metabolically stable position of the parent molecule such that it can stay associated with the metabolites for identification and quantification. Failure to do so not only affects the mass balance results but can also cause difficulties in differentiating endogenous compounds from the true metabolites [21]. Various radioisotopes can be used in mass balance studies, including tritium- ^3H , carbon- ^{14}C , phosphorus- ^{32}P , sulfur- ^{35}S , and iodine- ^{131}I [8,35–38]. The selected radiolabeled compound must have identical PK properties and metabolic fate as the unlabeled compound [39]. Prior knowledge of the metabolism of a compound (or known analogues) obtained from *in vitro* metabolism studies and/or *in silico* predictions can assist in determining the optimum radiolabeled positions and isotopes. Ideally, the same radiolabeled compound is used in both the preclinical and clinical mass balance studies, making human mass balance data more comparable to preclinical studies. ^3H and ^{14}C are the most commonly used isotopes in most of the mass balance studies. If heavier radioisotope are selected rather than ^{14}C and ^3H , the corresponding kinetic isotope effect needs to be considered because metabolic conversion can be affected by change in reaction

kinetics, as a result of the formation of strong chemical bonds by heavier isotopes [40]. ^{14}C is a preferred isotope due to its physiochemical stability, longer decay half-life, and higher radiation energy for better analytical sensitivity. Carbon ^{14}C has eightfold more energetic β -radiations (electrons) than that of tritium ^3H (0.156 vs 0.019 MeV), and thereby the detection sensitivity of ^{14}C is higher than ^3H . On the other hand, tritium-labeled isotope allows for a less expensive and more rapid chemical synthesis in most cases and a higher specific activity can be achieved (29.1 Ci/milliatom) than with ^{14}C (62.4 mCi/milliatom) [7,40]. These advantages may be useful for the early development stage and studies with a need of very small amounts of a compound. However, the major disadvantage of ^3H is its tendency to be exchanged with hydrogen in aqueous matrices, resulting in unlabeled compounds, especially when ^3H is bonded to a heteroatom or α -positioned to a double-bonded heteroatom [40,41]. If the drug compound has a large molecular weight (>700 g/mol), it is likely the molecule will divide into two metabolic moieties. The molecule can be dually labeled with ^3H and ^{14}C atoms to the designated moiety, respectively, and, thereby each moiety can be traced independently [21]. For either ^3H - or ^{14}C -labeled drug at the metabolically stable position, radiochemical purity is preferred $>98\%$ and should not be $<95\%$. The purity of the radiolabeled drug should be validated before the dose preparation, and stability of the radiolabeled drug under conditions of administered should be checked at predose and postdose time points [2].

9.6 DETERMINATION AND ANALYSIS OF MASS BALANCE AND PHARMACOKINETIC PARAMETERS

The main purpose for conducting the mass balance studies is to determine the mass recovery of administered radioactive dose, identify major metabolites and excretory pathways, and characterize the PK profile of the drug compound by means of measuring the concentration of parent drug/metabolites or total radioactivity in blood, plasma, urine, bile, and feces. In order to confirm the validity of mass balance results, these PK parameters obtained from the mass balance study can be compared with those from preclinical toxicity studies. All PK parameters for total radioactivity and parent drugs can be calculated with the software programs (e.g., WinNonlin), using non-compartmental or compartmental analysis method. For the p.o. dose, highest average plasma concentration ($C_{\max}$) and times to achieve this peak concentration ($T_{\max}$) are recorded directly from experimental observations for the drug and its metabolites. The radioactivity in blood and plasma is measured separately using LSC. These measured radioactivity parameters are also called *pseudo-pharmacokinetic parameters* and are calculated in the same manner as those of parent drug. The ratio of AUC of unchanged, intact parent drug versus the AUC of total radioactivity gives a measure of metabolites formation and their importance in drug disposition. The $\text{AUC}_{(0-\text{last})}$ is calculated using the linear trapezoidal rule, whereas $\text{AUC}_{(0-\infty)}$ is calculated using the Equation 9.7 as

$$\text{AUC}_{(0-\infty)} = \text{AUC}_{(0-\text{last})} + \frac{C_{(\text{last})}}{\lambda_z} \quad (9.7)$$

Here, the slope (λ_z) is derived from the slope of the log-linear line from at least the last three data points. This is used to calculate the half-life ($t_{1/2}$). Distribution half-life and elimination half-life are calculated to predict the accumulation in different

compartments and elimination time of the drug. The elimination half-life ($t_{1/2}$) of radioactivity in the plasma is important in selecting the time interval and further aid in determining the study end point, maximize the radioactive recovery in mass balance studies, and also assess the risk of accumulation of metabolites after multiple dosing [4]. Comparing the half-life of parent drug with that of total radioactivity gives a better understanding of the accumulation of the metabolites (*note*: detection sensitivity also needs to be taken into consideration). For the i.v. dose, the concentration of parent compound at time zero $C_{(0)}$, is back-extrapolated, based on a log-linear regression. The apparent volume of distribution at steady state (V_{ss}) is calculated from the i.v. data using expression given by Equation 9.8:

$$V_{ss} = \frac{\text{dose}_{(i.v.)} \times \text{AUMC}_{(0-\infty)}}{[\text{AUC}_{(0-\infty)}]^2} \quad (9.8)$$

where $\text{AUC}_{(0-\infty)}$ and $\text{AUMC}_{(0-\infty)}$ are the area under the concentration–time curve and area under the first moment concentration–time curve from time zero to infinity, respectively.

The total body clearance (CL_t), renal clearance (CL_r), biliary clearance (CL_b), and hepatic clearance (CL_h) can be calculated using following expressions.

The total systemic clearance (CL_t) is estimated from the ratio of dose divided by $\text{AUC}_{(0-\infty),(i.v.)}$ from the parent dose after i.v. dose and is given by Equation 9.9 [42]:

$$\text{CL}_t = \frac{\text{dose}_{(i.v.)}}{\text{AUC}_{(0-\infty),(i.v.)}} \quad (9.9)$$

In most cases, drug concentrations are measured in plasma rather than in blood, because sample preparation and drug analysis in plasma are simpler than in blood and this total systemic clearance is defined as equal to the total systemic plasma clearance, unless otherwise indicated in the text.

Renal clearance (CL_r) can be estimated from amount of unchanged parent dose excreted in urine over an extended period of time dividing by $\text{AUC}_{(0-t)}$ of parent drug after i.v. dose and is given by Equation 9.10 [42]:

$$\text{CL}_r = \frac{\text{Amount of parent drug excreted in urine up to time } t}{\text{AUC}_{(0-t),(i.v.)}} \quad (9.10)$$

The urine collection time should be long enough to account for almost the entire parent drug excreted in urine in order to get a reliable estimate of the CL_r . Additionally, it is desirable that $\text{AUC}_{(0-t)}$ is greater than 90% of $\text{AUC}_{(0-\infty)}$.

Biliary clearance (CL_b) of a drug is experimentally determined from the ratio of the amount of parent drug excreted in the bile versus the $\text{AUC}_{(0-t),(i.v.)}$ of parent drug after i.v. dose and is given by Equation 9.11 [42]:

$$\text{CL}_b = \frac{\text{Amount of parent drug excreted in bile from time 0 to } t}{\text{AUC}_{(0-t),(i.v.)}} \quad (9.11)$$

where $\text{AUC}_{(0-t),(i.v.)}$ is the AUC in plasma from the time 0 (zero) to t hours, and t is usually more than 24 h for sufficient bile collection after i.v. administration of a drug.

Clearance from different routes are additive and systemic or total clearance is given by the sum of individual organ clearances [43]. Assuming that the respiratory and extracorporeal clearances are negligible, hepatic clearance (CL_h) can be estimated by subtracting the sum of the renal and bile clearance ($CL_r + CL_b$) from the total clearance (CL_t) as expressed by Equation 9.12:

$$CL_h = CL_t - (CL_r + CL_b) \quad (9.12)$$

Equation 9.13 assumes that nonrenal and nonbiliary clearances may be due to liver metabolism. The cumulative percentage of collected radioactivity recovery from different matrixes (urine, feces, bile, cage wash, carcass, and expired air) gives the estimate of total mass balance recovery for any compound when compared with the dose administered, that is:

Total % radioactivity recovered = % of radioactivity recovered in urine (Eq. 9.1) + % of radioactivity recovered in feces (Eq. 9.2) + % of radioactivity recovered from cage wash (Eq. 9.3) + % of radioactivity recovered from bile (Eq. 9.4 is only applicable for BDC rats) + % of radioactivity recovered from carcass (Eq. 9.5) + % of radioactivity recovered from expired air (Eq. 9.6).

Table 9.6 summarizes the different methods for the calculation of various parameters from the mass balance study design with rationale and comments.

In addition, the mass balance studies can also determine the following other parameters from intact and BDC animals:

1. percent of radioactivity retention in different organs at any time during the study or at the end of the study,

TABLE 9.6 List of Factors to be Considered for Pharmacokinetic Parameters Analysis in Mass Balance and ADME Studies

Design	Calculations	Rationale and Comments
Pharmacokinetics of radiolabeled in blood and plasma	C_0 , $C_{\max}$, $T_{\max}$ observed; $AUC_{(0-168h)}$ and $AUC_{(0-\infty)}$; $t_{1/2}$ terminal half-life %; absorption (fraction absorbed, F_{abs})	With i.v. dosing determine C_0 ; With oral dosing determine $C_{\max}$, $T_{\max}$; Estimation of exposure; Prediction of accumulation of the drug-related radioactivity; Estimation of fraction absorbed; Comparison to toxicity studies; Comparison to other species
PK of unchanged drug in blood or plasma	C_0 , $C_{\max}$, $T_{\max}$ observed; $AUC_{(0-168h)}$ and $AUC_{(0-\infty)}$; Distribution and/or elimination half-life; CL , V_{ss} , MRT	Prediction of drug behavior Oral bioavailability First-pass estimate
Radioactivity in urine, bile, feces, cage wash, and carcass in rodents, if recovery in excreta is low	Cumulative % of dose % mass balance recovery % absorption (fraction absorbed, F_{abs})	Contribution of excretion route; Estimation of fraction absorbed for permeability classification needed for potential FDA biowaivers

Abbreviation: MRT, mean residence time.

2. level of parent drug and its metabolites in the target organs,
3. ratio of radioactivity exposure in organs (AUC_{organ}) and in plasma (AUC_{plasma}), and
4. fraction absorbed (F_{abs}) across the gastrointestinal barrier.

Oral bioavailability ($F\%$) gives an estimate for the extent of absorption and first-pass metabolism and it can be calculated from the ratio of exposure of parent drug after an p.o. dose to the parent drug exposure after an i.v. dose. Some reports have stated that the area under the curve (AUC) obtained from plasma radioactivity [21] can be used to calculate bioavailability, but it is not valid for the compounds with high first-pass metabolism. Tse and Laplanche [44] had already demonstrated the differences in the amount of drug-related material absorbed and the systemic bioavailability of the parent drug using the radioactivity.

The bioavailability ($F\%$) is estimated from the calculated AUC after the p.o. and i.v. administrations of parent drug using the Equation 9.13:

$$F\% = \frac{AUC_{(0-\infty), (p.o.)} \times \text{dose}_{(i.v.)}}{AUC_{(0-\infty), (i.v.)} \times \text{dose}_{(p.o.)}} \times 100 \quad (9.13)$$

The fraction absorbed or absorption (F_{abs}) can be calculated from the ratio of $AUC_{(0-\infty)}$ after the p.o. and i.v. administrations of total blood/plasma radioactivity using the same equation as for $F\%$.

This maximum fraction absorbed can be used to distinguish the drug on basis of permeability classification, which can be served as an important tool for potential FDA biowaivers. In some cases, the radioactivity excreted in urine can be used to calculate F_{abs} . Various other methods that can be used for the calculations of fraction absorbed are listed in Table 9.7 along with the limitations and recommendation.

TABLE 9.7 Different Methods for Estimation of Absorption and Bioavailability from Calculated Parameters of the Intact Parent Drug or Radiolabeled Drug, Total Radioactivity

Estimation of Percent Absorption or Fraction Absorbed (F_{abs})	Equation	Comments and Limitations
F_{abs} calculated from total blood/plasma radioactivity amount	$F_{\text{abs}} = \frac{AUC_{(0-\infty), (p.o.)} \times \text{dose}_{(i.v.)}}{AUC_{(0-\infty), (i.v.)} \times \text{dose}_{(p.o.)}}$	Not valid for a compound with high first-pass metabolism
F_{abs} calculated from total cumulative urinary radioactivity recovery ratio (p.o. vs i.v.)	$F_{\text{abs}} = \frac{D_{(\text{urine}), (p.o.)}}{D_{(\text{urine}), (i.v.)}}$	Minimal absorption; best suited for compounds with renal excretion >10%
F_{abs} calculated from bile-duct cannulated animals	$F_{\text{abs}} = \frac{D_{(\text{urine}), (p.o.)} + D_{(\text{bile}), (p.o.)}}{D_{(\text{urine}), (i.v.)} + D_{(\text{bile}), (i.v.)}}$	Value represents minimal absorption
F , oral bioavailability from intact parent drug, ideally in a crossover design	$F = \frac{AUC_{(0-\infty), (p.o.)} \times \text{dose}_{(i.v.)}}{AUC_{(0-\infty), (i.v.)} \times \text{dose}_{(p.o.)}}$	Assumes CL is independent of dosing routes and dose amount

D , fraction of the dose recovered from urine or bile.

9.7 CASE EXAMPLE FOR MASS BALANCE STUDY IN PRECLINICAL SPECIES

9.7.1 Compound A—Experimental Design

For compound A, preclinical mass balance studies were designed based on the methodology as described in Tables 9.1–9.5. The dose used was 5 mg/kg for the i.v. dose and 50 mg/kg for the p.o. dose for both the intact rat and BDC rat studies. The radiochemical purity was >99% for all study groups.

9.7.2 Compound A—Pharmacokinetic Parameters

All PK parameters for compound A were calculated based on the designs as discussed in Table 9.6. The time versus plasma concentration profile of unchanged compound A along with compound A-related total radioactivity after i.v. dosing and p.o. dosing are presented in Figs. 9.4 and 9.5, respectively. The maximum concentration ($C_{\max}$) for parent compound A observed in plasma is 950 ng/mL, which is achieved at 2 h post p.o. dosing, indicating a moderate absorption rate. Other observed and calculated PK parameters in different matrices are summarized in Table 9.8 for both p.o. and i.v. doses. The ratio of $AUC_{(0-\infty)}$ of parent drug versus $AUC_{(0-\infty)}$ of radioactivity is ~55%, indicating that some amount of metabolite(s) were formed from compound A and present in the blood circulation.

9.7.3 Mass Balance Parameters and Interpretations

The percentage of dose recovered for unchanged compound A in different matrices is shown in Table 9.9: All mass balance parameters were calculated from the derived equations (Eqs. 9.1–9.6) and using the methodology discussed in Table 9.6. As shown, compound A is predominantly eliminated as unchanged drug (57–69% of the total dose) in urine and feces. The major portion of the drug is recovered in feces with a

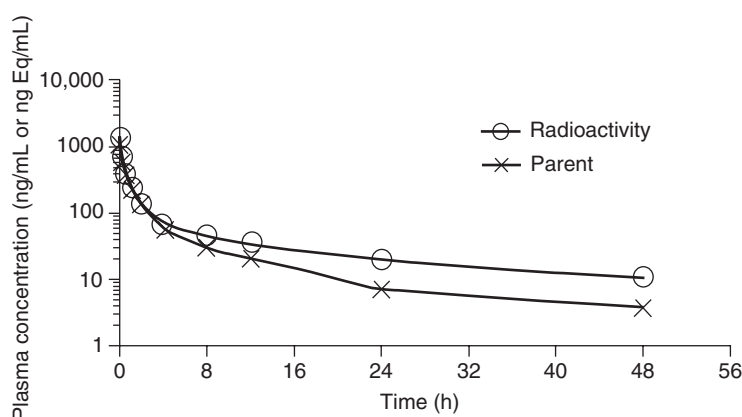


Figure 9.4 Plasma drug concentration versus time profile of parent (unlabeled) drug and radioactivity (labeled) drug after the intravenous dose. Concentration of unlabeled drug is given in units of ng/mL and concentration of labeled drug is given in units of ngEq/mL, respectively.

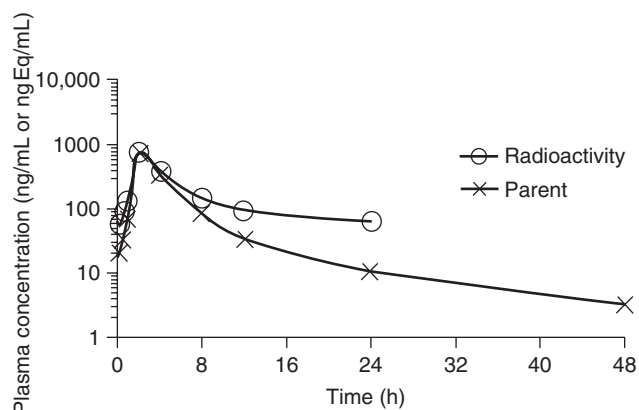


Figure 9.5 Plasma drug concentration versus time profile of parent (unlabeled) drug and radioactivity (labeled) drug after the oral dose. Concentrations of unlabeled drug is given in units of ng/mL and concentration of labeled drug is given in units of ngEq/mL, respectively.

TABLE 9.8 Pharmacokinetics Parameters of Unchanged Parent Compound A or Compound A-Related Radioactivity After Intravenous Dosing (5 mg/kg) and Oral Dosing (50 mg/kg) in Rats

PK Parameters	$C_{\max}$ (ng/mL or ngEq/mL) ^a		$T_{\max}$ (h)	AUC _(0-∞) (ng · h/mL or ngEq · h/mL) ^a		CL (L/h/kg)	V_{ss} (L/kg)	$F\%$ or F_{abs}	$t_{1/2}$ (h)	
	i.v.	p.o.	p.o.	i.v.	p.o.	i.v.	i.v.	p.o.	i.v.	p.o.
Plasma (parent)	1050	950	2	1500	3810	3.1	31	25	15	15
Plasma (radioactivity)	1350	1000	2	2700	6900	—	—	26	—	—
Blood (radioactivity)	1220	850	2	1680	4900	—	—	29	—	—

^aConcentration units are ng/mL for parent and ngEq/mL for radioactivity. $C_{\max}$ after intravenous dosing is referred to as C_0 .

TABLE 9.9 Unchanged Parent Compound A Recovered in Different Matrices

% Dose Recovery (Unchanged Drug)	Intact Rats		Bile-Duct Cannulated Rats	
	i.v.	p.o.	i.v.	p.o.
Excretion in urine	18	3.0	19	3.1
Excretion in feces	39	66	11	53
Excretion in bile	N/A	N/A	35	8.0
Total recovery (%)	57	69	65	64
CL _r (L/h/kg)	0.60	—	0.62	—
CL _t (L/h/kg)	3.1	—	—	—

Abbreviation: N/A, not applicable.

smaller amount in the urine following both routes of administration. The portion of unchanged parent compound A excreted in urine is approximately the same in intact and BDC rats after administration of both doses (19% for i.v. and ~3% for p.o.). As expected, the recovery in feces after i.v. dosing is lower in the cannulated compared to the intact rats (11% vs 39%), indicating biliary excretion of compound A, which was 35%. The renal clearance, CL_r , is comparable (~0.6 L/h/kg) between the intact and BDC rats.

The percentage of dose recovered as total radioactivity in different matrices is shown in Table 9.10. In intact rats, the fecal excretion of the radioactivity is dominant after both dosing routes accounting for 74% of dose after i.v. administration and 93% of dose after p.o. administration. The urinary radioactivity excretion is minor, accounting for 20% of the i.v. dose and 4% of p.o. dose, and is consistent in both intact and BDC rats. On the basis of combined biliary (21%) and renal excretion (5%), the absorption after p.o. dosing is estimated to be at least 26% for compound A, which is comparable to the values estimated from total plasma or blood radioactivity data (26–29%). The mass balance of radioactive dose recovered in excreta, including the cage wash is calculated using Equations 9.1–9.6 and is completed (~100%) within 168 h post dose. From these obtained parameters of the mass balance recovery, the calculation for various other parameters such as bioavailability, fraction absorbed, total clearance, renal clearance, biliary clearance, and hepatic clearance can be calculated based on Equations 9.9–9.13 and are listed as follows:

- The estimation of p.o. bioavailability of compound A, calculated from the parent drug using Equation 9.13, is ~25%, which is low.
- The estimation of fraction absorbed, calculated from the total radioactivity using Equation 9.13, is 26% (plasma) to 29% (blood), indicating that the absorption of compound A in rats is low and incomplete.
- The estimation of total clearance from the total exposure of the parent drug with time curve and the total dose administered i.v. calculated using Equation 9.9 is 3.1 L/h/kg, which indicates a high plasma clearance for compound A.
- The renal clearance is calculated by dividing percentage of parent drug recovered in urine after i.v. administration by the area of concentration under the curve with time, using Equation 9.10, and is 0.6 L/h/kg, which is 19% of the total clearance.

TABLE 9.10 Mass Balance Parameters for Compound A, Radioactivity Recovery in Different Matrices

% Dose Recovered (Radiolabeled Drug)	Intact Rats		Bile-Duct Cannulated Rats	
	i.v.	p.o.	i.v.	p.o.
Excretion in urine	20	4.0	21	5.0
Excretion in feces	74	93	16	74
Excretion in bile	N/A	N/A	63	21
Cage wash recovery	2.0	1.0	1.0	1.0
Total recovery (%)	96	98	101	101

Abbreviation: N/A, not applicable.

- The biliary clearance can be estimated from percentage of parent drug excreted in bile after i.v. administration, divided by the area of concentration under the curve with time, from the BDC rats, using Equation 9.11, and is 1.2 L/h/kg, indicating that 39% of total clearance is through bile.
- The hepatic clearance is calculated by subtracting the cumulative renal and bile clearance from the total clearance using Equation 9.12 and is 1.3 L/h/kg, suggesting that ~42% of the total clearance is hepatic clearance, suggesting that the drug is not extensively metabolized.

9.8 COMMON ISSUES DURING CONDUCT OF PRECLINICAL MASS BALANCE STUDIES

The most common challenge in mass balance studies is a low mass recovery of radioactivity. Theoretically, complete mass balance studies would require that 100% of the radioactivity administered should be recovered from excreta and cage wash, which is rarely possible. The most common reasons are that the excreta is not completely collected during the study, contributing to the loss of radioactivity balance collection, especially if the loss occurs within a period of high excretion rate of drug-related material [14]. As a rule of thumb, excreta samples need to be collected until the recovery is <1% of administered radioactivity in urine and feces for two consecutive days [4]. In the case for compounds with a long elimination half-life, as indicated by a prolonged retention of the radioactivity in blood, the sample collection period requires to be extended. Some other issues can influence the recovery of mass balance. Position of the radionuclide within the drug molecule should be carefully considered in order to prevent a labeled fragment of the drug from entering endogenous compound

TABLE 9.11 Reasons for Low Radioactivity Recovery with Proposed Recommendations

Reason for Low Recovery	Proposed Recommendation
<i>Artificial reasons</i>	
Inaccurate dose	Careful consideration in dose preparation and administration
Incomplete collection of excreta	Use of modified cages; collection of excreta until 1% recovery for consecutively two days
Portion of dose lost in vomiting (without collection)	Careful observation is needed after the oral administration
Long half-life ($t_{1/2}$) of radioactivity, long collection period, or diluted sample with drug concentration below the LOQ	Use of sophisticated analytical tools is recommended to obtain the maximum recovery
<i>Scientific reasons</i>	
Position of radioisotope in molecule, which is lost in expired air	If the recovery is below 80%, it is recommended to measure the radioactivity in the expired air
Tissue binding and noncovalent sequestration	Must be carefully studied for the parent drug and its metabolites from the preclinical studies

Abbreviation: LOQ, limit of quantification.

metabolism and also to prevent its conversion to a volatile metabolite as $^{14}\text{CO}_2$ [14], resulting in incomplete mass balance recovery. For lipophilic drugs that have low recoveries in urine and feces, the compounds can suffer from significant nonspecific adsorption to the collection containers. Mass balance studies provide limited information regarding the covalent binding to tissue macromolecules or to the blood proteins, which may account for a low mass recovery compared to total drug administered. The potential causes for low mass balance recoveries from preclinical and human studies are summarized in Table 9.11, and the reader is referred to a review article by Roffey

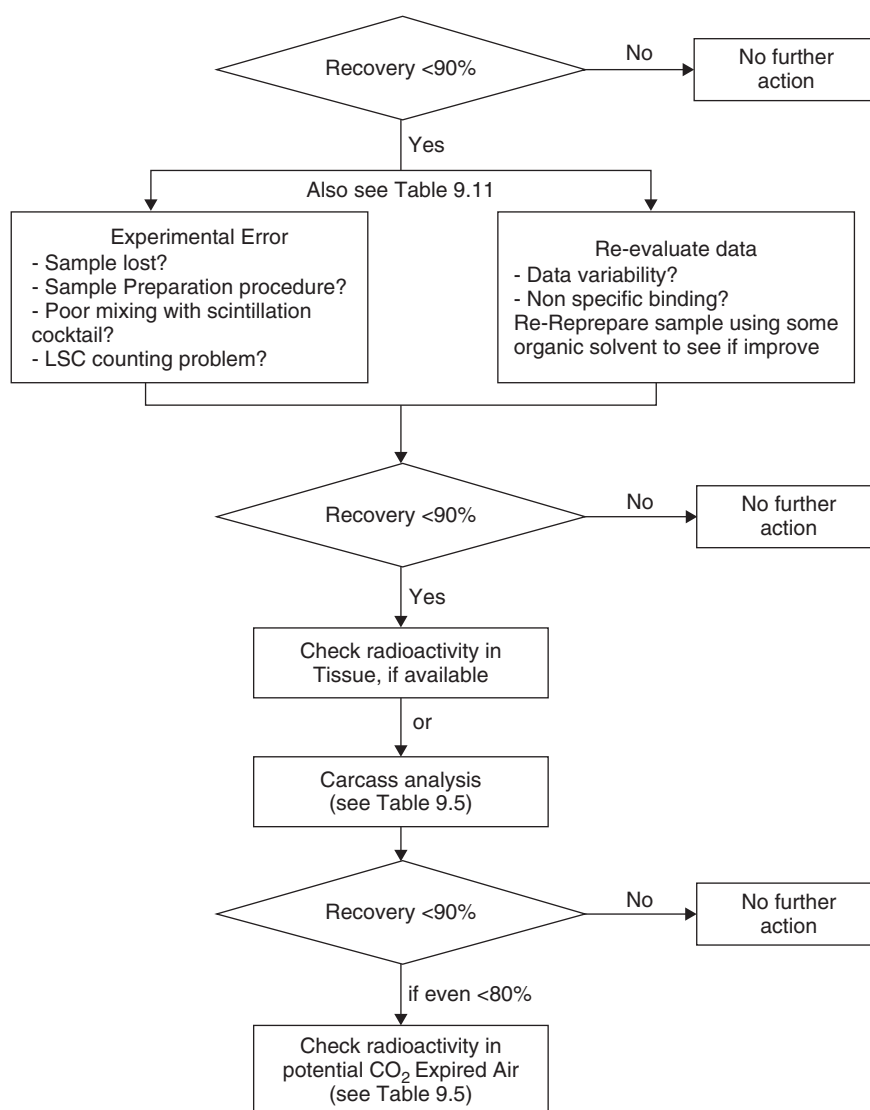


Figure 9.6 Basic considerations for the troubleshooting of low recovery issues in mass balance studies, usually conducted in rodents.

et al. [14]. Basic troubleshooting steps for mass balance studies with low recovery are listed in Fig. 9.6. In some BDC rat studies, the percent of radioactivity recovered in urine may be significantly higher when compared to that in intact rats even when the bile flow is in the normal range. This might be due to the alternation in the liver physiology (such as the changes in liver function, the expression of enzymes, and transporters) after bile-duct cannulation. In such cases, the data interpretation should be carried out with care and more analysis should focus on the results from the intact rats. As discussed, plasma samples may be taken for rat liver function tests before the bile study is conducted.

9.9 HUMAN MASS BALANCE STUDIES

After obtaining information of toxicity, PK, metabolite profiling, and organ/tissue radioactive exposure from preclinical mass balance studies, human mass balance studies can be conducted. These studies are required for small molecules by regulatory agencies before new drug application (NDA) filings and the mass balance data together with metabolite information is also important for the design of the clinical trials. The estimated timeline for conducting human mass balance studies is described in Fig. 9.1, which shows that the human mass balance studies are initiated once the phase 1 trials have been conducted and should be finished before the commencement of phase 2 trials. It is important to understand the metabolite profile of drugs in human and animal species as early as possible since both the ICH M3 (R2)[45] and the FDA [5] guidances highlight the need for providing safety data on human metabolites. Some metabolites are not much of toxicological concerns (e.g., most glutathione conjugates and glucuronides, except acylglucuronides). In general, ICH M3 (R2) recommends conducting the safety evaluation studies in preclinical species for metabolites that contribute more than 10% of total drug-related exposure and at significantly greater levels in human than the maximum exposure observed in the toxicity studies. The FDA guidance recommends that metabolites with an AUC exceeding 10% of the intact parent drug AUC will require toxicological evaluation in preclinical species. Because the use of radiolabeled drug in human mass balance studies adds more complexity, these studies are performed in accordance with the following codes and guidelines: Title 21, Part 56 CFR (Institutional Review Board Approval) [46]; Title 21, part 50 CFR (Protection of Human Subjects) [47]; the principles of the Declaration of Helsinki and its amendment; and Good Clinical Practice, in addition to standard International Conference on Harmonization (ICH) guidelines. For the studies to be conducted in the European Union (EU), European guidelines [48,49] are recommended to be followed. Before conduct all studies that include the use of radioisotope must be approved by the Administration of Radioactive Substances Advisory Committee (ARSAC). While these existing regulatory guidelines primarily focus on the safe and effective dose of radioactivity, in terms of exposure of ionizing radiations to various tissues/organs, to ensure “safe and effective” use of radioactive drugs [50], they do not state any acceptable criteria for total radioactivity recovery. Acceptable values for recovery, such as 85% [51] and 90% [4,14] have been proposed, but there is no widely accepted and defined cutoff value with a compelling underlying rationale [14]. The main objective of human mass balance studies is to establish the route of elimination of radioactive compounds and to compare the metabolites pattern in excreta, blood, and plasma with those obtained

from previously conducted preclinical mass balance studies for the safety exposure assessments. The nature of these studies is not intended for statistical evaluations, and ethics issues dictate the use of limited number of subjects [2]. Typically 3–8 subjects, aged 15–65 years, participate in human mass balance studies. It should be noted that conducting mass balance studies in children and pregnant women is virtually impossible due to the ethical issues, even though the drug disposition in these two groups differs significantly from the average population [52]. For anticancer drugs (mutagenic, carcinogenic, or teratogenic), healthy subjects are usually not used—patient subjects are needed for these mass balance studies. After at least an 8-h overnight fasting, in general, each subject receives a single clinically relevant dose of [^{14}C] drug containing 50–100 μCi of radioactivity. If [^3H] drug is administered the higher dose of radioactivity (50–1000 μCi) is needed to detect the low energy β -radiation of [^3H] [4]. The human radioactive dose must be evaluated for safety radiation exposure, using tissue distribution data from preclinical mass balance studies. Extrapolation of these data to humans allows the estimation of effective organ exposure to the radioactivity that is used for regulatory evaluation [50,53]. Mass balance studies in humans with the aid of the radiolabeled drug provide the total fate of drug-related material [7]. The projected human radiation exposure to selected radioisotope is estimated by direct extrapolation from the radioactivity exposure data obtained from the preclinical tissue distribution study (QWBA) based on body weight ratio [19] or drug exposure ratio (clinical plasma data compared to rat QWBA), with the assumption that drug-associated radioactivity is distributed and eliminated from the tissues in comparable manners between animal species and humans.

AMS can deliver analytical sensitivity far in excess of most other tools and has been applied to the preclinical and clinical mass balance studies [4,54]. The radiation risk, therefore, is not a concern for the study using AMS due to its low radioactive dose (tracer) and dosimetry data are not required. The high cost and low throughput are major disadvantages. The structure of metabolites should still be characterized by other analytical techniques unless the authentic reference compounds are available for coelution.

9.10 HUMAN DOSIMETRY CALCULATIONS

The purpose of the following section is to describe the various methods for the estimation of the human radioactive dose from preclinical mass balance studies using various calculation methods, which are also required for ARSAC approval (EU) to ensure the safety and efficacy after a human radioactive dose. The use of radiolabeled compounds in humans is also required to comply with the principles of As Low As Reasonably Acceptable (ALARA). International Commission on Radiological Protection (ICRP) [55] have modified the World Health Organization (WHO) exposure categories and made required the calculation of effective dose (E) [56]. This extrapolation of parameters obtained from preclinical mass balance studies to human organs is further based on the following factors:

1. the known human plasma level of the intact parent compound,
2. the observed ratio of radioactive compound relative to the parent compound in animals, and

3. the ratio of radioactivity in individual organs relative to the radioactivity in plasma for preclinical species.

The revised recommendations for radiological protection in ICRP publication-103 formally replace the previous ICRP publication-60 recommendations, and the reader is advised to follow the new guidelines to control the exposure from the radiation sources. The guidelines, provided by the U.S. Nuclear Regulatory Commission (USNRC), Part-20 of Standards for Protection against Radiation [56,57], which specify the list of different tissues/organs that are more susceptible to radiation damage and are required to be considered. Because of their susceptibility to damage, each organ is given a different weighing factor (W_T), by which the equivalent dose in various tissues/organs (T) is calculated. W_T represents the relative contribution of radioactivity in each defined tissue/organ and considers this information when calculating the total body radioactivity exposure, probability of fatal and nonfatal cancer, severe hereditary effects, and relative length of life lost. The ICRP publication-103 [58] and the U.S. Nuclear Regulatory Commission (USNRC), Part-20 of Standards for Protection against Radiation [56,57] have updated the radiation and tissue weighing factors based on the latest scientific information available and it is advised to follow these recommendations in both academia and industry. The list of specified tissues and their recommended weighing factor from ICRP-60, ICRP-103, and USNRC, Part-20 are listed in Table 9.12. For tissues/organs containing radioactivity, the exposure is calculated as the area under the radioactivity time curve (AUC) of the radiolabeled compound in the tissues/organs. Exposure in excretory organs, for example, gastrointestinal tract and bladder, is determined by the total radiation amounts in excreta (urine, bile, and intestinal contents) and the mean organ residence time in humans [20].

9.10.1 Dosimetry Calculations

The calculations for the human radiolabeled dose of [^{14}C] drug are carried out using the equations given below. The equations are derived based on the recommendations of the ICRP (Limits for intakes of Radionuclide by Workers, Radiation Dose to patients from Radiopharmaceuticals) [56,60,61]. The units of curies (Ci), as opposed to the International System of Units, Becquerel (Bq), usually express the radioactive dose. The conversion of these units is $1 \text{ mCi} = 37 \text{ MBq}$ and $1 \text{ Bq} = 60 \text{ DPM}$, where DPM means disintegrations per minutes. The fundamental equations derived for the calculation of the equivalent dose [59,62], which can be taken up in a given organ or tissue for [^{14}C] drug, are given as follows:

$$H_t = E \times f \times \text{AUC}$$

where

H_t = equivalent dose (mSv)

E = mean particle energy (MeV), and calculated for [^{14}C], $\text{MeV} = 0.050$

f = conversion factor = $\frac{\text{mSv} \times \text{g}}{\mu\text{Ci} \times \text{d} \times \text{MeV}}$ and calculated for [^{14}C], $f = 512$

AUC = time (d = day) integral of [^{14}C] drug concentration in target organ ($\mu\text{Ci d/g}$).

TABLE 9.12 List of Specified Tissues and Their Weighting Factors by ICRP Publication 60, ICRP Publication 103, and the US Nuclear Regulatory Commission Part-20^a

Name of the Tissue	Weighting Factor (W_T)		
	ICRP-60, Tables 2 and S-2	ICRP-103, Tables B.2 and B.3.5	USNRC Part-20, Sec. 20.1003
Gonads	0.20	0.08	0.25
Lower large intestine (colon)	0.12	0.12	NA
Lung	0.12	0.12	0.12
Red bone marrow	0.12	0.12	0.12
Stomach	0.12	0.12	NA
Bladder	0.05	0.04	NA
Breast	0.05	0.12	0.15
Liver	0.05	0.04	NA
Esophagus	0.05	0.04	NA
Thyroid	0.05	0.04	0.03
Bone surface	0.01	0.01	0.03
Skin	0.01	0.01	NA
Brain	NA	0.01	NA
Salivary glands	NA	0.01	NA
Kidney	NA	NA	NA
Remainder	0.05 ^b	0.12 ^c	0.30 ^d

Abbreviation: NA, not applicable.

The weighting factors are expressed as a fraction. The sum of the weighting factors is equal to 1.

^aRef. 57–59.

^bThe remainder is composed of the following additional tissues and organs: adrenals, brain, upper large intestine, small intestine, kidney, muscle, pancreas, spleen, thymus, and uterus.

^cThe remainder is composed of the following additional tissues and organs: adipose tissue, adrenals, connective tissues, extrathoracic airways, gallbladder, heart wall, kidney, lymphatic nodes, muscle, pancreas, prostate, small intestine wall, spleen, thymus, and uterus.

^dThe remainder 0.30 results from 0.06 for each of the five remainder organs that receive the highest dose (excluding the skin and lens of the eye).

After inclusion of the radiation energy, for practical use, the formula for the equivalent dose H_t for any organ or tissue is

$$H_t = k \times \text{AUC}_{(0-\infty)}$$

where k is the dose constant, and calculated for [¹⁴C], $k = \frac{\text{mSv} \times \text{g}}{\mu\text{Ci} \times \text{d}} = 25.6$.

The integration of the [¹⁴C] drug concentration–time function for the period zero to infinity is equal to the area under the [¹⁴C] drug concentration equivalent–time curve, $\text{AUC}_{(0-\infty)}$ ($\mu\text{gEq d/g}$), in the organ multiplied by the specific radioactivity (s):

$$\text{AUC}_{(0-\infty)} = s \times \text{AUC}_{(0-\infty)(\text{organ})}$$

where s is the specific activity of radioactivity ($\mu\text{Ci}/\mu\text{g}$).

Thus, the equivalent dose in any human organ or tissue (H_t) is calculated from the product of dose constant, specific radioactivity, and $\text{AUC}_{(0-\infty)(\text{organ})}$ as expressed in Equation 9.14:

$$H_t = k \times s \times \text{AUC}_{(0-\infty)(\text{organ})} \quad (9.14)$$

9.10.2 Dose Calculation for the Gastrointestinal Tract

The organs/tissues of the gastrointestinal tract are mostly exposed to the radioactivity present in the luminal contents of the organs and irradiating the organ wall. With the geometry factor of 0.5 and a defined organ residence time (τ) in the gastrointestinal tract segments, the organ equivalent dose, H_t (wall dose) is calculated by a modified formula from ICRP publication 53 [61] and expressed as Equation 9.15:

$$H_t = A \times F \times \frac{kt}{2m} \quad (9.15)$$

where

A = administering radioactivity dose (μCi)

F = fraction of the administered dose passing through the considered segment of the gastrointestinal tract

k = dose constant, and calculated for [^{14}C], $k = \frac{\text{mSv} \times \text{g}}{\mu\text{Ci} \times \text{d}} = 25.6$

τ = residence time given in days (d)

m = organ content weight given in grams (g).

As recommended by ICRP guidelines [60,61], the organ content weights and organ residence times in different organs for the human are summarized in Table 9.13. In the small intestine, the radioactivity exposure decreases due to absorption, and if absorption is a mono-exponential process, the decrease in radioactivity exposure in the small intestine is described by

$$A(t) = A^* e^{-\lambda t}$$

where A = radioactivity entering the small intestine (μCi) $A(t)$ = radioactivity remaining unabsorbed after time t , and passing to large intestine (μCi) λ = rate constant.

In that case, the equivalent dose of the small intestine is proportional to the time integral of the radioactivity present in the small intestine during the residence time τ and can be expressed as

$$H_t = A \times F \times \frac{k}{2m} \times \int_0^{\tau} e^{-\lambda t} dt$$

TABLE 9.13 List of Organ Content Weight and Organ Residence Time in Human

Organ	Organ Content Weight (g)	Organ Residence Time (h)	(d)	$k \cdot \tau / 2m^a$ (mSv/ μCi)
Stomach	250	1	0.0417	0.00213
Small intestine	1040	4	0.167	0.00205
Upper large intestine	220	13	0.542	0.03152
Lower large intestine	135	24	1.0	0.09481
Renal tract	1400	24	1.0	0.00914

^a m is organ content weight given in grams (g), τ is in units of days (d), k is 25.6 for ^{14}C .

From the limit 0 to τ after integration, this can be expressed as

$$H_t = A \times F \times \frac{k}{2m} \times \frac{1 - e^{-\lambda t}}{\lambda}$$

The rate constant, λ , is calculated by the following expression:

$$\lambda = \frac{\ln(A) - \ln(A\tau)}{\lambda}$$

9.10.3 Dose Calculation for Other Organs and Tissues

The equivalent dose (H_t) for any organ or tissue is calculated according to the general Equation 9.14. For the reference plasma, the equivalent dose H_{plasma} is

$$H_{\text{plasma}} = k^* s^* \text{AUC}_{(0-\infty)(\text{plasma})}$$

For individual organs and tissues in humans, the equivalent doses H_t are calculated based on the equivalent dose in plasma multiplied with the organ factor R_t , which is the ratio of $\text{AUC}_{\text{tissue}}$ to $\text{AUC}_{\text{plasma}}$ of radiolabeled drug from animal data. The factor R_t can be determined from the animal organ/tissue distribution data. For a detailed explanation, the R_t values from a rat tissue distribution study are summarized in Table 9.14, which are used to estimate tissue exposure in human, assuming comparable organ distribution patterns in rat and human. Thus, equivalent dose for human can be calculated from the modified Equation 9.16 and expressed as

$$H_{t(\text{human})} = H_{\text{plasma}(\text{human})} \times R_t \quad (9.16)$$

9.10.4 Effective Dose

According to the ICRP publication-60 and ICRP publication-103 [Recommendations of the ICRP, Annals of the ICRP (1991 and 2007)] [23,56,58], the whole-body effective dose (E , in units of mSv) is the sum of the weighted equivalent doses of individual tissue/organs. The effective dose is determined from the individual organs and tissues equivalent doses, H_t , using Equation 9.17. It is recommended to be <1 mSv to fall in the minor to intermediate exposure effect category set by WHO. The comparisons of weighing factors for different tissues/organs recommended by ICRP publication-60 and revised ICRP publication-103 and USNRC Part-20 is listed in Table 9.12. The ICRP publication-60 specifies 12 tissues/organs (Table 9.12) that must be taken into consideration because of their susceptibilities to radiation damage and the remainder is composed of adrenals, brain, upper large intestine, small intestine, kidney, muscle, pancreas, spleen, thymus, and uterus. In a more recent ICRP publication-103, brain and salivary glands were added to the mandatory organs (Table 9.12), thus making them a total of 14 and the remainder is composed of adipose tissue, adrenals, connective tissue, extrathoracic airways, gallbladder, heart wall, kidney, lymphatic nodes, muscle, pancreas, prostate, small intestine wall, spleen, thymus, and uterus. On the other hand, USNRC Part-20 only specifies six tissues required for consideration and an additional five others that receive the highest dose excluding skin and lens of the eye. Based on the principle of ALARA, it is recommended to use the weighting factor recommended

TABLE 9.14 Determination of Human Equivalent Dose in Tissues and Calculation of Whole Body, Committed Effective Dose

Organ/Tissue	R_t for Organs ^a	Equation Used ^b	Human Equivalent Dose (H_t) (mSv)	Weighting Factor (W_T)	Individual Organ Effective Dose ($W_T \cdot H_t$) (mSv)
Blood (reference organ)	5.05	14	0.00592	NA	NA
Gonads (testis)	1.8	16	0.01069	0.20	0.00214
Bone marrow	6.93	16	0.04103	0.12	0.00492
Lung	4.56	16	0.02698	0.12	0.00324
Stomach	—	15	0.107	0.12	0.0128
Lower large intestine (colon) ^c	NA	15	4.266	0.12	0.5122
Liver	87.16	16	0.516	0.05	0.0258
Esophagus	14.1	16	0.0831	0.05	0.00416
Fat (white) ^d	4.36	16	0.00112	0.05	0.0000558
Skin	37.47	16	0.2218	0.01	0.0022
Thyroid gland	24.53	16	0.1452	0.05	0.00726
Bladder/renal tract ^c	—	15	0.2743	0.05	0.0137
Bone mineral	6.67	16	0.00171	0.01	0.0000171
Eye (membrane) ^e	1518	16	8.987	0.025	0.225
Average of remainder	—	16	0.03271	0.025	0.00818
Sum of weighting factors = 1.0					
Committed effective dose, E (mSv)		17	—		0.822

Abbreviation: NA, not applicable.

^a R_t in Equation 9.16 is the ratio of tissue AUC relative to plasma AUC of ^{14}C , usually from the rat. Human plasma data used in the calculation are not shown.

^b For excretory organs (stomach, lower large intestine, and bladder), the H_t was also calculated by Equation 9.16 for the tissues. As Equation 9.15 for organ content provided a higher, more conservative H_t , this higher H_t value is reported.

^c Dose calculation is based on radioactivity expected to be present in the lumen of the organ.

^d Breast is not sectioned; the fat tissue is alternatively used with the weighting factor for the breast.

^e A weighting factor of 0.025 is applied to ocular uveal tract as its equivalent dose is in excess of the highest dose in any of the twelve organs for which a weighting factor is specified.

in ICRP publication-103 in Tables B-2 and B-3.5. The average radioactive dose for these tissues/organs of the body is used for calculations of effective dose (E) [56] and is given by the following expression:

$$E = \sum W_T \times H_{t(\text{human})} \quad (9.17)$$

where

W_T is the weighting factor for tissue or organ, T

$H_{t(\text{human})}$ is the human equivalent dose in tissue or organ, T .

The calculation of effective dose gives a prospective dose assessment for the planning and optimization of radiation safety and allows a retrospective dose assessment to demonstrate compliance with dose limits or dose constraints in radiological protection.

9.11 EXPERIMENTAL DESIGN OF HUMAN MASS BALANCE STUDIES

With the knowledge on the study designs and the results of the preclinical mass balance studies, the design for human mass balance studies can be appropriately established. Before conducting these studies, an independent institutional review board must approve the study protocol and the written informed consent documents should be obtained from all subjects before the enrollment into the study. The person preparing the dose must be well trained in pharmaceutical handling of the radionuclide, and both nonlabeled drug and radioactively labeled drug should be formulated together in the final dosage form. Both the dosage form and route of administration are recommended to be relevant to the intended use in clinical practice, as determined from the other previous studies. As mentioned previously in preclinical studies, the therapeutic efficacy/purity of the administered dose, including unlabeled and radiolabeled tracers must be determined exactly for the estimation of PK parameters and mass balance recovery parameters. Administration of a radioactive substance to humans should be performed in separate dedicated rooms, and only the qualified study personnel should be allowed with the subject [4]. Coadministration of other supplements such as high fiber diet, mild laxative, or regular fluid intake during the study period to allow sufficient defecation and adequate urination are recommended. In addition, it is advisable to rinse the container after the p.o. administration (if the dosage form is a solution or a suspension and is administered through a container), with suitable solvent to quantify the residual radioactivity [4]. The collection of radioactive biological samples (blood, urine, and feces) for the human studies should be long enough and optimum to allow the estimation of maximum recovery of the radiolabeled compound and its PKs without excessively burdening the patients. The collection interval of blood and urine samples can be designed based on the preclinical and clinical information, such as terminal half-life of the compound; otherwise, it is recommended to take more frequent samples during the distribution phase but less frequent during the terminal elimination phase. The date, time, and weight of the collection container should be recorded, before and after the collection period. Ideally, the collection of excreta should be stopped when all administered radioactivity has been recovered, which is not always the case with human mass balance studies. Assuming the elimination of the drug-related materials (metabolites) is similar to the parent drug, then plasma samples must be taken for at least four terminal half-lives [63], which can be established from previously conducted phase I studies. Under this assumption, 93% of the total radioactivity should be excreted after four half-lives. However, if it is taking more than the expected time to meet the criteria of the radioactivity recovery, then the study completion time may need to extend for more than seven days, sometimes up to 10 days or longer. Under certain circumstances (e.g., too long to hold them in the study center), the subjects under study can be allowed to go back to their homes and asked to bring the excreta (urine and feces) samples to the designated study center to increase the recovery of radioactive dose. For this, it is recommended to determine the levels of radioactivity recovery simultaneously in the collected samples, to get evidence-based extension of collection period. Usually, if there is no stability data available for the study compound and its metabolites in the urine and feces, it is recommended to store all samples at -20°C or less; otherwise, degradation products can mistakenly be identified as metabolites [4]. The sample analysis and the calculation of mass balance and PK parameters are usually performed in the same manner as for preclinical studies

discussed earlier and are summarized in Tables 9.6 and 9.7. It is recommended to calculate these PK parameters for the unchanged drug and its metabolites in human plasma matrix, in place of drug-related total radioactivity, as it composes of unchanged drug and various radioactive metabolites, which will result in pseudo-PK parameters. These total radioactivity-based PK parameters may be used to estimate the absorption in the human body, and after the metabolite profiling, the elimination pathways can be estimated based on the amount of radioactivity recovery in excreta and metabolite profile in each excreta. The contributions of each circulating component (including the parent) are estimated from the ratio of AUC of each component to the AUC of total radioactivity. These metabolites further characterize their contribution toward desired pharmacological and/or toxicological effects as needed.

9.12 CASE EXAMPLE FOR HUMAN MASS BALANCE

9.12.1 Drug B—Mass Balance Study Design

Three healthy, nonsmoking males were chosen from age 20 to 40 years, and a single p.o. dose of 100 mg containing labeled [^{14}C] plus unlabelled drug compound was administered p.o. The p.o. route was chosen because this was the proposed route of administration for human use in the clinical setting. The PKs and disposition of radioactivity in humans were cautiously predicted and the parameters obtained from the preclinical studies were used to calculate the radiation dose for human administration. The radiation doses (equivalent doses) for the individual organs following a single p.o. dose were calculated as described in Section 9.10 and listed in Table 9.14. All the calculations used in this example were based on the ICRP publication-60, and for future studies, as mentioned earlier, readers are advised to follow the revised recommendation from ICRP publication-103 for the calculation of committed effective dose for humans to get a minimum human radioactivity exposure. As shown in Table 9.14, the committed effective dose (human whole-body dose H_{wb}) was calculated to be 0.822 mSv for the radioactive dose of 47 μCi at a drug dose of 100 mg. This predicted effective dose is below the ICRP recommended dose (1 mSv); thus, the radiation health risk of the study volunteers was extremely low.

The radioactive dose given per subject was 47 μCi (1.85 MBq) for the 100-mg dose after calculation. Blood was collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144, and 168 h post dose by either direct venipuncture or an indwelling cannula inserted in a forearm vein. Eighteen milliliters of venous blood was collected at each time point in heparinized tubes. Plasma was separated from whole blood by centrifugation, transferred to a screw-top polypropylene tube, and frozen immediately. Urine samples were collected at pre dose and at 0–4, 4–8, 8–12, 12–16, 16–24, 24–36, 36–48, 48–72, 72–96, 96–120, 120–144, and 144–168 h post dose. Feces were collected as passed from time of dosing until at least of 168 h postdose. All these samples were stored at -20°C or less until analysis.

The radioactivity was measured in plasma, blood, urine, and feces by LSC with the same methodology discussed earlier in preclinical studies. The parent drug was measured using a validated LC/MS/MS assay and metabolites were measured semi-quantitatively using HPLC radiodetection with off-line microplate solid scintillation counting and structural characterization by LC/MS.

TABLE 9.15 Pharmacokinetic Parameters After a Single Oral Dose of 100 mg of Drug B in Humans

PK Parameters	Plasma Parent Drug	Plasma Radioactivity	Blood Radioactivity
$C_{\max}$ (ng/mL or ngEq/mL) ^a	397 ± 92	594 ± 153	470 ± 87
$T_{\max}$ (h)	1.1 ± 0.6	2.1 ± 1.3	2.0 ± 1.4
$AUC_{0-\infty}$ (ng · h/mL or ng Eq · h/mL) ^a	1610 ± 460	6000 ± 1610	3850 ± 1580
Apparent $t_{1/2}$ (h)	2.8 ± 1.0	4.6 ± 0.3	5.1 ± 2.5
CL/F (L/h)	65.2 ± 15.5	—	—
V_z/F (L)	269 ± 125	—	—

^aConcentration units are in ng/mL for the intact parent drug and ngEq h/mL for drug-related radioactivity. All values are presented as mean ± SD.

9.12.2 Pharmacokinetics Parameters for Drug B

The PK parameters of drug B were determined by conducting noncompartmental analysis using WinNonlin. The highest concentration in plasma ($C_{\max}$) was achieved at 2.1 h post dose with the mean value of 594 ng Eq/mL (total radioactivity) and 1.1 h post dose with mean value of 397 ng/mL for the intact, unchanged parent drug in all subjects, suggesting rapid gastrointestinal absorption of the drug. The mean plasma concentration–time profiles and other PK parameters of total radioactivity and unchanged drug compound after a single p.o. dose of [¹⁴C] drug are summarized in Table 9.15. The average terminal elimination half-life ($t_{1/2}$) of radioactivity and parent drug was calculated to be 4.6 and 2.8 h, respectively.

9.12.3 Mass Balance Parameters and Interpretations

After a single p.o. administration of 100-mg dose of [¹⁴C] drug B, radioactivity was excreted predominantly in urine (Table 9.16). After 168-h postdose, the excretion in urine and feces averaged 85.4 ± 4.4% and 14.8 ± 3.5% of the administered dose, respectively. The cumulative excretion of radioactivity was complete after 168 h postdose in all subjects averaging 100.2 ± 1.1%, as summarized in Table 9.16. The

TABLE 9.16 Cumulative Excretion of [¹⁴C] Radioactivity in Urine and Feces after Single p.o. dose of 100 mg of Drug B in Humans (Mean ± SD)

Time Period (h)	Urine (% Dose Excreted)	Feces (% Dose Excreted)
0–24	72.7 ± 4.8	1.37 ± 2.0
0–48	81.6 ± 4.2	9.93 ± 8.0
0–72	83.8 ± 4.4	13.2 ± 4.8
0–96	84.7 ± 4.4	14.3 ± 3.7
0–168	85.4 ± 4.4	14.8 ± 3.5
Total (% radioactive dose excreted in urine and feces)	100.2 ± 1.1	

elimination of radioactivity was rapid as more than 90% of the radioactivity was recovered in first 48 h after the dose was administered. Approximately 27% of the drug dosed was excreted as intact, unchanged parent drug (22.5% in urine and 4.54% in feces). The extent of absorption is estimated to be higher than 85.4% (amount of dose recovered in urine) and likely to be even higher since only 4.54% of dose was recovered in feces as unchanged drug. These results were in agreement with the absolute p.o. bioavailability (85%) observed in another clinical study with a single i.v. dose. Since ~22.6% of the dose in urine was recovered as unchanged compound, the renal clearance was estimated to be ~15 L/h.

9.13 CONCLUSIONS

Mass balance studies play an important role in the development of new drugs. These studies give in-depth understanding of absorption, bioavailability, routes (renal, biliary, hepatic, or gastrointestinal), and extent of excretion, metabolite profiling, metabolic pathways, and clearance mechanisms of a drug. The results from these studies aid in designing and conducting renal and hepatic impairment studies, DDI studies, and monitoring the metabolite(s) that may relate to pharmacology or toxicological concerns. The mass balance information will also help in understanding the mechanisms of intersubject variability of the drug exposure and potential DDI in terms of metabolism or transport. Moreover, across species mass balance studies can provide important information on whether a metabolite observed in human is required for separate safety testing in preclinical studies to fulfill the regulatory requirement as stated in the FDA and ICH guidances.

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10 Bioavailability and Bioequivalence

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10.1 Introduction	1
10.2 Bioavailability	2
10.3 Bioequivalence	8
10.4 Determinants of Oral Bioavailability	16
10.5 Summary	32
References	32

10.1 INTRODUCTION

To provide therapeutic benefit, pharmaceutical agents must gain access to their intended sites of pharmacological effect. Although pharmacologic effect is governed by interactions between drug and molecular targets such as cellular receptors or enzymes, drug transfer from the site of absorption and subsequent distribution to extravascular tissue also contributes to the extent and timing of drug effect. Thus, it follows that the magnitude of effect will generally be related to the amount of drug available to the site of action, whereby insufficient systemic drug levels may result in therapeutic failure and excessive levels could lead to toxicity.

To define safe and effective dosage regimens, an understanding of drug concentrations and their relationship to drug pharmacodynamics (PD) is required. Although some drugs may be administered directly to the site of action, such as by topical, pleural, or peritoneal routes, a majority of drug substances must enter the general circulation and be distributed to tissue before reaching their intended site of action. Therefore, the extent of drug transfer to the general circulation can be a function of drug distribution across successive membrane and cellular barriers. As a dose is transferred between the site of administration and its site of measurement in the circulation, the serial arrangement of physiological membranes and cellular elements may lead to cumulative drug loss. Thus, bioavailability is the pharmacokinetic (PK) parameter that describes the proportion of a dose to successfully reach the bloodstream as parent drug [1].

Bioavailability studies are largely concerned with determining systemic drug availability after oral administration, as the oral route is the intended mode of administration for the majority of drug substances. However, absorption of an oral dosage form is a complex process that is influenced by gastrointestinal (GI) physiology as well as the physicochemical properties of a drug. Following oral administration, drug must pass

from the GI lumen, across the gut wall, and into the hepatic circulation before systemic distribution. In addition to GI physiology, transfer of an oral dose to the systemic circulation also depends on drug solubility in aqueous and lipid environments as well as the potential for recognition by membrane transporters and metabolizing enzymes. From a PK perspective, bioavailability studies relate systemic exposure to drug absorption, while helping to understand mechanisms underlying drug distribution, transport, and metabolism.

Bioavailability studies are also conducted to evaluate the performance of one or more drugs or drug products against a previously defined reference. Such studies are referred to as *comparative bioavailability* studies and are designed to demonstrate bioequivalence of test and reference formulations for both new drug as well as generic drug products. While comparative studies are important elements of drug development, they may also be conducted after drug product registration in support of manufacturing changes [2]. Early in the drug development process, oral bioavailability of an initial dosage form might be determined relative to drug administered by the intravenous (IV) route or relative to an oral solution. As drug development progresses toward registration, improvements in oral delivery frequently lead to formulation changes and comparative bioavailability studies may be required in order to confirm equivalent PK and reestablish drug safety and efficacy of the reformulated dosage form relative to the earlier form. For marketed drug products, studies to demonstrate bioequivalence could be necessary following major changes in components, composition, and/or method of manufacture made after approval.

Determining bioequivalence is also a critical component for abbreviated new drug application (ANDA) submissions of generic drug products. Approval of generic drug products relies on the bioequivalence between the generic and innovator formulations. In order to be considered bioequivalent, drug products must contain the same active ingredient and display comparable bioavailability when studied under similar experimental conditions [3]. Thus, it is assumed that two bioequivalent drug products will be therapeutically equivalent and may be substituted for one another.

This chapter provides an introduction to the interrelated concepts of bioavailability and bioequivalence and their application to characterize drug disposition. While calculating bioavailability provides an assessment of the amount of drug reaching the systemic circulation relative to a reference dose, demonstration of bioequivalence for two drug formulations further permits an important assumption of therapeutic equivalence and potential for product substitution in clinical practice. As drug products are frequently designed for administration by the oral route, potential sources of drug loss that could contribute to incomplete oral bioavailability are also reviewed.

10.2 BIOAVAILABILITY

Bioavailability is characterized by the rate of drug absorption, how fast a drug enters the systemic circulation, and its extent of absorption, the quantity of dose to enter the body. These essential features of a nonvascular dosage form define the time course of circulating drug concentrations and their relationship to therapeutic effect, where the onset of drug effect is often a function of absorption rate and effect duration is typically related to the extent of absorption. Thus, the PK parameter bioavailability is

used to describe the proportion of a dose transferred to the site of drug measurement that becomes available for tissue distribution.

In experimental terms, drug bioavailability is determined by comparing the exposure levels of intact drug of a test dose to the exposure levels of a reference dose. When the entire reference dose is available at the site of sampling, as assumed for drugs administered by the IV route, then the systemic availability of a test dose is referred to as *absolute bioavailability*.

Drug concentrations are commonly measured in venous blood or plasma and therefore intravenously administered drugs are considered to be completely available within the systemic circulation. Although this is a valid assumption when intact drug reaches the arterial bloodstream without loss, experimental verification of the assignment of 100% bioavailability is not common. On IV administration, the entire dose becomes available to the pulmonary circulation during exchange to the arterial blood supply. While contributions to first-pass metabolism by the lung are difficult to quantify, the activities of several cytochrome P450 (CYP) enzymes are present in human lung [4,5]. In addition, recent evidence suggests that CYP activities localized in the right ventricle of the human heart may also play a role in pulmonary first pass [6]. Although the potential for pulmonary first-pass extraction exists for an IV dose, equivalent extraction is assumed for an oral dose during exchange to the arterial blood supply before sampling and therefore the net effect on estimating bioavailability is thought to be negligible.

Frequently, however, drug administration by the IV route cannot be considered, as the majority of drugs are not approved for IV use nor have they been monitored in a clinical development setting following IV administration. In order to evaluate human IV PK for absolute bioavailability determination, supportive toxicology and manufacturing data are also required to demonstrate drug safety associated with IV dosing. Additionally, dosing formulations developed for a nonvascular route may not be suitable for human IV use and thus identification of a formulation compatible with IV administration may also be required. Although the study of human PK following IV administration would generate valuable exposure data for future bioavailability characterization, approval to conduct clinical PK studies is not always sought.

When IV dosing is not plausible, both the reference dose and test dose of the same drug substance are introduced by nonvascular route(s) of administration. Consequently, systemic exposure following a test dose will be compared to a reference dose for which complete availability at the site of measurement cannot be assumed. The parameter *relative bioavailability* is used to characterize the availability of a test dose relative to a nonvascular reference dose.

Bioavailability studies to compare drug formulations dosed by nonvascular routes of administration are frequently undertaken to demonstrate generic drug bioequivalence to an innovator reference formulation. The calculation of relative bioavailability may also contribute to understand exposure–effect relationships for drug products when measurements of drug levels occur directly at the site of action. For instance, concentrations of the anticancer agent temozolomide were determined in brain interstitium by intracerebral microdialysis after oral temozolomide administration. Using this approach, temozolomide bioavailability within the central nervous system was determined to be 17.8% relative to systemic temozolomide concentrations after oral dosing [7]. Similarly, external γ -scintigraphy was used to determine relative bioavailability directly at a local site of drug action following nasal spray application of cromolyn sodium [8]. Although these specialized techniques for drug monitoring are not routine

approaches to determine drug bioavailability, knowledge of drug disposition at the site of therapeutic activity may further provide an understanding of exposure–effect relationships.

In other cases, however, alternate routes have been employed to facilitate drug application directly to the site of action when drug loss that may occur after oral dosing has not supported clinical use or when systemic exposure is to be minimized by design. Nonoral drug administration by topical [9], inhalation [10,11], and intranasal [12] routes are common. However, comparative bioavailability studies based on systemic exposure are not always possible. For instance, dermatological agents administered by topical application to the skin effectively maximize drug concentration at the site of action with minimal drug present in circulation. Consequently, systemic availability does not necessarily reflect local tissue exposure. Comparisons of drug formulations for bioequivalence determinations have instead utilized measures of *in vivo* tissue exposure such as tape stripping and microdialysis [9,13]. Recent efforts have also attempted to relate measures of *in vitro* permeability from an excised human skin model for *in vivo* efficacy predictions [14].

10.2.1 Calculation of Absolute Bioavailability

Following oral dose administration, maximum plasma concentration of drug ($C_{\max}$) is commonly used to evaluate the rate of absorption, while total area under the plasma concentration versus time curve (AUC) is used to evaluate the extent of absorption. However, in terms of estimating drug bioavailability, the proportion of dose reaching the systemic circulation as intact drug is determined by comparison of AUC exposures. When plasma drug concentrations are compared after administration of an extravascular test dose and IV reference dose, absolute bioavailability (F) is calculated as the AUC ratio of extravascular (AUC_{ev}) and IV (AUC_{iv}) exposures. As the IV route of administration is assumed to result in complete systemic drug availability, reference to AUC_{iv} provides an estimate of absolute bioavailability. Often the occurrence of high initial drug concentrations after IV dosing precludes evaluation of a matched dose by a nonvascular route. Accordingly, dose levels are taken into account when calculating absolute bioavailability [1]:

$$F = \frac{AUC_{\text{ev}}}{AUC_{\text{iv}}} \times \frac{(\text{Dose})_{\text{iv}}}{(\text{Dose})_{\text{ev}}} \quad (10.1)$$

Bioavailability may also be calculated from urinary excretion data for drugs that undergo renal elimination. When renal clearance accounts for the same proportion of total drug clearance after IV and extravascular administrations, absolute bioavailability can be estimated from urinary excretion data. This approach is feasible as the fraction of drug excreted unchanged in urine is independent of the route of administration within a linear dose range. However, a requirement for this approach is the complete collection of dose excreted in urine. Thus, by comparing the total amount of unchanged drug excreted in urine (Au_{inf}) after extravascular and IV dosing, the fraction of extravascular dose available in the systemic circulation can be determined by

$$F = \frac{(Au_{\text{inf}})_{\text{ev}}}{(Au_{\text{inf}})_{\text{iv}}} \times \frac{(\text{Dose})_{\text{iv}}}{(\text{Dose})_{\text{ev}}} \quad (10.2)$$

Inherent in Equations 10.1 and 10.2 is the assumption that drug clearance remains constant and independent of the route of administration. Clearance of drug from the systemic circulation is defined by the rate of drug elimination and drug concentration in plasma [15]. Therefore, in terms of mass balance, systemic clearance (CL) may be expressed as a ratio of amount of bioavailable dose and total AUC exposure in plasma such that

$$CL = \frac{F \times \text{Dose}}{AUC} \quad (10.3)$$

Equation 10.3 can be simplified for an IV dosing situation when complete bioavailability is assumed (i.e., $F = 1$). Thus, expressing an AUC ratio following extravascular and IV administrations in terms of bioavailable dose and systemic clearance, it follows that absolute bioavailability may be calculated by the following:

$$F = \frac{AUC_{ev}}{AUC_{iv}} \times \frac{CL_{ev}}{CL_{iv}} \times \frac{(\text{Dose})_{iv}}{(\text{Dose})_{ev}} \quad (10.4)$$

When the assumption of constant clearance holds after IV and PO dosing, Equation 10.4 may be simplified to Equation 10.1.

When plasma drug levels fall below the lower limit of assay quantitation soon after dose administration, insufficient concentration–time data may lead to unreliable estimates of AUC exposure and inaccurate bioavailability determinations. In such cases, parent drug bioavailability could be determined indirectly by monitoring metabolites present in circulation. Accordingly, determinations of plasma metabolite AUC (AUC_m) after extravascular and IV dosing can be used to calculate parent drug bioavailability when presystemic first-pass metabolism does not play a role in metabolite formation [16]:

$$F = \frac{AUC_{m,ev}}{AUC_{m,iv}} \times \frac{(\text{Dose})_{iv}}{(\text{Dose})_{ev}} \quad (10.5)$$

10.2.2 Variability in Bioavailability Estimates

Variation in oral bioavailability may arise from potential differences in drug absorption or first-pass elimination from the gut and liver across a patient population. However, it is generally accepted that variability will be greatest for drugs characterized by low bioavailability due to high first-pass extraction [17]. Accordingly, when variability in drug PK among individuals is large, predictions of PD effect at a given dose level are met with uncertainty. Moreover, PK variability between individuals is of particular concern for drugs with a narrow therapeutic index, as relatively small differences exist in drug concentrations associated with PD effect and toxicity or therapeutic failure.

Individual differences in drug PK can arise from physical differences in age, sex, and body size as well as disease state. In addition, there is increasing evidence that genetic differences in enzyme and transporter activities and expression also contribute to PK variability. In some cases, such as for anticancer agents, attempts to individualize treatment have involved calculating dose in terms of body surface area. Alternatively, bioavailability studies conducted in healthy subjects commonly employ a crossover design within the same group of individuals to minimize potential sources of PK

variability. This testing paradigm ensures all treatments evaluated in the study can be compared for each individual across the same panel of subjects. Thus, a crossover design avoids potential bias in comparisons of AUC exposure and decreases the effects of variability in drug elimination between subjects (intersubject). However, crossover studies do not account for PK variability within the same individual (intrasubject). Differences exhibited in systemic clearance within the same individual from one dose administration to the next can be difficult to distinguish experimentally and effects on bioavailability cannot readily be predicted.

Although identification of intrasubject differences in clearance would reduce variability in the estimation of bioavailability, determination of clearance differences in the same individual from one treatment to another is not possible using routine approaches without assumptions regarding other PK parameters. However, changes in elimination half-life ($t_{1/2}$) are measurable and may reflect changes in clearance when apparent volume of distribution is assumed to be constant between dose administrations. Expressing clearance in terms of elimination half-life and volume of distribution ($CL = 0.693 \times V_d/t_{1/2}$) and substituting into Equation 10.4 yields

$$F = \frac{AUC_{ev}}{AUC_{iv}} \times \frac{t_{1/2,iv}}{t_{1/2,ev}} \times \frac{(Dose)_{iv}}{(Dose)_{ev}} \quad (10.6)$$

Thus, in some circumstances, the accuracy of bioavailability estimates may be improved by incorporating half-life estimates to accommodate changes in clearance. However, experimentally derived estimates of half-life are a subjective measure of drug elimination that can be confounded by multiphasic concentration–time profiles as well as relatively slow rates of drug absorption that cannot be distinguished from the rate elimination.

The use of stable isotope methodologies provides an additional approach to decrease potential sources of variability in the calculation of bioavailability. To address potential within-subject differences in drug clearance that may arise from dosing on two separate occasions, stable labeled and nonlabeled drug have been administered simultaneously by two different dosing routes or by the same dosing route as two different formulations. These studies use drug substances prepared by incorporation of stable isotopes in order to take advantage of mass spectrometric analytical methods to distinguish between different isotopic forms of the drug in the same sample. Drug bioavailability studies have commonly employed the stable isotopes of hydrogen (deuterium, [2H]), carbon (carbon-13, [^{13}C]), and nitrogen (nitrogen-15, [^{15}N]). In this way, human PK for two simultaneous dose administrations can be determined from concentrations of stable labeled and nonlabeled drug quantified from the same plasma samples. Implicit to an assumption of similar PK is an expectation that both isotopically labeled and nonlabeled drug substances will distribute identically in the systemic circulation and be metabolized identically.

Stable isotope techniques have been used to determine the absolute oral bioavailability of many drugs, including ribavirin, an antiviral agent, the calcium channel blocker verapamil, and the anticonvulsant phenytoin. In the case of ribavirin, prolonged systemic exposure caused by intracellular sequestration of drug would require a long washout period between ribavirin dosing. A stable isotope study was planned with ribavirin in order to avoid the risk of insufficient washout between the treatment phases of a crossover design. To estimate absolute bioavailability, the study design involved

IV administration of [^{13}C]-ribavirin followed 1 h later by an oral dose of unlabeled drug [18]. The use of stable isotope methodology provided a robust estimate of ribavirin bioavailability (51.8%) without a period bias or a washout effect to confound the data. The absolute oral bioavailability of verapamil was also investigated using isotopically labeled drug. Deuterium-labeled verapamil was dosed orally to compare first-pass metabolism differences in patients with liver cirrhosis and healthy subjects. Dosage adjustments for patients with liver disease were recommended as a result of a nearly twofold increase in bioavailability associated with a reduction in oral clearance [19]. A stable isotope approach was also employed to estimate absolute bioavailability of phenytoin in patients with epilepsy. Conventional approaches to determine bioavailability are complicated by the slow absorption rate, extensive plasma protein binding, nonlinear PK, and narrow therapeutic window of phenytoin. Mean estimates of absolute bioavailability after simultaneous administration of an oral formulation and parenteral stable labeled phenytoin ranged from 86.4% in younger patients to 92.5% in older patients [20].

The use of stable isotopes has also facilitated relative bioavailability determinations in order to establish the bioequivalence of highly variable drugs. The oral bioavailability of the anti-arrhythmic moricizine was studied by simultaneous administration of nonlabeled tablet and [^{13}C]-moricizine oral solution. Initial studies determined that high variability within subjects due to the extensive first-pass metabolism of moricizine precluded a conventional crossover study design. It was estimated that a large number of study subjects would be required for a crossover study in order to pass average equivalence criteria with the desired statistical power. Instead, fewer subjects were studied by a stable isotope approach in order to confirm the bioequivalence of a moricizine oral solution to the marketed commercial tablet [21].

Although the use of stable label techniques for the study of bioavailability has afforded opportunities to minimize variability within subjects, inherent limitations regarding use in human studies have curtailed the broad application of this approach. Stable isotope use in evaluating bioavailability has been limited by requirements for custom syntheses of highly purified material conducted in accordance with good manufacturing practices (GMP), challenges associated with preparation of stable labeled formulations that are representative of clinical formulations and the need for a complement of nonclinical safety studies necessary to gain approval for IV dosing of stable labeled drug.

Advances in mass spectrometry have provided new opportunities for determining drug bioavailability with the use of trace levels of radioactive isotopes, obviating some of the drawbacks associated with the use of stable label approaches outlined above. Accelerator mass spectrometry (AMS) is a relatively new technique with high sensitivity, used to measure low concentrations of radiolabeled drug and metabolites in multiple matrices. The analytical sensitivity of AMS is derived from the fact that individual drug molecules are being detected by the mass spectrometer. Conventional radiolabel studies rely on detection methods such as scintillation counting to monitor the radioactive decay of the labeled drug. In contrast, AMS measures the ratio of individual radioactive atoms, commonly carbon-14 for human studies, and the naturally abundant carbon-12 atoms in samples after drug administration. A comparison of carbon-14/carbon-12 ratios in samples collected from subjects who received drug versus the same ratio in pretreatment samples allows very low levels of drug to be quantified in the femtomole to attomole concentration range.

The high sensitivity of AMS has made this technology amenable to mass balance studies and microdose scale PK studies [22,23]. Human bioavailability studies have also employed AMS technology to monitor low circulating plasma concentrations following subtherapeutic amounts of radiolabeled drug. Study designs to assess absolute bioavailability have involved either simultaneous IV administration of a microdose of radiolabeled drug with oral administration of nonlabeled drug or administration of a microdose of radiolabeled drug on two occasions separated by a suitable washout period between IV and extravascular dosing.

Although microdose studies represent an efficient approach to estimate absolute bioavailability in the clinic and are supported by an abbreviated safety dossier, drug PK after a microdose must accurately represent the kinetics at higher therapeutic dose levels. Accurate bioavailability determinations require that the PK are independent of drug concentrations across a range of dose levels. Therefore, studies have occasionally been conducted in nonclinical species to confirm linear PK at microdose and therapeutic dose levels before dosing to human subjects [24,25].

The bioavailabilities of several drugs following microdose administrations have also been conducted in human subjects in order to evaluate microdosing study designs. Results from a study in human volunteers indicated concordance between subtherapeutic and therapeutic dose PK for erythromycin, midazolam, and an experimental compound ZX253 [26]. Estimates of absolute bioavailability were determined for each compound by simultaneous administration of an IV carbon-14-labeled microdose plus a pharmacological oral dose and compared with estimates obtained in a conventional crossover study at therapeutic dose levels. Results of the study indicated relatively good agreement (i.e., differing by less than twofold) in the estimates of bioavailability obtained from microdose and conventional studies for midazolam and ZX253. The estimate of erythromycin bioavailability from a conventional crossover study at therapeutic dose levels was greater than twofold larger than the estimate of bioavailability obtained from a microdose—pharmacologic dose paring (35% vs 14%, respectively). However, the difference in absolute bioavailability was attributed in part to the acidic conditions of the upper GI tract and potential degradation of erythromycin following oral administration.

10.3 BIOEQUIVALENCE

The assessment of bioequivalence of two or more drug products offers important information regarding the safe and efficacious use of therapeutic agents. When measures of systemic exposure relate to drug efficacy and safety, bioequivalence confers therapeutic equivalence. As such, a common goal of all bioequivalence studies is to demonstrate that one drug product can be substituted for a second.

Studies to test for bioequivalence involve comparisons of bioavailability between two drug formulations that are either pharmaceutical equivalents or pharmaceutical alternatives of one another. That is, drug products that contain the same active ingredient and administered in the same dosage form (pharmaceutical equivalents) or in similar dosage forms prepared for the same route of administration (pharmaceutical alternatives). For pharmaceutical equivalents or alternatives to be considered bioequivalent, the active ingredient in a test product must not exhibit a significantly different rate and extent of absorption as that of the reference product when administered at the same

molar dose and under similar experimental conditions [3]. Inherent in the definition of bioequivalence is an assumption that once absorbed into the circulation, distribution, metabolism, and excretion of the active ingredient will be the same irrespective of formulation.

Two drug products are considered to be therapeutically equivalent if they are pharmaceutical equivalents and bioequivalent, thus leading to the same profile of clinical efficacy and safety. However, if test product levels in plasma are substantially lower than those of the reference product, concerns of a potential lack of therapeutic efficacy are raised. Alternatively, when test product levels are higher than those of the reference then concerns shift to a focus on safety. Although therapeutic equivalence is generally not tested directly, it is presumed based on tests of bioequivalence. When bioequivalent drug formulations share the same clinical effect and safety profile in patients, then they are considered to be therapeutically equivalent and may be used interchangeably.

Bioequivalence studies can be integral to the development of new drugs, as improvements in drug formulation may be required during clinical development. For instance, drug formulations used in clinical trials are often not suitable for commercialization. Thus, demonstrating bioequivalence of a drug product intended for market use will allow its substitution for the experimental formulation. Bioequivalence trials are also conducted to evaluate the effects of food, changes in the marketed formulation, or dose route of administration. Recent efforts to reformulate the antifungal agent amphotericin B represent attempts to reposition a previously approved drug that suffers from infusion-related side effects and renal toxicity. Amphotericin B has low solubility and permeability that result in negligible oral absorption. Reformulation of amphotericin B for oral administration would significantly lower treatment costs and improve its accessibility [27]. In contrast to oral formulations, therapeutic equivalence of drug products formulated for IV administration may be established by demonstrating pharmaceutical equivalence and does not require *in vivo* testing [28].

Bioequivalence studies are also conducted as components of an ANDA for a generic drug product. Because generic drugs are intended to be interchanged with innovator products, a demonstration of bioequivalence between formulations is essential for safe and efficacious use of the generic formulation. Thus, therapeutic equivalence to the innovator drug product is accepted on demonstration of bioequivalence of a pharmaceutically equivalent generic drug product.

The rules that govern generic drug substitution were defined by the Drug Price Competition and Patent Term Restoration Act of 1984 (Hatch–Waxman Amendments). Since adopting Hatch–Waxman, more than 11,000 generic drug products have been approved by the Food and Drug Administration (FDA). In order to judge the relative success of these approvals, a recent survey was conducted by FDA of generic drugs approved over a 12-year period spanning from 1996 to 2007 [2]. The survey revealed that in 2070 bioequivalence trials conducted over a 12-year period through 2007, the average difference in rate and extent of drug absorption between generic and innovator products was 4.35% and 3.56%, respectively [2].

In order to avoid complications that could arise from product substitution, the FDA maintains a list of generic drugs that can be safely and appropriately substituted for brand products. These drugs are listed in a federal publication entitled *Approved Drug Products with Therapeutic Equivalence Evaluations*, commonly known as the *Orange Book* [29]. To permit substitution of a brand drug, a generic equivalent must demonstrate bioequivalence as part of the approval process. However, the process by which a

brand drug is approved differs from the process required for generic product approval. A summary of the similarities and differences between new drug application (NDA) and ANDA dossiers are shown in Table 10.1. Both innovator and generic products must contain the same active ingredient in the same strength. Moreover, drug chemistry, manufacturing details and quality control measures must be documented for both products. However, after this point, the two processes begin to diverge. The Hatch–Waxman Amendments do not require generic drug applicants submitting an ANDA to provide nonclinical or clinical studies to establish the safety and efficacy of the active ingredient, as these characterizations have been established previously for the innovator product. Required of the generic product is demonstration of bioequivalence to the innovator product.

10.3.1 Study Design

Bioequivalence studies are generally conducted in a small number of healthy volunteers to determine the relative bioavailability of one or more test formulations following single dose administration. In most studies, the reference formulation is defined as the historical or innovator drug product and all individuals participating in the trial typically receive reference as well as test formulations. There are three basic study designs employed for determination of drug product bioequivalence—randomized crossover design, parallel design, and partial-block crossover design.

10.3.1.1 Randomized Crossover Design. A two-period randomized crossover design is one of the most commonly employed study designs to test the equivalence of two treatments. In accordance with this design, subjects are divided into two equal groups on random assignment to either a test or reference treatment group. After the first dosing period, a sufficient washout period equivalent to five or more drug half-lives is allowed to elapse before dosing resumes. For the second dosing period, subjects are crossed over into the opposite treatment group and receive either the test or reference dose depending on their dosing history in the first period. A randomized crossover study is intended to minimize intersubject variability by employing a Latin square design to allow each individual serves as a control subject. Bioequivalence studies to assess multiple regulatory requirements might employ a randomized

TABLE 10.1 Requirements of the New Drug Application (NDA) and Abbreviated New Drug Application (ANDA) Review Processes

NDA Requirements	ANDA Requirements
Chemistry	Chemistry
Manufacturing	Manufacturing
Controls	Controls
Labeling	Labeling
Testing	Testing
Animal studies	Bioequivalence
Clinical studies	
Bioavailability	

Source: Adapted from Ref. 30.

crossover of multiple treatments with a Latin square design. For instance, a study to compare test and reference treatments in fed and fasted subjects would generally be conducted as a single dose, randomized, four-period crossover design.

10.3.1.2 Parallel-Group Design. In a parallel study, subjects are divided into equal groups on random assignment to either a test or reference treatment group. Subjects receive the assigned treatment during the study and plasma concentrations are measured. A comparison of results allows an assessment of relative bioavailability. In contrast to the crossover design, higher intersubject variability is anticipated with a parallel design and larger numbers of enrolled subjects will be required. Consequently, testing for formulation differences could be insensitive due to a potential for larger statistical error. However, in some circumstances, a parallel-group study design may be a preferred option. For instance, parallel-group studies might be used to evaluate the therapeutic equivalence of two formulations in patients if treatment changes during the course of the study are precluded by consideration of patient welfare [31,32]. In addition, parallel designs might also be preferred for studying drugs with long half-lives or those requiring lengthy follow-up periods, thus making crossover designs impractical options.

10.3.1.3 Partial-Block Crossover Design. A partial-block design is an extension of the crossover design concept in which subjects receive a subset of formulations being tested. This design might be considered when a comparison of several formulations is planned for each subject in a time-consuming trial that potentially risks high numbers of subject dropouts. A partial-block crossover design could also be employed when studying a long half-life drug. For drugs with a long half-life, an extended duration of a washout period between treatment arms could potentially add several weeks to a complete crossover trial. Using a partial-block crossover design, each subject would experience fewer treatment washout periods, while maintaining within-subject comparisons that are intended to minimize variability. In general, the number of individuals enrolled in a partial crossover study is less than what is required for a parallel design but greater than that required for a complete crossover design.

As an example, a partial-block crossover design was employed to study the 5- α -reductase inhibitor dutasteride in a combination capsule with the α_1 -adrenoceptor antagonist tamsulosin due to the markedly different half-lives of these two compounds [33]. Dutasteride has a half-life of 7–9 days, much longer than the 9–11 h for tamsulosin, which required at least 28 days of washout between treatment periods. If conducted as a single four-way crossover study, the three long washout periods would have resulted in an excessively long study of approximately five months in duration. To avoid lengthy washout periods, a three-way partial crossover design was selected to evaluate two formulations in fed (groups A and B) and fasted (groups C and D) healthy volunteers, shown in Table 10.2. This design attempted to minimize the time spent on study for the subjects, while maintaining within-subject comparisons.

10.3.2 Statistics

The number of individuals enrolled in a bioequivalence study will be determined by the PK variability of the reference dose and based on prior experience. In general, the study size must be sufficient to detect a 20% difference in the measured exposure parameters

TABLE 10.2 Partial-Block Randomized Crossover Bioequivalence Study Design Used to Study Dutasteride–Tamsulosin Combination in Fed and Fasted Subjects

Subject No.	Drug Product ^a		
	Period 1	Period 2	Period 3
1	A	B	C
2	A	C	B
3	B	A	C
4	B	C	A
5	C	A	B
6	C	B	A
7	A	B	D
8	A	D	B
9	B	A	D
10	B	D	A
11	D	A	B
12	D	B	A

^aTreatments: (A) AVODART (dutasteride) plus Flomax (tamsulosin hydrochloride) in the fed state; (B) dutasteride–tamsulosin combination product in the fed state; (C) AVODART plus Flomax in the fasted state; and (D) dutasteride–tamsulosin combination product in the fasted state.

Source: Ref. 33.

with 80% certainty. The dose selected for study is often the highest strength being considered, as comparisons are most discriminating between treatments when exposures increase proportionally or greater than proportional to changes in dose level. When exposure increases are less than dose proportional, the lowest strength dose should be compared [2].

Data resulting from bioequivalence studies are evaluated statistically to analyze two one-sided confidence intervals. The first of two one-sided tests determines if a test product is 20% less bioavailable when substituted for the reference. A second test determines whether the reference is 20% less bioavailable when substituted for the test product. A difference of more than 20% for each of the statistical tests is considered significant, resulting in a lack of bioequivalence. In the case of the two one-sided tests, the null hypothesis is one of nonequivalence. Thus, incorrectly concluding bioequivalence for two nonequivalent drug products (type I error) places risk on the consumer. Alternatively, an incorrect conclusion of bioinequivalence for two equivalent drug products (type II error) places risk on the sponsor.

In practice, the two one-sided tests are each carried out at a 0.05 level of significance, with the combined tests yielding a 10% total error and 90% confidence interval of 80–125%. Therefore, the bioequivalence of two drug preparations can be concluded when the 90% confidence intervals of the geometric mean test/reference ratios of $C_{\max}$ and AUC fall within the limits of 80–125%. In other words, bioequivalence will be concluded when the test product differs from the reference product by $-20\%/+25\%$ or less in terms of the rate and extent absorption. Use of the $-20\%/+25\%$ interval was based on a medical decision that differences in systemic drug concentrations that fall within the $-20\%/+25\%$ range would not likely be clinically significant [34].

10.3.3 Approaches to Demonstrate Bioequivalence

Bioequivalence studies are designed to determine whether a test formulation may be substituted for an existing reference formulation. Successful substitution requires that the two drug products will provide equivalent therapeutic effects. For two oral drug products to be considered bioequivalent, each product must exhibit the same rate and extent of absorption. Thus, bioavailability must be measured by appropriate *in vivo* or *in vitro* methods in order to document bioequivalency. In accordance with accepted FDA methods, studies to determine the bioequivalence of two drug products may be conducted with PK, PD, clinical, or *in vitro* endpoints [3]. Thus, experimental methods to demonstrate bioequivalence will vary case by case, as the selection of appropriate study design and outcome measures will depend on the characteristics of drug products to be compared.

10.3.3.1 Pharmacokinetic Studies. Studies to quantify systemic exposure and characterize drug PK provide opportunities to compare drug bioavailability. Inherent to these studies is the assumption that systemic exposures will reflect drug concentrations at the site of action and thus will provide some assurance of comparable therapeutic effects. For orally administered drugs, the parameters of $C_{\max}$ and AUC provide measures related to the rate and extent of drug absorption, respectively. Bioequivalence studies commonly enroll healthy volunteers to receive a single dose of each formulation. A crossover study design is employed in order to minimize interindividual variability influences on bioavailability, as exposure measurements are assumed to have less interoccasion variability compared to the variability associated with formulation performance [3]. Differences in bioavailability will therefore likely reflect true differences in formulation.

Establishing bioequivalence is most often based on parent drug concentrations, as the parent compound is generally the entity most sensitive to formulation differences. However, when safety or efficacy is considered a function of all drug-related species present in circulation, then monitoring metabolite plasma concentrations to determine the bioequivalence of two drug products could be warranted. Metabolite exposure might be appropriate to monitor if parent drug is rapidly and extensively metabolized, pharmacological activity is correlated with metabolite levels, or if both parent drug and metabolite account for therapeutic effect [35].

Circulating levels of pharmacologically active metabolites are frequently monitored in comparative studies in order to estimate the relative bioavailability between formulations of high clearance drugs. For example, the anthelmintic agent albendazole undergoes rapid and essentially complete first-pass metabolism to a sulfoxide metabolite. Suspension and solution formulations were found to provide 4.3- and 9.7-fold improvements in oral bioavailability relative to tablets, respectively, as demonstrated by comparing AUC exposures of the sulfoxide metabolite [36]. Similarly, the potent desmethyl metabolite of tramadol, a centrally acting analgesic, was monitored to compare the PK of an extended-release tablet relative to an immediate-release reference product [37]. However, in the case of the antiplatelet agent clopidogrel, neither parent drug nor pharmacologically active metabolite is present in circulation at high concentrations following oral administration. Therefore, circulating levels of an inactive carboxylic acid metabolite of clopidogrel were monitored to compare different formulations in a randomized crossover study [38,39].

10.3.3.2 Pharmacodynamic Studies. PD studies are designed to establish bioequivalence of drug products based on measures of pharmacological effect. PD studies differ from PK studies in that circulating drug levels may or may not reflect concentrations at the site of action or correlate with therapeutic effect. PD endpoints are used to determine bioequivalence more often for drug products applied directly to the site of action where they may exert their desired effect, such as topical cream formulations [40,41]. In such cases, drug products are not intended to be absorbed into the systemic circulation. Therefore, bioavailability may be assessed by measurements intended to reflect the rate and extent to which drug becomes available at the site of action. Frequently, however, PD measurements are obtained in conjunction with PK measures in order to relate circulating drug concentrations with drug effect. Thus, the bioequivalence of drug products would typically be based on a measure of dose–response equivalence, where an $E_{\max}$ model is commonly assumed. Drug product comparisons might be made in terms of maximal effect, minimal effect, or average effect measures as well as the area under the effect versus time curve (AUEC). When PK data are available, drug concentrations associated with half-maximal effect (EC_{50}) may be compared when calculated from the model:

$$E = \frac{E_{\max} \cdot C}{EC_{50} + C} \quad (10.7)$$

where E is the effect and $E_{\max}$ the maximal effect [39,42].

10.3.3.3 Clinical Studies. Bioequivalence may also be based on a comparison of clinical outcomes between treatment groups. However, clinical assessments are generally not the sole determinant of equivalence when plasma drug concentrations or measures of PD effect are also available. Although clinical endpoints may be useful to conclude bioequivalence when quantitative measurements are not feasible, clinical assessments are also subjective and difficult to reproduce. Additionally, clinical studies frequently require large numbers of patients in order to ensure adequate statistical power to differentiate drug products.

Most clinical endpoint bioequivalence studies are limited to determination of whether a given treatment failed or succeeded. In a recent trial with the atypical antipsychotic clozapine, a subgroup of patients was randomly assigned to switch to a generic formulation or remain on the initial formulation. Both patient groups were monitored in parallel during the study and clinical outcomes determined from psychiatric tests administered before the switch and at time points after the switch were compared across treatments. A lack of significant differences in score results between the two treatment groups allowed investigators to conclude bioequivalence of generic clozapine based on therapeutic equivalence [31]. Clinical endpoints were similarly used to test the bioequivalence of two formulations of the antifungal agent amphotericin B. On the basis of comparison of clinical cure rates in patients with visceral leishmaniasis treated with each formulation, therapeutic equivalence of the test liposomal formulation of amphotericin B was concluded. In the trial, a single infusion of liposomal amphotericin B was determined to be noninferior to conventional therapy with amphotericin B deoxycholate [32].

10.3.3.4 In Vitro Studies. A fourth approach to characterize drug formulation bioequivalence incorporates knowledge of the key determinants of oral absorption. The biopharmaceutical classification system (BCS) provides a scientific approach to classify drug substances based on *in vitro* measurements of aqueous solubility and intestinal permeability [43]. Using BCS, drugs may be categorized into four classes according to *in vitro* estimates of aqueous solubility and permeability to predict *in vivo* bioavailability, as shown in Fig. 10.1. On the basis of this classification scheme, high solubility drugs may be characterized as having either high permeability (class I) or low permeability (class III), and low solubility drugs are similarly characterized as either high (class II) or low (class IV) permeability. Subsequently, BCS classification was adopted by FDA to allow waiver of *in vivo* bioavailability and bioequivalence testing of immediate-release solid dosage forms for drugs characterized by high solubility and high permeability (class I) that exhibit rapid dissolution [44]. Thus, characterization of drugs in terms of the fundamental properties governing oral absorption seeks to ensure similarities in systemic availability.

The utility of the BCS to classify drugs in terms of solubility and permeability has also provided a framework to predict the effects of food on oral absorption [46]. Physiological changes induced by the presence of food in the GI tract were predicted to similarly influence the oral absorption of drugs of the same BCS class. A relationship between drug disposition and BCS classification was later recognized, as drug substances characterized by high permeability (classes I and II) were eliminated primarily by metabolism and low permeability compounds (classes III and IV) were primarily excreted in urine and bile. On the basis of these observations, a modification was proposed to replace the permeability criterion of BCS with a designation of the major route of drug elimination [45]. Hence, the biopharmaceutics drug disposition classification system (BDDCS) was proposed with metabolism as an alternate measure of drug permeability (Fig. 10.1). BDDCS has also been used to predict the mechanistic effects of food on drug absorption [47].

In general, the *in vitro* determination of aqueous solubility is relatively straightforward and has led to accurate characterization of most drugs. As such, waiver of *in vivo* bioavailability and bioequivalence testing of rapidly dissolving drugs belonging to BCS class I is well accepted. A similar waiver for drugs belonging to BCS class III (high solubility and low permeability) that exhibit rapid dissolution has also been proposed [48,49]. However, *in vitro* predictions of intestinal drug permeability do not share the same level of consensus as do measures of aqueous solubility. This has led to increased acceptance of alternatives to the BCS measures of permeability, such as the

BCS		BDDCS	
Class 1 High permeability High solubility	Class 2 High permeability Low solubility	Class 1 High metabolism High solubility	Class 2 High metabolism Low solubility
Class 3 Low permeability High solubility	Class 4 Low permeability Low solubility	Class 3 Low metabolism High solubility	Class 4 Low metabolism Low solubility

Figure 10.1 Comparison of biopharmaceutics classification system (BCS) and biopharmaceutics drug disposition classification system (BDDCS). *Source:* Refs. 43 and 45.

incorporation of drug elimination by the BDDCS [45,50]. Although contrasting features of the BCS and BDDCS suggest differences in predictions of *in vivo* absorption should be anticipated, a recent comparison of drug classifications indicated that the two systems were largely in agreement. Concordance in the BCS and BDDCS classification of drugs with low solubility (classes 2 and 4) was observed with 87–92% agreement across 164 compounds. The level of concordance was slightly lower for high solubility drugs (classes 1 and 3) ranging from 64% to 72% being identically classified. The distinguishing feature leading to differences between the two systems was the selection of the BCS permeability reference drug. Overall agreement between BCS and BDDCS predictions was best when cimetidine was the reference permeability drug and weakest when metoprolol was used [51].

10.4 DETERMINANTS OF ORAL BIOAVAILABILITY

Given that the majority of drugs are administered as an oral dosage form, the role of the GI tract is significant in the disposition of many drugs. The GI tract serves many important functions involved in the digestion of food, absorption of dietary nutrients, exchanges of liquids, and excretion of waste products as well as providing a protective barrier to the entry of xenobiotics and other foreign matter such as bacteria [52]. Consequently, absorption into the portal circulation requires the physicochemical properties of a drug that govern solubility and permeability to be balanced with respect to physiology of the GI tract such as transit time, luminal content, pH environment, and absorptive surface area. The serial arrangement of the small intestine and liver also provide sequential barriers for drug transit to the systemic circulation.

For orally administered drugs, the amount of dose transferred from the site of administration to the systemic circulation will be determined by the fraction of absorbed dose as well as the available fraction not subjected to intestinal or hepatic first-pass elimination. Although some degree of passive permeability becomes a requisite feature for drug transfer across the gut wall, a component of bioavailability is also determined by drug affinity for transporters and metabolizing enzymes localized in the epithelial lining of the gut. Moreover, hepatocytes in the liver express a full complement of transporters and enzymes that may further limit drug transit to the systemic circulation. Thus, absorption from the GI lumen, metabolism by enzymes, and active transport processes localized in enterocytes, along with hepatic uptake and metabolism, are considered to be the major sources of drug loss following oral administration.

Oral bioavailability is a measure of the fraction of dose absorbed as intact drug from the GI lumen (F_a), the fraction transferred to the portal bloodstream without intestinal extraction (F_g), and the fraction not extracted on hepatic first pass (F_h) [1]. Thus, drug bioavailability can be estimated as a product of dose fractions that escape loss at each site during the sequential transit of intact drug from the GI tract to the liver and the systemic circulation. Accordingly, the determinants of oral drug bioavailability are related by the following expression:

$$F = F_a \times F_g \times F_h \quad (10.8)$$

The utility of this simple mathematic relationship can be illustrated by considering the example of raloxifene, a selective estrogen receptor inhibitor that exhibits low

oral bioavailability. After oral administration, $\sim 2\%$ of a raloxifene dose enters the systemic circulation as parent drug with the balance lost to malabsorption and first-pass elimination. Prior knowledge of the fraction of dose absorbed as intact raloxifene (63%), plus the fraction of dose that evaded hepatic extraction (59.3%), allows an estimate of the fraction of dose transferred across the gut mucosa. Using the above expression to solve for F_g , in which $F_g = (63\% \times 59.3\%)/(2\%)$, $\sim 5.4\%$ of the fraction of absorbed dose escaped intestinal extraction, suggesting that first-pass gut extraction is the major limitation to raloxifene oral bioavailability [53].

The bioavailability of orally administered drugs is determined by drug absorption and intestinal and hepatic first-pass elimination, as described by Equation 10.8. To understand the mechanisms responsible for incomplete drug bioavailability, limitations to absorption as well as first-pass elimination by the gut and liver must be determined separately. Experiments conducted in cannulated animal models have offered an approach to study drug loss in a physiologically intact system. Frequently, drug extraction or loss is measured by infusing drug at multiple sites of input (i.e., intra-arterial, IV, duodenal, and intraportal) and sampling from a single venous site in a rodent model. Alternatively, extraction can be determined by infusing drug at a single catheter site and sampling at multiple sites [54]. A five-catheter chronic rat model has also been used to assess drug bioavailability in animals that have fully recovered from surgery and anesthesia associated with catheter placement. This model records drug extraction under normal physiologic conditions as GI and liver functions are returned to presurgery status before experiments are initiated [55]. Although surgical approaches have frequently been adopted to elucidate the determinants of oral bioavailability in preclinical animal models, the use of invasive techniques is not common in human subjects.

10.4.1 Oral Drug Absorption

10.4.1.1 Approximation of F_a . In clinical studies, an estimate of F_a is frequently provided by monitoring the cumulative excretion of a single dose of radiolabeled drug. For drugs in which both parent and metabolites are eliminated by renal excretion, the proportion of total radioactive dose excreted into urine $[(Au,rad)/dose]$ can be used to approximate the fraction of absorbed dose. Comparison of total radioactivity excreted after oral (po) and IV dose administrations provides an estimate of F_a according to the following expression:

$$F_a \cong \frac{(Au,rad)_{po}}{(Au,rad)_{iv}} \times \frac{(Dose)_{iv}}{(Dose)_{po}} \quad (10.9)$$

Although Equation 10.9 requires determination of the proportion of IV dose eliminated in urine, IV data are frequently not available. However, in some cases, F_a can be estimated from oral dose data only, such as with the cyclooxygenase-2 inhibitor rofecoxib [56]. In the case of rofecoxib, F_a could be approximated from radioactivity excreted after oral dosing (Eq. 10.10) because the fraction of total radioactivity in urine represented a relatively large proportion of overall rofecoxib elimination:

$$F_a \cong \frac{(Au,rad)_{po}}{(Dose)_{po}} \quad (10.10)$$

Modest improvements in estimates of the fraction of absorbed dose have also been achieved by application of Equation 10.10 plus consideration of metabolite levels excreted in feces as a percent of dose. In the case of the antipsychotic drug olanzapine, the fraction of absorbed dose was approximated by the sum of total radioactive dose recovered in urine plus the fraction represented by excretion of the 10-*N*-glucuronide metabolite in feces [57]. However, a caveat to this approach assumes that metabolite was excreted into feces and not formed by biotransformation of either unabsorbed dose or excreted parent drug returned to the GI tract.

10.4.1.2 Dissolution and Solubility. The fraction of an oral dose absorbed from the GI tract is a function of the physicochemical properties of a drug substance, as absorption can be limited by incomplete dissolution of solid drug as well as low intrinsic solubility. When administered as a tablet or capsule, partial dissolution of solid drug or a slow rate of dissolution relative to transit times within the absorptive regions of the GI tract may limit drug absorption. The intestinal environment may also influence drug solubility. Because the luminal content of the small intestine is mostly water, aqueous solubility plays an important role in drug absorption. However, drug solubility and oral absorption can also be influenced by changes in gastric pH as well as the heterogenous composition of the intestinal lumen.

Most drug substances are either weak acids or weak bases that can become ionized under physiological conditions of the GI tract that ranges from low pH of the stomach (pH 0.8–1.8) to near neutral pH in the distal GI (pH 6.5–7.2) in the fasted state [58]. The degree to which a weak acid or weak base exists in an ionized or unionized state depends on pH of the local environment and the pK_a of the drug. Therefore, regional differences in pH along the GI tract can have an effect on the absorption of weak acids and weak bases caused by pH-dependent changes in solubility. The antifungal ketoconazole is an example of a weak base characterized by pH-dependent solubility and absorption. Owing to a low pK_a , weak bases such as ketoconazole have improved solubility under acidic conditions. Ketoconazole has two pK_a values (2.9 and 6.5) and is relatively insoluble near neutral pH, but solubility is dramatically improved at low pH conditions. Experimental evidence of the pH dependence of ketoconazole absorption was demonstrated in healthy volunteers. Oral ketoconazole bioavailability was increased when dosed in an acidic solution and decreased when gastric pH was raised by coadministration with the proton pump inhibitor cimetidine [59].

In addition to pH-dependent effects on solubility, bile salts in the intestinal lumen may also influence drug solubility and absorption. The aggregation of bile salts present in the small intestine form micelles that may enhance the solubility and absorption of some poorly water soluble drugs. The importance of bile salts to the dissolution and solubility of the antifungal agent griseofulvin and the steroid danazol have been proposed to explain enhancements in postprandial bioavailability of these agents [60]. Oral absorption of the cyclooxygenase-2 inhibitor rofecoxib is also believed to be facilitated by bile salts in the GI tract. Following oral [^{14}C]rofecoxib administration, the fraction of absorbed rofecoxib dose was lower in cholecystectomy patients than in healthy volunteers, suggesting enhanced rofecoxib intestinal solubility in the presence of bile [56].

10.4.1.3 Food Effects. The presence of food within the GI tract may further complicate processes leading to oral bioavailability, as changes in drug absorption and

systemic exposure may result from physiological changes caused by food. Following a meal, digestive processes initiate transient increases in gastric pH accompanied by slower gastric emptying as well as longer overall GI transit times. Changes in intestinal fluid composition which result from stimulated secretions from the pancreas, liver, and gallbladder may also affect drug absorption. Additionally, constituents of a meal such as fats, carbohydrates, and minerals, may interact directly with drug substances to influence absorption as well as with membrane transport proteins or enzymes responsible for drug disposition.

Predictions of the effect of food on drug absorption and systemic exposure have been afforded from estimates of relative solubility and permeability characteristics of different drug substances. Thus, grouping compounds based on BCS [43] and BDDCS [47] criteria provide a framework to predict the effects of a meal on bioavailability. However, although changes in physiology resulting from food intake are known, the mechanisms by which food may change the bioavailability of a given drug are not always straightforward. Moreover, the magnitude of effect due to individual mechanisms makes predictions of food effects even more challenging. For instance, the nonsteroidal anti-inflammatory drug ibuprofen is a weak acid characterized by low solubility and high permeability. With a meal, greater oral absorption of ibuprofen due to increased solubility at higher pH and prolonged gastric emptying time would be expected to improve bioavailability relative to fasting. However, following ingestion of a meal, ibuprofen bioavailability was decreased by ~20%, potentially due to drug binding to viscous chyme generated from the meal and rendering it unavailable for absorption in the proximal intestine [61].

The effects of a meal on oral absorption may result in an increase in drug exposure (positive food effect), a decrease in exposure (negative food effect), or an insignificant change in exposure. Although systemic exposures of many commonly prescribed drugs used in clinical practice can be altered when taken with a meal, dosing recommendations to instruct use with or without food are relatively uncommon. A representative sample of drugs having product inserts that contain dosing recommendations related to food intake are shown in Table 10.3. For many drugs that exhibit negative food effects, food restrictions are recommended in order to maximize systemic exposure. An extreme example is the bisphosphonate alendronate that requires dosing with water before the first food or beverage after an overnight fast in order to achieve ~1% oral bioavailability. For drugs that exhibit positive food effects, many are recommended with a meal as a means to improve bioavailability and achieve therapeutic concentrations. In many instances, variability in systemic exposure is decreased by food as the fraction of absorbed dose can be increased in the fed state. However, in the case of the tyrosine kinase inhibitor lapatinib, a positive food effect was associated with equivalent or slightly increased PK variability and therefore lapatinib dosing is recommended without food. The product insert of another tyrosine kinase inhibitor nilotinib also recommends a food restriction, although a high fat meal was shown to increase AUC exposure by 50% in patients and 82% in healthy volunteers [62]. Unique among the drugs with food interactions listed in Table 10.3 is the black box QTc safety warning of nilotinib that includes a description of food effects on drug exposure [63].

Interactions between drugs and multivalent cations present in foods and antacids can also lead to changes in drug absorption. The oral bioavailability of some drugs may decrease as a result of chelation, the formation of a complex between polar groups

TABLE 10.3 Representative Drugs with Package Inserts that Include Dosage and Administration Instructions Related to Food Intake

Drug	% <i>F</i> (Fasting) ^a	Package Insert Comments and Dosing Recommendations ^b
<i>Drugs with Positive Food Effects</i>		
Efavirenz	NA	Increased exposure when tablet was dosed with high fat meal; $C_{\max}$ (+79%; 1.79-fold) and AUC (+28%; 1.28-fold). Dosing recommended on an empty stomach. Dose: 600 mg q24h
Isotretinoin	40% (fed)	Owing to high lipophilicity, absorption is increased when dosed with a high fat meal; $C_{\max}$ (+170%; 2.7-fold) and AUC (+186%; 2.86-fold) for 80 mg dose. Dosing recommended with food
Lapatinib	NA	AUC is increased approximately threefold (low fat) to fourfold (high fat) with food. $C_{\max}$ increased ~2.5- to 3-fold. Dosing recommended at least 1 h before or after a meal. Dose: 1200 mg q24h
Lovastatin	≤ 5%	Increased exposure when dosed with food. Dosing recommended with meals. Dose: 10–80 mg q24h or 5–40 mg q12h
Nelfinavir Mesylate	— 20–80%	— Increased exposure and decreased PK variability with food. AUC is increased 2.2- to 5.2-fold with food and $C_{\max}$ is increased 2.0- to 3.8-fold with food. Dosing recommended with meals. Dose: 750 mg q8h or 1250 mg q12h
Nilotinib	NA	Black box safety warning to avoid food intake for at least 2 h before dosing due to associated QTc risk. Observed AUC increases of 50–82% when administered with a high fat meal. Dose: 400 mg q12h
Ziprasidone Hydrochloride	— 59% (fed)	— Increased bioavailability up to twofold when dosed with food. Dosing recommended with food. Dose (initial): 20 mg q12h
<i>Drugs with Negative Food Effects</i>		
Alendronate Sodium	— <0.7%	— Owing to poor solubility, absorption is decreased with food or beverages other than plain water. Dosing recommended a least 30 min before first food or beverage of the day
Lansoprazole	81%	Decreased exposure when dosed 30 min after meals; $C_{\max}$ (–50%) and AUC (–70%). Dosing recommended before a meal. Dose: 15–30 mg q24h
Riluzole	64%	Decreased bioavailability when dosed with food; $C_{\max}$ (–45%) and AUC (–20%). Dosing recommended at least 1 h before or 2 h after a meal. Dose: 50 mg q12h
Tegaserod Maleate	— 11%	— Decreased bioavailability when dosed with food; $C_{\max}$ (–20% to 40%) and AUC (–40% to 65%). Dosing recommended before a meal. Dose: 6 mg q12h
Valsartan	23%	Decreased exposure when dosed with food; $C_{\max}$ (–50%) and AUC (–40%). Dosing recommended with or without food. Dose: 80/160/320 mg q24h
Voriconazole	96% ^b	Decreased $C_{\max}$ (–34%) and AUC (–24%) with high fat meal. Dosing recommended at least 1 h before or 1 h following a meal. Dose: 200 mg q12h

Abbreviation: NA, not available.

^aSource: Ref. 64.^bSource: Ref. 65.

of a drug molecule and multivalent metal ions, such as aluminum, calcium, and magnesium. Chelates formed between drug substances and meal ions may impair tablet or capsule dissolution or may cause solubilized drug to precipitate in the gut lumen. The oral bioavailabilities of several antibiotic agents, such as the tetracyclines, oral cephalosporins, and quinolones, have been decreased by the formation of stable chelate complexes in the gut lumen. For example, decreased absorption of the quinolone antibiotic ciprofloxacin due to interaction with multivalent ions from dietary supplements, antacids, and phosphate-binding agents have been associated with 38–54% decreases in bioavailability [66,67].

10.4.1.4 Stability. Oral absorption may also be curtailed for drug substances prone to instability within the GI tract. The fraction of an oral dose that is absorbed from the GI tract can be limited by drug loss in the lumen due to decomposition or chemical degradation (e.g., pH-dependent processes) as well as by microbial or enzyme-mediated degradation.

Acid-labile drugs such as the immunosuppressive agent erythromycin and the reverse transcriptase inhibitor didanosine are unstable in the acidic environment of the upper GI tract. Didanosine undergoes acid-induced hydrolysis in the stomach and has been formulated with antacid in order to improve its oral absorption profile and bioavailability. More recently, introduction of didanosine as an enteric coated capsule has improved drug stability by avoiding contact with the acidic environment of the stomach and eliminated the digestive intolerance associated with the previous buffered oral formulation [68].

In addition to pH-dependent stability, the fraction of absorbed dose may also be modulated for drugs susceptible to metabolism by GI microflora. Bacteria residing in the large intestine contribute to normal digestive function by fermentation of carbohydrates and proteins that escape digestion in the upper GI tract. Drug substances may also be metabolized by enzymes in the gut microflora that typically catalyze chemical reduction and hydrolysis reactions [52,69]. In addition, metabolism of drugs by microbial enzymes may also be a function of the amount of intact drug reaching distal regions of the GI tract, consistent with an increased prevalence of microflora within the large intestine.

Drugs with low solubility and/or low permeability have a sufficiently long residence time in the gut to allow access to the distal GI tract. For instance, the peripherally acting μ -opioid antagonist alvimopan is hydrolyzed by amidases in intestinal flora to an equipotent metabolite that may contribute to PD effect. Evidence of a role for gut flora in alvimopan metabolite bioavailability was supported by coadministration of ciprofloxacin with alvimopan. Although alvimopan bioavailability, ~6%, was not changed by ciprofloxacin coadministration, metabolite concentrations were decreased by ~99% relative to the levels after alvimopan treatment alone. These findings were consistent with formation of a presystemic metabolite of alvimopan by amidases in intestinal flora [70]. Additionally, β -glucuronidases located in the distal region of the GI tract may catalyze the hydrolysis of excreted drug conjugates, leading to increases in systemic exposure by rendering parent drug available for reabsorption. Examples of drugs that undergo enterohepatic recirculation include indomethacin [71] and mycophenylatemofetil [72]. Mycophenylatemofetil is a prodrug that is completely absorbed after oral dosing and subsequently hydrolyzed to mycophenolic acid (MPA), the active immunosuppressive agent (Fig. 10.2). The elimination of MPA occurs mainly

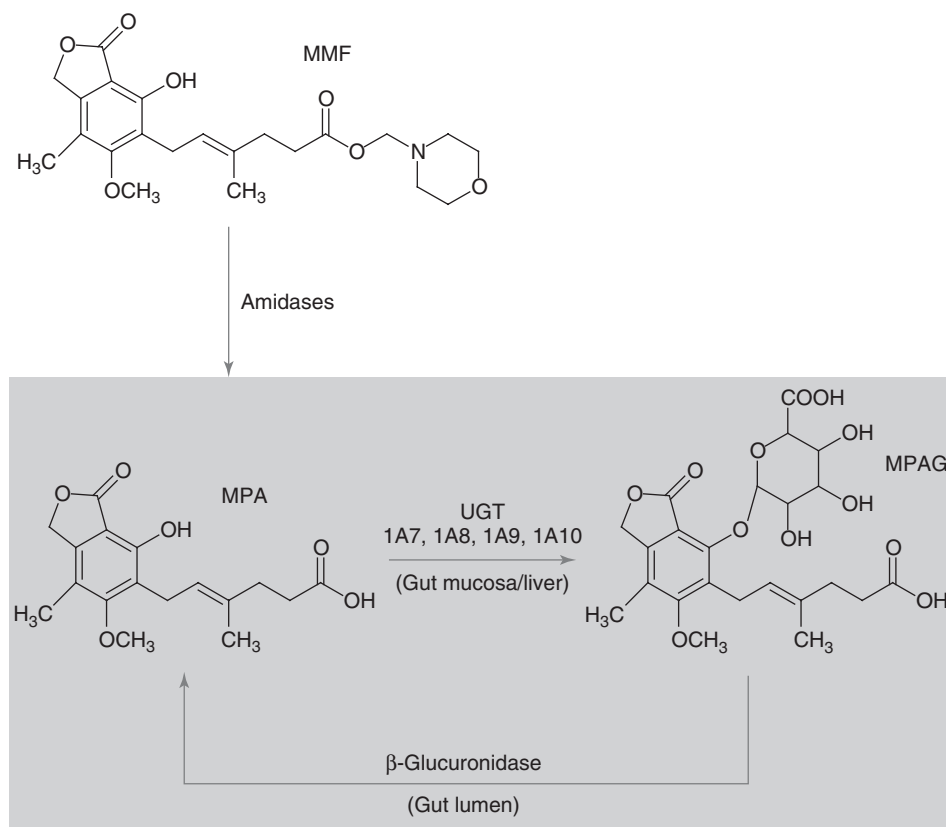


Figure 10.2 Proposed scheme of mycophenolate mofetil metabolism and enterohepatic recirculation of mycophenolic acid. Approximately one-third of MPA oral exposure is attributed to enterohepatic recirculation. *Abbreviations:* MMF, mycophenolate mofetil; MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide.

by conjugation with glucuronic acid, which is partially excreted in bile. On excretion, the conjugated metabolite may be deglucuronidated by the gut microflora and reabsorption of the aglycone contributes to overall systemic exposure and immunosuppressive effects. A mean increase of ~37% in mycophenylatemofetil bioavailability was attributed to enterohepatic recirculation of MPA from a crossover study conducted with the bile acid sequestrant cholestyramine in healthy volunteers [72]. The extent of MPA reabsorption was similarly decreased by concurrent antibiotic therapy. In healthy volunteers, concomitant treatment with the antibiotic metronidazole was associated with a 36% decrease in oral AUC exposure of MPA [73]. Taken together, the interaction studies with cholestyramine or metronidazole suggested that ~35% of oral MPA exposure and bioavailability may result from enterohepatic recirculation of MPA.

10.4.1.5 Intestinal Transporters. The fraction of absorbed dose following oral administration is also a function of drug permeability across the intestinal wall. The permeability of a drug may be influenced by passive diffusion as well as active transport mechanisms. Passive permeability typically occurs by transcellular

or paracellular processes in which drugs diffuse across the intestinal wall from a region of relatively high concentration in the gut lumen to lower concentrations in the portal circulation. Depending on the characteristics of individual drug substances, recognition by membrane transporters lining the GI epithelium may also affect overall drug permeability.

Membrane transport proteins also have a role in intestinal drug disposition, as both efflux and uptake transporters may determine drug bioavailability. Efflux transporters are part of a superfamily of ATP-binding cassette (ABC) transporters that extrude drug in energy-dependent processes and may prevent drug absorption into the portal blood circulation when expressed in enterocytes in the gut. Uptake transporters responsible for drug disposition belong to the two solute carrier superfamilies, SLC and SLCO, involved in drug uptake via mechanisms of cotransport. SLC and SLCO proteins transport drugs according to a concentration gradient and may function to improve the degree of intestinal drug absorption. Together, the activities of many ABC transporters and solute carrier proteins expressed within intestinal epithelium have proven to affect drug oral bioavailability. A summary of clinical interaction studies implicating the role of individual transporters in oral drug bioavailability is shown in Table 10.4.

The efflux transporter P-glycoprotein (P-gp; MDR1), encoded by *ABCB1*, is expressed in multiple tissues important for drug disposition, including epithelia of the GI tract and hepatobiliary canaliculi as well as endothelia of the blood–brain barrier. In the gut, P-gp is localized at the apical membrane of enterocytes and may function as a barrier to absorption by transporting drug back to the lumen. Thus, recognition by P-gp has been considered a key determinant of oral bioavailability for an array of structurally diverse drugs. Substrates for P-gp transport generally tend to be hydrophobic and are represented by high molecular weight drugs such as paclitaxol and cyclosporine A (CsA) as well as smaller sized drugs such as talinolol [90]. Although *in vitro* and *in silico* models are frequently used to identify potential P-gp substrates, the impact of P-gp on oral drug bioavailability has been predicted with varying levels of success.

In addition to broad substrate recognition, the effects of P-gp on oral bioavailability are largely due to the expression profile of P-gp along the GI tract. P-gp is expressed in the luminal membrane (apical) of the small and large intestines where it plays a central role in the export of drug from epithelial cells such as enterocytes. Gradually increased from the proximal to distal regions of the human intestine, P-gp expression is heterogeneous along the GI tract, as mRNA levels measured in colon have been shown to be approximately sixfold higher than levels in the duodenum [91]. The significance of heterogeneous expression levels of intestinal P-gp and the effect on oral bioavailability was considered in an exploratory study with CsA. In human volunteers, drug absorption from different regions of the GI tract was studied by infusion of CsA into specific regions of the GI tract. Plasma concentrations of CsA revealed an absorbance profile of CsA with a rank order (stomach > jejunum/ileum > colon) that was consistent with changes in P-gp expression level (stomach < jejunum/ileum < colon) [92]. Differential expression of P-gp due to polymorphisms in *ABCB1* could be a further source of variation across individuals; however, the specific role of individual *ABCB1* polymorphisms in drug PK continues to be an area of active research [90].

A mechanistic role for P-gp in the absorption of some drugs has been assessed in mice deficient in *mdr1a/b* genes. Using the genetic mouse model, differences in oral

TABLE 10.4 The Role of Selected Human Transporter Proteins Implicated in the Bioavailability of Orally Administered Drugs

Drug	Modulator	Effect	References
<i>P-glycoprotein (ABCB1)</i>			
Digoxin	Talinolol	AUC _{po} (0–6 h) increased by 23%, due to P-gp inhibition	74
Docetaxel	OC144-093	Bioavailability increased from 8% without to 26% with OC144-093 cotreatment, due to P-gp inhibition	75
	Cyclosporin A	Absolute bioavailability was increased from 8% without to 90% with cyclosporine A cotreatment, due to P-gp and CYP3A4 inhibition	76
	Ritonavir	AUC _{po} /AUC _{iv} = 1.31–1.61 range: PO docetaxel (100 mg) + ritonavir (100 mg) compared with IV docetaxel (100 mg) alone	77
Paclitaxel	Cyclosporin A	Bioavailability was increased from 4% without to 28% with CsA cotreatment, due to P-gp and CYP3A inhibition	78
	GF120918	Bioavailability was increased to 30% with GF120918 cotreatment, due to P-gp inhibition	79
Talinolol	Carbamazepine	Bioavailability decreased from 62% without to 53% with CBZ cotreatment, due to P-gp induction	80
	SJW	Bioavailability decreased from 52% without to 39% with SJW, due to P-gp induction	81
	Digoxin	Digoxin had no effect on talinolol bioavailability; contrary to the observed decrease in digoxin exposure due to talinolol inhibition (see above)	74
<i>BCRP (ABCG2)</i>			
Topotecan	GF120918	Bioavailability increased from 40% without to 97% with GF120918 cotreatment, due to BCRP inhibition	82
Sulfasalazine	BCRP polymorphism	Sulfasalazine AUC _{po} was 2.4-fold greater in subjects with ABCG2 (34GG/421CA) variant than subjects with (34GG/421CC) genotype	83

<i>OATP1A2 (SLCO1A2)</i> Fexofenadine	GFJ	Bioavailability decreased by 42% with GFJ compared to water, due to OATP1A2 uptake inhibition	84
<i>OATP2A1 (SLCO2A1)</i> Aliskiren	GFJ	AUC _{po} exposure was decreased by 61% with GFJ, due to enteric OATP2B1 uptake inhibition	85
<i>OATP1B1 (SLCO1B1)</i> Atorvastatin	Rifampin	AUC _{po} exposure of atorvastatin acid was increased by 6.8-fold with rifampin IV infusion, due to hepatic OATP1B1 uptake inhibition. Single rifampin dose unlikely to induce CYP3A4	86
Atorvastatin	SLCO1B1 polymorphism	Mean atorvastatin AUC _{po} was 1.44-fold (144%) greater in subjects with the <i>SLCO1B1</i> c.521CC genotype than in those with the c.521TT genotype	87
Simvastatin	SLCO1B1 polymorphism	Mean simvastatin AUC _{po} was 2.21-fold (221%) greater in subjects with the <i>SLCO1B1</i> c.521CC genotype than in those with the c.521TT genotype	88
<i>OATP1B3 (SLCO1B3)</i> MPAG	SLCO1B3 polymorphism	MPA enterohepatic cycling was decreased in patients with the OATP1B3 (334G-669A) haplotype, due to reduction in MPAG hepatic uptake	89

Abbreviations: GFJ, grapefruit juice; MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide; SJW, Saint John's wart.

absorption of the cytotoxic agent paclitaxel confirmed the importance of P-gp recognition and efflux [93]. The contribution of P-gp to oral paclitaxel PK was implicated by an observed increase in absolute bioavailability from 11% in wild-type mice to 35% in genetically modified mice. These predictions made from the mouse model were later corroborated in humans by demonstrating increased oral paclitaxel exposures on coadministration of transport inhibitors selective for P-gp. Consistent with the effect of P-gp efflux to limit oral absorption, the bioavailability of paclitaxel was approximately eightfold higher when dosed in combination with the P-gp inhibitor CsA than after oral paclitaxel alone [78]. Although CsA also inhibits metabolism by CYP3A4, the effect of CsA coadministration was attributed to an increase in paclitaxel absorption due to inhibition of P-gp efflux. This conclusion was supported by findings from a subsequent study in which a change in paclitaxel bioavailability of similar magnitude was observed on coadministration of the P-gp inhibitor GF120918 with paclitaxel [79]. Clinical interaction studies have also implicated P-gp efflux as a mechanism to limit the absorbed fraction of other oral agents such as digoxin, talinolol, and docetaxel, as shown in Table 10.4.

Breast cancer resistance protein (BCRP; encoded by *ABCG2*) is also expressed at the apical membrane of the small intestine. Similar to P-gp, BCRP may facilitate secretion of drugs such as the topoisomerase inhibitor topotecan back into the gut lumen, thereby reducing the absorbed fraction of an oral dose [82]. The modulating effects on drug absorption by uptake of SLCO transporters such as OATP1A2 and OATP2B1 expressed in the human small intestine have also been demonstrated. Inhibition of OATP1A2 uptake of fexofenadine by oral grapefruit juice was associated with a decrease in fexofenadine bioavailability [86]. Because fexofenadine is not metabolized by CYP3A, it was concluded that inhibition of OATP1A2 by grapefruit juice led to an observed decrease in fexofenadine oral bioavailability. Similarly, the effects of oral grapefruit juice leading to a 61% decrease in the oral AUC exposure of the antihypertensive agent aliskiren were attributed to inhibition of OATP2B1 uptake in the gut [85]. Although aliskiren is metabolized by CYP3A4, the net effect of concomitant grapefruit juice was to reduce exposure presumably due to inhibition of OATP2B1 uptake (Table 10.4).

10.4.2 Intestinal Extraction

10.4.2.1 Approximation of F_g . Drug extraction across the intestinal wall may also contribute to incomplete bioavailability after oral dose administration. The presystemic removal of drug on transfer from the GI tract to the systemic circulation is referred to as *first-pass drug elimination* [94]. Although the liver is generally assumed to be the major site of presystemic metabolism, the importance of first-pass intestinal extraction after oral dosing has been acknowledged for an increasing number of therapeutic agents. However, the contributions to first pass from intestinal extraction and hepatic extraction are not easily differentiated in clinical studies and therefore direct estimates of F_g are not always obtained experimentally. Instead, as described in the raloxifene example above, F_g is frequently determined algebraically from estimates of F_a , F_h , and F by Equation 10.8.

Experimental estimates of human first-pass intestinal metabolism have been obtained occasionally, generally following more invasive techniques. The relative contributions of intestine and liver to first-pass metabolism of cyclosporine and midazolam were

shown directly in anhepatic patients during liver transplant procedures [95,96]. In the study with midazolam, intestinal extraction (i.e., $1 - F_g$) was determined from arterial and hepatic portal venous concentrations of 1'-hydroxymidazolam and compared after IV and intraduodenal infusions of midazolam. The intestinal extraction of midazolam by the interduodenal route (0.43) was more than fivefold greater than that by the IV route (0.08), implicating a role for the small intestine in midazolam first-pass elimination. Nifedipine extraction by the intestinal mucosa has also been evaluated in patients with a portocaval anastomosis as well as in healthy volunteers using a multilumen perfusion catheter [97,98].

More recently, oral grapefruit juice has been used in clinical studies to implicate the role of the intestine in first-pass elimination of several drugs metabolized by CYP3A. Grapefruit juice contains furanocoumarin constituents known to irreversibly inhibit CYP3A in the gut mucosa [99,100]. A study in human volunteers with grapefruit juice demonstrated that the oral bioavailability of felodipine, a calcium channel blocker, was limited by intestinal first-pass elimination [101]. Relative to coadministration of water, chronic oral grapefruit juice consumption was associated with a 3.1-fold increase in felodipine AUC exposure. The observed increase in felodipine bioavailability was presumed to result from inhibition of intestinal CYP3A4, as a lack of effect on hepatic CYP3A4 activity by grapefruit juice was determined by the IV erythromycin breath test. Grapefruit juice interaction studies have indicated that intestinal metabolism may also contribute to the first-pass elimination of saquinavir [102] and atorvastatin [103]. A representative group of drugs that undergo presystemic elimination and the proportion attributed to intestinal first-pass metabolism is shown in Table 10.5.

10.4.2.2 Intestinal Metabolism. Drug-metabolizing enzymes expressed in human intestinal epithelial cells create a barrier to the oral bioavailability of several highly metabolized drugs. Consequently, drugs may be subjected to phase I biotransformation reactions as well as phase II conjugation reactions on absorption from the GI lumen. Among the enzyme activities present in the gut mucosa that are frequently involved in the metabolism of drugs are various members of the UDP glucuronosyltransferase (UGT), sulfotransferase (SULT), and CYP enzyme families.

Although multiple CYP enzymes expressed in the intestinal mucosa may contribute to presystemic drug metabolism, CYP3A is the most abundant in human intestine. On average, CYP3A (CYP3A4 and CYP3A5) accounts for ~80% of total immunoquantified CYPs in the proximal small intestine, with the expression of CYP3A5 being highly variable across individuals. The second most abundant CYP enzyme in the human intestine is CYP2C9, accounting for ~15% of intestinal CYPs [110]. In addition to the differential expression of various CYP enzymes, their heterogeneous distribution within the GI tract may also affect the bioavailability of some drugs. CYP3A is localized in epithelial cells, enterocytes, which largely compose the mucosal lining of the gut. Microsomal CYP3A content and catalytic activities vary along the length of the human intestine, generally highest in the duodenum, and decline progressively toward the distal region of the GI tract.

Conjugation reactions within the intestine may also contribute to first-pass metabolism and subsequent detoxification of many xenobiotics as well as endogenous compounds. The bioavailabilities of drugs such as raloxifene [111] and the antidiabetic agent troglitazone [112] are limited by glucuronidation that occurs as a result first-pass gut metabolism. Similar to liver, multiple human UGT enzymes are expressed in

TABLE 10.5 Clinical Interaction Studies of Representative Drugs That Undergo First-Pass Metabolism by Human CYP3A

Drug	Modulator	Effect	References
<i>First-Pass Metabolism</i>			
Atorvastatin $F_g = 0.24^a$	GFJ	Atorvastatin acid AUC _{po} increased by 2.5-fold with GFJ, due to gut CYP3A4 inhibition	103
Cyclosporin A $F_g = 0.41$	Ketoconazole	Bioavailability increased from 22% without to 56% with ketoconazole, due to gut and hepatic CYP3A4 inhibition	104
	Rifampin	Rifampin interaction showed CsA hepatic extraction (0.08) was much lower than intestinal extraction (0.60)	104
Felodipine $F_g = 0.45^a$	GFJ	AUC _{po} increased by 3.1-fold with GFJ, due to gut CYP3A4 inhibition	101
Midzalam $F_g = 0.57$	Erythromycin and GFJ	AUC _{po} was increased by 1.9 with GFJ (gut CYP3A4) and by 2.5-fold with erythromycin (gut and liver CYP3A4)	105
	IV/PO	Mean oral bioavailability was 30% (range 12–50%) with complete absorption and $F_h \sim 0.56$	106
Nilotinib $F_g > F_h$	GFJ	AUC _{po} exposure was increased by 29% with GFJ, due to gut CYP3A4 inhibition	107
	Ketoconazole	AUC increased by threefold with ketoconazole treatment, due to CYP3A4 inhibition	108
	Rifampin	AUC decreased by 80% with rifampin treatment, due to CYP3A4 induction	108
Tacrolimus $F_g = 0.14^a$	Ketoconazole	Bioavailability increased from 14% without to 30% with ketoconazole, due to gut and hepatic CYP3A4 inhibition	104
	Rifampin	AUC decreased twofold from 14% without to 7% with rifampin treatment, due to CYP3A4 induction	104
Saquinavir $F_g = 0.18^a$	GFJ	Bioavailability increased from 0.7% without to 1.4% with GFJ, due to gut CYP3A4 inhibition	102

Abbreviations: GFJ, grapefruit juice.

^aSource: Ref. 109.

the gut. Notably, the expression of two additional UGT enzymes, UGT1A8 and UGT1A10, is limited to the human small intestine [113,114].

Sulfate conjugation in the gut mucosa also constitutes a pathway of presystemic metabolism for endogenous compounds and drug substances. SULT expression levels in the small intestine are characterized largely by SULT1B1 and SULT1A3 [115,116]. For the adrenergic agonists albuterol, isoproterenol, and terbutaline, intestinal first-pass metabolism by sulfoconjugation is known to limit the bioavailability of each. Although these drugs are administered by inhalation to the lungs, a significant proportion of the dose is swallowed and subjected to gut first-pass metabolism. In the case of albuterol, enantioselective metabolism of the pharmacologically active (R) antipode indicated preferential bioavailability of the (S)-enantiomer from the intestinal tract [117]. In contrast to the higher expression levels of drug-metabolizing enzymes located in the liver, intestinal levels of SULT enzymes have been shown to exceed those of the liver [115].

10.4.2.3 Enzyme–Transporter Interplay. The efficiency of the human GI tract to limit the bioavailability of drug to the systemic circulation is due to a combination of factors that include GI physiology as well as the physicochemical attributes of drug substances. For many drugs, the extent of first-pass metabolism by CYP3A in the intestine is an important determinant of bioavailability. The relatively high expression level of intestinal CYP3A, ~80% of the total intestinal CYP content, localized at the epithelial layer of the gut mucosa make the proximal region of the small intestine well suited as a barrier to drug bioavailability. Additionally, the efflux transport protein P-gp functions to limit drug absorption from the gut lumen. Although CYP3A and P-gp are localized at the intestinal epithelium, the relative levels of each protein are differentially expressed in opposing direction along the GI tract. The expression pattern of CYP3A, highest in the proximal intestine and decreasing toward the distal regions, is complimentary to the progressive increase in P-gp levels toward the distal end of the GI tract.

In addition to the physiology of the human GI tract, bioavailability is also a function of the physicochemical properties of individual drug substances [118,119]. Although the active sites of CYP3A and P-gp may accommodate molecules that vary widely in size and chemical structure, substrates of these proteins share greater similarities with respect to the characteristics of hydrophobicity and permeability. Relative similarities in hydrophobic nature and membrane permeability that confer enzyme and transporter protein recognition are highlighted by the fact that most CYP3A substrates are also substrates or inhibitors of P-gp [120,121]. Moreover, the coordinate regulation of both CYP3A and P-gp by a single orphan nuclear receptor SXR has also been reported [122,123]. Taken together, the common physicochemical properties leading to overlapping substrate specificities and induction mechanism, plus coexpression in the epithelial layer of the gut mucosa with complimentary distributions along the GI tract, have led to a hypothesis that these proteins work together to coordinate an absorption barrier to drug transfer [104,124].

The relative importance of intestinal first-pass metabolism as a determinant of the bioavailability of some drugs has not been consistently predicted by CYP enzyme levels in human liver and intestine. Although estimates of total hepatic CYP content, ~25,000 nmol [125], far exceed estimates of enteric CYP levels, estimated at 78 nmol [126], intestinal metabolism has been found to account for a large proportion of the first-pass elimination of CYP3A substrates such as CsA [104,127] and midazolam

[96]. The importance of intestinal extraction has been explained in part by potential differences in unbound drug concentration within the gut lumen and portal bloodstream that could account for restricted access to hepatic enzymes due to plasma protein binding. Additionally, the functional interplay between intestinal CYP3A and P-gp has also been proposed to explain efficiencies of the gut mucosa to limit oral bioavailability of some drug substances [104,124,128]. CYP3A4, like all CYP enzymes, has a finite capacity to metabolize drugs. Following oral tablet dissolution and solubilization, high intraluminal concentrations of drug available for absorption could lead to CYP3A saturation and increased uptake of intact drug. However, the enteric localization of P-gp with CYP3A suggests that drug efflux to the lumen could provide a mechanism by which intracellular drug concentrations are maintained below levels that would otherwise saturate metabolism.

A simplified scheme depicting the coordinated interaction between CYP3A and P-gp in the human intestine is shown in Fig. 10.3. In this simplistic model, intact parent drug (P) absorbed into the enterocyte is presented with three available outcomes—(a) transcellular diffusion into the portal circulation as intact drug, (b) biotransformation to metabolite (M) with subsequent diffusion to portal blood, and (c) extrusion by P-gp out of the enterocyte and returned to the intestinal lumen. Effluxed drug (P') in the lumen may be reabsorbed by passive diffusion and the cycle begins anew. As luminal concentrations of drug are decreased along the GI tract, increasing proportions of absorbed drug are available for metabolism. In effect, the repeated cycle of absorption and efflux maintains low intracellular drug concentrations and thereby P-gp may control access of drug to intestinal CYP3A enzymes.

10.4.3 Hepatic Extraction and Uptake

10.4.3.1 Approximation of F_h . After incomplete dose absorption and first-pass metabolism within the gut mucosa, additional drug loss by hepatic extraction may further limit oral bioavailability. Once the absorbed fraction of parent drug enters the portal venous circulation, elimination by hepatic metabolism may occur on first passage through the liver. In clinical studies, the determination of drug clearance following

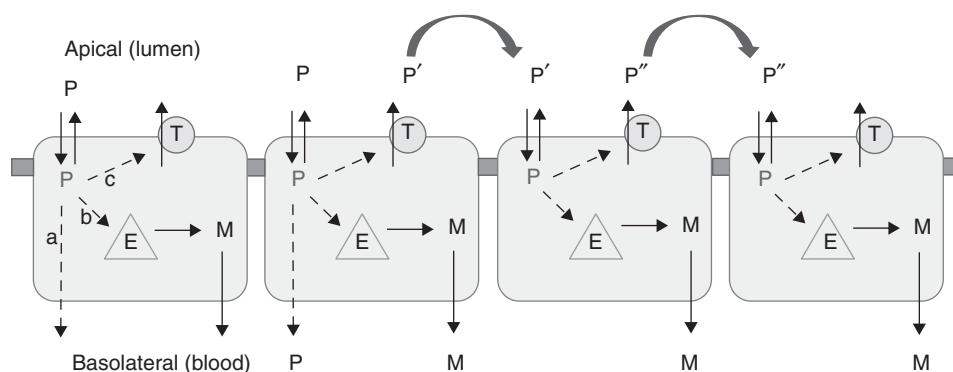


Figure 10.3 Proposed barrier to oral drug bioavailability involving P-glycoprotein and CYP3A localized at the intestinal epithelial cell layer. E, CYP3A4; M, metabolite; P, intact parent drug; P', effluxed parent drug; T, P-glycoprotein transporter. *Source:* Adapted from Ref. 124. (See color insert.)

IV administration can provide a measure of hepatic availability. An estimate of F_h is provided by determining hepatic extraction (E_h) as the proportion of hepatic plasma flow ($Q_{h,pl}$) represented by systemic drug clearance after IV dosing, according to the following expression [15]:

$$F_h = 1 - E_h = 1 - \frac{CL}{Q_{h,pl}} \quad (10.11)$$

in which $Q_{h,pl}$ is calculated from the product of liver blood flow and (1 - hematocrit), and the estimate of liver blood flow calculated from the product of body weight and 0.0216 L/min/kg [106]. Assuming that drug loss due to metabolism in the intestinal epithelium is negligible after IV dose administration, hepatic availability can be estimated by Equation 10.11. Although the estimation of F_h may be influenced by clearance within the intestine following IV dosing, it is generally assumed to be a minor contributing factor. For instance, when measured in patients during the anhepatic phase of liver transplantation, midazolam extraction across the gut mucosa was ~8% following IV dosing [96]. However, an assumption of negligible enteric metabolism after IV dosing could lead to incorrect estimates of F_h and consequently the role of gut extraction in limiting oral bioavailability might be underestimated [129].

Many of the drug-metabolizing enzymes localized in the gut mucosa which contribute to first-pass drug elimination are also expressed in the liver at much higher levels. Metabolism by CYP3A4, the most abundant human P450 enzyme, contributes to intestinal as well as hepatic first-pass elimination of several drugs. Although CYP3A4 metabolism may occur in each tissue, the proportion of oral bioavailability limited by either intestinal metabolism or hepatic metabolism varies for different drugs. For instance, intestinal first-pass metabolism by CYP3A4 is more significant for drugs such as the immunosuppressive agents tacrolimus and cyclosporine and the protease inhibitor saquinavir ($F_g < F_h$). Conversely, hepatic first-pass metabolism predominates for the lipid-lowering agent simvastatin and calcium channel blocker nifedipine ($F_g > F_h$) [109].

Contributions to hepatic first-pass extraction by the polymorphic CYP2D6 have also been shown by comparing drug disposition in extensive and poor metabolizer individuals. For instance, the antitussive agent dextromethorphan is rapidly absorbed and undergoes first-pass metabolism catalyzed largely by CYP2D6 with a lesser contribution by CYP3A4. The bioavailability of oral dextromethorphan has been shown to range from ~1% to 2% in extensive metabolizers to ~80% in poor metabolizers, indicating the importance of CYP2D6 in dextromethorphan first-pass metabolism [130]. Similarly, for the antimuscarinic agent tolterodine and the *N*-methyl-D-aspartate (NMDA) receptor antagonist traxoprodil, determinants of oral bioavailability included the CYP2D6 phenotype of individual subjects. Tolterodine exhibits first-pass metabolism, and oral exposures in CYP2D6 poor metabolizer individuals were ~30-fold higher than in extensive metabolizers [131]. Traxoprodil also undergoes hepatic first-pass metabolism by CYP2D6 and absolute oral bioavailability was ~80% in poor metabolizer subjects. Oral bioavailability appeared to be linear and independent of dose in poor metabolizer subjects, in contrast to extensive metabolizers in which oral bioavailability appeared nonlinear and dose dependent [132].

Limitations in oral drug bioavailability due to hepatic first-pass extraction may also occur for substrates of membrane transport proteins. The effects of hepatic uptake

transporters on oral bioavailability can be exemplified by the role of OATP1B1 in statin disposition. Although all statins are substrates for OATP1B1, polymorphisms in *SLCO1B1* expression have been associated with differential effects on the bioavailabilities of individual statins. For some statins, such as simvastatin, pitavastatin, and atorvastatin, variability in systemic exposure has been related to *SLCO1B1* genotype [87,88]. However, for other statins, such as fluvastatin, oral bioavailability appears to be independent of OATP1B1 genetics [133].

10.5 SUMMARY

The therapeutic use of a given drug is determined by factors associated with its pharmacological effect as well as safety. Inherent to both efficacy and safety is drug bioavailability, the proportion of dose to reach the systemic circulation, or site of pharmacological activity. For orally administered drugs, bioavailability may be limited by malabsorption from the GI tract as well as presystemic elimination by the gut mucosa and liver. Although the barriers to oral bioavailability have historically been considered as individual limitations, experimental research increasingly suggests that drug-metabolizing enzymes and transporters may provide a coordinated barrier to the portal circulation [124,134].

Estimation of bioavailability may be confounded by differences in drug PK, as low oral bioavailability has been associated with greater variability across patient populations [17]. Greater variability in PK can also affect bioequivalence testing as larger study groups are often required to provide sufficient statistical power to differentiate drug formulations. Although bioavailability studies frequently employ a crossover design within the same group of subjects in order to minimize PK variability between individuals, the use of isotopically labeled drug as well as microdosing approaches may also improve bioavailability estimation [22]. Moreover, accounting for sources of variability in enzyme as well as transporter expression and function may also improve the estimation of drug bioavailability.

While bioavailability studies relate systemic exposure to drug absorption and help understand mechanisms underlying drug distribution, transport, and metabolism, comparative bioavailability studies are conducted to evaluate the performance of one or more drugs or drug products against a previously defined reference. The bioequivalence of test and reference formulations for both new drug as well as generic drug products permits an assumption of therapeutic equivalence as well as the potential for product substitution.

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11 Interspecies Pharmacokinetic Scaling and the Prediction of Human Pharmacokinetics Using Animal and Cell Models

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11.1	Summary	1
11.2	Allometric scaling	2
11.3	Prediction of human pharmacokinetics using cell models	15
11.4	Conclusions and future perspectives	28
	References	29

11.1 SUMMARY

Early projection of human pharmacokinetic (PK) properties is of great significance for decision making at several stages in drug discovery and development (e.g., lead compound selection and optimization, first dose in human), and the interspecies allometric scaling is an important tool widely used in the industry for this type of prediction. The allometric scaling is an empirical method that has interested biological and pharmaceutical scientists for more than 100 years. It is developed based on cross-species similarities in anatomy, physiology, and biochemistry with a power function correlating physiological parameters with body size ($Y = aW^b$, where Y is the parameter of interest, W is the body weight, and a and b are the coefficient and exponent of the allometric equation, respectively). The allometric scaling method has been used successfully to describe a number of physiological and anatomical properties and has been applied to the projection of human PK for small-molecule drugs as well as therapeutic proteins. However, for most small-molecule drugs with high cross-species variability in hepatic metabolism, this method may not work well in the extrapolation of hepatic metabolic clearance (CL) from laboratory animals to humans. To improve the predictability of metabolic CL in humans, several modified scaling methods have been recommended and tested. Boxenbaum in 1983 proposed using maximum life span potential (MLP) and brain weight (BRW) as correction factors in allometric scaling since longevity

is frequently inversely correlated with hepatic cytochrome P450 drug oxidation rates. His work was later supported by the research of other scientists, and their work also demonstrated that the MLP and BRW correction improved the accuracy of human CL prediction when the allometry power exponent b was higher than 0.80—0.90. In 1997, Lave *et al.* integrated the *in vitro* data into the power function by normalizing the *in vivo* CL with *in vitro* metabolic CL and successfully applied their method to human CL prediction of 10 small-molecule drugs that are mainly hepatically eliminated. Nagilla and Ward in 2004 suggested using liver blood flow (LBF) as a correction factor for the scaling of small-molecule drugs. On the basis of their analysis of 103 compounds comparing simple allometry (SA) with LBF or MLP/BRW correction, they concluded that scaling with monkey LBF was the best approach among the methods tested (68% success rate). These modified scaling methods have improved the accuracy of prediction to some extent and are reviewed in this chapter. In recent years, there is an increasing interest in physiologically based pharmacokinetic (PBPK) and cell-based pharmacokinetic (CBPK) modeling. Both approaches are developed based on similar assumption that PK properties could be scaled-up from animals to humans according to fundamental physiological principles. These advanced methods allow the integration of input information from different sources (e.g., physiological and physicochemical properties of a drug molecule, permeability and binding affinity from *in vitro* experiments, PK profiles from animal tests and clinical trials). In addition to the prediction of PK parameters (e.g., CL, volume of distribution), the PBPK and CBPK models could also predict the concentration–time profiles in human blood as well as in tissues associated with efficacy and/or toxicity. The utility of PBPK and CBPK modeling goes beyond the prediction of “first-in-human trial,” as it has the potential to be continuously updated with new information (physiological and drug related). Some of the tools and techniques employed in PBPK and CBPK modeling are discussed in the second half of this chapter.

11.2 ALLOMETRIC SCALING

The allometry method was originally developed based on the assumption that mammals generally have similar anatomy, physiology, biochemistry, and cellular structure. This similarity would allow the extrapolation and scaling of physiologic properties (e.g., heart rate, blood flow, blood volume, organ size, longevity) from animals to humans (Fig. 11.1). A power equation $Y = aW^b$ is often used in allometric scaling, where Y is the physiologic variable of interest, W is body weight, and $\log a$ and b are the y -intercept and slope obtained from the plot of $\log Y$ versus $\log W$, respectively. Animals commonly used in preclinical tests (i.e., mouse, rat, rabbit, monkey, and dog) may not eliminate drugs with the same rate and pathway as humans, and small mammals usually eliminate chemicals faster than large mammals. Since the elimination of many drugs is associated with physiologic properties well described among species (e.g., size and blood flow for the elimination organs), it is assumed that drug elimination may be scaled among mammals, and many successful case studies have been reported in the literature [1–32]. However, the majority of the small-molecule drugs are eliminated via oxidative hepatic metabolism. If there are significant cross-species differences in substrate specificity and/or in enzymes activity, the animal to human extrapolation may

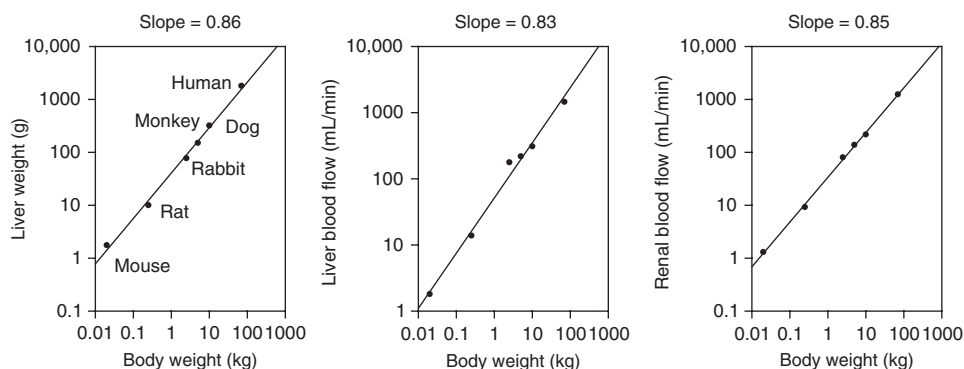


Figure 11.1 Correlation of liver weight, LBF, and renal blood flow versus total body weight in laboratory animals and humans.

fail. To reduce the error in the prediction of metabolic CL in humans, several correction factors have been introduced and are discussed in this section. In recent years, biotechnologically derived peptides and therapeutic proteins have been developed as mainstream therapeutic agents. Peptide and protein drugs (i.e., macromolecule drugs) now constitute a substantial portion of the compounds under preclinical and clinical development in the pharmaceutical industry. The distribution and metabolism of protein-based biotech drugs often follow the mechanisms of endogenous and nutritional proteins with nonspecific proteolysis as a major elimination pathway [33–35]. Owing to their structural similarity as polypeptides, it is generally much easier to predict how peptide and therapeutic proteins will be distributed, metabolized, and eliminated, and they typically have much faster development cycles. As the handling of peptides and proteins is relatively well preserved between different mammalian species, allometric scaling is usually much more successful for macromolecules than for the traditional small-molecule drugs. In this section, the scaling of these two types of drugs is discussed and compared. For some therapeutic proteins, the elimination process is associated with both nonspecific proteolysis and specific receptor-mediated endocytosis and degradation. Owing to the limited number of receptors expressed on the membrane or in the cell, the receptor-mediated degradation process could be saturated at high drug concentrations. For those proteins mainly eliminated with receptor-mediated degradation, saturation of this process would result in an over-proportional increase in systemic exposure, and a decrease of systemic CL with increasing dose (i.e., nonlinear PK). For example, dose-dependent elimination and prolonged half-life at higher doses have been reported for protein drugs erythropoietin, cetuximab, thrombopoietin, and trastuzumab (Herceptin) [36–40]. When this occurs, the systemic CL becomes dose dependent and is generally estimated using the Michaelis–Menten equation. Similar problem may also occur in the scaling of small molecules.

11.2.1 Prediction of Human Clearance Using Single Animal Species with a Fixed Exponent

As mentioned above, a power equation $Y = aW^b$, with Y as the physiologic variable of interest and W as body weight, is generally used in the allometric scaling. When a

log transformation of the power function is performed, the power equation becomes

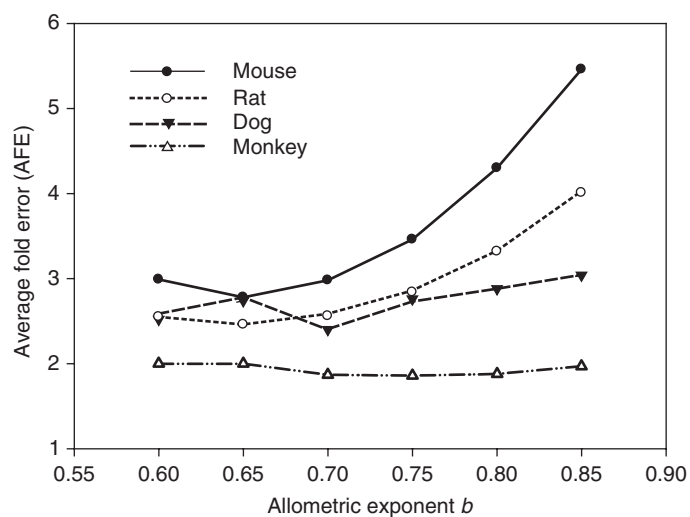
$$\log(Y) = \log(a) + b \times \log(W)$$

The coefficients a and b can be obtained from a plot of the body weight from several species against the PK parameter of interest on a log–log scale; $\log(a)$ is the y-intercept and b is the slope obtained from the plot of $\log(Y)$ versus $\log(W)$. In general, biological frequencies (e.g., heart rate per minute) have an exponent of -0.25 , biological time periods (e.g., circulation time, MLP) have an exponent of 0.25 , biological rates (e.g., CL, physiological flow rates, and metabolism) have an exponent of 0.75 , volume of distribution has an exponent of 1.0 , and body surface area has an exponent of 0.67 [2–4,32]. The allometric exponents for most drugs are in a similar range to those from the corresponding biological processes, with exponents of 0.60 – 0.80 for CL and 0.8 – 1.0 for volume of distribution (V_d). If the PK data are only available in a single animal species, the CL and V_d in the humans are often estimated using a fixed exponent of 0.75 and 1.0 , respectively. The authors have tested the single species (mouse, rat, dog, or monkey) scaling of CL using a fixed b value ranged from 0.60 to 0.85 , and the prediction errors from this analysis of 62 small-molecule and 32 macromolecule drugs are presented in Fig. 11.2 and Table 11.1. The results would suggest an optimal value of 0.70 – 0.80 for the exponent b and to use large animals (e.g., monkey or dog) for single species scaling [41]. In general, the error of human CL prediction is within twofold using monkey PK data and body weight. With the allometry exponent b fixed at 0.70 , 0.75 , or 0.80 , the average error was 1.88-, 1.87-, and 1.86-fold for small-molecule and 1.97-, 1.75-, and 1.61-fold, respectively, for macromolecule drugs. The average error of human CL prediction is higher for the small-molecule drugs using dog as a single species and ranged from 2.4- to 2.9-fold. For macromolecule drugs, the predicted human CL is generally within twofold of the observed value using dog data, with average error ranging from 1.6- to 1.8-fold.

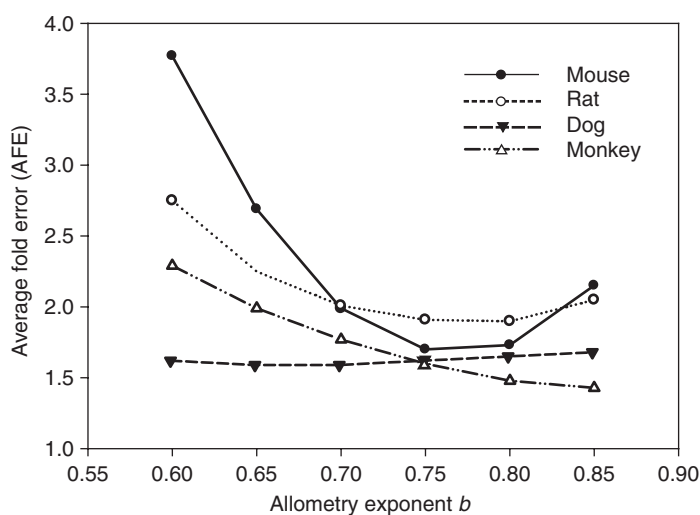
As mentioned previously, LBF is another physiological variable highly correlated to body size and was tested by several groups for its potential application in the extrapolation of hepatic metabolic CL from animals to humans [7,9,10]. The authors have evaluated recently [12] the use of LBF in single species scaling, and the results are summarized in Table 11.2. In general, the method works better for high CL drugs, and the average error of human CL prediction is 1.86- and 1.54-fold using monkey LBF and 2.9- and 2.3-fold using dog LBF for small- and macromolecule drugs, respectively. The average error of human CL prediction is much higher using mouse or rat data (body weight or LBF) for single species scaling [42].

11.2.2 Prediction of Human Clearance Using Two or More Animal Species

In general, allometric scaling is performed using two, three, or more animal species, and many successful case studies have been reported in the literature [1–32]. Conventional allometric scaling often works fairly well in human CL projection when the elimination process is dependent on kidney blood flow or LBF. However, for small-molecule drugs with significant cross-species differences in hepatic metabolism, simple allometric relationships may give poor prediction. Boxenbaum has suggested incorporating MLP into the allometric equation as a correction factor for the scaling of CL ($\text{MLP} \cdot \text{CL} = aW^b$) since longevity is frequently inversely correlated with hepatic



(a)



(b)

Figure 11.2 Cross-species comparison of the relationship between prediction error [average fold error (AFE)] and allometry exponent b for small-molecule (a) and macromolecule (b) drugs.

cytochrome P450 drug oxidation rates [2,3]. $MLP \cdot CL$ is the total volume from which drug would be cleared per MLP assuming constant drug exposure [2]. With regard to humans, it would appear that the relatively low CL (with respect to liver weight) is paced to their longevity, that is, activity is conserved so as to be extended over a relatively longer chronological MLP . Boxenbaum also explored the possibility of using BRW as a correction factor ($BRW \cdot CL = aW^b$) since MLP is closely correlated to BRW [$MLP = 10.839 \cdot (BRW)^{0.636} \cdot (W)^{0.225}$].

Consistent with Boxenbaum's proposal, Feng and others [5–7] also reported that correction with MLP or BRW could help to reduce the error in human CL prediction

TABLE 11.1 Prediction of Human CL Using Single Species Scaling with Fixed Allometry Exponent b

		Dog		Monkey	
		Small-Molecule Drug ($N = 60$)	Macromolecule ($N = 10$)	Small-Molecule Drug ($N = 45$)	Macromolecule ($N = 11$)
Average	$b = 0.60$	2.52	1.57	2.0	2.63
fold error	$b = 0.65$	2.74	1.73	2.0	2.28
	$b = 0.70$	2.4	1.75	1.87	1.97
	$b = 0.75$	2.76	1.57	1.86	1.75
	$b = 0.80$	2.88	1.6	1.88	1.61
	$b = 0.85$	3.04	1.63	1.97	1.64

TABLE 11.2 Prediction of Human CL Using Liver Blood Flow (LBF) Scaling Method

		Dog		Monkey	
		Small-Molecule Drug ($N = 60$)	Macromolecule ($N = 10$)	Small-Molecule Drug ($N = 45$)	Macromolecule ($N = 11$)
Average fold error		2.93	2.26	1.86	1.54

^aThis approach predicts human CL according to the following equation: Predicted $CL_{\text{human}} = CL_{\text{monkey}} (LBF_{\text{human}}/LBF_{\text{monkey}})$, where liver blood flow is 21 and 45 mL/min/kg in human and monkey, respectively, as reported in the literature. For a human weighing 70 kg and a monkey weighing 5 kg, the liver blood flow is 1470 and 225 mL/min, respectively.

for small-molecule drugs with SA exponent b higher than 0.85. Examples from Feng's research are presented in Table 11.3. For the seven small-molecule drugs overpredicted using SA ($CL = aW^b$), incorporating BRW into the power equation ($BRW \cdot CL = aW^b$) increased the accuracy of human CL prediction and reduced the average error from 9.13- to 1.82-fold. Additional research from Mahmood [8,13–17] and Tang *et al.* [18] also supported the use of MLP and BRW as correction factors. Mahmood *et al.* and Balian [17] analyzed several series of small-molecule drugs and proposed three approaches for human CL prediction based on the value of the exponent b derived from the power equation $CL = aW^b$. They suggested incorporating MLP into the allometric relationship ($MLP \cdot CL = aW^b$) when the power exponent b is between 0.71 and 1.0 (0.75–0.90 from analysis of nine anticancer drugs) and correcting with BRW ($BRW \cdot CL = aW^b$) when b is higher than 0.90 or 1.0.

On the basis of a similar mechanism, a combined method of SA and multiexponential allometry (MA) was proposed to improve the accuracy of human CL prediction [19]. For the MA approach, human CL was estimated by the equation $CL = aBW^b + cBW^d$, where a and b are the coefficient and slope generated from the SA scaling and c and d are the coefficient and slope for MA. The parameter d is derived based on the slope from the scaling of blood flow rate, organ volume, and organ weight of liver or kidney versus body weight in preclinical species. The slopes of weight, volume, and flow rates of these major excretory organs (liver or kidney) are very similar and approximately

TABLE 11.3 Human CL Prediction with (CL·BRW = aW^b) or without (CL = aW^b) Brain Weight (BRW) Correction

Drug	Exponent b From SA	Actual Human CL (mol/min/kg)	Predicted/Actual	
			SA	CL × BRW
PD-7	0.97	0.0043	5.6	0.74
PD-8	1.1	8.5	5.7	1.5
Antipyrine	0.93	0.46	11.5	1.8
Theophylline	0.96	0.87	3.6	0.80
Diazepam	1	0.35	32.9	3.8
Valproate	1.16	0.11	9.9	1.9
Midazolam	2.15	11	12.3	2.0
Average fold error			9.13	1.82

equal to 0.9. Therefore, the slope d was fixed as 0.9. The coefficient c was defined as a function of a and b :

$$c = a \left[\frac{(1 - \frac{3}{2}b)}{(1 - \frac{1}{2}b)} \right]$$

As reported by Goteti *et al.* [19], the proposed method helped to reduce the error of human CL prediction for small-molecule drugs with allometry exponent b higher than 0.7. The authors have evaluated the SA + MA method for the human CL prediction of 62 small-molecule drugs and compared with the SA approach with the results presented in Table 11.4. It appears that the SA + MA combination, although still empirical, reduced the average prediction error from 4.2- to 3.1-fold. However, the prediction errors still appear high for these small molecules using the SA + MA approach.

Although the prediction of human CL appears complicated and sometimes problematic for the small-molecule drugs, the SA method generally could predict human CL within twofold for the macromolecule drugs (i.e., therapeutic proteins). The authors have analyzed 32 macromolecule drugs and the outcome is presented in Table 11.4. The average error of human CL prediction is 2.1-fold using the SA, suggesting that the predicted values are normally within twofold of the observed human CL for these protein drugs, probably because the major elimination pathway for these therapeutic proteins is nonspecific proteolysis that is not species-dependent and very different compared to the complicated oxidative metabolic pathway for the small-molecule drugs. The results also recommend no correction factors for the scaling of these protein drugs since the SA method produced the lowest average error for human CL prediction (Table 11.4).

11.2.3 Integration of *In Vitro* Metabolic Clearance into Allometric Scaling

With the recent increase in the availability of human liver tissue, metabolic CL in humans may be estimated by a physiological approach using liver microsome, hepatocytes, expressed liver enzymes, or liver perfusion techniques [1,20–28]. Using the below equation, the hepatic blood CL ($CL_{b,H}$) in humans is calculated based on the intrinsic CL (CL_{int}) determined from an *in vitro* system, the LBF (Q_H), and the free fraction in human blood (f_{u_b}).

TABLE 11.4 Multiexponential Allometric Scaling

Drug Molecule <i>N</i>	SA		MA		SA + MA		SA + MLP	
	Small 61	Macro 18	Small 61	Macro 18	Small 61	Macro 18	Small 61	Macro 18
Average fold error	4.19	2.06	3.52	2.51	3.05	2.79	3.20	3.13
Fold error < 2	10 (34%)	14 (78%)	19 (38%)	11 (61%)	30 (50%)	10 (55%)	32 (71%)	9 (50%)
Fold error > 2	19	4	40	7	30	8	13	9

^aSA = simple allometry, $Y = aW^b$. MA = multiexponential allometry, $CL = aW^b + \left[\frac{1-1.5b}{1-0.5b}\right]aW^b$. SA + MA = rule of exponent—exponent >0.7, MA; exponent <0.7, SA. SA + MLP = rule of exponent—exponent >0.7, corrected by MLP; exponent <0.7, SA.

$$CL_{b,H} = \frac{Q_H \cdot fu_b \cdot CL_{int}}{Q_H + fu_b \cdot CL_{int}}$$

The *in vitro* systems (i.e., liver microsome, hepatocytes) possess many major drug-metabolizing enzymes and are thus useful tools for the *in vitro* to *in vivo* extrapolation and the prediction of the hepatic metabolic CL in humans. The details of this methodology are discussed in the chapters titled **Review of Drug Metabolism in Drug Discovery and Development**, **In Vitro and In Vivo Models of Drug Metabolism**, and **Enzyme Kinetics of Drug-Metabolizing Reactions and Drug–Drug Interactions** of this encyclopedia.

As mentioned previously, if there are significant cross-species differences in drug metabolism, the allometric scaling method may not work well. A modified power equation was proposed by Lave *et al.* in 1997 by integration of the *in vitro* data into the allometric relationship to improve the accuracy of hepatic CL prediction in humans. As listed in the following equation, the *in vivo* CL was normalized by the ratio of CL_{int} between the corresponding animal species and man, where CL_{int} is obtained from the *in vitro* experiment using liver microsome or hepatocytes and W is the body weight [29]:

$$CL_{man} = CL_{animal} \left(\frac{CL_{int,man}}{CL_{int,animal}} \right) \left(\frac{W_{man}}{W_{animal}} \right)^b$$

Comparing to the purely empirical approaches, Lave's method using both *in vitro* and *in vivo* experiment data added a physiological exponent to the conventional allometric scaling and gave an opportunity to integrate some of the complexities as discussed above. Lave successfully applied his method for the prediction of human hepatic metabolic CL for 10 compounds mainly eliminated hepatically [29]. Four small-molecule drugs (antipyrine, mibefradil, propranolol, and theophylline) from Lave's list with an allometry exponent $b > 0.80$ and an overpredicted human CL from SA are summarized in Table 11.5. The results suggest that correction with BRW or CL_{int} could improve the accuracy of human CL prediction and Lave's approach appears slightly better than the BRW method. The average error of human CL prediction is 4.84-, 1.86-, and 1.61-fold for SA, BRW, or CL_{int} correction, respectively. The scaling plots for propranolol with a comparison of all three methods are presented in Fig. 11.3. Propranolol human CL was nearly 3.5-fold overpredicted using SA (Table 11.5), with a scaling exponent of 0.82. The predicted propranolol human CL was 10.0 and 7.7 mL/min/kg using BRW and CL_{int} correction, respectively, and both are within twofold of the actual value reported in the literature (13.0 mL/min/kg).

11.2.4 Prediction of Volume of Distribution in Humans

Volume of distribution (V_d) or steady-state volume of distribution (V_{dss}) in humans is usually well predicted based on animal data. When we use the allometric power equation ($V_d = aW^b$, $V_{dss} = aW^b$) to perform human extrapolation, a plot is normally constructed of total (or unbound) volume of distribution in preclinical species (in units of liter or milliliter) versus animal body weight on a log–log scale for a test compound (Fig. 11.4). Linear regression is performed to determine the values of a and b , and these are then used, along with a standard value for human body weight (e.g., 70 kg), to predict the volume of distribution in humans. As mentioned previously, the allometric exponents for most drugs are in the range of 0.8–1.0 for the scaling of volume of

TABLE 11.5 Human CL Prediction for Antipyrine, Mibefradil, Propranolol, and Theophylline with Simple Allometry (SA), *In vitro* Metabolic Clearance (W_h), or Brain Weight (BRW) Correction

Drug	Exponent b from SA Scaling	Human CL (mL/min/kg)				Predicted/Actual		
		Actual	SA	CL $\times$ BRW	CL $\times$ W_h	SA	CL $\times$ BRW	CL $\times$ W_h
Antipyrine	0.93	0.46	5.3	1.2	0.25	11.5	2.6	0.54
Mibefradil	0.90	7.0	42.0	6.3	3.8	6.0	0.90	0.54
Propranolol	0.82	13.0	45.0	7.7	10.0	3.5	0.59	0.77
Theophylline	0.92	0.61	1.4	0.25	0.93	2.3	0.41	1.52
Average fold error						4.8	1.9	1.6

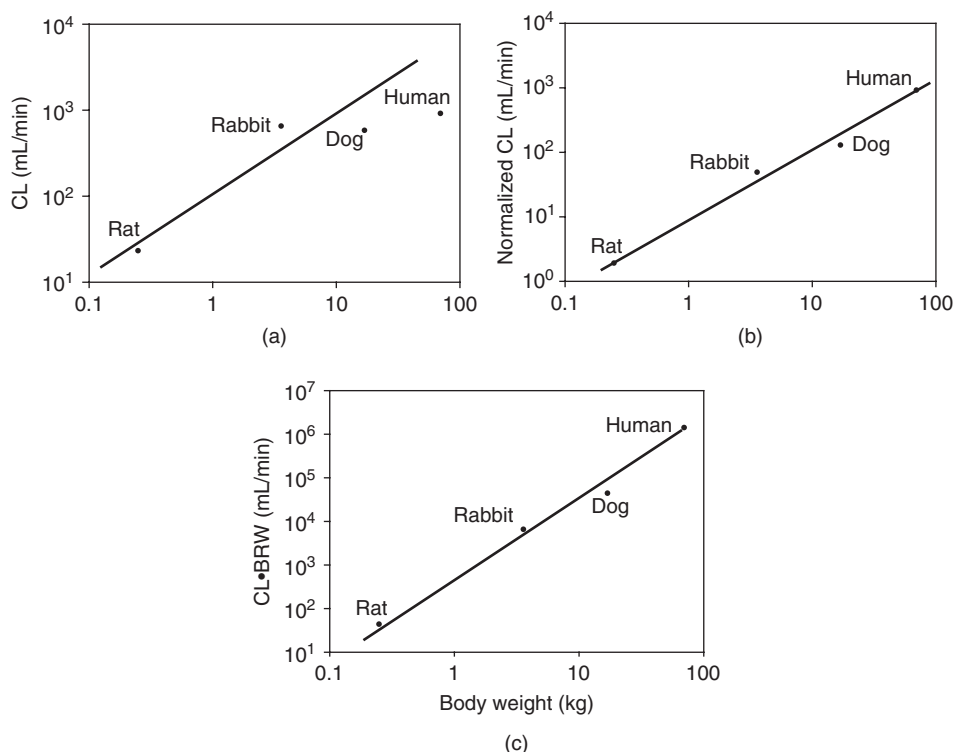


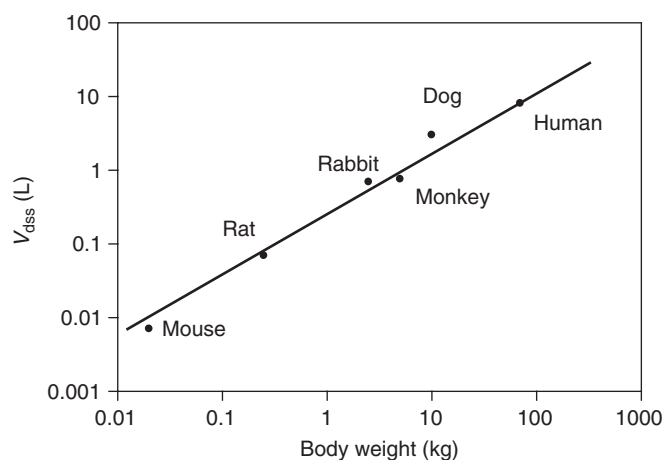
Figure 11.3 Prediction of propranolol human CL with (a) SA (no correction), (b) correction using *in vitro* CL_{int} (normalized CL), and (c) brain weight ($CL \cdot BRW$).

distribution, similar to those from the corresponding biological processes. Boxenbaum in 1982 presented a close relationship ($V_d = 0.756 \cdot W^{0.961}$) between antipyrine volume of distribution and body weight in 11 species including human. His results indicated that the volume of distribution in humans could be successfully predicted using SA [3]. Research from Feng *et al.* [5–7] and other groups [14,31] also support the scaling of V_d or V_{dss} using allometry exponents of 0.8–1.0 with examples presented in Table 11.6 and Fig. 11.4. For the majority of the compounds, the steady-state volume of distribution (V_{dss}) in humans could be estimated using SA with the predicted values within twofold of the actual one (Table 11.6). Although the prediction of diazepam did not work well with the allometric analysis, the error was reduced to twofold when another method using the “average fraction unbound in tissues” was applied, and the details of this method are discussed below.

Obach *et al.* [31] have reported several methods for the prediction of volume of distribution in 1997. In addition to the SA, they also evaluated the contribution of protein binding, and the potential of using the dog–human proportionality or the average fraction unbound in tissues for human extrapolation. The dog–human proportionality method is developed based on the assumption that tissue binding of drugs is similar in dogs and humans, and the physiological parameters, such as extracellular fluid volumes, are similar between the two species on a per-weight basis. Therefore, proportionality could be set up between the free fraction of drug in plasma and the volume

TABLE 11.6 Prediction of Steady-State Volume of Distribution in Humans for 14 Drugs

Drug	Exponent	V_{dss} (L/kg)		
		Predicted (<i>P</i>)	Actual (<i>A</i>)	Ratio (<i>P</i>)/(<i>A</i>)
Cefotetan	0.98	0.21	0.13	1.56
Cefmetazole	0.85	0.15	0.14	1.03
Cefoperazone	0.93	0.17	0.14	1.21
Moxalactam	0.94	0.17	0.14	1.24
Cefpiramide	0.82	0.12	0.11	1.04
Cefazolin	0.98	0.17	0.11	1.60
Erythromycin	0.75	1.33	0.94	1.42
Oleandomycin	0.79	1.30	0.81	1.59
CI-921	0.67	0.29	0.32	0.91
Amsacrine	0.80	1.38	1.56	0.88
SK&F	0.93	0.20	0.24	0.80
Sumatriptan	0.97	2.53	1.64	1.55
Tolcapone	0.94	0.17	0.11	1.47
Diazepam ^a	1.36	7.05	0.89	7.93
Average	0.91	—	—	1.73

^aCorrected by protein bonding.**Figure 11.4** Allometric scaling of steady-state volume of distribution (V_{dss}) for cefotetan.

of distribution at steady state in these two species. The equation for the dog–human proportionality method is listed below, where the term “fu” designates the fraction of drug unbound in the plasma (or serum) in dog or human, and V_{dss} (human) and V_{dss} (dog) represent the volume of distribution at steady state in human (predicted) and in dog (actual), respectively, in units of L/kg.

$$V_{dss(\text{human, predicted})} = \left(\frac{fu_{(\text{human})} \cdot V_{dss(\text{dog})}}{fu_{\text{dog}}} \right)$$

For the method named as *average fraction unbound in tissues*, experimentally determined values for volume of distribution (in units of L/kg) and unbound fraction in plasma (f_u) for each species were used, along with standard values for extracellular fluid volumes, plasma volumes etc., to calculate the fraction unbound in tissues (f_{ut}) in animal species [43]. After f_{ut} was calculated for each of the preclinical species, all values for a given compound were averaged. This averaged animal value for f_{ut} is assumed to be equal to the f_{ut} in humans and, along with the experimentally determined f_u value for humans, was used in the prediction of human V_{dss} .

As listed in Table 11.7, the average error of human V_{dss} prediction was 1.6-, 1.6-, 1.8-, and 2.8-fold for the methods of “dog–human proportionality,” “average fraction unbound in tissues,” “SA with correction for f_u ,” and “SA without correction for f_u ,” respectively. On the basis of the results, Obach *et al.* recommended using the “dog–human proportionality” or the “average fraction unbound in tissues” method for the prediction of human V_{dss} in drug discovery and development.

As summarized in Fig. 11.5, early projection of CL and volume of distribution in humans is of great significance during drug discovery to assist in the optimization of drug PK characteristics. CL, when combined with the volume of distribution, gives the estimation of elimination half-life ($t_{1/2}$) of a drug. Hepatic metabolic CL, when combined with the fraction absorbed, determines the oral bioavailability ($\%F$) of a drug. Half-life and the oral bioavailability are key determinants of the dosing regimen of oral drugs, that is, size of dose and frequency of administration. Thus, the accurate prediction of CL and volume of distribution in humans is critical in the selection of

TABLE 11.7 Comparison of Various Methods Used for the Prediction of Steady-State Volume of Distribution in Humans

Method	<i>N</i>	Average Fold Error
Average fraction unbound in tissues	16	1.56
Dog–human proportionality	16	1.56
SA without correction of free fraction in plasma	14	2.78
SA with correction of free fraction in plasma	12	1.83

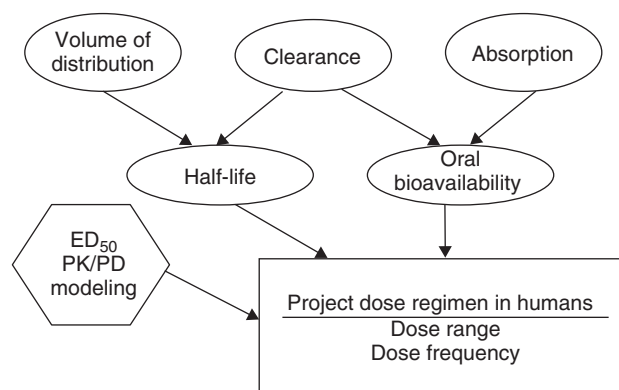


Figure 11.5 Projection of dosing regimen in humans.

new lead compounds for progression into development, as new drugs on the market must not only be efficacious and safe but also be convenient to use for patients and physicians.

11.2.5 Prediction of Pharmacokinetic Profiles in Humans with Nonlinear Mixed Effects Modeling

The use of a nonlinear mixed effects model (NONMEM) as a single-step approach for interspecies scaling has offered certain advantages. This approach could estimate all the allometric parameters in one step and assess interindividual variability in the population. The PK parameters (e.g., CL or volume of distribution) for each species (including human) will then be calculated based on the body weight. The NONMEM equations often used for a two-compartment PK modeling following a single intravenous dose are listed below:

$$\begin{aligned} \text{TV}V_1 &= \theta_1(W)^{\theta_5} \\ \text{TV}V_2 &= \theta_2(W)^{\theta_6} \\ \text{TV}CL &= \theta_3(W)^{\theta_7} \\ \text{TV}Q &= \theta_4(W)^{\theta_8} \\ V_1 &= \text{TV}V_1 \cdot \exp(\eta_1) \\ V_2 &= \text{TV}V_2 \cdot \exp(\eta_2) \\ CL &= \text{TV}CL \cdot \exp(\eta_3) \\ Q &= \text{TV}Q \cdot \exp(\eta_4) \end{aligned}$$

where TV is the population estimate (or typical value) of the PK parameter; θ represents each of the allometric parameters; W is the body weight; CL represents the systemic CL; V_1 and V_2 are the volume of distribution for the central and the peripheral compartments, respectively; Q is the intercompartment CL; and η is the estimate of interindividual variability for each of the PK parameters.

A population pharmacokinetic model was developed and applied to the interspecies PK scaling of pegylated r-HuEPO (PEG-EPO). With NONMEM, the PK profiles of pegylated r-HuEPO (PEG-EPO) and the associated variability were appropriately described in all species (rat, rabbit, monkey, dog, and human) [44]. Another example is the interspecies PK scaling of sumatriptan using mixed effect modeling. The predicted kinetic parameters in each species were close to the actual values from the literature. The mixed effect modeling approach allowed the assessment of covariates associated with the physiologic status of animal (e.g., pregnancy) in the model, which resulted in an improved accuracy in the estimation of human PK parameters and concentration–time profiles [45].

The authors have assessed the interspecies allometric scaling of SK&F and propranolol using the NONMEM approach and the results are presented in Fig. 11.6 [45–50]. Animal data from rat, monkey, and dog were pooled and analyzed in one step using a mixed effect modeling approach (population), and the predicted PK parameters (CL, volume of distribution, etc.) and the plasma concentration–time profiles in humans are closely correlated with the observed values.

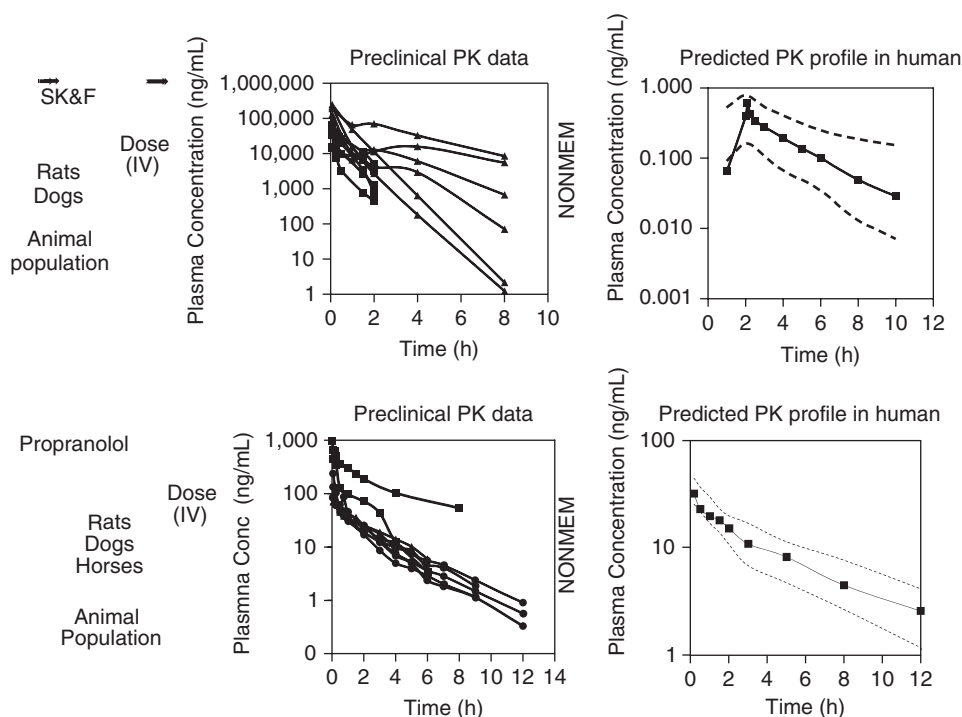


Figure 11.6 Prediction of human PK profiles using nonlinear mixed effects modeling (NONMEM). Symbols used for the preclinical PK plots: square, rat; triangle, dog; and circle, horse. Symbol and line used for the predicted human PK plots: square, observed human PK data and dashed line, upper and lower boundaries of the predicted human PK profile.

Recently, Tabata *et al.* reported a population pharmacokinetic (e-PPK) model for predicting human PK using exploratory ADME (absorption, distribution, metabolism, and excretion) data during early drug discovery research. The model was developed based on data of 11 internal compounds and validated with 12 external compounds. Among the data sets (internal and external references), the predictive performance has shown good consistency [51]. With the NONMEM approach, it is possible to model successfully more complicated allometric equations, to evaluate the potential contribution of physiological variables in the model, and to handle sparse and unbalanced data (quite frequent with animal data). However, allometric scaling using NONMEM is not applicable in all cases. Some of the reasons for failure arise from the differences in enzyme or transporter activities or in metabolic pathways across the species tested.

11.3 PREDICTION OF HUMAN PHARMACOKINETICS USING CELL MODELS

The human body consists of heterogeneous organs that differ in phospholipid contents, organelle densities, and macromolecule distribution patterns. Enhanced distribution to a specific organelle in the target tissue helps to improve the efficacy while undesired accumulation in a subcellular organelle is usually associated with adverse effects

[52–54]. Many of the newly developed drug molecules are inhibitors or stimulators of subcellular enzymes, receptors, or other targets. The subcellular distribution of these drugs in a target tissue is an important determinant of their efficacy or adverse effects. For example, the mechanism of highly active antiretroviral (ARV) therapy through the blockade of different steps of the retrovirus life cycle is now well established. The nucleoside reverse transcriptase inhibitors (NRTIs), integrase inhibitors, and protease inhibitors (PIs) all act on intracellular targets, and the intracellular drug concentrations are closely correlated to the efficacy and toxicity of ARV drugs [55]. The mitochondria and lysosomes are membrane-bound organelles common to virtually all types of eukaryotic cells. Mitochondria-selective accumulation of certain cationic lipophilic drugs accounts for drug retention in target organs as well as a favorable pharmacological profile in preclinical studies. Lysosomal trapping comprises a large portion of total drug uptake of psychotropic drugs, resulting in a more profound drug uptake in lysosome-rich tissues. For therapeutic proteins and monoclonal antibodies, lysosomal degradation is an important elimination pathway following receptor-mediated endocytosis or nonspecific pinocytosis.

The subcellular distribution of a drug molecule may be estimated to some extent by physicochemical properties and unique organellar characteristics (e.g., membrane potential, intraorganellar acidity, bimolecular composition, and organelle density). In recent years, cell-based physiological models have been developed to help enhance our ability to incorporate the subcellular accumulation/degradation of drug molecules into PK and pharmacodynamic (PD) modeling, and this type of modeling is discussed in this section.

11.3.1 Drug Distribution in Lysosomes

Lysosomes (and endosomes) form a complex intracellular recycling system in all nucleated cells, with relatively high abundance in liver, kidney, spleen, and in certain types of cells in the lung [56]. They are characterized by acidic luminal pH (between pH 5 and 6) and a variety of hydrolases (e.g., glucosidases, lipases, proteases, and nucleases) capable of degrading biomolecules and are involved in the normal turnover of organelles [57]. Morphologically, lysosomes and endosomes generally appear as membrane-delimited spheres but can be elongated tubules in fibroblasts [58] and macrophages [59]. The size and number of lysosomes (and endosomes) are maintained by strict regulation of fusion and biogenesis to keep the cellular complement of lysosomes at a relative constant level. Basic amines and weak bases often have a high volume of distribution resulting from their accumulation in the acidic compartments in the cell, usually the lysosomes [60].

Drug-induced lysosomal swelling can be detected in mammalian cells *in vitro* when incubated with high concentration of lysosomotropic compounds, and large swollen vacuoles can often be identified under electron microscopy, laser scanning microscopy, or fluorescent microscopy (Fig. 11.7a–c). Accumulation of lysosomotropic compounds in the lysosomes is also observed *in vivo* in various tissues including liver, kidney, and lung, with the process spanning several weeks [61–63]. There are over 50 marketed pharmaceuticals that induce lysosomal swelling, and they are all weak bases. Several prescribed drugs, amiodarone, azithromycin, chlorpromazine, gentamicin, imipramine, propranolol, and, most notable of all, chloroquine (CQ) are known to cause lysosomal swelling and severe organ impairment in animals and humans [64–66]. CQ, an

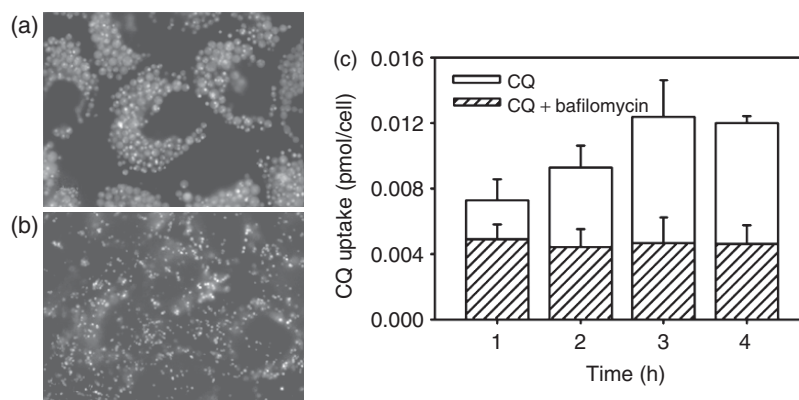


Figure 11.7 Chloroquine (CQ)-induced lysosomal swelling and bafilomycin-inhibited CQ uptake in Madin–Darby canine kidney (MDCK) cells. (a) The swollen lysosomes in cells treated with 50 μM CQ for 4 h, as illustrated by vital staining with 0.5 μM LysoTracker® Green (Molecular Probes, L7526) for 30 min. (b) In cells cotreated with 50 μM CQ and 10 nM bafilomycin A1 for 4 h, lysosomal swelling was inhibited. (c) Intracellular CQ accumulation was inhibited by cotreatment with 10 nM bafilomycin A1.

antimalaria medicine, also known as an *inhibitor of autophagic proteolysis*, inhibits the acidification of lysosomes and endosomes leading to disruption of lysosomal function that impairs maturation of viruses. This mechanism is closely associated with CQ's inhibition effects on human immunodeficiency virus (HIV) and hepatitis C virus (HCV) replication by targeting virus-associated autophagy [67].

A lysosomal trapping model has been developed [68] to describe the dynamic process of lysosomal accumulation induced by basic compounds (Fig. 11.8). In brief, assuming constant pH in the lysosomes and minimum permeability of a protonated molecule, the lysosomal to cytosol concentration ratio (F) of a lysosomotropic compound can be estimated as

$$F = \frac{H_i}{H_e} \quad \text{for monobasic drugs}$$

$$F = \left(\frac{H_i}{H_e} \right)^2 \quad \text{for dibasic drugs}$$

where H_i and H_e are the concentrations of hydrogen ion in the lysosome and the extracellular medium, respectively [56]. Assuming that the intralysosomal pH is 5 and the extracellular pH is 7.2, the F value for a dibasic drug such as CQ ($pK_{a,1} = 10.0$, $pK_{a,2} = 7.5$) can be estimated and appears very high (2.5×10^4). Thus, basic drugs may exhibit a pK_a -dependent accumulation in the lysosomes in cell culture or *in vivo* in lysosome-rich tissues, and this is supported by research from several groups. In an experiment from Orton *et al.* [69], the basic compounds with $pK_a > 8.5$ accumulated extensively in the perfused lung, while amines with $pK_a < 7.0$ showed no sign of accumulation. In a lung perfusion experiment by Anderson *et al.* [70], a pK_a -dependent decrease in replacing amphetamine by other basic drugs was reported. Ishizaki *et al.* [71] discovered a significant reduction of tissue to blood ratio (K_p)

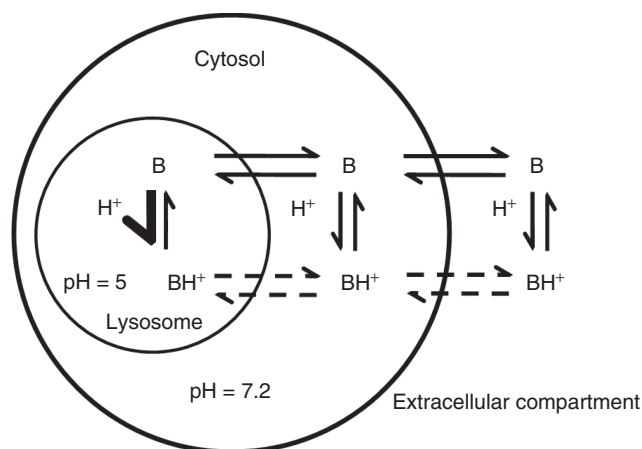


Figure 11.8 Mechanism of lysosomal trapping. Basic drugs in their neutral form may diffuse through the plasma and lysosomal membranes and are then protonated in the acidic lysosomal lumen. These molecules are trapped in the lysosomes due to the poor permeability of the charged form. The lysosomal to extracellular concentration ratio F could be roughly estimated as $F = H_l/H_e$ for a monobasic or $F = (H_l/H_e)^2$ for a dibasic compound, where H_l and H_e are concentrations of hydrogen ion in the lysosome and the extracellular medium, respectively [54,66].

of biperiden and trihexyphenidyl in rat lung, heart, and kidney in the presence of ammonium chloride, a compound that abolishes the pH gradient across lysosomal membranes. They also found a strong positive correlation between lysosomal density and the reduction of K_p caused by ammonium chloride, suggesting an important contribution by lysosomes to the total tissue uptake of both drugs. Similar findings were reported by Hayeshi and collaborators [72] with a significant reduction of intracellular uptake of amodiaquine (a weak base drug) in Caco-2 cells in the presence of the basic compounds CQ and ammonium chloride, both induced an increase of intralysosomal pH.

Lysosomal accumulation significantly affects drug distribution in tissues and organs that contain cells with different lysosomal structures and densities. For example, structural integrity of the lysosomal compartment is required for the uptake of CQ in different tissues [73]. In the mammalian central nervous system, the neuron, astroglia, and oligodendroglia cells form the gray and white matter of the brain. In general, the neuronal cells are highly concentrated in the gray matter, while more glial cells are found in the white matter. Studies in the literature suggested a higher lysosomal density in the neuronal cells (i.e., the gray matter) than in the astroglia and oligodendroglia cells (i.e., the white matter) [74,75]. Daniel *et al.* [76] tested a variety of basic psychotropic drugs and measured the tissue uptake by vertically cut brain slices and cellular uptake by primary neuronal and glial cell cultures. They reported that the steady-state uptake of these psychotropic drugs was more pronounced in the neuronal cells (i.e., gray matter) than in the glial cells (i.e., white matter). In the tissue slices or cell culture experiments, the uptake of these drugs was inhibited by ammonium chloride, a weak base interrupting lysosomal luminal acidity, and the inhibitory effect was more prominent in the neuronal than in the glial cells.

As cited above, basic psychotropic drugs tend to accumulate in the acidic subcellular compartment (i.e., lysosomes), and the accumulation of these drugs in the lysosomes could result in an increase in the intralysosomal pH. A positive correlation was reported between the extent of lysosomal trapping and the therapeutic efficacy of those drugs. In addition, several biochemical processes associated with these pharmacological activities were found altered at enhanced intralysosomal pH [77]. For drugs that function by binding to neurotransmitter receptors and/or transporters, the replacement of a less lysosomotropic compound with a more lysosomotropic psychotropic drug would enhance drug efficacy via distributive interaction with lysosomes [78].

11.3.2 Mitochondrial Sequestration of Drug Molecules

Mitochondria are the energy generators in eukaryotic cells, converting oxygen and nutrients into ATP via aerobic respiration. They appear rod shaped under electron microscopy but form a mobile tubular network *in vivo*. Perhaps, as a result of their prokaryotic origin, mitochondria have two functionally distinct membranes with high lipid and protein composition, as well as an independent genome that codes for a variety of inner membrane proteins [79]. The importance of this organelle is confirmed by an increasing number of diseases associated with the mutation of the mitochondrial genome, malfunction of the respiration pathway, and deregulation of the apoptotic machinery [80–85].

Molecules that exert pharmacological effects via accumulation in the mitochondria or by interaction with mitochondrial proteins represent a structurally diversified group. Among these, lipophilic compounds with delocalized positive charges constitute the largest subgroup with selective mitochondrial accumulation because of the highly negative mitochondrial membrane potential [86]. These molecules can diffuse through lipid bilayers because of their high lipophilicity and are driven into the mitochondrial matrix by the membrane potential gradient [87] (Fig. 11.9). Basic amines can be attracted to mitochondria by the electrical potential and can accumulate in this subcellular organelle; however, the selectivity is relatively low unless specific binding to mitochondrial phospholipids occurs [88–90].

Selective mitochondria-tropic molecules may have applications in anticancer therapy because the mitochondrial membrane potential is notably lower in carcinoma cells than in normal epithelial cells. Therefore, lipophilic cation compounds (mitochondria-tropic) may highly concentrate in carcinoma cells and stimulate an anticancer effect. Chen *et al.* investigated the anticarcinoma activity of two lipophilic cations, dequalinium and MKT-007 [92,93] (Fig. 11.10), and found that both compounds could inhibit the growth of human breast carcinoma cells *in vitro* in a clonogenic assay and prolong the survival rate in mice implanted with tumors. Fluorescent microscope studies further confirmed the selective mitochondrial localization of these two compounds and their toxic effect on mitochondria morphology.

In the field of rational drug design, a series of selective mitochondria-targeted prodrugs have been synthesized by adding a lipophilic cation [e.g., triphenyl phosphonium (TPP)] to the drug molecule (Fig. 11.11) [94–100]. For example, Smith *et al.* [101] covalently attached the TPP cation to the natural antioxidant coenzyme Q to form a prodrug. After oral administration, this mitochondria-targeted coenzyme Q (MitoQ) was rapidly taken up by tissues that are severely affected by mitochondrial dysfunction (the heart, liver, brain, and skeletal muscle), reaching a very high (several hundredfold

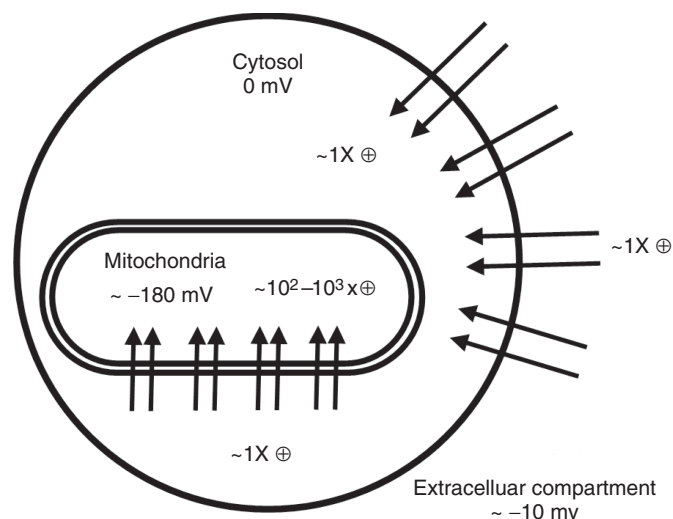


Figure 11.9 Mechanism of mitochondrial selectivity. Driven by electropotential across the membrane, a lipophilic cationic molecule will cross the phospholipid bilayers toward the negatively charged mitochondria lumen. The extent of accumulation can be described by the Nernst equation: $E_{\text{out}} - E_{\text{in}} = \frac{RT}{zF} \ln \frac{[\text{ion}]_{\text{in}}}{[\text{ion}]_{\text{out}}}$, where E is the membrane potential in volt, R is the universal gas constant, T is the absolute temperature, z is the number of positive charge of the ion, F is the Faraday constant, and $[\text{ion}]$ is the concentration of the ion. Assuming a negative mitochondrial membrane potential of 180 mV at 37°C [91] and a negative plasma membrane potential of 10 mV compared to the cytosol, a positively charged mono-ion may accumulate in the mitochondria with a concentration approximately 10^2 - to 10^3 -fold higher than that in the extracellular environment [89,90].

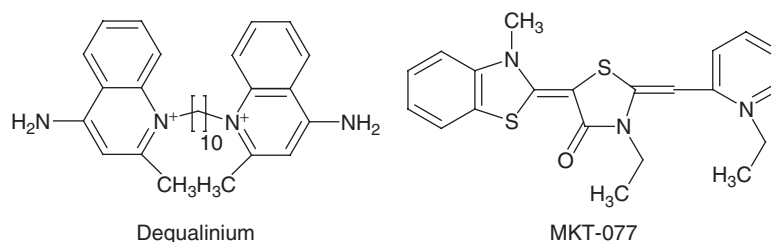


Figure 11.10 Two representative mitochondria-selective lipophilic compounds with delocalized charge(s).

higher) concentration in the mitochondria of these tissues. Meanwhile, the MitoQ was timely cleared from other tissues in the body, leaving no systemic adverse effects. It was also reported that the MitoQ prodrug protected rats from heart dysfunction and cell death caused by ischemia-reperfusion [102], decreased inflammatory response in a rat model with sepsis [103], maintained mitochondrial membrane potential against fluoroquinolone-induced oxidative stress [104], and reduced systolic blood pressure and cardiac hypertrophy in stroke-prone spontaneously hypertensive rats [105]. The synthesis and characteristics of TPP-conjugated antioxidants have been reviewed by Smith and Murphy [106,107].

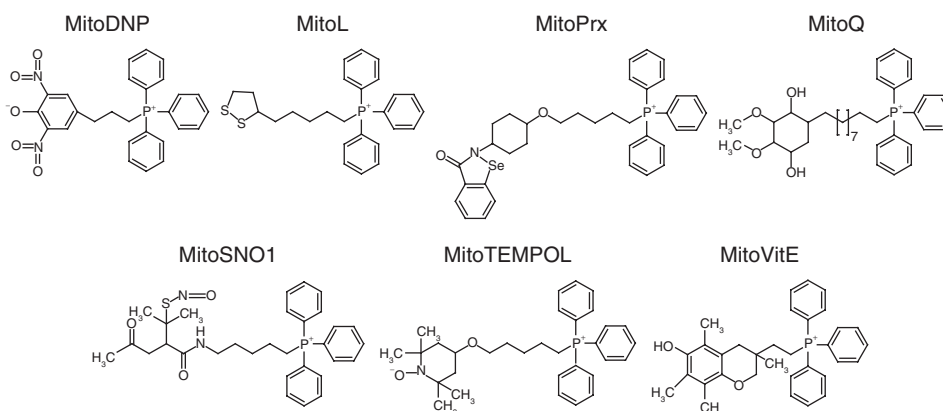


Figure 11.11 Lipophilic molecules with delocalized positive charges targeted to the mitochondria by conjugation to triphenyl phosphonium.

11.3.3 Binding of Drugs to DNA and Other Nonorganelle Components

The cellular composition of proteins, DNA, and phospholipids in plasma, liver, heart, lung, kidney, and many other tissues varies in response to dietary and disease conditions [108]. This could cause high variation in drug–protein (or drug–DNA, drug–phospholipid) interaction in different tissues and affect the disposition, PK, and PD of drug molecules. For example, Nishiura and collaborators [109] revealed that the phosphatidylserine (PhS) content is much higher in the nucleus and membrane fractions than in other organelles in the lung. If a drug molecule preferentially binds to a specific type of lipid component in a subcellular organelle in the tissue, the distribution pattern of this drug may highly correlate with the lipid composition in that organelle. Murata *et al.* [110] reported that the distribution volume of grepafloxacin (GPFX), a quinolone antibacterial agent, is much higher than that of other quinolone antibacterials such as ofloxacin. Suzuki *et al.* have conducted additional studies [111] and identified a positive correlation between the amount of GPFX in subcellular fractions and the PhS content in these fractions (Fig. 11.12). Many anticancer drugs act by interacting with DNA and blocking the biosynthesis of macromolecules. Terasaki *et al.* [112] investigated the tissue distribution of doxorubicin in rats and rabbits and demonstrated a close correlation between tissue uptake of doxorubicin and the levels of DNA in the tissue. Additional studies confirmed the exclusive subcellular localization of doxorubicin associated with the nucleus and a weak correlation between doxorubicin concentration and lipid components. A similar correlation between DNA binding and tissue distribution has also been reported for doxorubicin analogs daunomycin, adriamycinol, daunorubicinol, and actinomycin D [113].

11.3.4 Cell-Based Pharmacokinetic Modeling to Predict the Absorption and Disposition Kinetics in Humans

PBPK models are developed with a series of organ/tissue spaces arranged in a flow diagram incorporating anatomical, physiological, and drug-specific parameters into the model. The whole-body PBPK modeling could help us to predict the kinetic profiles in plasma and in any tissues associated with pharmacological activity or toxicity,

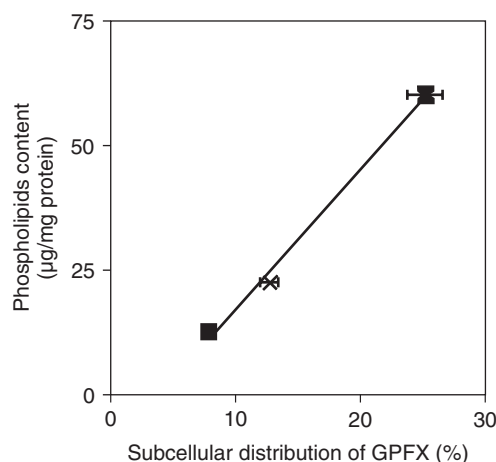


Figure 11.12 Phospholipids composition and subcellular distribution of grepafloxacin (GPFx) in the lung. The contents of phosphatidylserine (PhS) in different subcellular fractions were measured [107] and plotted against the fraction of GPFx discovered in the corresponding subcellular fraction [109]. The majority of GPFx was distributed in the nucleus and plasma membrane fraction (60.3%), followed by the lysosome and mitochondria fraction (22.7%) and the microsome fraction (12.9%). The distribution of GPFx showed good correlation with PhS contents in these subcellular fractions.

and it is a valuable tool to assist the extrapolation of PK/PD information from animals to humans and in decision making in pharmaceutical research and development [114–116]. As discussed previously, the disposition of a drug molecule in the body is controlled by a variety of factors: (i) the organ blood flow and size, (ii) membrane permeability and the physicochemical properties of a drug molecule, and (iii) the binding affinity of the molecule to the plasma/tissue proteins, membrane transporters, and the cytosol/subcellular organelles. Physiologically realistic CBPK models have been developed in recent years to incorporate all these factors in the modeling, and several examples are listed in Tables 11.8 and 11.9. A representative whole-body PBPK model incorporating cell-based mechanism is presented in Fig. 11.13 correlating the cellular events with the disposition of a drug molecule in the body. Single-cell-based pharmacokinetic models were developed in 2005–2008 to simultaneously predict passive transcellular permeability and subcellular distribution of small molecules in polarized epithelial cells [117–119], and one example is presented below. This model consists of several compartments [cellular (cytosol, mitochondria, lysosomes) and extracellular (apical and basolateral)] as described in Equations 11.1–11.4:

$$\frac{dm_c}{dt} = A_a J_{a,c} - A_m J_{c,m} - A_l J_{c,l} - A_b J_{c,b} \quad (11.1)$$

$$\frac{dm_m}{dt} = A_m J_{c,m} \quad (11.2)$$

$$\frac{dm_l}{dt} = A_l J_{c,l} \quad (11.3)$$

$$\frac{dm_b}{dt} = A_b J_{c,b} \quad (11.4)$$

TABLE 11.8 Molecular Characteristics and Drugs with Subcellular Accumulation or Binding

Subcellular Organelles		Distribution	Drugs with Subcellular Accumulation or Binding	Molecular Properties	Mechanisms Involved
Lysosomes		High abundance in liver, kidney, spleen, and some types of cells in the lung [56]	Amiodarone, azithromycin, chlorpromazine, gentamicin, imipramine, propranolol, biperiden, trihexyphenidyl, and chloroquine [62–64]	Weak bases (including some moderate to strong basic compounds)	Ion trapping, electrostatic interactions
Mitochondria		High abundance in muscle	Dequalinium, MKT-007, TPP-conjugated molecules, such as MitoQ [89,90, 92–99]	Lipophilic cations	Passive diffusion, and highly negative mitochondrial membrane potential
Nonorganellar component	DNA	All tissues	Doxorubicin, daunomycin, adriamycinol, daunorubicinol, actinomycin D, [112,113]	Positively charged at physiological pH	Electrostatic interaction
	Lipids	High abundance in adipose [124]	Lipophilic molecules	Lipophilic	Partitioning or electrostatic interaction

TABLE 11.9 Examples of Cell-Based PBPK Models and Application in PK Prediction

Compound	Model Features	References
Napsagatran	Permeability-limited distribution was assumed in the liver; active transport parameters were obtained from <i>in vitro</i> hepatocyte experiment, and passive diffusion was negligible based on hepatocyte data; transport proteins involved are still unknown; biliary excretion rate was estimated from bile-duct-cannulated rats.	123
Fexofenadine	Permeability-limited distribution assumed in the liver; active transport parameters were obtained from <i>in vitro</i> hepatocyte experiment, and medium passive diffusion was derived from hepatocyte data; it is a substrate of Oatp1a1 and 1a4 (hepatic uptake), Mrp3 (hepatic efflux), Mdr1 and Abcc11 (biliary excretion); biliary excretion rate was estimated from bile-duct-cannulated rats.	123
A variety of bases, acids, neutrals, and zwitterions	Passive diffusion and steady state were assumed; binding to plasma protein, partitioning between neutral and ionic species and neutral lipid, neutral phospholipids, and acidic phospholipids were considered in all major tissues.	124,125
A variety of monovalent molecules	Passive diffusion was assumed; Fick's First Law and Nernst–Planck equation were applied to model the transport of neutral and ionic species across biomembrane. Subcellular accumulation and transcellular permeability of small molecules can be calculated.	117,118
Pravastatin	Active transport with organic anion-transporting polypeptide (OATP) 1B1 and multidrug-resistance-associated protein (MRP) 2 included	126
Methotrexate	Model features permeability-limited compartment for bone marrow, GI tract, spleen, liver, kidney, muscle, and skin; extracellular and intracellular binding were included for those tissues. Permeability-limited tumor compartment was included in a later model.	127,128
SDZ IMM 125	Cell-based PBPK model with three major components: (i) vascular blood cells, (ii) combined interstitial and tissue plasma space, and (iii) tissue cells (rapidly interacting pool and moderately interacting pool).	129
Cyclosporine A	Model features nonlinear intracellular binding and tissue distribution.	130,131
Terbinafine	Cell-based PBPK model with permeability-limited distribution in skin and testis.	132
Fingolimod (FTY720)	Cell-based PBPK model with permeability-limited distribution in brain, thymus, and lymph nodes.	133

Everolimus (RAD001)	Cell-based PBPK model with nonlinear partitioning into red blood cells, nonlinear tissue binding, and degradation in plasma.	120
ISIS 1082	Cell-based PBPK model with permeability-limited distribution in tissues.	134
Domperidone	P-gp-mediated efflux was explored in mouse brain and heart.	135
β -Endorphin	Cell-based PBPK model with capillary-membrane-limited distribution in lung, heart, muscle, skin, liver, gut, kidney, brain, and adipose.	136
Monoclonal antibodies	Cell-based PBPK model with lymph circulation. Two-pore model was employed for capillary-membrane-limited step. Binding components were added and specified for different mAbs.	91,137
Monoclonal IgG1		
BFA		138
FcRn		139
scFv-Fc		140
Panacarcinoma mAb		121
CC49		

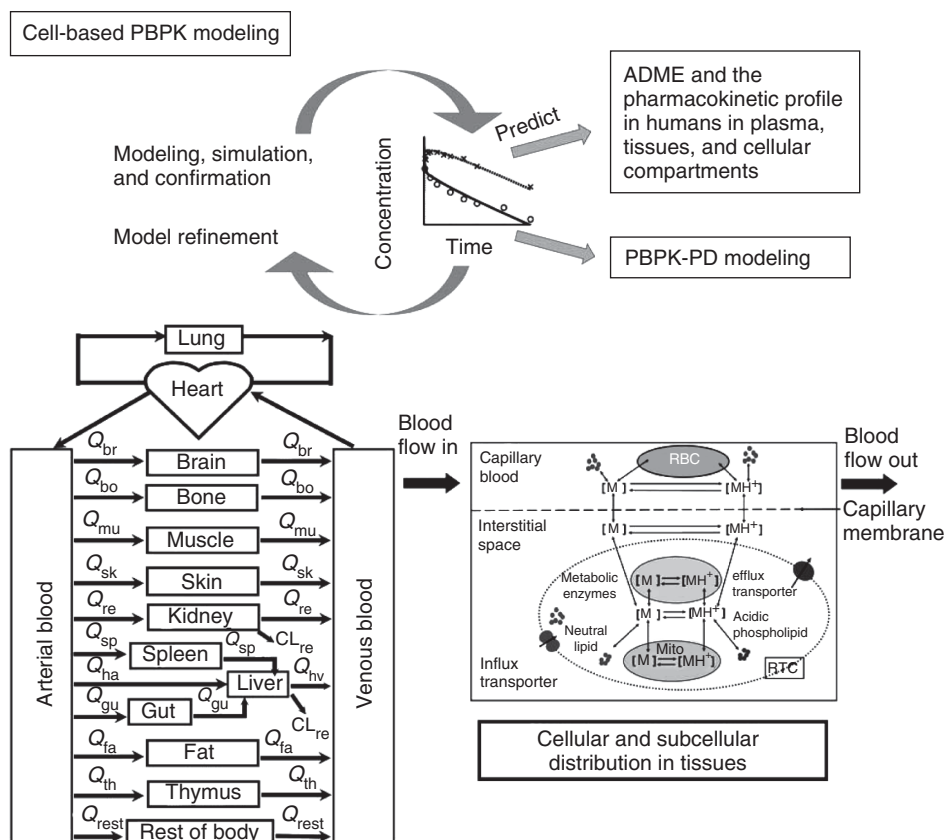


Figure 11.13 Cell-based PBPK modeling and application to human PK/PD prediction. The anatomical/physiological [e.g., size and blood flow (Q) for each tissue] and drug-specific parameters (e.g., ionization, lipophilicity, number of hydrogen bonds, molecular size, polar surface area, red blood cell partitioning, binding to proteins, DNA, and other subcellular components) are incorporated into the model. RBC, red blood cell; Lyso, lysosome; Mito, mitochondria; RTC, representative tissue cell.

where J is the net flux, m is the total molecular mass, t is time, A is membrane surface area, and the subscripts c, m, l, a, and b represent the cytosol, mitochondria, lysosomes, and extracellular (apical and basolateral), respectively. The concentration in the apical (donor) is assumed to be constant, and the concentration in the basolateral (receiver) compartment is kept at sink condition. The passive diffusion of neutral molecules across the membranes is described by Fick's First Law along the concentration gradient (C_o or C_i) as listed in Equation (11.5):

$$J = P_m(C_o - C_i) \quad (11.5)$$

where P_m is the permeability of the molecules across the cellular membrane.

For electrolytes, the driving force across cellular membrane may be the differences in electrochemical gradient (described by the Nernst–Planck equation). Assuming trans-membrane electrical potential to be constant along the membrane, the flux of an ionic

molecule across the membrane is expressed by Equation (11.6);

$$J = -D \left[\frac{dC(x)}{dx} + C(x) \left(\frac{zF}{RT} \right) \left(\frac{E}{d} \right) \right] \quad (11.6)$$

where D is the diffusion coefficient (area per time unit); F , R , T , and z are the Faraday constant, molar gas constant, temperature (in Kelvin), and electric charge, respectively; E and d are the membrane potential and thickness, respectively; and $C(x)$ is the concentration of the drug molecule. Equation (11.7) is then derived by integrating Equation (11.6) from 0 to d for x ,

$$J = P \frac{N}{e^N - 1} (C_o - C_i e^N) \quad (11.7)$$

where $P = D/d$ (diffusion coefficient/membrane thickness) and $N = zEF/RT$.

Several other CBPK models have been developed to include subcellular binding (e.g., the binding to tubulins/microtubules) [141–143], the efflux and uptake membrane transporters [e.g., P-glycoproteins (P-gps)], and the hepatic metabolic CL as listed in Table 11.9. For example, paclitaxel's cytotoxicity is closely related to its binding to subcellular tubulins, and a CBPK model was developed based on experiments using human breast MCF7 tumor cells with negligible P-gp expression and human breast carcinoma BC19 cells derived from *mdr1* (i.e., P-gp) transfected MCF7 cells [142]. The outcome from the model analysis indicates that the P-gp-mediated drug efflux accounts for more than 70% of total drug efflux when the extracellular drug concentrations are lower than 200 nM, but the efflux accounts for less than 30% of total efflux when the extracellular drug concentration is 1000 nM. These findings suggest that if patients have P-gp expression in the tumor similar to those in the BC19 cells, the effect of P-gp-mediated drug efflux may not be significant since clinically relevant concentrations are generally higher than 200 nM.

Poirier *et al.* [144] reported a mechanism-based model including active uptake [e.g., via organic anion-transporting peptide (OATP)], nonspecific binding, and saturable hepatic metabolism. Compared to the conventional two-step approach, the CBPK model greatly improved the estimation accuracy and precision of hepatic metabolic CL.

The elimination of pravastatin is mediated by OATP and the multidrug-resistance-associated protein 2 (MRP2), while the pharmacological activities and the side effects are associated with the hepatic disposition kinetics and/or nonspecific accumulation in the muscle and brain tissues. A perfusion limited cell-based PBPK model was developed by Watanabe *et al.* to predict the exposure of pravastatin in plasma, liver, muscle, and brain by integrating data from both *in vitro* and *in vivo* experiments into the model (e.g., active uptake by multiple transporters, hepatic metabolic CL, active biliary excretion) [126]. The liver was segmented into five subcomponents, each consisting of extracellular and hepatocyte compartments. Overall, the cell-based PBPK model gave reasonable predictions, and the model-predicted kinetic profiles of pravastatin in the plasma and tissues were highly correlated with the observed values in rats and humans [126].

In summary, many important biological events at the cellular levels may affect the disposition of drug molecules in the organ/tissue and the whole body. CBPK models may be linked to whole-body PBPK models to predict the effects of cellular events on drug absorption and disposition.

11.4 CONCLUSIONS AND FUTURE PERSPECTIVES

The ability to predict the PK properties (i.e., CL, volume of distribution, elimination half-life, bioavailability, concentration–time profile) during early discovery and development is of great significance for both small-molecule and protein drugs. The interspecies scaling based on animal data and the allometric relationship between body size and biological function is a useful tool widely used in the pharmaceutical industry for this type of projection. The SA method has been successfully applied to the estimation of human CL and volume of distribution for many small- and macromolecule drugs. However, for small-molecule drugs with complicated oxidative metabolic pathway and significant cross-species differences in hepatic metabolism, the SA power equation may not work well for the estimation of human CL, and correction factors (e.g., MLP, BRW, LBF, *in vitro* metabolic CL, free fraction in blood, binding affinity to receptors or subcellular components) have been proposed to enhance the accuracy of prediction. Each of these techniques mentioned above has its own merits and drawbacks, and some of them have had only partial success in predicting human CL. Research work using *in vitro*, *in vivo*, and *in silico* models are still ongoing to further improve the estimation accuracy of human PK profiles to help reduce the risk and the tremendous financial costs associated with failed clinical trials. Cross-species differences in plasma protein binding is another potential problem in interspecies scaling because only the unbound drug molecules are filtered and secreted in the kidney or metabolized and eliminated in the liver. Literature published in this area has demonstrated that human CL would be more accurately predicted using unbound plasma CL. However, accurate measurement of the free fraction (f_u) in plasma or blood is critical especially for drugs highly bound to plasma proteins. Experimental error in f_u estimation could compromise the scaling results.

Recent advances in computational chemistry have provided additional tools (i) to calculate molecular descriptors (e.g., Log P , Log D , polar surface area and other polar descriptors, free rotatable bonds, atomic charge or free energy associated parameters) to help improve the projection accuracy of the absorption, elimination, and target tissue distribution of drug molecules in humans and (ii) to facilitate the development of cell-based PK/PD models to identify the mechanism associated with biological membrane penetration and drug-receptor/enzyme interaction. For example, high correlation has been reported between the polar surface area (PSA) of a drug molecule and the fraction absorbed in humans [145], or between the PSA and the distribution to brain tissue [146]. Biological mechanism-based models (e.g., PBPK or CBPK models) allow separation of biological and compound-specific components and are thus, by design, capable of integrating information from various processes. By using measurable physiological, anatomical, and biochemical parameters (e.g., organ volume, blood flow rate, binding affinity, and biomembrane permeability), the PBPK or CBPK models can successfully predict the concentration–time profiles in the blood and tissues associated with safety or efficacy in humans [120,121,134,122,147]. The power and utility of PBPK or CBPK models is further increased when linked with mechanistically based PD models [138,148,139,149]. This effective combination is shifting the endpoint of modeling/simulation from a concentration versus time curve toward the time course of a PD outcome. Use of such models can have a major impact during all phases of drug discovery and development and may ultimately result in significant cost reductions for the pharmaceutical industry. In addition, mechanistically based models are

valuable tools for hypothesis testing and mechanistic understanding of the compound's properties and could provide an opportunity to make uncertainty and variability more transparent. With the availability of these models at the preclinical stage, early estimates of the PK/PD profiles in the relevant target (human) population can be obtained to reduce the uncertainty and to improve decision making at early drug discovery and development. This type of combined PBPK/PD or CBPK/PD assessment could provide critical information on the efficacy and safety of drug candidates *in vivo* and bridge the PK/PD concept established during the preclinical development to clinical phase I–III trials. In summary, additional research and investigation are in progress to help increase our ability in performing interspecies scaling and the projection of PK and PD to humans. We believe that the advantage of these newly developed computational models will gradually be recognized and that they will be widely utilized in early drug discovery and development.

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12 The Significance and Determination of Plasma Protein Binding

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12.1	Introduction	1
12.2	Estimation of protein binding	6
12.3	Summary	16
	Acknowledgments	16
	References	16

12.1 INTRODUCTION

Investigations pertaining to profiling the pharmacokinetics (PK) of a drug, require that a drug dose be administered either intra- or extravascularly, followed by measurement of blood, plasma, or serum drug concentrations to follow and mathematically model the fate of the drug. Following drug administration, the drug finds itself directly in the systemic circulation (intravascular dosing) or has to reach the systemic circulation via the process of absorption (extra-vascular administration). The drug then undergoes the process of distribution (defined as the reversible transfer of drug from and to the systemic circulation) that results in the drug reaching the sites of action, the sites of adverse effects, and the organs of elimination. Biotransformation or excretion by the organs of elimination subsequently leads to the loss of drug from the system and the termination of biological activity [1,2].

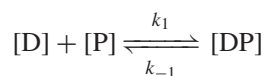
The drug in the systemic circulation can interact with many of the components of the blood and likewise may also interact with non-target components in the tissues. These interactions generally involve reversible binding to blood or tissue proteins mediated by noncovalent interactions that include ionic, electrostatic, hydrophobic, and van der Waals forces [1,3]. The consequence of these interactions is increase in the apparent

molecular mass and molecular size of the bound drug. This leads to the inability of the complex (or drug) to permeate across membranes by simple diffusion or by glomerular filtration thereby affecting the rate and extent of drug distribution and renal excretion. Furthermore, these interactions also result in an inability to enter the active site of the target and/or drug-metabolizing enzymes thus impacting the pharmacological activity and/or the first-pass or systemic elimination or clearance of the drug.

The components involved in these interactions in the blood are plasma proteins and include human serum albumin (HSA), alpha acid glycoprotein (AAG), globulins (α , β , and γ), lipoproteins [including chylomicrons, very low density lipoproteins (VLDLs), low density lipoproteins (LDLs), high density lipoproteins (HDLs), and other related forms of lipoproteins), transcortin, to name a few [3]. In addition, the drug may also be sequestered in blood cells such as erythrocytes, leucocytes, and platelets. Binding of drugs can also occur to tissue proteins and result in differential nonhomogenous distribution of drug within and among tissues [1,3].

Among all of the interactions listed, the interaction with plasma proteins has arguably the maximum impact on both the PK and the pharmacodynamics of drugs, in addition to the very specific binding to the target molecule involved in drug action. HSA and AAG, in this regard, have a greater impact on plasma protein binding than other plasma components. HSA is a single polypeptide chain with a molecular mass of about 66 kDa (585 amino acids). HSA is present in plasma at concentrations as high as 50 mg/mL (about 700 μ M). It is known that HSA has six different types of binding sites that have been delineated based on the binding properties. For example, type I and II sites (present up to three times on an HSA molecule) bind warfarin and diazepam, respectively. Similarly, type III site (present at six or more regions on an HSA molecule) binds phenytoin in an apparently nonsaturable manner. Some of other drugs known to bind to HSA are NSAIDs (nonsteroidal anti-inflammatory drugs), digoxin, imipramine, theophylline, caffeine, probenecid, tamoxifen, and so on [1,3,4]. Both the high concentrations in the plasma and the presence of a multitude of binding sites make HSA the most versatile of proteins that bind drugs. AAG is a single polypeptide with a molecular mass of 44 kDa and present in the plasma in concentrations up to 1–2 mg/mL (45 μ M). AAG is generally known to have high affinity for basic drugs such as imipramine and propranolol. Other proteins that are present in lower concentration in the plasma have much lower significance in terms of the impact on the PK of the drug.

The drug–protein binding phenomenon is most commonly characterized by fraction of drug unbound (f_u) (or referred to as the free fraction) in plasma or tissue and by the dissociation rate constant (K_s) that is indicative of the strength of the binding [1–4]. For a drug (D) and protein (P), the interaction is depicted below



where k_1 and k_{-1} are the rate constants associated with the formation and breakdown of the DP complex. At equilibrium,

$$k_1[D_f][P_f] = k_{-1}[DP]$$

The dissociation constant (K_s) may be defined as follows:

$$K_s = \frac{k_{-1}}{k_1} = \frac{[D_f][P_f]}{[DP]}$$

$$[DP] = \frac{[D_f]P_f}{K_s}$$

By applying law of mass action that the total drug concentration $[D]$ is the sum of $[D_f] + [DP]$ and that the free fraction of the drug is defined as the relative concentration of free to the total concentration of the drug,

$$f_u = \frac{\text{Free drug concentration}}{\text{Total drug concentration}} = \frac{[D_f]}{[D]} = \frac{[D_f]}{[D_f] + [DP]}$$

Therefore,

$$f_u = \frac{[D_f]}{\left([D_f] + \frac{[D_f][P_f]}{K_s}\right)}$$

Dividing the numerator and denominator by $[D_f]$,

$$f_u = \frac{1}{\left(1 + \frac{[P_f]}{K_s}\right)} = \frac{K_s}{K_s + [P_f]}$$

This indicates that the fraction unbound (free fraction) is directly proportional to the dissociation rate constant. The higher the tendency of the DP complex to dissociate to release free drug, the higher will be the fraction unbound. The fraction unbound is also inversely related to the free protein concentration, in that more the concentration of free protein, the lower will be the value of fraction unbound. The unbound fraction may be expressed as values ranging from 0 to 1 or as a percentage ranging from 0% to 100%. In general, drugs with a f_u of 0.9 (90%) or more are referred to as *highly protein-bound drugs*. Although, not explicitly indicated, f_u is also dependent on the total drug concentration and in situations where the dissociation constant is small (less free drug concentration) and the plasma total drug concentrations are high, the binding sites on the protein may be saturated and f_u may increase. However, for most drugs, the f_u is essentially constant over the therapeutic drug concentration range.

Since it is only the free or unbound drug that is able to cross membranes and interact with the target tissues, distribution equilibrium is achieved when the free concentrations in both the plasma and tissue are identical. However, at this juncture, the total concentrations of drug in the plasma and tissues may be nonidentical. For example, if the fraction unbound in the plasma is 0.9 (unbound concentration of 9 units relative to a total concentration of 10 units) and that in the tissue is 0.1 (unbound concentration of 9 units relative to a total concentration of 90 units), one can appreciate that at distribution equilibrium when the free concentrations of drug are same and equal to 9 units in both the plasma and tissue, the total tissue concentration is ninefold higher. A reversal of this situation can result in plasma concentrations being ninefold higher than tissue concentrations. In general, lower the free fraction in the plasma, less is the propensity for the drug to go to tissue compartments. The primary PK parameter that

reflects this interplay is the apparent volume of distribution (V) [1,2]. The equation that states this relationship is

$$V = V_p + V_{TW} \left(\frac{f_u}{f_{uT}} \right)$$

where V_p and V_{TW} are the plasma and tissue water volumes, respectively, and f_u and f_{uT} are the fraction of drug unbound in the plasma and tissue water, respectively.

Thus, other factors being equal, drugs that have a lower free fraction in plasma tend to be sequestered in the plasma compartment (showing a low V) relative to drugs with a higher free fraction in plasma. Likewise, drugs that have a lower free fraction in tissues tend to be sequestered in the tissue compartment (showing a high V) relative to drugs with a higher free fraction in tissue.

The free drug is also the entity that is amenable to metabolism by drug-metabolizing enzymes, glomerular filtration, active secretion and active reabsorption.

The determinants of the organ metabolic clearance [1–4] are indicated by the following equation.

$$CL_{org} = \frac{Q_{b,org} \times f_{u,b} \times CL_{int}}{Q_{b,org} + [f_{u,b} \times CL_{int}]}$$

where CL_{org} and CL_{int} are organ clearance and intrinsic clearance (volume/time), Q_{org} is the organ perfusion rate or organ blood flow (volume/time) and $f_{u,b}$ is fraction unbound in blood.

For drugs that are efficiently metabolized by drug-metabolizing enzymes and have high intrinsic clearance values, the above equation collapses to the following:

$$CL_{org} = \frac{Q_{b,org} \times f_{u,b} \times CL_{int}}{f_{u,b} \times CL_{int}}$$

$$CL_{org} = Q_{b,org}$$

The organ clearance is thus perfusion rate limited and independent of both protein binding and intrinsic clearance.

For drugs that are not good substrates for drug-metabolizing enzymes (low intrinsic clearance), the equation for organ metabolic clearance then rearranges to the following:

$$CL_{org} = \frac{Q_{b,org} \times f_{u,b} \times CL_{int}}{Q_{b,org}}$$

$$CL_{org} = f_{u,b} \times CL_{int}$$

The free fraction in blood or $f_{u,b}$ is now a crucial determinant of hepatic metabolic clearance in addition to the low intrinsic capacity (intrinsic clearance) of drug-metabolizing enzymes that can biotransform the drug.

As an extension, organ clearance can also be expressed as

$$CL_{org} = Q_{b,org} \times ER$$

where ER is the extraction ratio and is the fraction of drug that is removed by the eliminating organ per single pass of the blood through the organ [1,2,4]. As a corollary,

the fraction that escapes the elimination organ is given by $[1 - ER]$. This relationship allows one to estimate the expected bioavailability (F) of an orally administered drug.

$$F = f_a \times f_g \times f_h$$

where f_a , f_g , and f_h are fractions that escape the lumen, gut wall metabolism, and hepatic metabolism, respectively. If we assume that all of drug escapes the lumen and gut wall metabolism, f_h will then determine the lower limit of the oral bioavailability. Thus,

$$F = 1 \times 1 \times f_h = f_h$$

But,

$$\begin{aligned} F &= f_h = 1 - ER \\ F &= 1 - \left[\frac{f_{u,b} \times CL_{int}}{Q_{b,org} + (f_{u,b} \times CL_{int})} \right] \\ F &= \frac{Q_{b,org}}{[Q_{b,org} + (f_{u,b} \times CL_{int})]} \end{aligned}$$

For a low clearance drug (low CL_{int}), this equation collapses to

$$F = \frac{Q_{b,org}}{Q_{b,org}} = 1$$

For a high clearance drug (high CL_{int}), this equation collapses to

$$F = \frac{Q_{b,org}}{(f_{u,b} \times CL_{int})}$$

Thus, a higher fraction unbound can lower the overall bioavailability of the drug. Overall, f_u can significantly impact hepatic clearance, oral bioavailability, and total body clearance (CL).

Similarly, the overall filtration clearance of a drug in the kidney is given by $f_u \cdot GFR$ (glomerular filtration rate) [1,2]. Furthermore, active secretion and active reabsorption that are mediated by specific efflux or uptake transporters are also able to accept only unbound drug for transport, and thus, the unbound fraction of drug impacts these processes as well.

To summarize, polytherapy or disease states that alter the drug–plasma and/or drug–tissue binding due to changes in the concentration of the plasma/tissue-binding proteins or displacement of bound drug from its binding sites can result in changes in the plasma free concentrations of the drug and potentially lead to changes in primary PK parameters (V and CL) and consequently the derived PK parameters, such as half-life and AUC, of a victim drug.

During the discovery and development of an NCE (new chemical entity), determination of the plasma protein binding or HSA binding of a NCE is thus crucial to

aid the complete PK profiling of the drug and evaluation of the drug–drug interaction potential of the NCE.

12.2 ESTIMATION OF PROTEIN BINDING

Several *in vitro* methods are used for the estimation of the unbound drug fraction (f_u) [5,6] and include equilibrium dialysis, ultrafiltration, ultracentrifugation, and so on (Fig. 12.1). Among them, equilibrium dialysis and ultrafiltration are most commonly used for the estimation of f_u in plasma, serum, or diluted tissue homogenate because of their simplicity and high throughput compatibility. However, neither of these methods is free of biological, chemical, or physical artifacts, and the advantages and disadvantages of each method are discussed in this section.

Often, in drug discovery and development, the choice of method is based on progress stage of the compound. As the compound progresses in its life cycle, the methods become more robust and reliable (Fig. 12.2). In early drug discovery, rapid screening methods are required, which allow classification of compounds in low, medium, and high binding categories. As the compound moves forward and more quantity is available, more definitive methods such as equilibrium dialysis and ultrafiltration are used, which allow the study of sex or species differences in plasma protein binding, linearity in binding with respect to drug and protein concentration and identification of proteins to which drugs bind. It is recommended that protein binding should be studied by at least two methods for compounds in this stage and the binding estimations are supported by sensitive and specific analytical methods [e.g., liquid chromatography tandem mass spectrometry (LC-MS/MS)]. For compounds in clinical development,

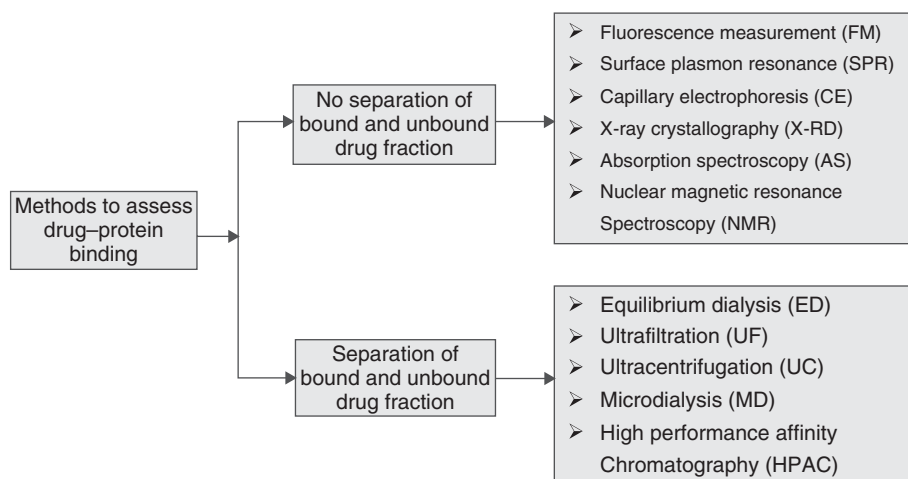


Figure 12.1 Methods used to assess protein binding of drugs. Methods for estimation of plasma protein binding involve either prior separation of bound and unbound drug [bottom right quadrant of the figure] or determination without prior separation of bound drug from free drug [top right quadrant of the figure]. Routine methods use the separation of free and protein-bound drugs for estimation of the fraction unbound.

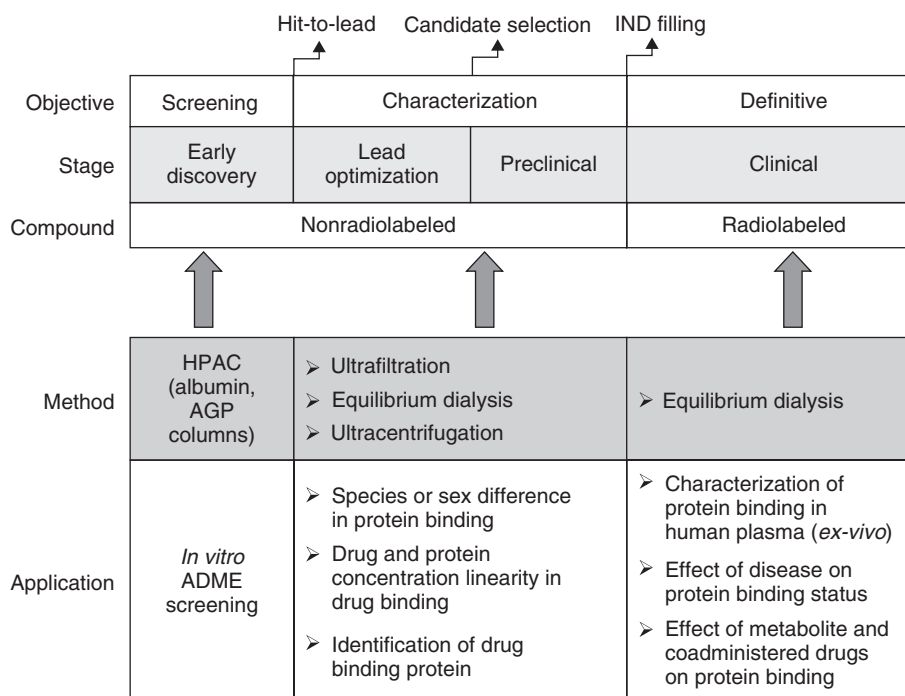


Figure 12.2 Protein binding methods and their application in various stages of the drug development. The method used to assess protein binding is dependent on the depth of information required. At early stages, quick and semiquantitative estimation of the fraction unbound is sufficient, but during the clinical development phase, an accurate estimate of protein binding is done using equilibrium dialysis—“the gold standard” as it also has the potential for evaluation of protein-binding-based drug–drug interaction.

radiolabeled compounds that provide a more reliable and robust assessment of protein binding are used.

12.2.1 Equilibrium Dialysis

Equilibrium dialysis method is often considered as the “gold standard” for measuring protein binding. It is based on the establishment of an equilibrium state between a protein compartment and a buffer compartment, separated by a semipermeable membrane. This membrane (pore size/molecular weight cutoff (MWCO) is selected depending on the compound to be studied or application *viz.* 12–14K MWCO, 6–8K MWCO, 10K MWCO, 3.5K MWCO, etc.) and allows only low molecular weight ligands such as drug molecules to be transported between two compartments. The experimental method is depicted in Fig. 12.3.

Briefly, drug-spiked biological matrix (plasma, serum, or tissue homogenate) is placed in one compartment and buffer (typically sodium or potassium phosphate buffer, pH 7.4) is placed in the other, and the assembly is incubated at 37 °C until equilibrium between free drug concentration in matrix and that in the buffer is reached (incubation period usually lasts longer than 2 h). During the incubation period, in addition to the

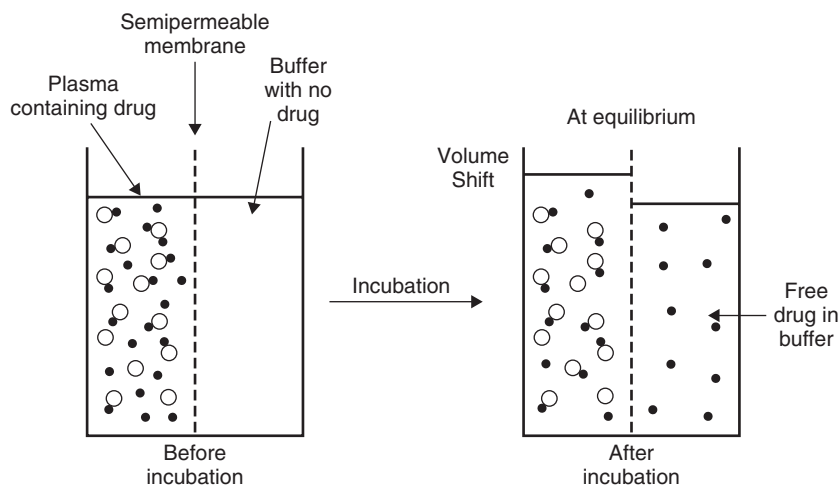


Figure 12.3 Equilibrium dialysis method for estimating protein binding of drugs. The figure indicates the movement of free drug (closed circle) from the left reservoir that contains both free drug and drug bound to protein (open circle), through the semipermeable membrane, to the right reservoir that contains only buffer. At equilibrium, the free drug concentrations on both sides are the same. Note the volume shift due to movement of buffer to the left side protein-containing chamber due to osmotic pressure and/or Donnan ion effect.

transport of unbound drug molecules, water molecules from the buffer compartment are continuously moving to the matrix compartment because of the differences in osmotic pressure between the two compartments and/or the Donnan ion effect. This results in an increase in volume in the matrix compartment and decrease in volume in the buffer compartment; this phenomenon is known as the *volume shift*, which is usually about 15–20% and needs to be corrected during the estimation of protein binding. At equilibrium, aliquots of matrix and buffer are sampled for analysis of drug concentrations. Also volumes in the matrix and buffer compartments are measured. Protein binding is estimated as shown in Equation 12.1.

$$\text{Protein binding (\%)} = \frac{(C_{pe} - C_{be}) \times \frac{V_{pe}}{V_{pi}}}{\left[(C_{pe} - C_{be}) \times \frac{V_{pe}}{V_{pi}} \right] + C_{be}} \quad (12.1)$$

where C_{pe} is the peak area of analyte in matrix at equilibrium, C_{be} the peak area of analyte in buffer at equilibrium, V_{pe} the volume of matrix at equilibrium, and V_{pi} the initial volume of matrix.

Maintenance of physiological pH and temperature is important for accurate estimation of protein binding [7,8]. High throughput equilibrium dialysis apparatus have become increasingly popular and are widely accepted today. These include the Rapid Equilibrium Device (RED, Thermo Scientific) [9], Microequilibrium Dialysis Device HTD 96 (HTDialysis) [10], 96-Well Equilibrium Dialyzer (Harvard Apparatus) [11], and so on. These require shorter preparation and dialysis times and are amenable to automation. For bioanalysis, after attainment of equilibrium, an aliquot of buffer

(90 μL) from the buffer compartment is mixed with blank matrix (10 μL) and similarly, an aliquot of matrix (10 μL) from the matrix compartment is mixed with blank buffer (90 μL) before sample processing. This approach eliminates the need for preparation of separate calibration curves of drug in buffer and matrix and can save valuable analytical instrumentation time. For calculation of protein binding, the values are corrected for sample dilution by applying respective dilution factors, that is, values from matrix side are multiplied by 10 and values from the buffer side are multiplied by 1.1. The fraction of unbound drug (f_u) is calculated as shown in Equation 12.2.

$$f_u = \frac{C_u}{C_b} \quad (12.2)$$

where C_u is the concentration of drug on buffer side and C_b the concentration of drug on matrix side.

The percentages of drug unbound or free and bound to protein are calculated using Equations 12.3 and 12.4.

$$\% \text{ Drug Unbound} = f_u \cdot 100 \quad (12.3)$$

$$\% \text{ Protein binding} = (1 - f_u)100 \quad (12.4)$$

The correction for volume shift is not generally used in the 96-well setup because of the large error associated with withdrawal and measurement of small volumes required for the correction. When nonradiolabeled drug is used, drug concentrations in matrix and buffer are typically analyzed by LC-MS/MS, and when a radiolabeled drug is used, analysis is performed by liquid scintillation counting (LSC).

Equilibrium dialysis can also be used to study the binding interactions by dialyzing free drug through a semipermeable membrane until its concentration across the membrane is at equilibrium (Harvard Apparatus). Data obtained under different experimental conditions can then provide information on various binding parameters such as binding constants and number of binding sites or binding capacity. Binding to HSA and AAG can be studied by this method using purified protein solutions [12].

Equilibrium dialysis is theoretically the most accurate method for determining plasma protein binding because the equilibrium is not shifted when aliquots are taken from either side of the membrane [13]. It is a suitable method for drugs that are highly protein bound (>98%). Some advantages and disadvantages of equilibrium dialysis method are listed in the following.

Advantages

- Easy to setup and use;
- Needs small amount of sample;
- Temperature controlled;
- Thermodynamically sound;
- Reproducible results can be obtained even for low affinity compounds;
- Meaningful results can be determined for compounds with nonspecific binding (NSB) [14];
- Compatible to high throughput with robotic systems;
- Binding interactions can be studied.

Disadvantages

- Requires long time to reach equilibrium;
- Volume shift [15];
- Donnan effects (hindering of the passage of free ligand) [16];
- NSB;
- Potential degradation of compound in matrix due to thermal or metabolic instability;
- Shift of pH due to continuous loss of carbon dioxide [17];
- Overestimation of free fraction as a result of slight leakage of protein into buffer compartment [18].

12.2.2 Ultrafiltration

Ultrafiltration method is based on the separation of unbound drug from the protein-bound drug by filtering the protein–drug matrix through a semipermeable membrane under positive pressure generated by centrifugation. The experimental method is depicted in Fig. 12.4.

Briefly, drug-spiked matrix is placed in the sample reservoir, and the reservoir is capped and fitted onto a sample collection tube. The entire ultrafiltration assembly is then centrifuged at $1000\text{--}2000\times g$. The incubation time is fixed based on the flow rate such that the ultrafiltrate (plasma water, PW) obtained is about one-fifth of the initial volume of the sample. The centrifugation can be carried out at room temperature or at 37°C ; however, the chosen temperature should be maintained constant during the centrifugation process as drug binding decreases with rising temperature. A major

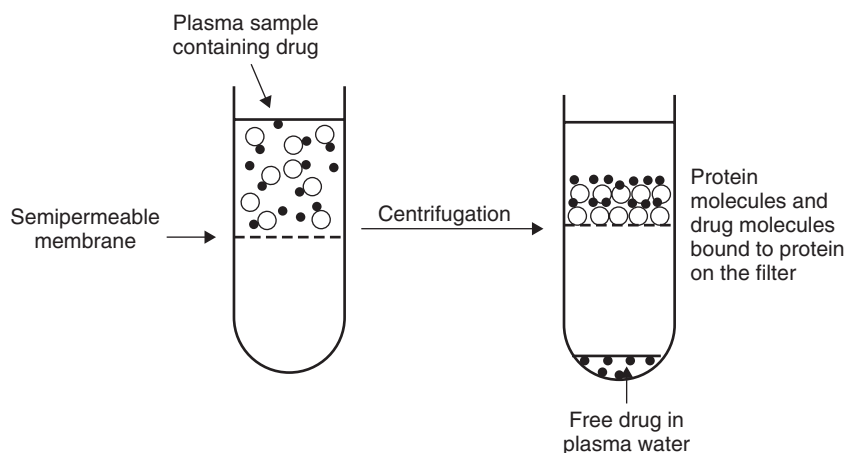


Figure 12.4 Ultrafiltration method for estimating protein binding of drugs. The figure indicates the movement of free drug (closed circle) from the top chamber that contains both free drug and drug bound to protein (open circle), through the ultrafiltration membrane, to the bottom chamber. The movement is assisted by the application of a centrifugal force. The ultrafiltrate is analyzed to obtain the free drug concentration.

drawback of the ultrafiltration method is the potential NSB of the drug to the plastic tube and ultrafiltration membrane, which will lead to an underestimation of the free drug concentration. This is usually assessed and corrected when calculating the protein binding by running a parallel control sample using drug-spiked blank PW or physiological phosphate-buffered saline and using this as sample instead of plasma. The concentration of the drug in the ultrafiltrate and plasma can be quantified using a LC-MS/MS. If radiolabeled drug is used, LSC can be used. The fraction of drug unbound can be calculated by using Equation 12.5 (employs correction factor for NSB, that is, ratio between drug concentration in the original PW (C_{pw}) and the concentration of the drug in PW ($C_{pw,f}$) after ultrafiltration).

$$f_u = \frac{C_f (C_{pw} / C_{pw,f})}{C_p} \quad (12.5)$$

where C_f is the concentration of drug measured in the ultrafiltrate after centrifugation of the matrix containing the drug and C_p is the concentration of the drug in the matrix before centrifugation.

The percentage of drug bound or unbound can be calculated by using Equations 12.3 and 12.4 described earlier.

To reduce NSB of drugs, pretreatment of the filter membrane with 5% Tween 80 or benzalkonium chloride has been suggested [19]. Equilibrium dialysis is considered to be more suitable for protein binding estimations if the NSB of the drug to the ultrafiltration apparatus is greater than 5%. It has also been suggested that if NSB exceeds 5%, correction for NSB for estimating the protein binding of drugs should not be used, as the presence of plasma may alter its extent.

Ultrafiltration can be performed in a single ultrafilter unit or in a 96-well plate high throughput ultrafiltration system: Ultrafiltration unit (Millipore).

Some advantages and disadvantages of ultrafiltration method are listed in the following.

Advantages

- Fast, simple, and efficient technique;
- Lack of sample dilution and volume shifts;
- No requirement of nonphysiological buffer;
- Compatible to high throughput with robotic systems.

Disadvantages

- NSB of drug to plastic tube or ultrafiltration membrane;
- Constriction of membrane pores during ultrafiltration;
- Leakage of bound drug through the membrane;
- Donnan ion effect;
- Volume of ultrafiltrate may not be sufficient for assay;
- Deviation of drug–protein binding equilibrium because of the change in protein concentration due to loss of plasma water; especially in case of high protein-bound drugs.

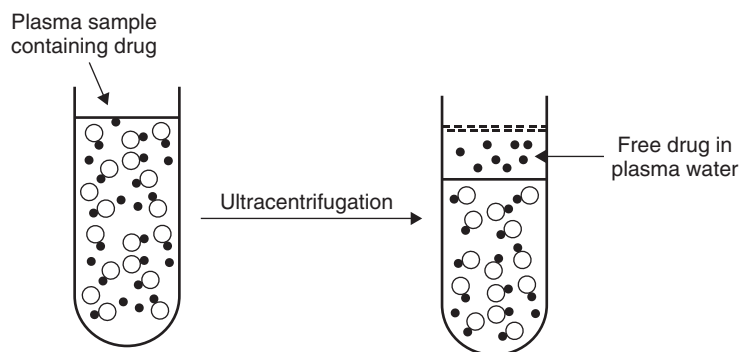


Figure 12.5 Ultracentrifugation method for estimating protein binding of drugs. The figure indicates the sedimentation of drug bound to protein (open circle) from the free drug (closed circle) under the influence of an extremely high centrifugal force ($150,000 \times g$) and a positive air pressure of up to 30 psi. Sampling of supernatant yields the free drug concentration.

12.2.3 Ultracentrifugation

Ultracentrifugation is based on the separation of free and protein-bound drug by application of a high centrifugal force without a membrane. The method is based on differential sedimentation of plasma constituents depending on their molecular mass. The experimental method is depicted in Fig. 12.5.

Briefly, drug is spiked in plasma and aliquots are transferred to centrifuge tubes for centrifugation at $150,000 \times g$ at an air pressure of 30 psi. The remaining plasma is maintained during the time of centrifugation. The centrifugation time is predetermined by stopping the centrifuge at different time intervals and measuring the drug concentration in the top layer. The time at which the concentration is constant is chosen for the centrifugation of the study samples. At the end of the centrifugation period, the plasma separates into distinct layers: the sedimented plasma protein layer, the supernatant PW layer, and a thin white lipoprotein layer at the surface. The drug concentrations are estimated in the supernatant PW layer ($C_{\text{supernatant}}$) and the stored spiked plasma (C_{initial}) by LC-MS/MS analysis. The fraction of drug unbound can be calculated by using Equation 12.6. The percentage of drug bound or unbound can be calculated by using Equations 12.3 and 12.4 described earlier.

$$f_u = \frac{C_{\text{supernatant}}}{C_{\text{initial}}} \quad (12.6)$$

Depending on the centrifuge available, different centrifugation times have been used. Also a microscale ultracentrifugation was evaluated by Nakai *et al.* [20], which utilized only 200 μL of plasma sample with a bench top ultracentrifuge ($436,000 \times g$, 140 min). They showed a very good correlation in the protein binding estimated for 10 compounds with this method and that determined by equilibrium dialysis or ultrafiltration. Ultracentrifugation is an alternative method to equilibrium dialysis and ultrafiltration and can be used for compounds having high NSB, as it eliminates problems associated with free membrane effects. Also, it is the best method for highly hydrophobic compounds for which other methods prove inadequate. Some advantages and disadvantages of ultracentrifugation method are listed in the following.

Advantages

- Fewer NSB issues;
- No dilution of sample.

Disadvantages

- Expensive instrumentation (high speed ultracentrifuge);
- Time consuming (up to 12–24 h centrifugation time);
- Low throughput as limited samples can be centrifuged simultaneously;
- No automation possible;
- Larger volume of sample required;
- Self-sedimentation of large-molecular-weight compounds;
- Interferences due to thermal back diffusion and floating lipoprotein in the supernatant.

12.2.4 High Performance Affinity Chromatography

High performance affinity chromatography (HPAC) for estimation of protein binding of drugs is based on the use of a liquid chromatographic technique that uses a protein as the stationary phase for the analysis of sample containing drug. The retention of the drug is based on its reversible interaction with the protein (affinity ligand). The earliest work using HSA columns was carried out by Domenici *et al.* [21]. Later, different strategies for the development of columns immobilized with HSA have been reported. The epoxy method used the epoxy groups of a copolymer (glycidyl methacrylate and ethylene dimethacrylate) for direct immobilization of HSA through its amine residues. In other approaches, the epoxy groups were converted to diols for later use in the carbonyldiimidazole, disuccinimidyl carbonate, and Schiff base methods [22]. Stable and selective chiral stationary phases were also prepared by covalent binding of HSA to silica particles via reactive polymers. Stationary phases obtained by immobilization of HSA on (C8) and (C18) reverse phases and on poly(1-vinylimidazole)-coated silica have also been reported [23]. In addition to HSA, other columns using immobilized proteins such as AAG and lipoproteins of different species are now available.

HPAC can be used to study protein binding of drugs using zonal elution and frontal analysis techniques. In both these methods, the protein of interest is used as an immobilized ligand and an injection or application of analyte is made onto the affinity column in the presence of only buffer or buffer plus a mobile phase modifier/competing agent. By examining the elution time or volume of the analyte after it has passed through the column, it is possible to obtain information on the extent of drug–protein binding, the equilibrium constants for these processes, the ability of the drug to compete with other compounds, the effects of temperature or solvent composition on these reactions, and the structure and location of the drug binding sites on the immobilized protein [24].

Some advantages and disadvantages of HPAC method are listed in the following.

Advantages

- Easy setup;
- Low cost;
- Low sample consumption.

Disadvantages

- Only binding to one protein at a time is possible that may lead to underestimation of protein binding of drug;
- For UV-inactive compounds, LC-MS/MS is needed.

12.2.5 Microdialysis

Microdialysis is a technique for *in vivo* sampling of drugs that has been used to estimate the concentrations of unbound drug present in the extracellular fluids in various tissues or organs or in the blood. The data obtained from microdialysis sampling is continuous unlike that obtained from blood sampling at specific time points, and thus, it is best represented on a time axis at the midpoint of the sampling interval [25]. A schematic describing the functioning of a microdialysis probe is shown in Fig. 12.6.

The basic setup for a microdialysis experiment consists of a microdialysis probe, a perfusion pump, and an analytical method with the required sensitivity to quantify small concentrations of substances in small volumes of sample. Briefly, a microdialysis probe, consisting of a capillary (typical outer diameter of 500 μm) with a dialysis membrane covering its tip, is implanted into a blood vessel, tissue, or organ. The probe is then perfused with a physiological buffer such as Ringer's solution at a constant flow rate

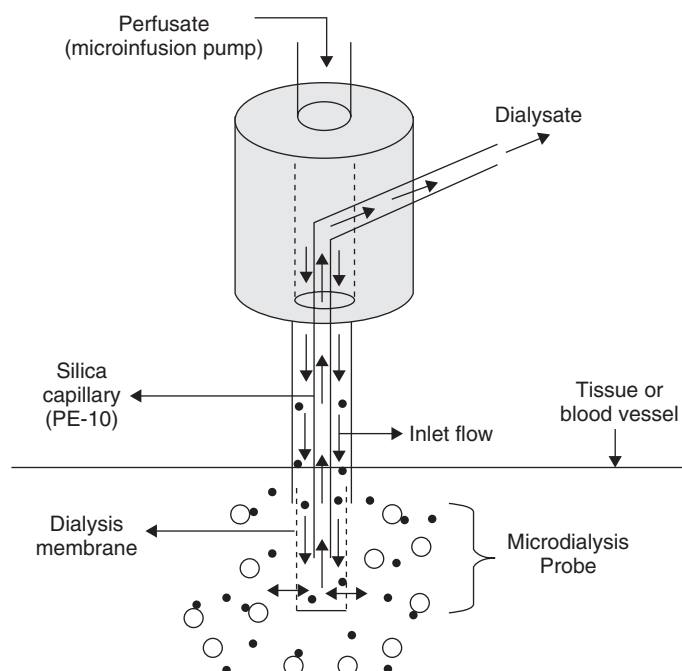


Figure 12.6 Microdialysis probe design and functioning. The figure indicates the movement of free drug from the tissue/blood vessel through the tip of the microdialysis probe that has semipermeable membrane. Using this method, after drug administration, the change in concentration of free drug can be monitored continuously over time akin to blood sampling during traditional pharmacokinetic studies.

(usually $<2\ \mu\text{L}/\text{min}$). Owing to the semipermeable membrane of microdialysis with a typical MWCO of 20 kDa, only small molecules diffuse along the concentration gradient toward the probe lumen into the perfusate. The concentration of the drug in the perfusate is analyzed by LC-MS/MS and represents the unbound drug concentration.

When using microdialysis, it is important to assess the recovery of drug. Microdialysis is not performed under equilibrium conditions because the perfusate is constantly being pumped through the probe; thus, the concentration of the drug in the perfusate is always lower than that of the extracellular fluid surrounding the dialysis probe. The ratio between these concentrations is defined as relative recovery of the analyte. The relative recovery decreases as the flow of the perfusate increases. To improve recovery of drugs, bovine serum albumin, glycerol [26], or β -cyclodextrin [27] have been added in the perfusate by some researchers.

Microdialysis allows continuous sampling from awake, freely moving animals in small numbers. Moreover, multiple site sampling can provide detailed PK information and drug transport equilibration across membranes such as blood-to-brain distributions. The microdialysis technique is devoid of any loss of biological fluid, thus enables use of the same animals as their own control in a cross-over design.

Some advantages and disadvantages of microdialysis method are listed in the following.

Advantages

- Can be used to estimate unbound drug concentrations (and kinetics) in blood or tissue in awake animals thus maintaining physiological conditions;
- Very clean dialysate requiring no sample clean-up;
- Unbound drug in the dialysate is chemically stable as the dialysate is free of any plasma or tissue components.

Disadvantages

- Low recovery of hydrophobic and highly protein-bound drugs;
- *In vitro* recovery may not translate to *in vivo* recovery due to interaction of plasma or tissue components with membrane material;
- Requirement of sensitive analytical methods that can work with low sample volumes;
- Tissue damage by microdialysis probe implantation.

12.2.6 Other Methods

Many other nonseparative methods have been used to assess protein binding of drugs. These include fluorescence measurement, surface plasmon resonance, capillary electrophoresis (CE), X-ray crystallography, absorption spectroscopy, and nuclear magnetic resonance spectroscopy.

The fluorescence measurement technique [28] uses the detection of intrinsic fluorescence of tryptophan (Trp) residues of HSA and AAG, which are the primary plasma proteins. In tryptophan fluorescence quenching (TFQ), the Trp is excited at 285 nm and the emission fluorescence is monitored as the drug is titrated into the system. The fluorescence of the intrinsic Trp in HSA is quenched by the drugs that

interact closely to the Trp. The binding constant is measured by fitting the drug concentration versus fluorescence profile. TFQ is a domain-specific binding assay that is susceptible to interference from fluorescent compounds. It cannot measure the binding constants for compounds that bind at sites distant from the Trp such that quenching is not detected. The dissociation constant K_D of analyte can then be calculated. This method can be used as a 96-well assay and is high throughput compatible.

Another high throughput method uses surface plasmon resonance biosensors [29], such as Biacore®, to study drug–plasma protein binding. In this method, one interacting partner (“ligand”) is attached to the surface of a chip and the sample containing the second interaction partner (“analyte”) is passed over the surface of the chip. Binding of molecules to the sensor surface generates changes in the resonance angle in real time, which is proportional to the bound mass. Drugs can be classified as low, medium, or high binding, and this process can be applied to large numbers and classes of compounds. When additional rigor is desired and sufficient compound is available, definitive K_D constants can be determined. The limitations of Biacore® are nonaqueous solvent effects and lack of ruggedness.

Drug–protein binding can also be studied using CE. Advantages of CE methods over chromatographic methods include its resolving power and small amounts of proteins and drugs required. The major limitation of the CE methods is their low sensitivity. In the electrophoretic techniques, the separation of the free drug, the free protein, and the drug–protein complex is based on their differences in electrophoretic mobility due to the net charge and size differences. It is simple, robust, and easy to implement; can deal with multiple equilibria; and requires less reagents than all the other methods [30].

12.3 SUMMARY

The phenomenon of plasma and tissue binding is the important determinant of the overall properties of the drug that are of importance in evaluating the PK and PD of drugs and the design of the dosage regimen. There are several methods for the estimation of plasma protein binding, and one must carefully choose the method that is commensurate with the level of information regarding plasma protein binding that is desired based on the developmental stage of the NCE or drug.

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13 Excretion Systems

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13.1	Vectorial transport of xenobiotics in tissues	2
13.2	Kinetic consideration of key factors determining the organ clearance	10
13.3	Physiologically based pharmacokinetic modeling	22
13.4	Conclusion	27
	References	27

In previous chapters, the expression and function of metabolic enzymes and drug transporters have been overviewed as important factors dominating ADME (the absorption, disposition, metabolism, and excretion) of drugs. As common features, each metabolic enzyme and transporter consists of a wide variety of isoforms, and the substrate specificity of each isoform is very broad, thereby providing an evolutionary ability to protect the body against numerous kinds of xenobiotics. Thus, a single compound can often be recognized by multiple metabolic enzymes and transporters as a substrate because it partly overlaps with different isoforms of enzymes and transporters. These molecules are appropriately located at several tissues in the body and increase efficiency in the detoxification of xenobiotics. For example, in the liver, efficient detoxification can be achieved by the sequential processing of compounds, such as in the cellular uptake from the blood circulation to hepatocytes via influx transporters, phase I and II metabolism, and biliary excretion via efflux transporters.

In this chapter, we present an overview of how combinations of enzymes and transporters work concertedly as a detoxification “system”. Moreover, when evaluating the efficiency of detoxification quantitatively, because these molecules have different roles and do not function in parallel in this system, clearance of xenobiotics in each tissue cannot be expressed simply as the sum or product of an intrinsic clearance of each isoform of metabolic enzymes and transporters. Therefore, we will also discuss theoretical considerations and experimental methods to evaluate the influence of the function of each molecule on the overall efficiency of a detoxification system from the viewpoint of pharmacokinetics.

13.1 VECTORIAL TRANSPORT OF XENOBIOTICS IN TISSUES

Most xenobiotics are transferred to the liver and kidney, extensively metabolized in the liver, and excreted into bile and urine as an unchanged form or as metabolites for detoxification. In those processes, compounds must pass through both the basal and the apical membranes of hepatocytes and kidney tubular epithelial cells to reach the bile and urine compartments: this is called *transcellular transport* [1]. Such transcellular transport of drugs can also be seen in various other tissues such as intestinal epithelial cells as a site of drug absorption and the brain capillary endothelial cells as a major component of the blood–brain barrier. In general, because gap junctions seal the intercellular space tightly and transport between cells is very limited, transcellular transport is a major contributor to drug movement across cells, although paracellular transport sometimes plays a partial role in the intestinal absorption of small hydrophilic compounds according to Renkin's equation [2]. To enhance the efficiency of detoxification, basal-to-apical directional transcellular transport ("vectorial transport") of compounds is achieved with the aid of uptake and efflux transporters. For example, in the liver and kidney, several uptake transporters are expressed on the basal membrane facing the blood circulation and efflux transporters are expressed on the apical membrane facing the bile or urine. This allows the compounds to be concentrated into the bile and urine from the systemic blood.

For lipophilic compounds, because they can penetrate the plasma membrane easily, vectorial transport can be achieved only by the expression of efflux transporters. However, hydrophilic compounds cannot access efflux transporters because of their poor membrane permeability, and thus, both uptake and efflux transporters are required for their transcellular transport. For example, the bile-to-plasma concentration ratio of pravastatin, a nonmetabolized type of HMG-CoA reductase inhibitor (statin), is calculated to be about 960 in rats [3]. In fact, pravastatin is well known to be a substrate of the hepatic uptake transporter, OATP (organic anion transporting polypeptide) 1B1 and the efflux transporter, MRP2 (multidrug resistance-associated protein 2), which contribute to its concentrative transport in the bile and subsequent efficient excretion [4]. Such vectorial transport can be utilized in other tissues such as the small intestine and blood–brain barrier to strictly regulate the flux of necessary and unnecessary compounds in the body [1].

As shown in Fig. 13.1, vectorial transport is mediated by two different types of transporters, the ABC (ATP-binding cassette) family and the SLC (solute carrier) family transporters. The ABC family transporters are driven by the hydrolysis of intracellular ATP and mediate efflux transport from the inside to the outside of cells. Conversely, the SLC family transporters can transport compounds bidirectionally, depending on the concentration gradient of the driving force. For example, hepatic OATP family transporters generally take up compounds from the blood to hepatocytes, although the endogenous driving force has not been fully clarified [5], whereas the apically localized renal MATE (multidrug and toxin extrusion) transporters are considered to be involved in the efflux of cationic drugs into the urine driven by an exchange of H^+ ions [6]. The combinations of transporters for the transcellular transport of compounds differ depending on the physicochemical properties of compounds. In the case of organic anions, because the intracellular compartment is negatively charged and it is difficult for anions to gain access to the inside of cells from the outside via facilitated diffusion, active transport machineries must be involved in their uptake. Their efflux involves

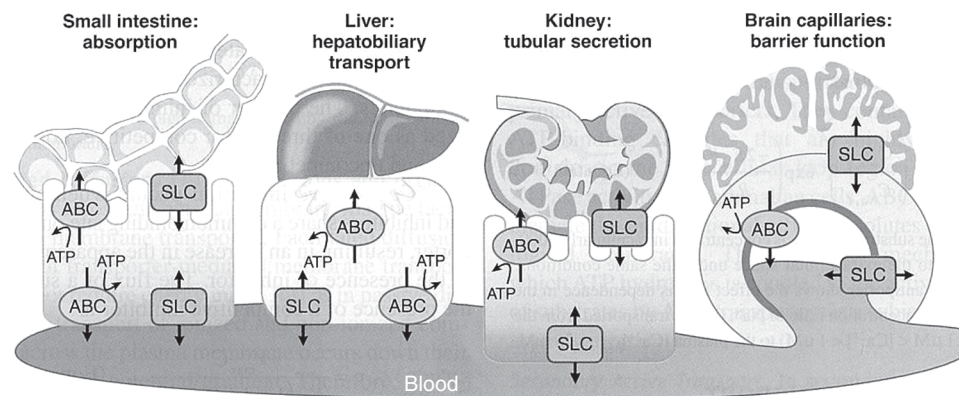


Figure 13.1 Role of SLC and ABC transporters in the transcellular transport of compounds in various tissues. Key: ABC, ATP-binding cassette transporter; SLC, solute carrier-type transporter. *Source*: This figure is cited from Ref. 1.

both facilitated diffusion-type transporters, such as OST α/β (organic solute transporter α/β), and ABC transporters, such as MDR1 (multidrug resistance 1) and MRP2. On the other hand, in the case of cationic compounds, their uptake is mediated by facilitated diffusion-type transporters such as OCTs (organic cation transporters), whereas active transport mechanisms such as MATEs are mainly involved in their efflux.

Table 13.1 shows some examples of vectorial transport of endogenous and exogenous compounds mediated by the cooperation of uptake and efflux transporters [7]. Bile acids are distributed predominantly in the gastrointestinal tract and liver. This is achieved by the efficient enterohepatic circulation, consisting of both a transcellular transport system in the liver mediated by NTCP (Na⁺-taurocholate cotransporting polypeptide) and BSEP (bile salt export pump), and in the intestine mediated by ASBT (apical Na⁺-dependent bile acid transporter) and OST α/β [8]. This type of enterohepatic circulation also benefits the pharmacological and toxicological effects of statins. For example, the transcellular transport of pravastatin in the liver is dominated by OATPs (uptake) and MRP2 (efflux), and its reuptake in the small intestine is thought to be mediated by the OATP family transporters [4]. The enterohepatic circulation contributes to the long-term accumulation of statins in the liver and to the subsequent sustained exposure to intrahepatic HMG-CoA reductase, a pharmacological target of statins. It also contributes to their low level in the blood circulation and a decreased risk of myopathy, which is one of the major side effects of statins.

TABLE 13.1 Transporter-Mediated Vectorial Transport of Compounds in the Tissues

Organ	Substrates	Basal Transporter	Direction	Apical Transporter
Liver	Organic anions	OATP1B1/OATP1B3/ OATP2B1	→	MDR1/MRP2/BCRP
Kidney/small intestine	Bile acids	NTCP	→	BSEP
	Dipeptide/peptide mimetics	(not identified)	←	PEPT1/PEPT2
Kidney	Organic anions	OAT1/OAT3	→	RST/NPT1/NPT4/ MRP4/MRP2/ OAT4/BCRP/ MDR1
	Organic cations	OCT2	→	OCTN1/OCTN2/ MATE1/MATE2/ MDR1
	Glucose	GLUT1/GLUT2	←	SGLT
	Nucleobase	ENT	←	CNT
	Carnitine	(not identified)	←	OCTN2
	Urate	OAT1/OAT3	→	NPT4
	Urate	GLUT9	←	RST
	Bile acids	OSTa/b	←	ASBT
	Folate/folate analogs	(not identified)	←	PCFT/RFC
Intestine				

Direction: →, basal-to-apical transport, ←, apical-to-basal transport.

Please note that combinations of transporters involved in the vectorial transport largely depends on the compounds.

This table contains some transporters whose *in vivo* role in the vectorial transport of compounds has not been fully clarified.

Source: This table is cited from Ref. 7 with some modifications.

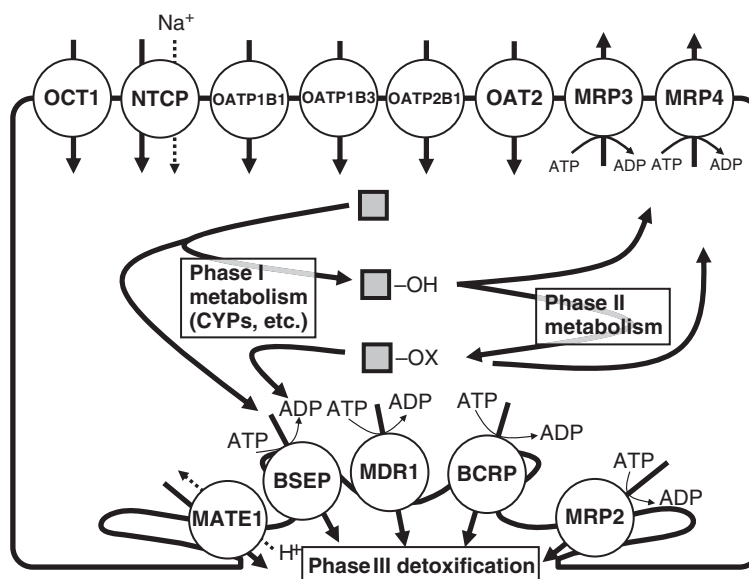


Figure 13.2 Major systems of drug detoxification in hepatocytes.

13.1.1 Vectorial Transport Machineries in the Liver

Figure 13.2 illustrates the major transporters expressed in hepatocytes. As mentioned above, transcellular transport of bile acids is mainly dominated by NTCP and BSEP. Clinically important anionic drugs including statins and angiotensin II receptor antagonists (sartans) are taken up into hepatocytes mainly via OATP1B1 and OATP1B3. Because the substrate specificities of OATP1B1 and OATP1B3 are very broad and are similar to each other, one compound is often recognized by both transporters. Previous reports suggested that the relative contribution of OATP1B1 and OATP1B3 to the hepatic uptake differs between substrates, although these anionic compounds accumulate preferentially in the liver [9]. For example, the hepatic uptake of valsartan is mediated by both OATP1B1 and OATP1B3 to the same extent [10], but that of telmisartan is exclusively dominated by OATP1B3 [11] even in the same category of drugs (sartans). These differences in the contribution of each transporter affect the extent of change in the overall intrinsic clearance of these compounds when the function of a single transporter is altered by genetic polymorphisms or drug interactions. OAT2 is also expressed on the basal membrane in the hepatocytes and accepts various structurally unrelated compounds such as bumetanide, zidovudine, 5-fluorouracil (5-FU), paclitaxel, and prostaglandin E₂ [12]. However, the relative contribution of OAT2 to the overall *in vivo* hepatic uptake of substrates is not fully understood.

OCT1 mediates hydrophilic organic cations with a relatively low molecular weight such as metformin and TEA (tetraethylammonium) [13]. Various kinds of ABC transporters expressed on the apical membrane are involved in biliary excretion. Generally, MDR1 preferentially transports various kinds of bulky hydrophobic and cationic compounds, MRP2 mainly transports anionic compounds including several conjugated metabolites such as glucuronide and glutathione conjugates, and BCRP can also

transport a wide variety of compounds, especially sulfated conjugates [14]. However, this rule is not always applicable to some compounds. MDR1 can sometimes transport anionic and zwitterionic drugs such as E₂17 β G (estradiol-17 β -glucuronide) and fexofenadine, and BCRP can transport cationic (cimetidine), zwitterionic (mitoxantrone), and neutral (topotecan) compounds as well as anionic compounds. Recent reports have shown that MATE1 is also expressed on the apical membrane of hepatocytes and is involved in the biliary excretion of cationic drugs such as metformin, since coadministration of pyrimethamine, a potent and specific inhibitor for MATEs, or gene knockout of MATE1 expression increased the hepatic distribution of metformin [15,16].

The vectorial transport of compounds mediated by uptake and efflux transporters in the liver was mimicked experimentally by the use of double-transfected cells. Originally, Cui *et al.* and Sasaki *et al.* constructed OATP1B3/MRP2 and OATP1B1/MRP2 double-transfected MDCKII cells, respectively, and they demonstrated the basal-to-apical vectorial transport of several organic anions in the monolayer of these cell systems on a transwell membrane insert [17,18]. Sasaki *et al.* have demonstrated that *in vivo* biliary clearance was predicted quantitatively from the vectorial transcellular transport of several compounds in rat Oatp1b2/Mrp2 double-transfected cells [19]. Subsequently, a set of double-transfected cells expressing OATP1B1/OATP1B3 (uptake) and MDR1/MRP2/BCRP (efflux) were constructed for rapid screening of the machineries involved in the vectorial transport of compounds [20]. For example, basal-to-apical vectorial transport of E₂17 β G and pravastatin was largely enhanced in OATP1B1/MRP2 double-transfected cells compared with OATP1B1/MDR1 and OATP1B1/BCRP cells (Fig. 13.3), suggesting that the combination of OATP1B1-mediated uptake and MRP2-mediated efflux controls the efficient biliary excretion of these compounds [20]. This is supported by the drastic decrease in the biliary excretion of these compounds in the Mrp2-hereditary-deficient rat strain, EHBR (Eisai hyperbilirubinemic rat) [21,22]. Kopplow *et al.* have also constructed a quadruple-transfected cell line expressing OATP1B1, OATP1B3, OATP2B1, and MRP2 to better understand the substrate selectivity of MRP2 [23], because most of the substrates for that OATP family transporters are also substrates for MRP2, although they do not share homology with each other. For organic cations, double-transfected MDCKII cells expressing OCT1 and MDR1 were constructed and vectorial transport of berberine, a cationic plant alkaloid, was confirmed in this system [24]. Sato *et al.* have constructed OCT1/MATE1 double-transfected MDCK cells, and several cations such as TEA, metformin, and cimetidine could be transported vectorially from the basal to the apical sides of cell monolayers [25,26].

13.1.2 Vectorial Transport Machineries in the Kidney

Figure 13.4 illustrates the major transporters expressed in the proximal tubular epithelial kidney cells. OAT1 and 3 are thought to be involved mainly in the uptake of organic anions [12,27]. OAT1 accepts several kinds of small and hydrophilic organic anions such as PAH (*p*-aminohippurate) and nucleotide analog reverse-transcriptase inhibitors (adefovir, tenofovir, and cidofovir). On the other hand, OAT3 accepts a wide variety of bulky hydrophobic/amphipathic organic anions and its substrate specificity generally overlaps that of the OATP family transporters, although the protein sequences of these transporters are not similar. OAT3 can also transport cationic drugs such as histamine

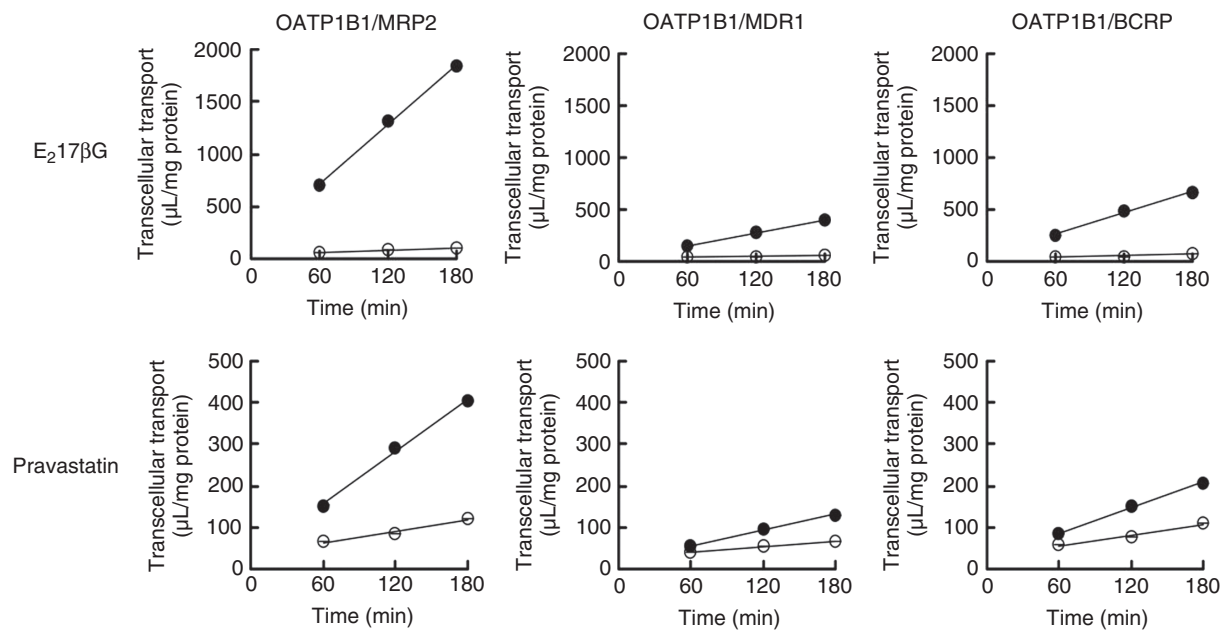


Figure 13.3 Transcellular transport of estradiol-17β-glucuronide (E₂ 17βG) and pravastatin in double-transfected cells expressing OATP1B1 and MDR1, MRP2, or BCRP. *Source:* This figure is cited from Ref. 20.

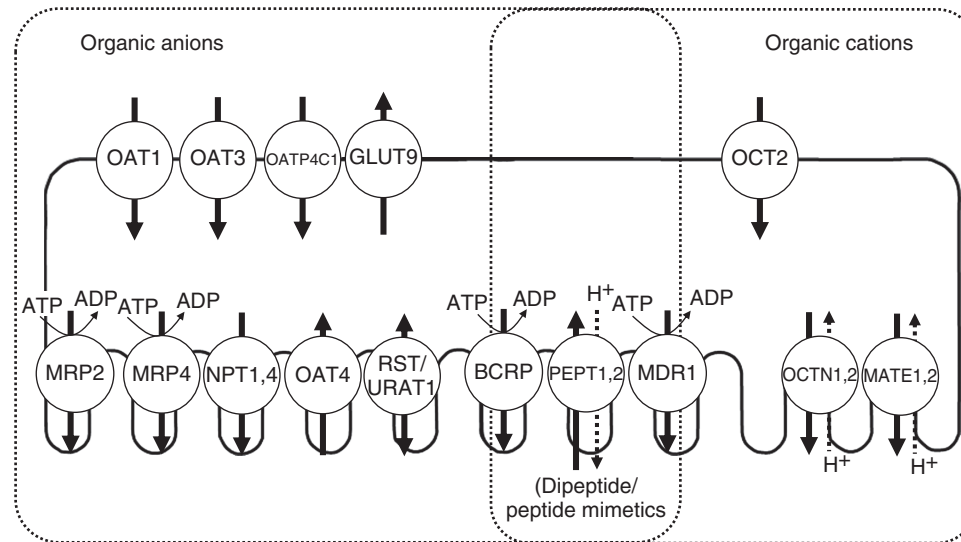


Figure 13.4 Major drug transporters for the transcellular transport of compounds in the kidney.

H₂ blockers (cimetidine, famotidine, ranitidine). As for the luminal efflux of intracellular anionic compounds to urine, considering the inside negative electrical potential, facilitated diffusion as well as active transport can be involved in the efflux of anionic compounds in the kidney. On the apical membrane, both ABC transporters such as MDR1, MRP2, MRP4, and BCRP and facilitative diffusion-type transporters such as RST [renal-specific transporter (in rodents); URAT1 (urate transporter) (in humans)], NPT (Na⁺-dependent phosphate transport protein) 1, and NPT4 are known to be expressed [27]. Previous *in vitro* experiments using BBMVs (brush border membrane vesicles) suggested that at least two different transport systems, electroneutral exchange of organic anions and voltage-dependent facilitated diffusion, are involved in the luminal efflux of anions. However, there are few lines of evidence showing the importance of these transporters in the renal efflux of anions *in vivo*. The use of transporter knockout mice revealed that Bcrp is responsible for the luminal efflux of methotrexate, E3040 sulfate, and edaravone sulfate [28,29] and Mrp4 is involved in the luminal efflux of diuretics (hydrochlorothiazide and furosemide), antiviral drugs (adefovir and tenofovir), cephalosporins (ceftizoxime and cefazolin), and edaravone glucuronide [27]. In *in vitro* experiments using OAT3/RST double-transfected cells, basal to apical vectorial transport of benzylpenicillin, urate, and 2,4-dichlorophenoxyacetate was observed clearly, suggesting the role of these transporters in the urinary excretion of these compounds, although the significance of RST (URAT1) *in vivo* has not been demonstrated directly [30]. Urate is both secreted and reabsorbed in the kidney, and 90% of urate excreted via glomerular filtration is ultimately returned to the body. Recent clinical genetic studies and *in vitro* experiments suggested that the renal secretion of urate is thought to be dominated by uptake via OAT1/OAT3 and efflux via NPT4, while its reabsorption is mediated by uptake via URAT1 and efflux via GLUT (glucose transporter) 9 [31]. This hypothesis is partly supported by previous *in vivo* phenotypic observations of transporter knockout mice, although the metabolic fate of urate is totally different in rodents and humans. *In vitro* studies showed that NPT1 can transport some other organic anions such as PAH, benzylpenicillin, faropenem, and E₂17βG and that NPT4 can transport PAH, estrone-3-sulfate, E₂17βG, bumetanide, and ochratoxin A [32,33]. Thus, these NPT transporters might also contribute to the secretory vectorial transport of anionic compounds in cooperation with OATs.

Conversely, the renal uptake of hydrophilic and small organic cations is mainly mediated by OCT2 [34]. OCTN1, OCTN2, and MATEs, as well as MDR1 are expressed on the apical side of renal tubular epithelial cells. However, the transporter responsible for the renal efflux of cationic compounds *in vivo* is still under investigation, as it presumably acts in cooperation with OCT2. Ohashi *et al.* demonstrated that the renal secretion clearance of TEA was reduced to half in OCTN2-deficient *jvs* (juvenile visceral steatosis) mice, suggesting the importance of OCTN2 in the efflux of TEA in the kidney [35]. MATE family transporters (MATE1, MATE2, and MATE2-K) accept various kinds of organic cations such as metformin, fexofenadine, antiviral drugs (acyclovir, ganciclovir), anticancer drugs (oxaliplatin, cisplatin, and topotecan), and antibiotics (cephalexin, cephadrine, and levofloxacin) [36,37]. Recent studies *in vivo* indicated that the kidney-to-plasma concentration ratios of TEA and metformin were increased by the coadministration of pyrimethamine, a potent selective MATE inhibitor, in mice. Moreover, the renal clearance of metformin, cephalexin, and cisplatin was decreased significantly in MATE1 gene knockout mice [15,16]. Kusuvara *et al.* also demonstrated that the renal clearance of metformin was decreased by 35%

when it was coadministered with pyrimethamine in healthy human subjects [38]. Thus, MATEs as well as OCT2 are considered to be responsible for the efficient secretory transport of metformin in the human kidney. *In vitro* experiments using OCT2/MATE1 double-transfected MDCK cells showed that the basal-to-apical transcellular transport of several cations such as TEA, metformin, cimetidine, procainamide, and quinidine was significantly higher than that in the opposite direction [25,26]. Thus, renal secretion of these compounds is thought to be dominated by uptake via OCT2 and efflux via MATE1.

As for reabsorption process in the kidney, although it is well known that several nutrients (e.g., amino acids, sugars, and vitamins) extensively undergo renal reabsorption from the lumen side, the combinations of uptake and efflux transporters dominating apical-to-basal vectorial transport of xenobiotics have not yet been fully clarified. Knockout of PEPT2, a peptide transporter, increased the renal clearance of cephadoxil and carnosine, suggesting that PEPT2 is involved in their renal reabsorption [39]. However, the transporters responsible for the basal efflux have not been determined. Oatp1a1 is also expressed on the apical renal membrane in male rats but almost not in female rats [40,41]. Previous studies demonstrated that the renal clearance of E₂17 β G, dibromosulfophthalein, and zenarestat was significantly higher in male than in female rats, suggesting a role for Oatp1a1 in the uptake of compounds at the apical side of the kidney [40,41]. In contrast, there is no information about the corresponding OATP transporter gene expressed on the apical membrane of human kidneys or about the gender differences in the renal clearance of compounds in humans.

13.2 KINETIC CONSIDERATION OF KEY FACTORS DETERMINING THE ORGAN CLEARANCE

Excretion systems in the liver and kidney consist of multiple uptake and efflux transporters and metabolic enzymes. Because these molecules do not work sequentially one by one or in parallel for the detoxification of xenobiotics, organ clearance, an indicator for the performance of the detoxification system, cannot be simply defined by the sum or product of the intrinsic activity (transport/metabolism) of each molecule. In each process such as uptake, efflux, and metabolism, multiple molecules are involved simultaneously, thanks to their broad and overlapping substrate specificities. Thus, we must consider the relative contribution of each molecule to the overall activity of each step. Organ intrinsic clearance is dominated by uptake, efflux, backflux to the blood circulation, and metabolism and can be described theoretically by using the intrinsic activity of each step. Then, organ clearance can be estimated from multiple factors such as the overall intrinsic clearance, blood flow, and protein unbound fraction in a model-dependent manner. In this section, we describe how the intrinsic activity of each molecule affects the performance of detoxification system in the *in vivo* situation quantitatively, from the viewpoint of pharmacokinetic theory. Such a consideration is essential to estimate the extent of change in the total clearance and exposure of drugs in the body when the expression and function of each molecule are altered by several factors such as genetic polymorphisms, interacting drugs, and pathophysiological conditions.

13.2.1 Contribution of Each Isoform to the Overall Metabolism/Transport

In each step of detoxification such as uptake, efflux, backflux, and metabolism, multiple transporters or metabolic enzymes often work in parallel. For metabolism, metabolic intrinsic clearance (CL_{met}) can be simply described as the sum of the intrinsic clearance of each metabolic enzyme ($CL_{\text{enzyme},i}$) as

$$CL_{\text{met}} = CL_{\text{enzyme},1} + CL_{\text{enzyme},2} + \cdots + CL_{\text{enzyme},i} \quad (13.1)$$

On the other hand, for the membrane transport of lipophilic compounds, we must also think about the contribution of passive permeation as well as active transport. Thus, intrinsic membrane transport clearance ($PS_{\text{transport}}$) is the sum of the intrinsic clearance of passive transport (CL_{passive}) and active transport mediated by each transporter ($CL_{\text{TP},i}$).

$$PS_{\text{transport}} = CL_{\text{passive}} + CL_{\text{TP},1} + CL_{\text{TP},2} + \cdots + CL_{\text{TP},i} \quad (13.2)$$

The impact of the relative contribution of each molecule on the overall intrinsic clearance can be tested virtually in a model case. Assuming that drug A is eliminated only by the hepatic metabolism and that its pharmacokinetics follow the well-stirred model, when drug A is orally administered, the area under the plasma concentration–time curve (AUC) can be described by the following equation.

$$\begin{aligned} AUC_{\text{oral}} &= \frac{F_a F_g \cdot F_h \cdot \text{Dose}}{CL_h} = \frac{F_a F_g \frac{Q_h}{Q_h + f_B CL_{\text{int},h}} \text{Dose}}{\frac{Q_h f_B CL_{\text{int},h}}{Q_h + f_B CL_{\text{int},h}}} = \frac{F_a F_g \cdot \text{Dose}}{f_B CL_{\text{int},h}} \\ &= \frac{F_a F_g \cdot \text{Dose}}{f_B (CL_{\text{enzyme},1} + CL_{\text{enzyme},2} + \cdots + CL_{\text{enzyme},i})} \end{aligned} \quad (13.3)$$

Here, $F_a F_g$ is the intestinal availability, F_h the hepatic availability, CL_h the hepatic clearance, Q_h the hepatic blood flow rate, f_B the protein unbound fraction in blood, and $CL_{\text{int},h}$ the hepatic intrinsic clearance.

Under this situation, if the function of enzyme 1 is decreased to $1/R$, the fold increase in the AUC_{oral} value largely depends on the relative contribution of intrinsic clearance mediated by enzyme 1 to the overall metabolic clearance ($f_{m,1}$).

$$\begin{aligned} AUC_{\text{oral ratio}} &= \frac{f_B (CL_{\text{enzyme},1} + CL_{\text{enzyme},2} + \cdots + CL_{\text{enzyme},i})}{f_B \left(\frac{CL_{\text{enzyme},1}}{R} + CL_{\text{enzyme},2} + \cdots + CL_{\text{enzyme},i} \right)} \\ &= \frac{f_{m,1} \cdot CL_{\text{int},h} + (1 - f_{m,1}) CL_{\text{int},h}}{f_{m,1} \cdot \frac{CL_{\text{int},h}}{R} + (1 - f_{m,1}) CL_{\text{int},h}} = \frac{1}{f_{m,1} \cdot \frac{1}{R} + (1 - f_{m,1})} \end{aligned} \quad (13.4)$$

Following Equation 13.4, the fold increase in the AUC_{oral} is plotted against the inhibition potency of enzyme 1 (R) in Fig. 13.5. If enzyme 1 is inhibited potently, the AUC_{oral} ratio shows large difference even when the relative contribution ($f_{m,1}$) changes slightly. Thus, the quantitative estimation of the contribution of each molecule to the overall intrinsic clearance is important if we correctly predict the AUC change caused by the functional change of a single molecule.

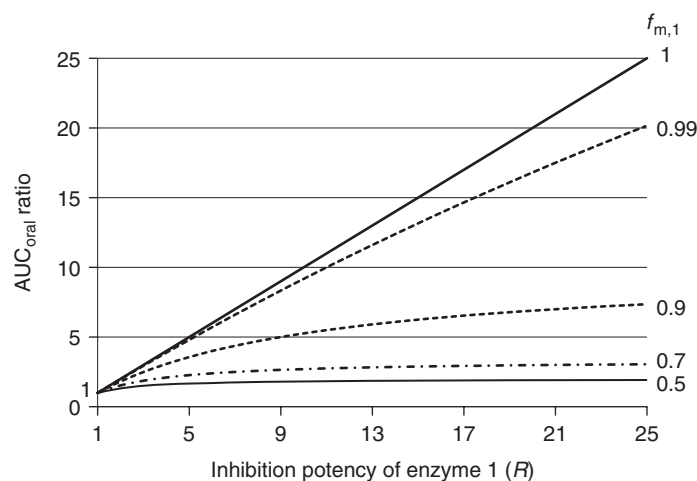


Figure 13.5 Importance of the contribution of a specific enzyme to the overall metabolism in the increase of the AUC_{oral} value. Theoretical calculation of the AUC_{oral} ratio ($AUC_{oral} (+inhibitor)/AUC_{oral} (control)$) is performed when enzyme 1 contributes $f_{m,1}$ values of 1.0, 0.99, 0.9, 0.7, and 0.5 of the total metabolic activity and the metabolic activity of enzyme 1 is specifically decreased to $1/R$.

Niemi *et al.* [42] showed that the AUC of repaglinide, a substrate of CYP2C8 and CYP3A4, was increased by 8.1-fold in coadministration with gemfibrozil, a potent inhibitor of CYP2C8, and increased by 1.4-fold in coadministration with itraconazole, a potent inhibitor of CYP3A4. On the other hand, in coadministration with both gemfibrozil and itraconazole, the AUC of repaglinide increased greatly by 19.4-fold [42]. This seems to be a synergistic inhibitory effect of gemfibrozil and itraconazole. However, this kind of drug interaction can be explained only by conventional pharmacokinetic theory, considering the contribution of each molecule. We assume that drug B is eliminated by three different metabolic pathways mediated by enzymes X, Y, and Z, and that the relative contributions (f_m) of enzymes X, Y, and Z to the overall metabolism of drug B are 0.5, 0.4, and 0.1, respectively. In this situation, when drug B is coadministered with a potent inhibitor of enzyme X, according to Equation 13.4, the AUC of drug B is expected to be increased by 2-fold, while its AUC is increased by 1.7-fold when coadministered with a potent inhibitor of enzyme Y. On the other hand, when both enzymes X and Y are completely inhibited by coadministration of both inhibitors, the AUC of drug B is increased drastically up to 10-fold because the metabolic intrinsic clearance becomes one-tenth of that in a control situation. Therefore, it must be noted that a combination of inhibitor drugs for different metabolic pathways can sometimes increase the exposure of the victim drug significantly, although the complete inhibition of a single metabolic pathway does not affect the drug concentration so much.

A similar story is also discussed in a recent paper by Kusuvara and Sugiyama [43] concerning the apparent synergistic effect of the P-gp and BCRP on the brain distribution of substrates. There is some evidence demonstrating that the double knockout of P-gp and Bcrp expression in mice produced remarkable increases in the concentration of some drugs in the brain, whereas single knockouts of P-gp or Bcrp showed only

A hypothetical common substrate drug with following kinetic parameters:
 $PS_{2,u} = 0.5$, $PS_{BCRP} = 2$, $PS_{P-gp} = 5$

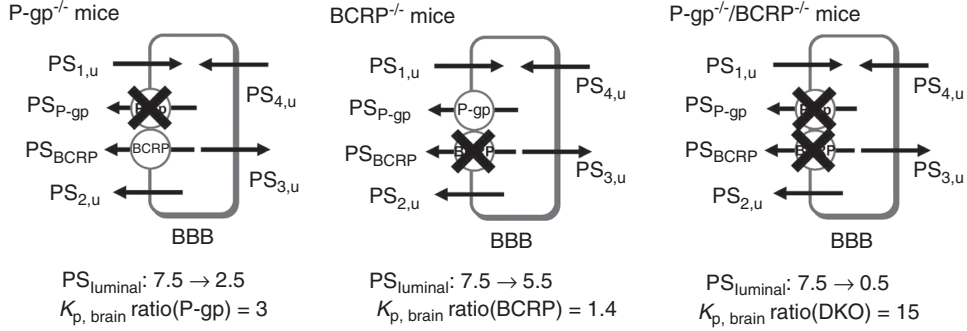


Figure 13.6 Theoretical consideration of the apparent synergistic effects of P-gp and BCRP knockout on the brain distribution of substrates. The simple model for the transcellular transport of compounds at the blood–brain barrier was constructed and $K_{p,brain}$ was estimated when each parameter was set at the designated values shown in this figure. Then, $K_{p,brain}$ values were compared between wild type, P-gp knockout, BCRP knockout, and P-gp/BCRP double-knockout mice. *Source:* This figure is cited from Ref. 43.

limited effects [44–46]. This apparent synergism can be explained by simple pharmacokinetic theory without considering any interplay between the P-gp and Bcrp [43,47]. Following the schematic diagram of the membrane transport in brain endothelial cells, brain-to-blood concentration ratio ($K_{p,brain}$) can be described by the parameters shown in Fig. 13.6 according to the following equation.

$$K_{p,brain} = \frac{PS_{blood \rightarrow brain}}{PS_{brain \rightarrow blood}} = \frac{PS_{1,u} \frac{PS_{3,u}}{PS_{P-gp} + PS_{BCRP} + PS_{2,u} + PS_{3,u}}}{PS_{4,u} \frac{PS_{2,u}}{PS_{P-gp} + PS_{BCRP} + PS_{2,u} + PS_{3,u}}} = \frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} (PS_{P-gp} + PS_{BCRP} + PS_{2,u})} \quad (13.5)$$

Thus, the ratio of the $K_{p,brain}$ value in knockout mice to that in normal mice is

$$K_{p,brain} \text{ ratio(P-gp)} = \frac{\frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} (PS_{BCRP} + PS_{2,u})}}{\frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} (PS_{P-gp} + PS_{BCRP} + PS_{2,u})}} = 1 + \frac{PS_{P-gp}}{PS_{BCRP} + PS_{2,u}} \quad (13.6)$$

$$K_{p,brain} \text{ ratio(BCRP)} = \frac{\frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} (PS_{P-gp} + PS_{2,u})}}{\frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} (PS_{P-gp} + PS_{BCRP} + PS_{2,u})}} = 1 + \frac{PS_{BCRP}}{PS_{P-gp} + PS_{2,u}} \quad (13.7)$$

$$K_{p,brain} \text{ ratio(P-gp/BCRP)} = \frac{\frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} \cdot PS_{2,u}}}{\frac{PS_{1,u} \cdot PS_{3,u}}{PS_{4,u} (PS_{P-gp} + PS_{BCRP} + PS_{2,u})}} = 1 + \frac{PS_{P-gp} + PS_{BCRP}}{PS_{2,u}} \quad (13.8)$$

Therefore, when the absolute value of each parameter is set according to Fig. 13.6, the $K_{p, \text{brain}}$ ratios in P-gp and Bcrp single-knockout mice increase slightly by 3- and 1.4-fold, respectively, whereas that in P-gp/Bcrp double-knockout mice largely increases by 15-fold, which reproduces the apparent synergism of the *in vivo* observation [43].

Predictive methods to estimate the relative contribution of each molecule to the overall intrinsic clearance have been established and validated in the fields of both metabolic enzymes and transporters. For metabolism, Crespi and Penman [48] originally published the concept of the RAF (relative activity factor) method. In this, the metabolic clearance of a test compound and a selective substrate for each enzyme is measured in liver microsomes, and the recombinant enzyme expression system and the ratio of the clearance of each selective substrate in liver microsomes to that in the expression system are estimated for each enzyme ($R_{\text{enzyme}, i}$). Then, the contribution of enzyme 1 to the overall metabolic enzyme ($f_{m, \text{enzyme}, 1}$) can be described as follows.

$$f_{m, \text{enzyme}, 1} = \frac{R_{\text{enzyme}, 1} \cdot \text{CL}_{\text{test}, \text{enzyme}, 1}}{\sum_i R_{\text{enzyme}, i} \cdot \text{CL}_{\text{test}, \text{enzyme}, i}} \left(= \frac{R_{\text{enzyme}, 1} \cdot \text{CL}_{\text{test}, \text{enzyme}, 1}}{\text{CL}_{\text{int}, h}} \right) \quad (13.9)$$

Here, $\text{CL}_{\text{test}, \text{enzyme}, i}$ is the metabolic clearance of a test compound in a recombinant expression system for enzyme i .

Now, an *in vitro* selective substrate for each CYP isoform has been established almost completely. Another method is to use a selective inhibitor for each isoform. In the field of CYP enzymes, chemical selective inhibitor and functional neutralizing antibody for each major CYP isoform have also been established. The metabolic clearance of a test compound is measured in the absence and presence of a selective inhibitor and then the contribution of enzyme 1 to the overall metabolic enzyme ($f_{m, \text{enzyme}, 1}$) can be described as follows.

$$f_{m, \text{enzyme}, 1} = 1 - \frac{\text{CL}_{\text{test}}(+\text{inhibitor})}{\text{CL}_{\text{test}}(-\text{inhibitor})} \quad (13.10)$$

Similar methods can be used in the field of drug transporters. Kouzuki *et al.* [49,50] applied the RAF method to estimate the relative contributions of Ntcp and Oatp1a1 to the overall uptake of compounds in rat hepatocytes. E₂17βG and taurocholate were used as selective substrates for Oatp1a1 and Ntcp, respectively, although E₂17βG is no longer used as a reference compound for Oatp1a1. Hirano *et al.* [51] have established a method for estimating the relative contribution of OATP1B1 and OATP1B3 to the overall uptake of E₂17βG and pitavastatin in human hepatocytes by using estrone-3-sulfate (E-sul) and cholecystokinin octapeptide (CCK-8) as selective substrates for OATP1B1 and OATP1B3, respectively. They calculated the ratio of the uptake clearance of the reference compounds in human cryopreserved hepatocytes to that in the transporter expression systems ($R_{\text{act}, \text{transporter}}$). The uptake clearance of test compounds mediated by a specific transporter in hepatocytes can be calculated by multiplying the $R_{\text{act}, \text{transporter}}$ value by the uptake clearance of test compounds in the expression system ($\text{CL}_{\text{test}, \text{transporter}}$). If we can assume that test compounds are taken up into hepatocytes exclusively by OATP1B1 and OATP1B3 then the uptake clearance of test compounds in hepatocytes ($\text{CL}_{\text{test}, \text{hep}}$) should be described by the following equation.

$$CL_{\text{test, hep}} = R_{\text{act, OATP1B1}} \cdot CL_{\text{test, OATP1B1}} + R_{\text{act, OATP1B3}} \cdot CL_{\text{test, OATP1B3}} \quad (13.11)$$

Using this method, they demonstrated that both $E_217\beta G$ and pitavastatin were taken up into hepatocytes mainly via OATP1B1. The use of a selective inhibitor for each isoform is also available to estimate the contribution of OATP1B1 and OATP1B3 to the uptake of compounds in human hepatocytes [11]. Thus, E-sul can be used as a specific inhibitor for OATP1B1. The uptake of $E_217\beta G$ and pitavastatin in human hepatocytes was almost inhibited in the presence of an excess of E-sul, which further supports the major contribution of OATP1B1. Conversely, telmisartan uptake was observed only in OATP1B3-expressing cells but not in OATP1B1-expressing cells and the uptake of telmisartan in human hepatocytes was not inhibited by E-sul, suggesting that telmisartan is transported predominantly by OATP1B3 [11]. Figure 13.7 summarizes our findings of the contributions of OATP1B1 and OATP1B3 to the hepatic uptake of substrates. Even in the same category of drugs, the relative importance of OATP1B1 and OATP1B3 is different between valsartan (OATP1B1 = OATP1B3) and telmisartan (OATP1B3 $\gg$ OATP1B1). This kind of information is useful when considering the effect of genetic polymorphisms and drug interactions on the changes in pharmacokinetics of substrate drugs. Previous clinical pharmacogenetic studies indicated that the pharmacokinetics of some statins are clearly affected by SNPs (single nucleotide polymorphism) in OATP1B1 but not telmisartan [52,53].

A similar type of investigation was also performed by using rat and human kidney slices to estimate the contribution of OAT1 and OAT3 to the renal uptake of compounds. Hasegawa *et al.* [54,55] have established a RAF method for renal uptake by using PAH and pravastatin as selective substrates for rat Oat1 and Oat3, respectively, and demonstrated that renal uptake clearance predicted using the RAF method was similar to that observed in rat kidney slices in most cases. Using this method, the contributions of Oat1 and Oat3 to the renal uptake of several uremic toxins have been determined, and these results were further validated by observing the inhibitory effects of PAH (an OAT1-selective inhibitor), PCG, and pravastatin (an OAT3-selective inhibitor) on their uptake in rat kidney slices [56].

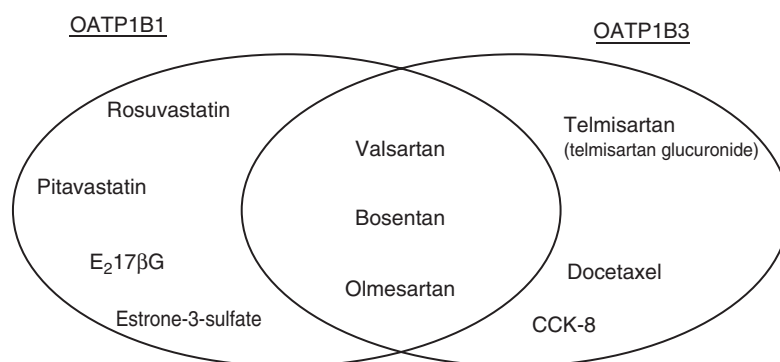


Figure 13.7 Relative contribution of OATP1B1 and OATP1B3 to the overall hepatic uptake of compounds estimated in human cryopreserved hepatocytes. This figure indicates which compounds are taken up in the hepatocytes, mainly mediated by OATP1B1 (left), OATP1B3 (right), or both OATP1B1 and OATP1B3 (center).

Sandwich-cultured hepatocytes have been utilized for the characterization of efflux from hepatocytes to the bile. LeCluyse *et al.* [57,58] have demonstrated that hepatocytes cultured between layers of collagen gel or Matrigel could efficiently repolarize and form what they termed a *bile pocket* between adjacent cells, which mimics the bile canalicular compartment in the liver and depletion of Ca^{2+} from the incubation medium rapidly disrupted the closed bile pocket by opening tight junctions. Thus, the amounts of compounds excreted into the bile pocket can be estimated by their differential cumulative uptake in sandwich-cultured hepatocytes preincubated with Ca^{2+} -containing and Ca^{2+} -free buffers. Recent studies succeeded in the use of siRNA for inhibiting each efflux transporter in determining its contribution to the overall efflux of compounds into the bile pocket. Tian *et al.* [59] demonstrated that knockdown of Mrp2 by siRNA in rat sandwich-cultured hepatocytes resulted in a 50% decrease in the protein expression and a 45% decrease in the biliary excretion index (BEI) of carboxydichlorofluorescein (CDF), which is reported to be excreted predominantly into the bile under the control of Mrp2 in rats. Bcrp knockdown by adenoviral vector-mediated RNAi (RNA interference) also succeeded, and the protein expression of Bcrp was drastically decreased to 5% of control at day 6 after starting the culture of rat hepatocytes, whereas the BEI value of nitrofurantoin, a selective substrate of Bcrp, was also decreased to 11% of control [60]. The BEI value of $^{99\text{m}}\text{Tc}$ -sestamibi, which is used for hepatobiliary scintigraphy, in the bile pocket was not decreased by treatment with a Bcrp siRNA-expressing adenovirus, but its efflux was totally abolished in the presence of GF120918, a typical inhibitor for both Mdr1 and Bcrp, suggesting that $^{99\text{m}}\text{Tc}$ -sestamibi can be used as a selective substrate for Mdr1 in rat hepatocytes [61].

Renal BBMVs are useful tools for characterizing efflux transport systems in the kidney [62]. However, because the responsible transporters have not been well characterized and the driving force of efflux transporters is required to be known for the transport assay using BBMVs, it is difficult to estimate the contribution of each transporter to the overall efflux of compounds in the kidney. For reabsorption, we can understand the involvement of reabsorption in the renal clearance qualitatively by observing the reduction in reabsorption by diuresis induced by mannitol [63]. However, there is no appropriate *in vitro* experimental system for the quantitative analysis of transporters involving reabsorption at present.

13.2.2 Rate-limiting Step of the Overall Intrinsic Clearance in a Tissue

In traditional pharmacokinetic theory, we normally regard the metabolic intrinsic clearance to be the same as the hepatic intrinsic clearance. Previous reports suggested that the *in vivo* hepatic clearance of various CYP substrate drugs could be well predicted from the *in vitro* metabolic intrinsic clearance estimated by using liver microsomes. However, this model cannot be applied to the transporter substrates, which are extensively excreted into bile as unchanged forms with minimal metabolism, such as some statins (pravastatin, pitavastatin, rosuvastatin) because the hepatic clearance is relatively large, while metabolic clearance is too low to be estimated. Lam *et al.* [64] suggested that the *in vivo* intrinsic clearance of digoxin could be better predicted from the *in vitro* intrinsic clearance with primary rat hepatocytes rather than that observed with liver microsomes. Frassetto *et al.* have also demonstrated in humans that the results of the erythromycin breath test, a typical clinical test to evaluate the CYP3A4 activity *in vivo*, were altered by coadministration of rifampicin, a typical

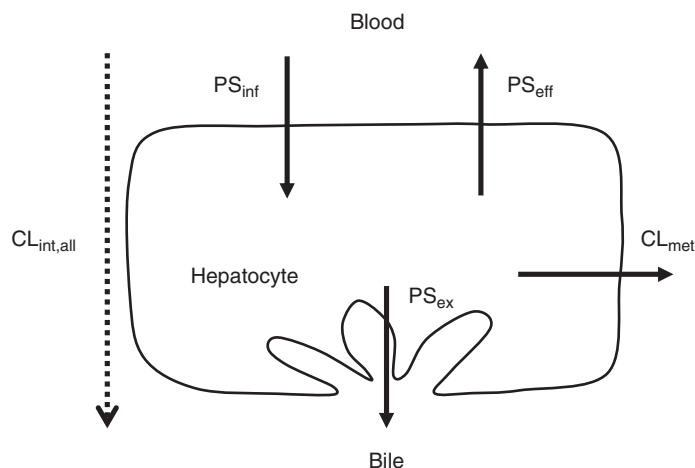


Figure 13.8 Scheme of hepatic transport and metabolism in hepatocytes for the theoretical consideration of overall hepatic intrinsic clearance ($CL_{int,all}$).

OATP inhibitor, and lansoprazole, an MDR1 inhibitor. These findings indicate that the uptake and efflux transport activity of erythromycin affects its overall hepatic intrinsic clearance [65].

For the transporter substrates, detoxification in the liver consists of (1) hepatic uptake from the blood to hepatocytes (PS_{inf}), (2) backflux from hepatocytes to the blood (PS_{eff}), (3) biliary excretion from hepatocytes to bile (PS_{ex}), and (4) metabolism in hepatocytes (CL_{met}) (Fig. 13.8) [66]. In this case, the apparent overall hepatic intrinsic clearance ($CL_{int,all}$) can be described by the following equation.

$$CL_{int,all} = PS_{inf} \frac{PS_{ex} + CL_{met}}{PS_{eff} + PS_{ex} + CL_{met}} \quad (13.12)$$

If PS_{eff} is much lower than the sum of PS_{ex} and CL_{met} , according to Equation 13.12, the overall hepatic intrinsic clearance approximates the intrinsic uptake clearance (PS_{inf}).

$$CL_{int,all} \sim PS_{inf} \quad (13.13)$$

This equation means that the overall hepatic intrinsic clearance is solely dominated by the uptake activity of drugs, even if they are eliminated from the body mainly by metabolism. This situation is often called “uptake-limited clearance.” On the other hand, if PS_{eff} is much greater than the sum of PS_{ex} and CL_{met} , the overall intrinsic clearance can be described as

$$CL_{int,all} = PS_{inf} \frac{PS_{ex} + CL_{met}}{PS_{eff}} \quad (13.14)$$

In this case, the intrinsic clearances of all the processes affect the overall hepatic intrinsic clearance. If drugs can rapidly penetrate the plasma membrane by passive diffusion, we can assume symmetrical membrane transport ($PS_{inf} = PS_{eff}$) and PS_{eff} is

much larger than $PS_{ex} + CL_{met}$. Under these assumptions, the overall hepatic intrinsic clearance described in Equation 13.12 can approximate the metabolic intrinsic clearance (CL_{met}).

$$CL_{int,all} \sim CL_{met} \quad (13.15)$$

It must be noted that the prediction of hepatic intrinsic clearance from *in vitro* metabolic clearance with liver microsomes or cytosol fractions can succeed only in limited situations, even if drugs are metabolized extensively in the liver.

We sometimes define “ β value” according to the following equation.

$$\beta = \frac{PS_{ex} + CL_{met}}{PS_{eff} + PS_{ex} + CL_{met}} \quad (13.16)$$

On the basis of Equation 13.12, $CL_{int,all}$ can be described as

$$CL_{int,all} = PS_{inf} \times \beta \quad (13.17)$$

Thus, β is a kind of indicator representing whether the rate-limiting step of hepatic intrinsic clearance is likely to be an uptake process or not. The β value affects the change in the overall intrinsic clearance when each parameter is altered. Figure 13.9 shows the impact of the β value on the change in the overall intrinsic clearance when PS_{inf} or $CL_{met} + CL_{ex}$ are decreased. When the β value is close to 1, $CL_{int,all}$ approximates PS_{inf} as in Equation 13.13, and thus, a small decrease in the $CL_{met} + CL_{ex}$ value has little effect on the reduction of $CL_{int,all}$. On the other hand, when the β value is small, $CL_{int,all}$ becomes as described in Equation 13.14 and it becomes proportional to the $CL_{met} + CL_{ex}$ value. Therefore, the decrease in the $CL_{met} + CL_{ex}$ value affects the reduction of $CL_{int,all}$ directly. Conversely, a decrease in PS_{inf} value always affects $CL_{int,all}$ to the same degree regardless of the β value because $CL_{int,all}$ is proportional to PS_{inf} as shown in Equation 13.12. These simulation results tell us that the inhibition of influx transporters decreases $CL_{int,all}$ irrespective of the β value, while the inhibition of metabolic enzymes and/or biliary efflux transporters do not always have a direct

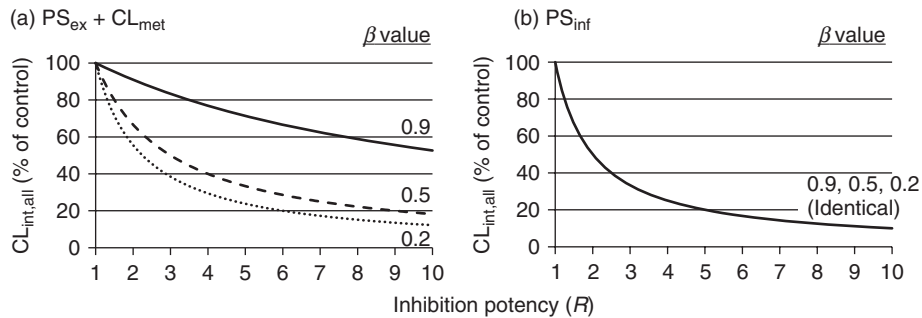


Figure 13.9 Impact of the β value on changes in the overall intrinsic clearance. Theoretical calculation of the change in the overall intrinsic clearance ($CL_{int,all}$) of a drug, whose β value is set at a designated value (0.9, 0.5, or 0.2) and is performed when $PS_{ex} + CL_{met}$ (a) or PS_{inf} (b) is specifically decreased to $1/R$.

effect on $CL_{int,all}$, if drugs are substrates of uptake transporters. We must also note that the intrinsic clearance of drugs with a β value of almost 1 can be decreased if CL_{met} or CL_{ex} are suppressed drastically and the β value itself becomes less than 1 after a change in CL_{met} or CL_{ex} (Fig. 13.9).

Watanabe *et al.* [67] have demonstrated that rat *in vivo* biliary and renal clearances with regard to the plasma concentration of several transporter substrate drugs were well predicted from *in vitro* uptake experiments using isolated rat hepatocytes and kidney slices, respectively, except for a few compounds whose renal clearance is very small, possibly because of the involvement of reabsorption. This concept was also successfully applied to human's case [68]. It has been also shown that the *in vivo* $CL_{int,all}$ values of four kinds of statins (pravastatin, pitavastatin, atorvastatin, and fluvastatin), two of which are eliminated from the body by extensive CYP-mediated metabolism, were similar to the uptake intrinsic clearance estimated using the multiple indicator dilution (MID) method in rats or in an uptake assay using cryopreserved human hepatocytes in humans. By contrast, the metabolic intrinsic clearance obtained from an *in vitro* metabolism assay with rat or human liver microsomes considerably underestimated the *in vivo* $CL_{int,all}$ value (Fig. 13.10) [69]. This evidence suggested that the rate-limiting step of hepatic intrinsic clearance of these statins is in the hepatic uptake process and that their β values are expected to be close to 1. Soars *et al.* also indicated that *in vitro* clearance estimated from the disappearance of compounds from the incubation media in an *in vitro* assay using hepatocytes ("media loss" assay) could explain their *in vivo*

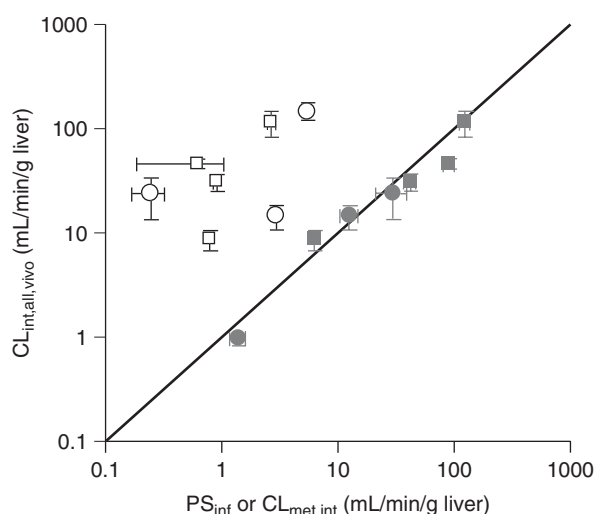


Figure 13.10 Rate-determining process of the hepatic intrinsic clearance of statins. The *in vivo* hepatic intrinsic clearances ($CL_{int,all,vivo}$) of four kinds of statins (pitavastatin, fluvastatin, atorvastatin, and pravastatin) in rats and humans are plotted against the uptake clearance or metabolic clearance. Closed circles, predicted from uptake clearance in human cryopreserved hepatocytes; open circles, predicted from metabolic clearance in human liver microsomes; closed squares, predicted from uptake clearance in rats determined using an MID (multiple indicator dilution) method; open squares, predicted from metabolic clearance in rat liver microsomes. The straight line indicates a 1:1 correlation. *Source:* This figure is cited from Ref. 69.

TABLE 13.2 Drugs That are Substrates of Both Metabolic Enzymes and Uptake Transporters

Substrates	Transporter	Metabolic Enzymes
Atorvastatin	OATPs	CYP3A4
Cerivastatin	OATPs	CYP2C8, 3A4
Fluvastatin	OATPs	CYP2C9
Repaglinide	OATPs	CYP2C8, 3A4
Nateglinide	OATPs	CYP2C9, 3A4
Glibenclamide	OATPs	CYP2C9, 3A4
Bosentan	OATPs	CYP2C9, 3A4
Torsemide	OATPs	CYP2C9
Gimatecan	OATPs	CYP3A4
Lopinavir	OATPs	CYP3A4
Docetaxel	OATP1B3	CYP3A4
Telmisartan	OATP1B3	UGTs

hepatic clearance better compared with the use of a conventional assay measuring the metabolite formation clearance with regard to the intracellular unbound concentration of compounds in the hepatocytes. This suggests the importance of uptake transporters in the determination of the overall intrinsic clearance in hepatocytes for some compounds [70,71].

The number of drugs that are substrates of both uptake transporters and are metabolic enzymes and eliminated from the body by extensive metabolism has been increasing gradually, as shown in Table 13.2. The pharmacokinetics of most of these OATP substrate drugs are altered by the coadministration of potent OATP inhibitors such as cyclosporine A and rifampicin and by genetic polymorphisms of OATP1B1 (e.g., T521C) [72,73]. For predicting changes in the pharmacokinetics of these drugs, their β values are essential, but they have not yet been estimated correctly.

Maeda *et al.* [74] have performed a clinical study in humans *in vivo* to demonstrate the rate-limiting step of the hepatic clearance of atorvastatin, a substrate of both OATP transporters and CYP3A4. In this study, a cocktail of a microdose of atorvastatin and of probe drugs for CYP3A4 (midazolam) and hepatic OATPs (pravastatin) was administered simultaneously to healthy volunteers. Changes in pharmacokinetics induced by the coadministration of a selective inhibitor for CYP3A4 [200 mg itraconazole (i.v.)] or for OATPs [600 mg rifampicin (single p.o.)] were observed. As expected, the plasma AUC of midazolam was increased only when it was coadministered with itraconazole, but not with rifampicin, whereas that of pravastatin was increased only by coadministration with rifampicin, but not with itraconazole. These results suggest the selective inhibition of CYP3A4 and OATPs by itraconazole and rifampicin, respectively. Under such conditions, the plasma AUC of atorvastatin was drastically increased by coadministration of rifampicin, but not itraconazole, although the plasma concentration of 2-hydroxy atorvastatin, one of the major metabolites of atorvastatin produced by CYP3A4, was decreased. This suggested that the hepatic clearance of atorvastatin is mainly dominated by hepatic uptake processes mediated by OATP transporters in humans.

13.2.3 Relationship Between Intrinsic Clearance and Organ Clearance

Organ clearance, defined as the rate of elimination of drugs divided by their blood concentration, is dominated not only by tissue intrinsic clearance but also by the blood flow rate in tissues and by the blood unbound fraction. Thus, a change in the tissue intrinsic clearance discussed above does not always lead to a change in organ clearance and subsequent alterations in the blood concentrations of drugs. To consider the effect of change in the intrinsic clearance on the pharmacokinetics of drugs, one of the important factors is the relative contribution of organ clearance to the total clearance (CL_{tot}). In general, drugs are eliminated mainly from the liver and kidney. Therefore, the total clearance can be described as in Equation 13.18.

$$CL_{tot} = CL_h + CL_r + CL_{others} \quad (13.18)$$

Here, CL_h , CL_r , and CL_{others} , respectively, represent the hepatic clearance, renal clearance, and clearance from other organs (if applicable). It is understood that the pharmacokinetics of a drug mainly eliminated from the kidney are changed little even if the hepatic clearance of the drug is largely decreased.

There are several models that relate the intrinsic organ clearance to organ clearance, such as the well-stirred model, the parallel tube model, and the dispersion model. The well-stirred model is used most frequently because of its simple mathematical handling. In this model, there is rapid and complete mixing, hence the term *well stirred*, of drugs coming from the blood circulation and blood in the tissue, and the blood concentration of drugs at the exit of tissue is assumed to be equal to that inside the tissue. In this condition, hepatic clearance (CL_h) can be expressed as in Equation 13.19.

$$CL_h = \frac{Q_h \cdot f_B \cdot CL_{int,h}}{Q_h + f_B \cdot CL_{int,h}} \quad (13.19)$$

Here, Q_h , f_B , and $CL_{int,h}$ represent the hepatic blood flow rate, blood unbound fraction of a drug, and the hepatic intrinsic clearance of a drug, respectively. For the kidney, we must consider the contribution of glomerular filtration, secretion, and reabsorption, and the renal clearance is described as

$$CL_r = \left(f_B \cdot GFR + \frac{Q_r \cdot f_B \cdot CL_{int,r}}{Q_r + f_B \cdot CL_{int,r}} \right) (1 - FR) \quad (13.20)$$

Here, GFR , Q_r , $CL_{int,r}$, and FR represent the glomerular filtration rate, renal blood flow rate, intrinsic renal secretion clearance, and fraction of drug reabsorbed from the urine, respectively.

Let us consider the relationship between intrinsic organ clearance and organ clearance by selecting two extreme cases. When Q_h is much smaller than $f_B CL_{int,h}$, Equation 13.19 is approximated by Equation 13.21.

$$CL_h \sim Q_h \quad (13.21)$$

Thus, hepatic clearance is only dominated by the hepatic blood flow rate, and a change in the hepatic intrinsic clearance does not alter the hepatic clearance

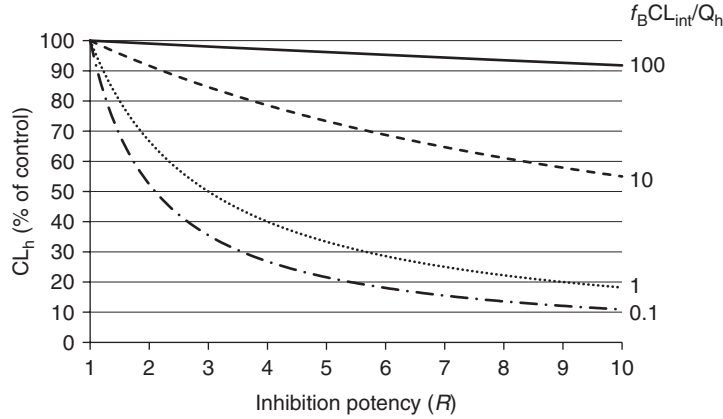


Figure 13.11 The impact of the ratio of $f_B \text{CL}_{\text{int},h}$ to Q_h values on changes in the hepatic clearance (CL_h). This shows a theoretical calculation of changes in the CL_h of a drug, whose $f_B \text{CL}_{\text{int},h}/Q_h$ value is set at a designated value (100, 10, 1, or 0.1) when $\text{CL}_{\text{int},h}$ is decreased to $1/R$.

(“blood-flow-limited clearance”). In contrast, when Q_h is much larger than $f_B \text{CL}_{\text{int},h}$, Equation 13.19 is approximated by Equation 13.22.

$$\text{CL}_h \sim f_B \text{CL}_{\text{int},h} \quad (13.22)$$

In this situation, hepatic clearance is proportional to the hepatic intrinsic clearance. Thus, the change in the hepatic intrinsic clearance should affect hepatic clearance directly (“intrinsic clearance-limited clearance”). Figure 13.11 shows the impact of the ratio of $f_B \text{CL}_{\text{int},h}$ to Q_h on the change in the CL_h value when $\text{CL}_{\text{int},h}$ is decreased. If $f_B \text{CL}_{\text{int},h}$ is larger than Q_h , CL_h approximates Q_h (Eq. 13.21) and it is not changed so much when $\text{CL}_{\text{int},h}$ is decreased. On the other hand, if $f_B \text{CL}_{\text{int},h}$ is larger than Q_h , CL_h approximates $f_B \text{CL}_{\text{int},h}$ (Eq. 13.22) and it is affected directly by a decrease in $\text{CL}_{\text{int},h}$. When drugs are administered orally, if they are eliminated only from the liver, the blood AUC of drugs (AUC_{oral}) can be described by the following equation.

$$\text{AUC}_{\text{oral}} = \frac{F_a F_g \cdot F_h \cdot \text{Dose}}{\text{CL}_h} = \frac{F_a F_g \frac{Q_h}{Q_h + f_B \text{CL}_{\text{int},h}} \text{Dose}}{\frac{Q_h \cdot f_B \text{CL}_{\text{int},h}}{Q_h + f_B \text{CL}_{\text{int},h}}} = \frac{F_a F_g \cdot \text{Dose}}{f_B \text{CL}_{\text{int},h}} \quad (13.23)$$

Here, $F_a F_g$ and F_h represent intestinal availability and hepatic availability, respectively. From Equation 13.23, it should be noted that after the oral administration of drugs, their blood AUC is solely dominated by the hepatic intrinsic clearance, but not by the hepatic blood flow rate, regardless of the rate-limiting step of hepatic clearance.

13.3 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING

As discussed above, organ clearance is regulated by multiple factors such as functions of various uptake/efflux transporters and metabolic enzymes. Because the overall

clearance in each organ cannot be described simply as the sum or product of the activity of each molecule, it is necessary to consider the detoxification machineries in each organ and in the body as a *system*. PBPK (physiologically based pharmacokinetic) modeling is one powerful tool that enables us to reconstruct detoxification systems in the body, based on the quantitative functions of multiple molecules and physiological parameters as a mathematical model. Rowland *et al.* [75] first described the clearance concept and a PBPK model for the liver in 1973. Then, as molecules dominating pharmacokinetics have been rapidly cloned and characterized, whole-body PBPK models for several drugs based on the accumulated knowledge of molecules have been constructed and analyzed by many pharmaceutical studies. In a PBPK model, the organs and circulating blood are expressed as compartments and movements of drugs from and to the compartment such as influx, efflux, and metabolism are represented as arrows, and these compartments are connected with the blood flow. Then, several physiological parameters such as organ volume and blood flow rate and drug-dependent parameters such as kinetic parameters for each enzyme and transporter are determined, and differential equations for individual compartments are formulated. Normally, in humans, we can only collect the blood and urine samples to quantify the drug concentration, and the number of sample collections should be limited. Moreover, it is almost impossible to measure the drug concentration in human tissues, which makes it difficult to predict the pharmacological/toxicological effects of drugs. In such situations, once we can construct an appropriate PBPK model, the time-dependent drug concentration in the plasma and all the tissues incorporated into the model can be simulated easily *in silico*. The US FDA clearly recommends the use of dynamic models for the accurate prediction of drug–drug interactions in the process of drug development in its recently published draft guidance about drug–drug interactions (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf>). Recently, several FDA-based researchers have published papers showing the importance of PBPK modeling for the drug development. For example, Zhao *et al.* [76] described a number of examples for the use of PBPK models in efficient decision making during a regulatory review at the FDA, and Huang and Malcolm [77] discussed the use of simulation with PBPK modeling to address regulatory questions in clinical pharmacology reviews.

Figure 13.12 shows examples of organ models [78]. If membrane transport of drugs is mainly determined by passive diffusion, and rapid equilibrium of drugs between the blood and organ compartments can be assumed, a single compartment is enough to represent the tissue concentration of drugs as shown in Fig. 13.12(a). Assuming that drugs are well mixed inside a compartment, the blood concentration of drugs inside the tissue is thought to be equal to that at the exit of the compartment. Thus, the differential equations for tissue and the blood compartment can be described as

$$\text{Blood : } V_B \frac{dC_B}{dt} = -Q_T \cdot C_B + Q_T \cdot \frac{C_T}{K_p} \quad (13.24)$$

$$\text{Tissue : } V_T \frac{dC_T}{dt} = Q_T \cdot C_B - Q_T \frac{C_T}{K_p} - f_B \text{CL}_{\text{int}} \frac{C_T}{K_p} \quad (13.25)$$

Here, V_T is the tissue volume, C_T the drug concentration in the tissue, C_B the drug concentration in the blood, Q_T the blood flow rate in the tissue, K_p the tissue-to-plasma

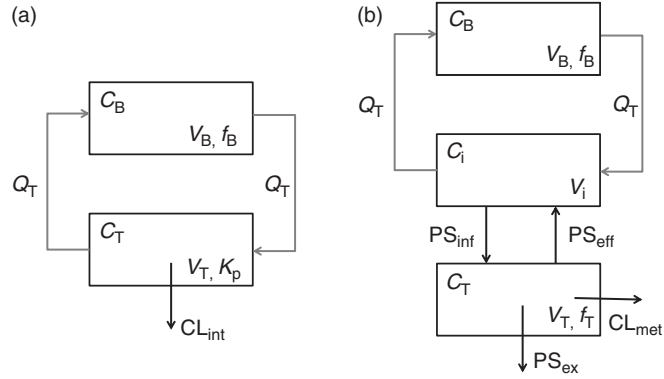


Figure 13.12 Examples of simple models expressing the detoxification ability of organs. (a) Model for drugs whose membrane transport is mainly determined by passive diffusion and where rapid equilibrium between blood and organ can be assumed. (b) Model for drugs that are taken up into organs by active transporters.

concentration ratio, f_B the protein unbound fraction in the blood, and CL_{int} the tissue intrinsic clearance.

On the other hand, if active transport is involved in the membrane permeation of drugs, the inlet and tissue compartments should be separated to represent the membrane transport clearance as shown in Fig. 13.12(b). In this case, the differential equations for blood, inlet, and tissue concentrations are described as in Equations 13.26–13.28.

$$\text{Blood : } V_B \frac{dC_B}{dt} = -Q_T \cdot C_B + Q_T \cdot C_i \quad (13.26)$$

$$\text{Inlet : } V_i \frac{dC_i}{dt} = Q_T \cdot C_B - Q_T \cdot C_i - f_B PS_{inf} \cdot C_i + f_T PS_{eff} \cdot C_T \quad (13.27)$$

$$\text{Tissue : } V_T \frac{dC_T}{dt} = f_B PS_{inf} \cdot C_i - (PS_{eff} + PS_{ex} + CL_{met}) f_T C_T \quad (13.28)$$

Here, V_i is the inlet volume, C_i the drug concentration in the inlet compartment, and f_T the protein unbound fraction in the tissue.

By integrating these equations, the blood and tissue AUCs of drugs are represented as follows [79].

$$AUC_B = \frac{\text{Dose}}{f_B \cdot PS_{inf} \frac{PS_{ex} + CL_{met}}{PS_{eff} + PS_{ex} + CL_{met}}} = \left(\frac{\text{Dose}}{f_B \cdot CL_{int,all}} \right) \quad (13.29)$$

$$AUC_T = \frac{\text{Dose}}{f_T (PS_{ex} + CL_{met})} \quad (13.30)$$

These equations demonstrate important messages about the different changes in blood and tissue concentrations when each intrinsic clearance is altered. As mentioned

in Section 13.2.2, if $PS_{ex} + CL_{met}$ is much larger than PS_{eff} , Equation 13.29 approximates $Dose/f_B PS_{inf}$. Under this condition, when the hepatic uptake activity (PS_{inf}) is decreased, the blood AUC is increased, while the tissue AUC is not changed. Conversely, when the intrinsic clearance of biliary excretion (PS_{ex}) and/or metabolism (CL_{met}) are decreased, the blood AUC is unchanged, whereas the tissue AUC is increased inversely with $(PS_{ex} + CL_{met})$.

Watanabe *et al.* [79] have constructed a PBPK model of pravastatin, which is a substrate of OATPs and MRP2 in the liver (Fig. 13.13(a)). In this model, the liver compartment was divided into five compartments to mimic the dispersion model because the hepatic clearance of pravastatin is very close to the hepatic blood flow rate. The same intrinsic clearance of hepatic influx, backflux, biliary excretion, and metabolic clearance were set up in each segment. Then, all the kinetic parameters were set up only from the results of *in vitro* experiments using a simple scaling-up method. The intrinsic clearances of influx and passive diffusion of pravastatin were determined from uptake studies using isolated hepatocytes, intrinsic clearance of biliary excretion was determined from ATP-dependent transport using canalicular membrane vesicles (CMVs), and metabolic intrinsic clearance was determined from a metabolism assay using the liver S9 fraction. They confirmed the time profiles of the plasma concentration and cumulative biliary excreted amount of pravastatin at several doses in rats and the plasma concentrations after intravenous and oral administration of pravastatin in humans were well reproduced by simulation in this PBPK model. One of the advantages of a PBPK model is in performing sensitivity analysis. This is a good method to identify critical processes dominating the phenotype (e.g., plasma and tissue concentrations) by monitoring any alteration of the target phenotype when specific process is changed *in silico*. Watanabe *et al.* performed the same type of simulation to understand the effects of changes in each intrinsic clearance on the plasma and hepatic concentrations of pravastatin. As shown in Fig. 13.13(b), changes in the influx intrinsic clearance largely altered the plasma concentration but not the hepatic concentration. However, changes in the biliary excretion intrinsic clearance greatly altered the hepatic concentration but not the plasma concentration, which is very similar to the results obtained from the simple model discussed above (Fig. 13.12(b)). A PBPK model also enables us to simulate the severity of drug–drug interactions accurately by connecting the PBPK models of substrate drugs and inhibitors to their target enzymes/transporters. For example, Kanamitsu *et al.* [80] predicted a lethal drug–drug interaction between 5-FU and sorivudine by connecting the PBPK models of 5-FU, sorivudine, and its metabolite, (E)-5-(2-bromovinyl) uracil (BVU). This drug–drug interaction is caused by the mechanism-based inactivation of dihydropyrimidine dehydrogenase (DPD), which is a major metabolizing enzyme of 5-FU, by BVU. As a result, they succeeded in reproducing a great decrease in the hepatic content of active DPD and a subsequent dramatic increase in the plasma concentration of 5-FU by coadministration of sorivudine in an *in silico* simulation. As a whole, by utilizing a PBPK model, we can simulate the plasma and tissue concentrations of drugs in any virtual situation easily by changing the parameters in the model without any additional clinical studies. This greatly helps pharmaceutical researchers to optimize the pharmacokinetic properties of drugs because it is very difficult to estimate the target outcome intuitively from the complicated whole-body drug detoxification system.

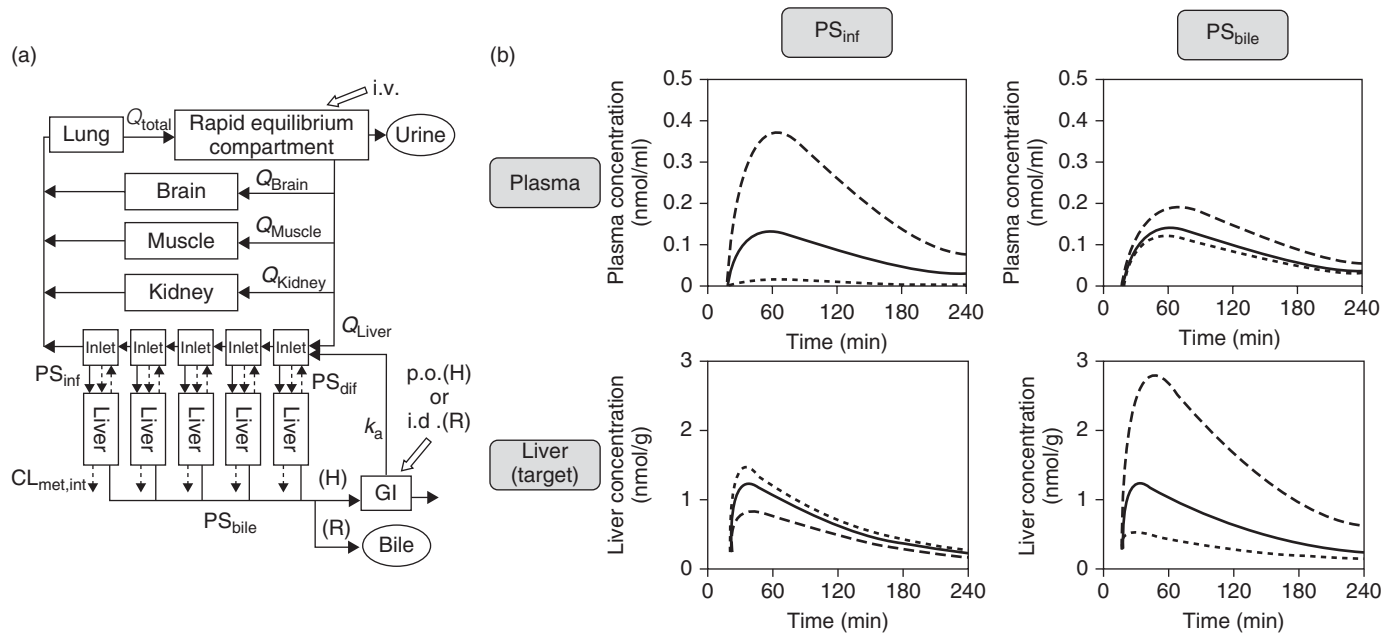


Figure 13.13 Simulation of plasma and liver concentration of pravastatin by using a PBPK (physiologically based pharmacokinetic) model. (a) Structure of PBPK model for pravastatin. (b) Simulated time profiles of plasma and liver concentrations of pravastatin when PS_{inf} or PS_{bile} values are increased threefold (broken line) or decreased by one-third (dotted line) compared with the control value (solid line). *Source:* This figure is cited from Ref. 79.

13.4 CONCLUSION

In this chapter, we have overviewed the combinations of molecules responsible for the detoxification of drugs in the liver and kidney and have discussed the quantitative importance of each intrinsic process in the overall performance of detoxification systems from a pharmacokinetic viewpoint. We have also discussed the significant role of the PBPK model for the better understanding of complex systems and quantitatively predicting target outcomes in the process of drug development. Major molecules involved in the detoxification of drugs have already been identified and characterized by *in vitro* assay systems. Thus, we can construct a PBPK model simply by integrating the kinetic parameters of all the molecular-based processes without any empirical parameters. However, the general procedures to set up parameters in any PBPK model from *in vitro* data must be investigated further and validated. Because molecular mechanisms and their networks of pharmacodynamic (PD) processes are very complicated and still not fully understood for most drugs, molecular-based PD models have not been used often, although the concept of mechanistic PK/PD modeling has been established. To construct the correct PD model, it is necessary to have a wide variety of real data sets in different conditions for a thorough validation of the entire complex model because so many parameters dominate the model. Systems biological approaches such as proteomics and metabolomics might help overcome this issue. During the drug discovery and development process, such models representing the molecular systems of detoxification and pharmacological actions of drugs greatly save time and human resources by skipping unnecessary clinical studies. They enable us to predict the pharmacokinetics of drugs in special situations such as with genetic polymorphisms of molecules, drug–drug interactions, and pathophysiological conditions without using any clinical studies. Therefore, general procedures of the construction, data acquisition, and validation of models need to be established.

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14 Pharmacokinetics and Toxicokinetics in Drug Discovery and Development

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14.1	Introduction	1
14.2	Pharmacokinetic (PK) assessment in discovery and development	2
14.3	Toxicity assessment in drug discovery and development	3
14.4	Parameters that define PK/TK profile	4
14.5	PK/TK modeling in predicting clinical dose	8
14.6	Toxicogenomics and biomarkers	12
14.7	Species differences in drug disposition	13
14.8	MIST (metabolites in safety testing)	14
14.9	Conclusions	16
	References	17

14.1 INTRODUCTION

Pharmacokinetics (PK) is the science that describes the time course of drug concentration in the body, resulting from administration of a certain drug dose. In comparison, pharmacodynamics (PD) is the science that describes the relationship of the time course of drug concentration and the drug effects in the body [1]. Similarly, toxicokinetics (TK) reveals the toxicity effects as a function of drug exposure. Therefore, PK and TK can be considered biomarkers of drug exposure as well as markers of efficacy and safety. Key determinants of the PK/TK of a drug include absorption, distribution, metabolism, and elimination (ADME) [2]. The current practice of drug discovery and development requires PK and TK analyses from the earliest stages of the program. This assessment is helpful in (i) selection of dose for preclinical toxicity testing and dosage route, (ii) understanding drug exposure, and (iii) identifying dose for first-in-human (FIH) studies and the follow-up clinical trials. Accurate quantitation of drugs in biological media such as blood (usually plasma or serum) and tissues has become more or less a routine with much improved sensitivity via the bioanalytical tools. However,

significant challenges to bioanalysis of potential new chemical entities (NCEs) still lurk in the background, with analytical chemists innovating to address issues.

The purpose of this chapter is to introduce to the fundamentals of PK and TK and their applications in drug discovery and development. It also presents the fundamentals of computational analysis of the data derived from the estimated concentrations in the biological matrices such as plasma. Finally, it discusses the implications of species differences, genomics, and exposure of the metabolites in determining the safe dose in the FIH clinical trials and further identifying clinical dosage regimen.

14.2 PHARMACOKINETIC (PK) ASSESSMENT IN DISCOVERY AND DEVELOPMENT

It is well established that poor drug PK is one of the leading causes of compounds failure in preclinical and clinical drug development [3]. Compounds with poor PK profile tend to have low oral systemic plasma exposure and high interindividual variability, which limits their therapeutic utility [4]. Therefore, a better understanding of the PK profile early on enables the discovery of compounds with druglike properties [5]. In drug discovery settings, the main outcomes of PK/TK assessments are to

- select compounds with the maximum potential of reaching the target
- determine the appropriate route of administration to deliver the drug (typically oral)
- understand how the drug blood levels relate to efficacy or toxicity in order to choose efficacious and safe doses
- facilitate appropriate dose sections for rodent and/or nonrodent species in toxicology testing and drug safety evaluation
- decide on the frequency and duration of dosing in order to maintain adequate drug concentration at target for disease modification
- accurately predict the PK in humans' profile before clinical studies.

A PK/TK study involves dosing animals or humans with NCE and collecting blood samples at predefined time points. After sample preparation and quantification, a concentration–time profile is generated (Fig. 14.1). In drug discovery, preliminary PK studies are usually conducted in rodents to evaluate the extent of drug exposure *in vivo*. This is commonly followed by studies in nonrodents, such as dogs or monkeys, to better characterize the PK profile of the compound and to support safety risk assessment studies. PK scaling, also known as *allometry*, is a discipline that is used to predict human PK profile using preclinical data and is widely used in predicting the drug's half-life, dose, and extent of absorption in humans. This approach is based on empirical observations that various physiological parameters are a function of body size. The allometric methods assume that the same metabolic and disposition processes in the species evaluated are correlated with those observed in humans. However, it has become increasingly realized that, for example, the cytochrome P450 enzymes in the rat are not the same as those in humans and thus may exhibit altered disposition of the compound or even produce different metabolite patterns (Section 14.7). Similarly, uptake and efflux transporters in the animal species may differ in substrate specificity or rate,

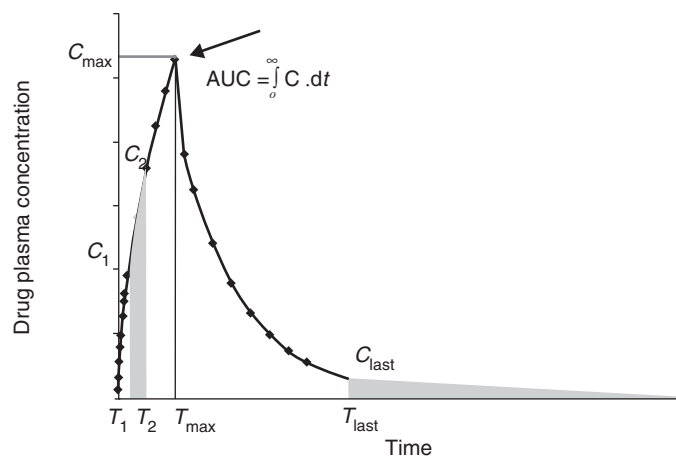


Figure 14.1 Estimation of the area under the plasma concentration–time curve (AUC).

as compared to humans, and thus may confound predictions of human PK. Accurate prediction of human PK profile is imperative to minimize drug failure in development because of the PK liability. More detailed description of methods in predicting human PK is beyond the scope of this chapter but can be found in many excellent reviews [6]. An in-depth discussion of various PK concepts and their application can be found in various references [7].

14.3 TOXICITY ASSESSMENT IN DRUG DISCOVERY AND DEVELOPMENT

Several toxicology studies are conducted during early drug discovery and all the way to the late stages of drug development before a New Drug Application (NDA) filing is made. In spite of comprehensive toxicity assessment in early- and late-stage discovery, attrition of NCEs in clinical studies is not uncommon owing to disconnect in predictions of risk in humans on the basis of preclinical data obtained from cell culture and animal models. Nevertheless, extensive preclinical assessment and appropriate scaling and modeling tools will improve predictions. In general, the correlation between human and animal toxicities is good for conditions such as cardiovascular, hematological, and gastrointestinal diseases and the poorest correlation for adverse drug reactions such as, idiosyncratic reactions, skin rash, hypersensitivity, and hepatotoxicity. Toxicology testing in drug discovery is initiated by the high throughput screening, which is followed up by definitive tests. Screening refers to the methods that yield rapid and comprehensive data often using *in vitro* tools. The origin of any toxicological or safety outcome is multifactorial and complex and thus demands for use of sophisticated systems for definitive assessment. Thus, many pharmaceutical companies are also introducing *in vivo* (i.e., animals) toxicology studies as early as possible, quite often in the lead optimization (LO) stage. Extensive and appropriate toxicology studies of varying duration ranging from acute, single dose to chronic, repeat dose in rodent and nonrodent species are needed to establish safe human clinical trials. Acute

toxicity (single dose ranging) studies in preclinical species are performed to support selection of a drug candidate for potential advancement to repeat-dose toxicology studies and ultimately to enable initial FIH clinical trials. The objective of such studies is to identify a dose at which the major adverse effects are observed. These studies are usually carried out in rodents, following a single dose up to a limit of 2000 mg/kg. The information obtained may be translated to select the dose levels for the FIH studies and also to give an indication of potential effects of acute overdose in humans.

Early drug development starts with candidate compound selection. Repeat-dose toxicity studies (7–14 days in duration) in both rodent and nonrodent species are used to better refine safety margins and PK/PD modeling, as well as set appropriate dosages for the subsequent good laboratory practice (GLP) one-month general toxicology and safety pharmacology (i.e., cardiovascular testing in a nonrodent; CNS and respiratory function tests in a rodent) studies that proceed the Investigational New Drug (IND) application before starting FIH clinical trials. TK assessment is based on the multiple samples obtained throughout the duration of the study along with the PK data. Such data are critical in defining a margin of safety between the no observed adverse effect level (NOAEL) and the projected plasma concentrations achieved in human. It is generally considered that a 100-fold safety factor (rodent-to-human exposure ratio) from the most sensitive species NOAEL provides good safety margin in clinical studies. However, our enhanced capability of understanding interspecies sensitivity and detecting more and more subtle effects may warrant a more flexible approach. The toxicology assessment profile includes, for example, the maximum tolerated dose (MTD), safety margins and therapeutic index, target organ toxicities, most sensitive preclinical species, and reversibility of an effect/toxicity. Biomarkers characterization and preclinical to clinical translation can also be investigated in these GLP toxicology studies.

Later drug development includes phases I–IV. Phase I (FIH) starts with a single dose escalation and then multiple dosing in normal healthy subjects. It is used to establish human safety profile and MTD. Phase II defines the efficacy/safety of candidate profile in target patient population (e.g., rheumatoid arthritis), drug–drug interactions, and proof of concept (POC) before proceeding into phase III. Several repeat-dose toxicology studies (general toxicology, embryo-fetal and developmental, fertility, juvenile, carcinogenicity) of longer duration (three months and up to two years) in both rodent and nonrodent species are conducted to support clinical trials of longer duration in patients.

14.4 PARAMETERS THAT DEFINE PK/TK PROFILE

14.4.1 Area Under the Curve (AUC)

AUC is a primary measure of the extent of drug availability to the systemic circulation, that is, it reflects the total amount of unchanged drug that reaches the systemic circulation following intravenous or extravascular administration. Mathematically, area under the plasma (or blood) concentration–time curve (AUC) can be calculated from the obtained concentration–time profile:

$$AUC = \int_0^{\infty} C \cdot dt \quad (14.1)$$

The unit for AUC is concentration per unit time (e.g., ng h/mL). AUC is determined using simple integration method as shown in Equation (14.1) or often by linear trapezoidal method (Fig. 14.1).

The area of each trapezoid is calculated using the following equation:

$$\text{AUC}_{t_1 \rightarrow t_2} = \frac{(C_2 + C_1)}{2} \times (t_2 - t_1) \quad (14.2)$$

The extrapolated area from t_{last} to ∞ , is estimated as

$$\text{AUC}_{t_{\text{last}} \rightarrow \infty} = \frac{C_{\text{last}}}{K_e} \quad (14.3)$$

where C_{last} is the last observed concentration at t_{last} and K_e is the slope obtained from the terminal portion of the curve, representing the terminal elimination rate constant. The total AUC ($\text{AUC}_{0 \rightarrow \infty}$) is determined as

$$\text{AUC}_{0 \rightarrow \infty} = \text{AUC}_{0 \rightarrow t_{\text{last}}} + \text{AUC}_{t_{\text{last}} \rightarrow \infty} \quad (14.4)$$

AUC is used in the calculation of clearance (CL), apparent volume of distribution, and bioavailability (see following sections) and reflects the general extent of exposure over time.

14.4.2 Maximum Plasma Concentration (C_{max}) and Time of Maximum Concentration (T_{max})

C_{max} is defined as the maximum observed drug concentration in the plasma concentration–time profile following intravenous or oral dosing. Most commonly, C_{max} is obtained by direct observation of the plasma concentration–time profile (Fig. 14.1). For some drugs, the pharmacological effect is dependent on the C_{max} ; for example, aminoglycosides, which are widely used antibiotics, need to achieve a C_{max} that is at least 8- to 10-fold higher than that of the minimum inhibitory concentration (MIC) to obtain a clinical response >90% [1]. The unit of C_{max} is concentration unit (e.g., ng/mL).

T_{max} is the time required to reach C_{max} . As with C_{max} , T_{max} is usually determined from direct observation of the plasma concentration–time profile. The unit of T_{max} is time (e.g., h).

14.4.3 Clearance (CL)

CL is a primary parameter that describes the process of irreversible elimination of a drug from the systemic circulation. It is defined as the volume of blood or plasma that is totally cleared of its content of drug per unit time. Thus, it measures the removal of drug from blood or plasma. However, CL does not indicate the extent of drug is being removed but represents the rate, and thus the units are given as mL/min or mL/min/kg (normalized to body weight).

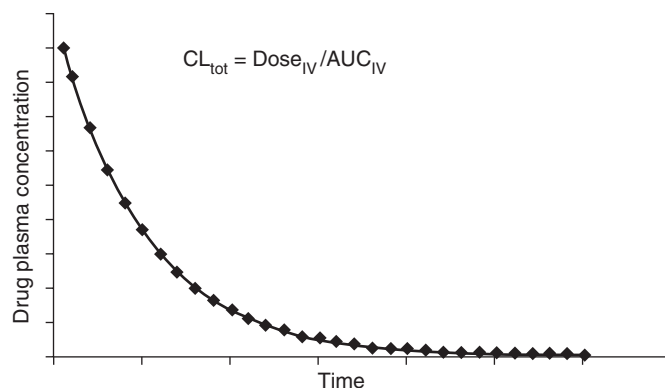


Figure 14.2 Plasma concentration–time profile following IV dosing.

The most widely used approach to evaluate plasma (total) CL involves intravenous administration of a single dose and measuring its plasma concentration at different time points (Fig. 14.2).

$$CL_{tot} = \frac{\text{Dose}_{IV}}{AUC_{IV}} \quad (14.5)$$

In general, a drug is either eliminated unchanged through excretion in the urine and/or bile or by metabolic conversion into more polar metabolite(s) that can be readily excreted in urine and/or bile. Since all these CL mechanisms operate independently, total body CL is a sum of all CLs by individual mechanisms and can be expressed as

$$CL_{tot} = CL_{hep} + CL_{ren} + CL_{bil} \quad (14.6)$$

where CL_{tot} is the total body CL from all different organs and mechanisms, CL_{hep} is the hepatic CL, CL_{ren} is the renal CL, and CL_{bil} is the biliary CL [7].

It is interesting to note that around three-quarters of the top 200 prescribed drugs in the United States are primarily cleared by hepatic metabolism [8]. The hepatic extraction ratio (E_h) is a PK parameter that is widely used to assess the liver's ability to extract drug from the systemic circulation [7]. E_h is defined as the fraction of a drug in the blood that is cleared (extracted) on each passage through the liver and is a function of hepatic blood CL (CL_{hep}) and the hepatic blood flow (Q) [7].

$$E_h = \frac{CL_{hep}}{Q} \quad (14.7)$$

If the predominant CL mechanism for a compound is via hepatic metabolism, then it is reasonable to assume that the CL_{tot} is equal to CL_{hep} . Thus

$$E_h = \frac{CL_{hep}}{Q} = \frac{CL_{tot}}{Q} \quad (14.8)$$

Compounds that undergo hepatic metabolism can be categorized according to their E_h . Compounds with $E_h < 0.7$ are considered high extraction drugs, whereas

compounds with $E_h < 0.3$ are considered low extraction drugs. E_h has a major impact on oral drug bioavailability (see below).

Renal CL (CL_{ren}) can be obtained from the unchanged amount excreted (Ae_{IV}) and plasma AUC (AUC_{IV}) following intravenous dosing [9].

$$CL_{ren} = \frac{Ae_{IV}}{AUC_{IV}} = \frac{Ae_{Oral}}{AUC_{Oral}} \quad (14.9)$$

Similarly, the unchanged amount excreted in urine (Ae_{Oral}) and plasma AUC (AUC_{Oral}) following oral administration can also be used for estimating CL_{ren} .

14.4.4 Apparent Volume of Distribution (V_d)

Volume of distribution (V_d) is a proportionality factor that relates the amount of a drug in the body to its blood or plasma concentrations:

$$\text{Amount of drug in the body at time } t = V_d \times Ct \quad (14.10)$$

Similar to CL, V_d is a primary PK parameter and is represented as volume by unit body weight (e.g., L/kg). V_d is used to assess the extent of drug distribution in the body. This is usually achieved by comparing the drug V_d to the total body water. If the drug has a V_d smaller than the total body water (human total body water = 42 L per 70 kg human body weight), the drug has limited tissue distribution (e.g., naproxen has a $V_d = 11$ L per 70 kg human body weight) [7]. Being proportionality constant, V_d ranges from 3 to more than 40,000 L per 70 kg human body weight and therefore is usually referred to as *apparent volume of distribution*.

14.4.5 Apparent Volume of Distribution at Steady State (V_{dss})

V_{dss} is the volume of distribution that is determined when plasma concentrations are measured at steady state and in equilibrium with the drug concentration in the tissue compartment.

$$V_{dss} = \frac{\text{Amount of drug in the body at equilibrium conditions}}{\text{Steady - state plasma concentrations } (C_{ss})} \quad (14.11)$$

Although V_{dss} is a steady-state parameter, it can be calculated using non-steady-state data as

$$V_{dss} = CL \times MRT \quad (14.12)$$

where mean residence time (MRT) is the average time for all drug molecules to exist in the body. Following intravenous dosing, MRT is calculated as

$$MRT = \frac{AUMC}{AUC} = \frac{\int_0^{\infty} C \cdot t \cdot dt}{\int_0^{\infty} C \cdot dt} \quad (14.13)$$

where AUMC is the area under the first moment versus time curve from time $t = 0$ to ∞ and calculated using trapezoidal rule similar to AUC.

14.4.6 Half-Life ($t_{1/2}$)

Half-life, usually expressed in hours, is the time that is required for the amount (or concentration) of a drug in the plasma to decrease by one half.

$$t_{1/2} = \frac{0.693 \times V_d}{CL} \quad (14.14)$$

Half-life is a dependent PK parameter that is determined by the independent PK parameters, CL and V_d . It is the most widely reported PK parameter since it may constitute a major determinant of the duration of action after single and multiple dosing. In addition, half-life plays a key role in determining the time that is required to reach steady state following multiple dosing and the frequency with which doses can be given.

14.4.7 Bioavailability ($F\%$)

According to the European Medicines Evaluation Agency (EMA), bioavailability ($F\%$) is “the rate and extent to which an active moiety is absorbed from a pharmaceutical form, and becomes available in the systemic circulation.” It is usually determined by calculating the respective AUC after oral and intravenous administrations as

$$\text{Absolute bioavailability} = \frac{AUC_{PO}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{PO}} \quad (14.15)$$

Oral bioavailability is determined by the fraction of dose absorbed (F_a) in the gastrointestinal tract and fraction of dose that does not undergo metabolism in the intestinal tract (F_g) and liver (F_h) (Fig. 14.3) and is expressed as

$$F = F_a \cdot F_g \cdot F_h \quad (14.16)$$

F_h is calculated using the following equation:

$$F_h = 1 - E_h = 1 - \frac{CL_h}{Q} \quad (14.17)$$

Thus, if a drug has a high hepatic extraction ($E_h > 0.7$), then its bioavailability will be low when it is given orally ($F < 0.3$). On the other hand, if a drug has low hepatic extraction ($E_h < 0.3$), then the extent of bioavailability will be high, provided it is completely absorbed and not significantly metabolized by the intestine.

14.5 PK/TK MODELING IN PREDICTING CLINICAL DOSE

PK/TK is an area of science dealing with the exposure of test compound and metabolites, which is determined by the kinetics of exposure, absorption, distribution, metabolism, and excretion. Generally, the extent and duration of exposure are related to the pharmacological or toxicological effects, and thus, the change of the occurrence of the observed effects can be optimized by altering the dose or exposure period.

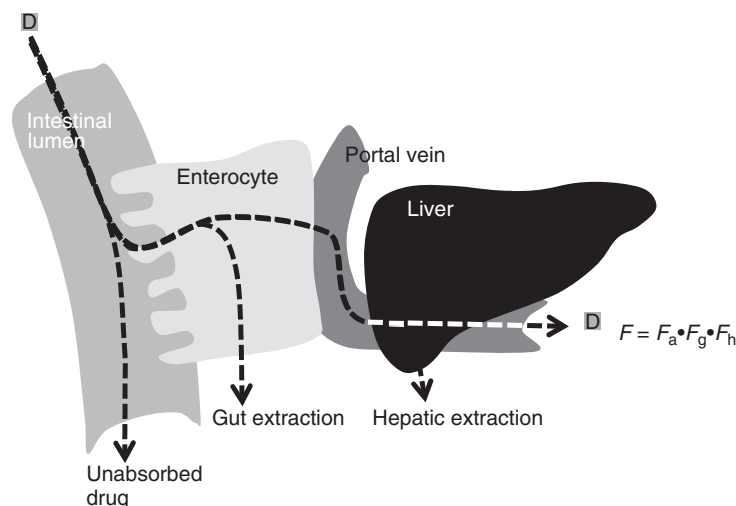


Figure 14.3 Oral bioavailability is a function of intestinal absorption (F_a), the fraction escaping intestinal metabolism (F_g), and the fraction escaping hepatic extraction (F_h), which occur in series. (See color insert.)

A basis for toxicity assessment is the NOAEL or no observed effect level (NOEL) or low observed effect level. NOAEL represents the highest dose at and below which no significant adverse effects are seen. Owing to ethical and practical reasons, assessment of the NOAEL is derived only from animal toxicity data and extrapolated to identify a clinical dose that is significantly lower than the NOAEL. The same strain and species used in the toxicology studies should be used in the TK studies. Initial studies may involve one sex of each species; however, the use of multiple species is relevant to build confidence in models predicting human effects. Using dose–response relationship, the statistical confidence limits of dose at which the incidence or frequency of a toxic effect is established.

Developing mathematical models is of value only when the mechanisms of toxicity are understood and/or the parameters that are being used in model building have established relationships with the observed toxicity effect. While it is important to understand whether the parent molecule or its metabolite is responsible for toxicity, an estimate of whether humans are of less, equal, or greater sensitivity in comparison to the test species is needed for translating the preclinical data to predict human effects. To establish relationship between exposure and dosimetry, a PK/TK model should incorporate the rate and extent of absorption, compound and/or metabolites distributed in the body, metabolism and kinetics of metabolites (if appropriate), elimination rate and elimination route(s), and the influence of dose on all the above processes (dose dependency).

14.5.1 Noncompartmental Analysis

Various PK parameters such as CL, V_d , $F\%$, MRT, and $t_{1/2}$ can be determined using noncompartmental methods. These methods are based on the empirical determination of AUC and AUMC described above. Unlike compartmental models (see following

section), these calculation methods can be applied to any other models, provided the drug follows linear PK. However, a limitation of the noncompartmental method is that it cannot be used for the simulation of different plasma concentration–time profiles when there are alterations in dosing regimen or when multiple dosing regimens are used.

14.5.2 Compartmental Analysis

Compartmental models of PK analysis are widely used to describe drug distribution and disposition. In these models, the body is assumed to be composed of one compartment or more and the drug kinetics can be defined by differential equations generally of first-order process. These compartments do not have any physiological significance. However, they may represent a group of tissues or organs with similar distribution characteristics. For example, highly blood-perfused body organs such as liver, lungs, and kidney often have different drug distribution than fat tissue.

14.5.2.1 One-Compartment Open Model. One-compartment model, which has gained wide acceptance in TK because of its simplicity, describes the whole body as a single compartment in which the compound is homogeneously distributed following administration (Fig. 14.4). Here, the body is assumed to be a homogeneous unit where the compound is rapidly distributed throughout the body and follows a monoexponential decline. Following intravenous dosing, the plasma drug concentration can be calculated as

$$C = \frac{\text{Dose}}{V_c} \cdot e^{-K_e t} = C^0 \cdot e^{-K_e t} \quad (14.18)$$

where V_c is the volume of compartment, K_e is the rate of elimination, t is time, and C^0 is concentration at time zero.

However, following extravascular administration, the peak plasma concentration is a function of the rate and extent of the absorption as well.

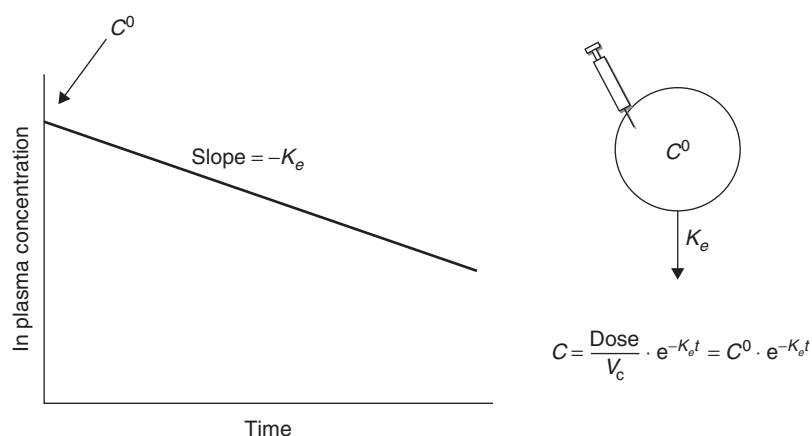


Figure 14.4 One-compartment model.

14.5.2.2 Two-Compartment Open Model. In the two-compartment model, the drug is assumed to distribute into two compartments, the central and the tissue compartments. The central compartment represents the highly blood-perfused body organs where the drug distributes rapidly and uniformly. On the other hand, in the tissue compartment, the drug distributes more slowly (Fig. 14.5).

When the drug concentration versus time profile demonstrates a biexponential decline following intravenous dosing, a two-compartment model that is the sum of two first-order processes (distribution and elimination) will better describe the data (Fig. 14.5). For a drug that follows the two-compartment model, the rate of drug plasma concentration change following intravenous dose can be determined as

$$C = Ae^{-\alpha \cdot t} + Be^{-\beta \cdot t} \quad (14.19)$$

where A and B are functions of the administered dose and α and β are the first-order constants for the distribution and elimination phases, respectively.

In this chapter, only the one- and two-compartment models following intravenous dosing were described. Other models with extravascular dosing have an additional compartment with an absorption rate constant describing input into the central compartment. Models with three or greater compartments may be used if the drug concentration versus time may be described better with additional exponential terms. However, these models have greater complexity.

14.5.3 Physiology-Based Pharmacokinetic (PBPK) Modeling

The accuracy of human prediction will depend on the degree of physiological and biochemical fidelity captured by the model. The advent of structured physiology-based pharmacokinetic (PBPK) models had a dramatic influence on the ability to predict tissue exposure. Such modeling is governed by more fundamental physiological and biochemical processes and is especially useful in estimating tissue exposure, which may be vital in predicting the organ specific toxicity. The major advantages of such modeling are twofold. First, unlike the empirical models (e.g., allometry), the obtained PK parameters are physiologically relevant, and second, concentration–time profiles of

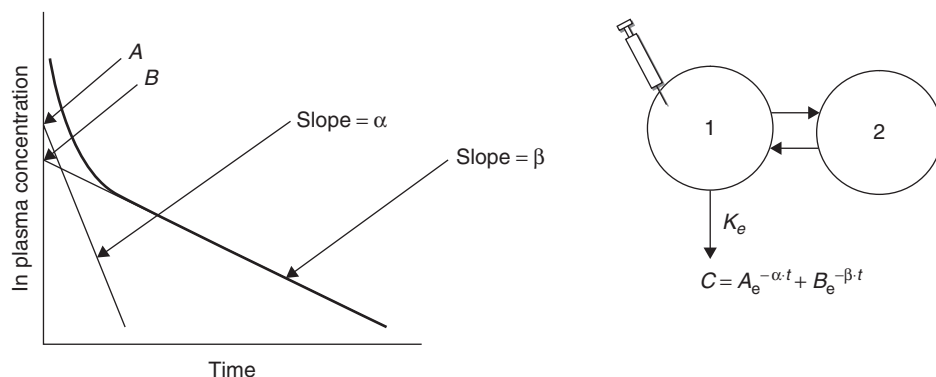


Figure 14.5 Two-compartment open model.

each tissue can be obtained simultaneously. With the exploratory relationships between tissue concentration–time profiles and the pharmacological or toxicological effects, PBPK modeling provides framework for mechanistic PK/PD modeling. Mathematically, the model constitutes of multiple compartments, each corresponding to different body organs or tissues and linked together based on their anatomical placement with respect to blood flow. The vital physiological information in building these models are the tissue volumes, blood flow to the tissue, and the tissue composition. Partition coefficients are measured by equilibrating with tissue homogenates [10]. PBPK models are also most reliable in dose–response and tissue exposure assessments under various physiological conditions (e.g., age, disease condition) [10]. Data collected during a preclinical TK study with focus on time–concentration profiles of compound and its metabolites in plasma and other tissues, facilitates development and validation of comprehensive PBPK models. Such models are useful in assessing the toxicological effects on repeated dose exposure, enzyme induction and inhibition, age- and sex-related physiological factors, and drug–drug interactions, as well as enhance our understanding of interspecies differences.

14.6 TOXICOGENOMICS AND BIOMARKERS

Toxicology safety biomarkers are functional or structural measurements that correlate with a morphologic histopathologic or clinical pathologic change in an organ system such as the liver or heart. Having a translatable biomarker is critical in drug discovery and development. Toxicogenomics is the integration of the *omics* technologies genomics, proteomics, and metabonomics, bioinformatics, and toxicology to better understand drug- or toxicant-induced alterations in biochemical networks (gene, protein, and metabolite) of drug candidate development in a pharmaceutical setting. Therefore, toxicogenomics data can be used as drug toxicity/exposure biomarkers or signatures that provide insights into the toxic mechanism of action of a drug candidate and support safety risk assessment. Such approach can be used in high throughput screening in discovery research. In fact, gene expression is used in the clinic to predict pathologic conditions (e.g., breast cancer), prognosis, and response to therapy. Issues with toxicogenomics experiments are variability in experimental design, strain, gender, age of experimental animals, husbandry, nutrition; interanimal variation; clinical health effects of test compounds; and organs heterogeneity. For example, although the liver appears heterogeneous, it is composed of ~60% hepatocytes, 20% endothelial cells, 15% Kupffer cells, 5% biliary epithelium, and 5% stellate cells.

A good example of the application of toxicogenomics was its use to identify a transcriptional biomarker of the histopathological liver change of oval cell-mediated bile duct hyperplasia (BDH). BDH is a histopathologic finding that occurs in both rodent and nonrodent species. BDH can progress to cholangiocarcinoma with low margins of safety, which can lead to costly, late-stage compound terminations and increased risk to patient safety. Thus, interpretation of the significance of BDH requires consideration of a number of variables, including duration of drug exposure, margins of safety, intended drug indication, and availability of biomarkers to monitor patient safety. The specificity and sensitivity of the discovered candidate biomarker, called *deleted in malignant brain tumor* (DMBT1), was evaluated in livers of rats treated with more than 30 different compounds comprising hepatotoxicities that ranged from BDH and

hepatocyte proliferation to phospholipidosis, hepatocellular vacuolation, apoptosis, and inflammation [11]. Multidisciplinary collaboration between toxicologic pathologists, toxicologists, biologists, personnel in toxicokinetics, and statisticians is needed for successful toxicogenomic efforts.

14.7 SPECIES DIFFERENCES IN DRUG DISPOSITION

Oral exposure of drug molecules is a product of their absorption and hepatic and intestinal first pass. Species differences were reported in the oral exposure of various drug molecules. Several investigators attributed these findings mainly to differences in anatomical and physiological factors such as hepatic blood flow, metabolizing enzyme type, and expression or extent of protein binding. There are significant species differences in bile flow rate and hepatic blood flow (Table 14.1), which may explain some of this variation. In addition, bile composition (acid, ions, electrolytes) also varies between species and may further explain reported species differences in drug biliary excretion rate and thus differences in PK disposition of various drugs [12].

Nelson *et al.* [13] reported that so far 14 CYP gene families have been identified in mammals with significant variations in the primary sequence of amino acids across species. However, these members of the superfamily had highly conserved regions of amino acid sequence. Similar findings were also reported with uridine diphosphoglucose transferases and carboxylesterases [14,15]. Overall, these small differences in the amino acid sequences can lead to significant differences in substrate affinity and specificity, which translate into differences in the metabolism rate and metabolism profiles. As a general rule, compounds with good passive absorption, high rat hepatic extraction ratio, and poor oral bioavailability tend to have better oral bioavailability in higher species such as dogs, monkeys, and humans. There are many cited examples that are consistent with this trend. For example, atomoxetine is a CYP2D6 substrate with an absolute human oral bioavailability of 94% and 63% in poor and extensive metabolizers of CYP2D6, respectively [16]. The moderate to high human oral bioavailability suggests nearly complete oral absorption of atomoxetine. However, preclinical evaluations showed that the absolute oral bioavailability of atomoxetine in rat was only 4% [17] but was 74% in dog [17]. Overall, the disposition of atomoxetine is similar in rats, dogs, and humans with a primary oxidative metabolite of 4-hydroxyatomoxetine that is subsequently conjugated to form 4-hydroxyatomoxetine-*O*-glucuronide. In a

TABLE 14.1 Mean Hepatic Blood Flow and Bile Flow in Selected Species^a

Species	Hepatic Blood Flow (mL/min/Kg)	Bile Flow (mL/min/Kg)
Mouse	73	100
Rat	39	90
Monkey	33	25
Dog	23	12
Human	17	5

^aRef. 18.

radiolabeled study in rats administered ^{14}C -atomoxetine, atomoxetine AUC following oral administration accounted for only 2% of the total ^{14}C AUC as compared to 30% of the ^{14}C AUC following intravenous administration, indicating extensive first-pass metabolism in rats [17]. In a corresponding radiolabeled study in dogs, atomoxetine AUC following oral administration accounted for 33% of the total ^{14}C AUC as compared to 39% of the ^{14}C AUC following intravenous administration, indicating considerably less pronounced first-pass metabolism [17]. This example clearly illustrates the importance of understanding not only the species differences in a drug's metabolic fate but also the extent of species differences in the first-pass metabolism when utilizing preclinical data to project human oral bioavailability.

Indinavir, a CYP3A4 substrate, is an HIV protease inhibitor for which variable oral bioavailability have been observed in preclinical species, ranging from 72% in dogs to 19% in monkeys, and was 24% in rats [18]. This variability was mainly attributed to species differences in the extent of hepatic first-pass metabolism. Chemical and immunochemical inhibition studies indicated the potential involvement of CYP3A isoforms in the metabolism of indinavir in rats, dogs, and monkeys [19], which is consistent with the observation that CYP3A4 is the main isoform responsible for the oxidative metabolism of indinavir in human liver microsomes [19]. The *in vitro* profile of indinavir metabolism was qualitatively similar across species [19]. In addition, an *in vitro*–*in vivo* correlation was established in rats and dogs using the *in vivo* hepatic CL and hepatic first-pass extraction ratio obtained from *in vitro* rat and dog metabolic data, respectively. Based on the *in vitro*–*in vivo* correlation established in rats and dogs, the *in vitro* intrinsic CL of indinavir in human liver microsomes projected a small first-pass metabolism in humans ($E_h = 0.25$), which was consistent with indinavir's high oral bioavailability (60–65%) observed in humans at clinically relevant doses [20]. This example depicts the importance of establishing an *in vitro*–*in vivo* correlation in tested preclinical species so as to use it as a basis to project human CL and oral bioavailability. Overall, these successful medications would not be on the market if the discovery team solely depended on rat oral bioavailability to evaluate their metabolism in humans.

Species differences in the extent of protein binding of various xenobiotics were also reported. It is interesting to note that albeit their structural and functional homologies, there are minor differences in the amino acid sequence in plasma protein molecules such as albumin among various mammals. This may be another contributing factor to the differences in both the binding affinity and sites of drugs in protein molecules among different species [21].

14.8 MIST (METABOLITES IN SAFETY TESTING)

Guidance for Industry on Safety Testing of Drug Metabolites, MIST (METABOLITES IN SAFETY TESTING), was published in 2008 from the Center for Drug Evaluation and Research (CDER) defining the threshold for human metabolites that raise safety concerns as 10% of total drug exposure. Additional guidelines issued from the International Conference on Harmonization (ICH) on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, ICH M3[R2], define the threshold as 10% of total drug-related exposure, which supersedes when in conflict with the CDER guidance. Others have suggested that

total metabolite burden that accounts for dose is a more relevant threshold [22,23]. Early clinical assessment of human metabolites in plasma or urine enables identification of significant human metabolites and whether they are present at equal or greater exposure levels (AUC) in any one of the preclinical safety species is critical for addressing MIST implications. While rare, this is exceedingly important if the metabolite is formed in humans only. Traditionally, preclinical measurement of circulation concentrations of metabolites only occurs to address specialized cases such as when extensive metabolism of parent drug is expected. When the human metabolite profile is expected to be qualitatively similar to at least one of the preclinical safety testing species, measurement of circulating parent in plasma is adequate in preclinical PK and TK studies. Drug development history has taught us that human drug metabolites can play significant roles in primary and secondary pharmacology, general toxicity, and potentially idiosyncratic drug toxicity (Table 14.2). Therefore, one must develop fit-for-purpose metabolite assessment strategy and use the knowledge of the chemical space of the drug candidate in question, structural activity relationship for both primary and related secondary pharmacology, metabolic CL pathways, and potential target organ toxicity to evaluate if measuring metabolites in preclinical PK/TK samples is warranted and would provide the discovery team with knowledge to design a best-in-class compound. Once a compound has progressed through early development, usually post POC, definitive radiolabeled ADME studies are conducted to identify metabolites and assess the need to monitor these in the clinic or qualify in toxicology studies. Realistically, successful drug discovery teams know well in advance the outcome of these studies.

14.8.1 Pharmacologically Active Metabolites

Metabolism of drug candidates can occur at sites of the molecule that do not alter the active pharmacophore, sometimes even resulting in metabolites that have greater pharmacological activity or longer PD duration. This is usually first suspected when existing PKPD relationships fail to predict the accurate dose required for the desired effect and thus may serve as a trigger for metabolite scouting activities. Prodrug strategies are the intentional design of active pharmacological species from an inactive precursor. Where prodrug approaches achieve quantitative conversion of inactive precursor to the active species, circulating metabolite analysis is the only manner to confirm that dose was administered.

14.8.2 Reactive Metabolites

The MIST guidance addresses a majority of direct or indirect conjugates; the latter class of metabolites is considered harmless, but toxicity continues to be a major challenge

TABLE 14.2 Examples of Human Drug Metabolites that are Known to be Responsible for General Toxicity and Potentially Idiosyncratic Drug Toxicity

Parent Drug	Metabolite	Suspected Metabolite Activity
Acetaminophen	<i>N</i> -acetyl- <i>p</i> -benzoquinone imine (NAPQI)	Toxicity
Troglitazone	GSH conjugation/ring scission	Idiosyncratic toxicity
Losartan	5-Carboxylic acid (10–40 × potency)	Pharmacologically active
Dexfenfluramine	D-Norfenfluramine	Pharmacologically active

leading to drug development failures. Reactive metabolites are frequently suspected when hepatotoxicity or idiosyncratic toxicity is observed. By the very nature of these species, circulating concentrations of reactive metabolites often occur at trace levels, making them difficult to detect and quantitate. Recent strategies to identify reactive metabolites as conjugates to biomolecules such as glutathione or cysteine offer an approach to confirming the formation of these species *in vivo* [24]. Confirming suspected reactive metabolites is informative, and also designing enzymology studies to understand pathways leading to their formation allows an understanding of the potential for species-specific reactions.

14.8.3 Enabling Technologies

Plasma pooling methods were compared to the traditional approaches of obtaining quantitative information on the levels of circulating metabolites in preclinical species. The exposure values obtained via sample pooling are comparable to those obtained by traditional methods of analyzing samples individually. When kinetic information is not needed, this approach allows metabolite identification and exposure burden estimates with a modest resource investment. While metabolite analysis without authentic standards can be accomplished using high resolution or accurate mass LCMS techniques, new techniques using chromatographic measures such as retention time shifting or background subtraction of vehicle-dosed samples offer methods to identify metabolites [25].

There are various approaches to assess the metabolite exposure margin between toxicology species and humans: either by direct or indirect comparison. The preferences in when and how to pursue metabolite assessment is based on the overall development strategy. Therefore, it is important to understand the utility and limitations of analytical instruments in order to apply an appropriate analytical tool to address specific questions posed at different stages of drug development. The urgency of metabolite monitoring depends on the intrinsic nature of the compound, therapeutic intent, and objective of the clinical development; there is not a unified approach that provides efficient resource utilization.

14.9 CONCLUSIONS

PK/TK are important biomarkers of drug exposure, efficacy, and safety in early- and late-stage drug discovery and development. Determinants of a drug PK/TK include ADME. Several toxicity assessment studies (e.g., acute, single, repeat dose studies, GLP) in both rodent and nonrodent species are used to better refine safety margins and PK/PD modeling and set appropriate dosages before starting FIH clinical trials. Critical parameters that define PK/TK profile are AUC, $C_{\max}$, $T_{\max}$, V_d , V_{dss} , $t_{1/2}$, and F . Mathematical modeling can be of great value in the PK/TK evaluation. There is an emerging and increased utility of toxicogenomics to better define drug toxicity/exposure to support compound selection and safety risk assessment. There are significant species differences in the oral exposure to various drugs that must be taken into consideration. Such differences are related to various anatomical and physiological factors including hepatic and renal blood flow, metabolizing enzymes (i.e., CYPs), and protein binding. A valid strategy should be a fit-for-purpose metabolite assessment strategy that uses

the knowledge of the chemical space of a drug candidate being developed, structural activity relationship to pharmacology and toxicology and metabolism CL pathways. Ultimately, a well-designed PK/TK evaluation would provide the discovery team with knowledge to design and advance a best-in-class compound.

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15 Pharmacokinetic Data Analysis and Modeling of ADME Processes

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15.1	Introduction	1
15.2	Recovery of the radioactive dose in excreta	2
15.3	Pharmacokinetic analysis of blood data using noncompartmental analysis	4
15.4	Pharmacokinetic analysis of urine data	5
15.5	Pharmacokinetic analysis of plasma data with compartmental model	10
15.6	Summary	35
	References	36

15.1 INTRODUCTION

Human absorption, distribution, metabolism, and elimination (ADME) studies are routinely conducted for small molecule drug candidates mostly during the stage of early clinical development to characterize their ADME in the body. Since biological drug candidates, in general, contain only the naturally occurring components of amino acids or nucleic acids, there is no need to perform a human ADME study to understand metabolism and/or elimination for these drug candidates. However, a human ADME study may be desirable for the biological drug candidates that contain chemically modified components such as unnatural amino acids, nucleic acid analogs, or antibody–drug conjugate with small molecule chemical drug complexes.

Pharmacokinetic (PK) evaluation in human ADME study is intended to define the systemic exposure of a drug and its metabolite(s), in particular, pharmacologically active metabolites in the body after dose administration. The most important information of drug and/or metabolite concentration–time profile is to provide the rate and extent of ADME.

Plasma and urine data obtained in human ADME studies can be used to calculate standard PK parameters for unchanged drug, such as time of maximum observed plasma concentration ($T_{\max}$), maximum observed plasma concentration ($C_{\max}$), area under the plasma concentration–time curve from time zero to infinite time ($AUC_{0-\infty}$) or to time of last quantifiable concentration (AUC_{last}), clearance (CL), volume of distribution (V), elimination half-life ($t_{1/2}$), renal clearance (CL_R), and percentage of dose excreted

unchanged in urine. These PK results confirm appropriateness of ADME study design and study conductivity if these results are comparable with the results for the same drug from other previously conducted phase I trials.

To date, two different methods have been used to analyze drug and metabolite kinetics. The first is noncompartmental analysis (NCA), also called *model-independent analysis* of concentration–time profile. This model is not necessarily restricted to the assumption of compartments and is a simple first-order distribution or elimination process. The second approach is compartmental analysis and utilizes mathematical models to describe drug/metabolite behavior in the body, with assumptions of well-mixed compartments and following certain mathematical equations. With the limited assumptions required, NCA allows the estimation of the most basic PK parameters characterizing the ADME processes of a drug. NCA methods are relatively simple to use prevailing in drug development and regulatory evaluation. Nearly all PK analysis of phase I intensive data are completed using NCA. Although NCA provides useful summary assessments of PK parameters, it does not provide some important PK parameters such as rate constants and distribution of drug to different compartments that could help researchers understand mechanisms of drug disposition and action. On the other hand, the mechanistic, model-dependent approach can provide information for a more quantitative understanding of drug action in living cells, organisms, or disease-related targets. Considering that drug development is an information-gathering process of learn–confirm cycles, mechanistic modeling plays an important role in knowledge-based drug development and eventually regulatory approval.

In addition to identifying the major excretory pathways, much can be learned from ADME studies about blood, bile, urine, and feces data of unchanged drug and total radioactivity (TRA). The goal of this chapter is to demonstrate how important information relating to drug ADME may be obtained from the analysis of PK data. The theory and application of PK data analysis and modeling to characterize the PK of a drug and its metabolite(s) based on blood and urine data are presented. The discussion focuses on conventional methods that have been utilized in assessing PK data in human studies.

15.2 RECOVERY OF THE RADIOACTIVE DOSE IN EXCRETA

One of the objectives of a human ADME study is to evaluate mass balance of an administered drug. This type of study provides information on the amount of drug that is recovered over time via the different elimination routes of the body after dose administration. The analyses of radioactivity in excreta sample such as urine, feces, and bile offer information regarding elimination routes of the drug. In human ADME studies, the cumulative excreta samples of urine and feces across the entire study period in each subject are collected following a single oral or intravenous (IV) administration of a radiolabeled drug. For some orally administered drugs, bile samples may also be collected if biliary excretion is expected to be a major route for elimination of drug or its direct conjugates/unstable metabolites that will be converted back to the parent by hydrolysis of the conjugate in the gut microflora. With these samples analyzed, the recovery of the radioactive dose in excreta can be estimated.

It is ideal to have mass balance, that is, near complete recovery with at least 90% of the total administered radioactivity recovered in excreta. A case study using a

Bristol-Myers Squibb drug candidate (BMS-690514) is used as an example of a typical human ADME study and also used to discuss noncompartmental PK data analysis later in this chapter. In the human ADME study of [^{14}C]BMS-69051 [1], an orally administered selective inhibitor of human epidermal growth factor receptors (HERs) and vascular endothelial growth factor receptors (VEGFRs), a single 200-mg dose of [^{14}C]BMS-690514 containing 80 μCi of radioactivity (0.39 $\mu\text{Ci}/\text{mg}$) in a solution of 50 mM citric acid was administered orally to a total of nine healthy male subjects, who were randomly assigned to group 1 ($n = 6$) without bile sample collection or to group 2 ($n = 3$) with bile sample collection. The blood, urinary, and fecal samples were collected up to 216 h postdose and bile samples were obtained from 3 to 8 h postdose by positioning the terminal end of an oral gastroduodenal tube at the vertical limb of the duodenal loop near the ampulla of Vater (confirmed via fluoroscopy). The TRA in excreta was determined by liquid scintillation counting and the concentration of drug-derived ^{14}C radioactivity in individual plasma samples was determined by accelerator mass spectrometry. Following oral administration of [^{14}C]BMS-690514, the mean cumulative recovery of radioactivity over the 0- to 264-h collection interval was 99.98% (Fig. 15.1). The majority of radioactivity, 50%, was recovered in the feces and $\sim 34\%$ was recovered in the urine. For subjects with bile collection, an additional 16% of the dose was recovered in the bile over the 3- to 8-h postdose interval. The excretion rate reached a plateau at 96 h postdose with $<2\%$ daily incremental changes thereafter.

Although there are many ways to increase the chance to achieve a high recovery such as by preventing a labeled fragment of the drug from entering endogenous compound metabolism and prohibiting conversion of the label to a volatile metabolite, it is not unusual to obtain $<100\%$ recovery of the radiolabeled dose in a mass balance study. Beumer *et al.* [2] suggest that total recovery should be at least 90% of the

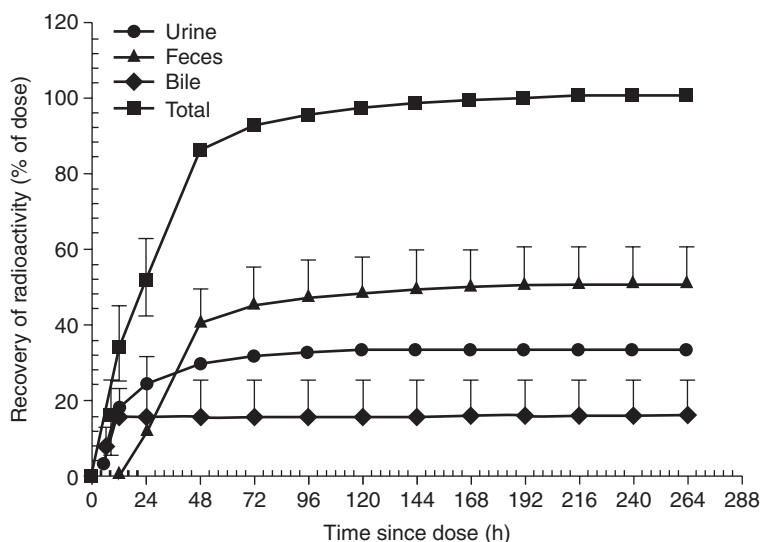


Figure 15.1 Mean $\pm$ SD cumulative recovery of radioactivity after administration of a single 200-mg (80- μCi) PO dose of [^{14}C]BMS-690514 to healthy male subjects—time course for excretion of radioactivity in urine, feces, bile, and total excreta [1].

administered dose with a 10% margin ($100 \pm 10\%$) as being acceptable. Roffey *et al.* [3] reviewed 171 human mass balance studies and found that 42% had overall recovery values below 90%. If the criteria were dropped to 80%, 15% of mass balance studies would have been considered failures. Low recovery of radioactivity can be explained by biological factors such as binding to specific proteins in tissues, binding to melanin, binding to phospholipids, and covalent and noncovalent binding to tissue. Noncompliance by subjects/study personnel to meticulously collect all excreta samples can also contribute to a low recovery. In general, the drugs with a long half-life tend to show low recovery of administered radioactivity. The success or failure of a mass balance study is dependent on the value of absolute recovery. The acceptance of a mass balance study is ultimately based on achievability of the primary objective of human mass balance study to determine the profile of parent drug and metabolites using excretory and circulatory samples obtained in this study.

15.3 PHARMACOKINETIC ANALYSIS OF BLOOD DATA USING NONCOMPARTMENTAL ANALYSIS

Typically, serial blood samples are collected after single-dose administration of radiolabeled drug in order to characterize the PK of TRA and the drug from plasma or serum concentration versus time profiles in human ADME studies. This allows pharmacokineticists to determine the PK parameters of TRA and a drug by an NCA method without the assumptions of mixed compartments and following certain mathematical equations.

The single-dose PK parameters assessed in human ADME studies, in general, include $T_{\max}$, $C_{\max}$, AUC_{last} , $AUC_{0-\infty}$, $t_{1/2}$, CL/F (apparent total body clearance), and V_{ss}/F (apparent steady-state volume distribution) for an orally administered radiolabeled drug. If a radiolabeled drug is administered via IV injection, the same set of PK parameters will be estimated as for an oral drug, except absolute CL and V_{ss} will be estimated.

The $C_{\max}$ and $T_{\max}$ are obtained from experimental observations. The terminal log-linear phase of the concentration–time curve is identified by least-square linear regression of at least three data points that yielded a maximum G-criterion, which is also referred to as *adjusted R^2* , using no weighting factor. The $t_{1/2}$ value was calculated as $\ln 2/k_{el}$, where k_{el} was the absolute value of the slope of the terminal log-linear phase. The AUC_{0-T} was calculated using the mixed log-linear trapezoidal algorithm with a NCA method. The $AUC_{0-\infty}$ was estimated by summing AUC_{0-T} and the extrapolated area, computed by the quotient of the last observable concentration and k_{el} .

As an example, the mean plasma concentration–time profiles and PK parameters for TRA and BMS-690514 are shown in Fig. 15.2 and Table 15.1, respectively. BMS-690514 was rapidly absorbed with $T_{\max}$ values ranging from 0.5 to 2 h postdose. $T_{\max}$ values for TRA occurred between 1.5 and 4.0 h postdose and reflected a composite plasma concentration–time profile of BMS-690514 and other metabolites in plasma. On the basis of the $AUC_{0-\infty}$ values, the unchanged parent drug, BMS-690514, accounted for $\sim 3.2\%$ and 7.6% of the circulating radioactivity in group 1 (without bile sample collection) and group 2 (with bile sample collection) subjects, respectively, suggesting that metabolites contributed significantly to the circulating radioactivity. Plasma TRA

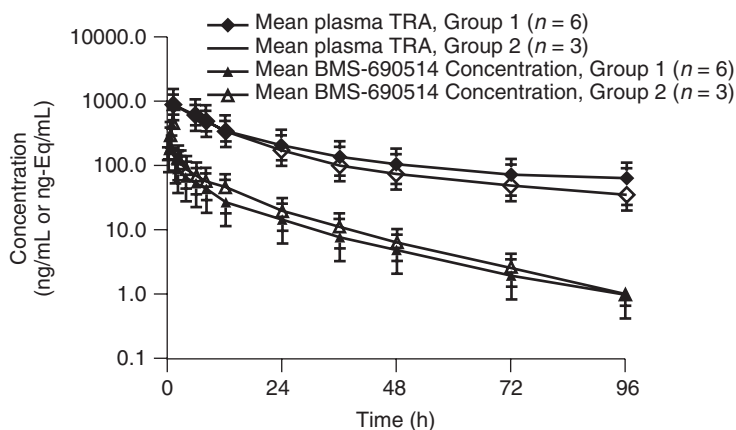


Figure 15.2 Mean $\pm$ SD plasma concentration–time profile for total radioactivity (TRA) and BMS-690514. Concentrations of drug-derived radioactivity in plasma were determined by accelerator mass spectrometry (AMS); BMS-690514 concentrations were measured with a validated LC-MS/MS assay [1].

was eliminated from the systemic circulation at a slower rate than parent drug, which had a mean $t_{1/2}$ of 16.7 h for the two groups combined. Subjects who went through bile collection had generally lower systemic exposures to TRA than those who did not have bile collection.

15.4 PHARMACOKINETIC ANALYSIS OF URINE DATA

Since renal excretion is an important route of elimination for many drugs, analysis of urinary data can provide useful PK information. Although urine data are less reliable than plasma or serum data for the calculation of PK parameters, together with plasma or serum data, they may allow the calculation of renal clearance. The ratio of a drug excreted unchanged to the TRA excreted in urine is also informative. Comparison of this ratio to the ratio of an unchanged drug to the TRA in plasma or serum may generate insight into drug–metabolite differences in passive/active excretion and reabsorption in the kidneys.

15.4.1 Renal Clearance

Renal excretion is an important route of elimination for many drugs, including unchanged drugs that are primarily excreted in urine and many drug metabolites that are formed by hepatic metabolism of the parent drug. Renal clearance, a useful PK parameter, can be calculated from analysis of urinary data together with plasma data obtained in the human ADME study.

Renal clearance is the hypothetical volume of plasma from which the substance is eliminated by the kidney appearing in the urine per unit time. A physiologic definition of renal clearance (CL_R) is a ratio of the instantaneous urinary

TABLE 15.1 Pharmacokinetic Parameters of Total Radioactivity and BMS-690514 After a Single 200-mg PO Dose of [^{14}C]BMS-690514 to Healthy Subjects in Group 1 (without Bile Collection) and Group 2 (with Bile Collection)

	BMS-690514					Total Radioactivity			
	$T_{\max}$ (h)	$C_{\max}$ (ng/mL)	$\text{AUC}_{(\text{INF})}$ (ng h/mL)	$t_{1/2}$ (h)	UR (%)	$T_{\max}$ (h)	$C_{\max}$ (ng Eq/mL)	$\text{AUC}_{(\text{INF})}$ (ng Eq/mL)	$t_{1/2}$ (h)
Group 1 ($n = 6$)	0.5 (0.5, 1.0)	173.55 (44)	1205.07 (37)	15.98 ± 2.31	2.97 ± 0.83	2.01 (1.50, 4.00)	913.41 (23)	37,579.68 (45)	$327.14 \pm$ 287.78
Group 2 ($n = 3$)	0.52 (0.45, 2.12)	142.49 (26)	1698.52 (10)	18.13 ± 2.22	4.19 ± 1.14	2.12 (1.92, 4.00)	724.33 (18)	22,246.77 (28)	$170.46 \pm$ 72.36
All ($n = 9$)	0.5 (0.45, 2.12)	162.52 (38)	1351.13 (31)	16.70 ± 2.39	3.37 ± 1.06	2.02 (1.50, 4.00)	845.45 (24)	31,554.24 (49)	$274.92 \pm$ 243.33

Data are presented as median (minimum, maximum), geometric mean (percent coefficient of variance), or mean $\pm$ SD.

excretion rate (dA_e/dt) and the plasma concentration of drug at the time when the urinary sample is collected (C_p):

$$CL_R = \frac{dA_e/dt}{C_p} \quad (15.1)$$

This equation assumes that urine is collected over a finite period during which the plasma concentration of drug is changing following first-order kinetics. Practically, renal clearance is calculated as the ratio of the amount of drug excreted unchanged ($A_{e(\Delta t)}$) during a urine collection interval (Δt) to the plasma concentration of drug at the midpoint of the interval (C_{mid}):

$$CL_R = \frac{A_{e(\Delta t)}/\Delta t}{C_{mid}} \quad (15.2)$$

By rearranging Equation 15.2, the slope of a linear regression of multiple urine collections ($A_{e(\Delta t)}/\Delta t$) versus C_{mid} is equal to average renal clearance. A short collection period approximates the change in plasma concentration of drug linearly with time but increases the inaccuracy in the estimate of excretion rate due to incomplete bladder emptying. On the other hand, prolonging the urine collection interval can avoid the problem of incomplete emptying. However, the change in drug plasma concentration during a sample collection interval may no longer follow a linear function because the drug plasma concentration is in fact changing exponentially with time under the conditions of Equation 15.1. In practice, a lower limit and upper limit for a collection interval, dt in Equation 15.1 and Δt in Equation 15.2 is ~ 0.5 h [4] or less than an elimination half-life [5].

When taking integration of Equation 15.1 from time 0 to infinity, the numerator dA_e becomes $A_{e(\infty)}$ and the denominator $C_p dt$ is the corresponding area under the plasma concentration–time curve of drug ($AUC_{0-\infty}$):

$$CL_R = \frac{A_{e(\infty)}}{AUC_{0-\infty}} \quad (15.3)$$

Clinically, severe difficulties with compliance in plasma and urine sample collection may occur, especially for drugs with a long terminal elimination half-life as typically a sample collection period of at least five terminal elimination half-lives can be considered to be equivalent to infinite term. Equation 15.3 is practically modified as follows:

$$CL_R = \frac{A_{e(t_2-t_1)}}{AUC_{(t_2-t_1)}} \quad (15.4)$$

On the basis of the plasma and urine data obtained in a human ADME study as presented in Table 15.2 and Figs. 15.3 and 15.4, renal clearance of a drug can be calculated by applying Equation 15.2 or 15.3. The results of the renal clearance calculation using the methods described above are reasonably close to each other at 6.4 L/h. The terminal elimination half-life of this drug is between 10 and 20 h. No practical difficulties occurred in the urine collections taken over an adequate period of five to six terminal elimination half-lives. However, for drugs with a prolonged terminal elimination half-life, more than one month of urine collection will cause compliance issues in the clinical study. In this case, the methods stated by Equations 15.2 and 15.4 gain an obvious advantage over the method approaching infinity.

TABLE 15.2 Plasma and Urine Data and Renal Clearance of a Drug Following a Single IV Administration to a Normal Healthy Volunteer

Time Interval (h)	Amount of Unchanged Drug Excreted in Urine (μg)	Urinary Excretion Rate ($\mu\text{g/h}$)	Mid Plasma Concentration Between Time Interval (ng/mL)	AUC in Plasma Within Time Interval (ng h/mL)	Renal Clearance ^a (L/h)
0–12	5869	489	75.2	929	6.32
12–24	1866	156	24.4	299	6.37
24–48	1540	64.2	12.2	203	7.58
48–72	499	20.8	6.87	78.2	6.38
72–96	223	9.30	3.60	41.1	5.44
>96	202	—	—	10.3	—
0– ∞	9997	—	—	1561	6.40

^aCalculated by equation $\text{CL}_R = \frac{A_{e(t_2-t_1)}}{\text{AUC}_{(t_2-t_1)}}$.

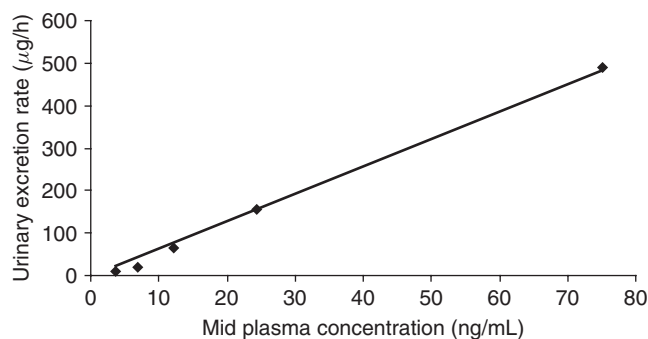


Figure 15.3 Relationship between the urinary excretion rate of a drug and the plasma concentration of the drug at the midpoints of each sample collection interval. The slope of the regression line is equal to the average renal clearance of the drug over 96-h sample collection period. The intercept of the regression line was forced to zero during regression analysis. Renal clearance = 6.43 L/h; $R^2 = 0.9939$.

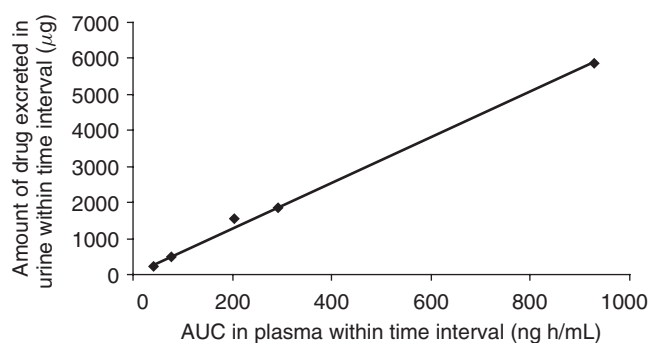


Figure 15.4 Relationship between the amount of unchanged drug excreted in urine and AUC of drug in plasma within each sample collection interval. The slope of the regression line is equal to the average renal clearance of the drug over 96-h sample collection period. The intercept of the regression line was forced to zero during regression analysis. Renal clearance = 6.37 L/h; $R^2 = 0.9969$.

15.4.2 Fraction of Unchanged Drug Excreted in Urine

The fraction of the amount of drug entering the systemic circulation that is excreted unchanged in urine (f_e) is an important PK parameter that indicates the major pathway of drug elimination through either the excretion of unchanged drug via the kidney or metabolism in the liver followed by the excretion of the metabolite(s) in urine. The value of f_e ranges between 0 and 1.0 depending on the extent to which a drug is renally excreted compared to the overall drug elimination. When the value is low, excretion is a minor pathway of drug elimination, suggesting that the most amount of the drug administered is metabolized. If renal excretion is the major route of elimination, the value of f_e will be higher with an upper limit of 1.0, which means that the renal excretion is the only route of drug elimination. The value of f_e for a particular drug will remain constant independent of the dose of the drug administered via IV injection and is estimated by

$$f_e = \frac{U^\infty}{\text{Dose}} \quad (15.5)$$

where U^∞ is total drug excreted unchanged. If the drug is administered orally, f_e is defined by

$$f_e = \frac{U^\infty}{F \times \text{Dose}} \quad (15.6)$$

where F represents the absolute bioavailability of a drug. In the case of a hypothetical drug with urine and plasma data listed in Table 15.2, the amount of unchanged drug excreted in urine is calculated by multiplying urine drug concentration and the urine volume collected within a time interval, and summing the amount from all intervals, giving the value of 9.997 mg. If the IV dose is 200 mg, the f_e is equal to 0.05. In practice, care should always be taken to ensure collecting urine for at least five elimination half-lives.

In those situations in which total urine collection is impossible, f_e may be estimated by the ratio of renal and total clearances,

$$f_e = \frac{k_{\text{ren}}}{\lambda_z} = \frac{\text{CL}_R \cdot C}{\text{CL} \cdot C} = \frac{\text{CL}_R}{\text{CL}} \quad (15.7)$$

where k_{ren} is the urinary excretion rate constant, λ_z the first-order elimination rate constant, C the plasma drug concentration, CL_R the renal clearance, and CL the total clearance. The CL_R can be estimated from the urine data described in the previous section, while CL can be determined from the plasma data,

$$\text{CL} = \frac{\text{Dose}}{\text{AUC}} \quad (15.8)$$

where AUC is the total area under the plasma concentration–time curve. This calculation of clearance is independent of the shape of the concentration–time profile. For a drug with linear PK, the value of clearance is also independent of dose.

15.5 PHARMACOKINETIC ANALYSIS OF PLASMA DATA WITH COMPARTMENTAL MODEL

The compartmental model approach for analyzing PK data is typically to fit a concentration–time profile with a mono-, bi-, or triexponential equation. Computer fitting concentration–time data obtained from a clinical study, including human ADME study, will provide disposition rate constants, which can be used for estimating more PK parameters such as CL, V , absorption rate constant (k_{01}), and elimination rate constant (k_{10}). Since the number of subjects in human ADME study is limited, the PK data from this type of study are frequently pooled with the data from the other human studies of the drug for determining these PK parameters which can be further used in exposure–response analysis. In this case with a large PK dataset, a population PK approach can be applied to estimate inter- and intrasubject variability in addition to the classic PK parameters. With the variability, the PK of a drug and further the exposure–response correlation, if there is any, can be simulated and predicted for certain dose levels and study outcomes that have not been studied in humans. The most frequently used one-, two-, and three-compartment models are listed in Table 15.3. Since data fitting of concentration–time data is out of the scope of this chapter, readers can reference relevant book [6].

15.5.1 Evaluation of Absorption Kinetics from Plasma Concentration–Time Data

Drug absorption process in the gastrointestinal (GI) tract following oral administration is complex because it involves several processes, including disintegration of a solid dosage form, dissolution of a drug into the gastric fluid, gastric emptying, and the diffusion of a drug molecule across the gut wall. In general, absorption of many drugs after oral administration is assumed to follow a first-order process of which the absorption rate is described by

$$\frac{dA}{dt} = F D k_a e^{-k_a t} \quad (15.9)$$

where A denotes the amount of drug in the body, F the bioavailability, D the dose administered, k_a the first-order absorption rate constant, and t the time. This model predicts fast absorption, a peak concentration with an intermediate plateau around it followed by an exponential decrease showed in a hypothetical plasma concentration–time profile (Fig. 15.5).

Under certain circumstances, plasma concentrations following oral administration sharply increase to a peak at which absorption slows and quickly declines with no intermediate plateau, of which this absorption of drugs is better described by zero-order kinetics:

For $t \leq T$,

$$C = \frac{k_0}{k_{10} V_d} [1 - e^{-k_{10}(t-t_0)}] \quad (15.10)$$

TABLE 15.3 Basic Pharmacokinetic Compartmental Models

Model	Input	Compartment	Elimination Rate	Equation	Compartmental Scheme
1	IV bolus	1	First order	$C(t) = \frac{D}{V} e^{-k_{10}t},$ $k_{10} = \frac{CL}{V}$	<pre> graph LR Input -- Bolus IV --> 1((1)) 1 -- k10 --> Elimination </pre>
2	First-order extravascular	1	First order	$C(t) = \frac{D \cdot k_{01}}{V(k_{01} - k_{10})} (e^{-k_{10}t} - e^{-k_{01}t})$ $k_{10} = \frac{CL}{V}$	<pre> graph LR 0((0)) -- k01 --> 1((1)) 1 -- k10 --> Elimination </pre>
3	IV bolus	2	First order	$C(t) = Ae^{-\alpha t} + Be^{-\beta t},$ $k_{10} = \frac{CL}{V_1}, \quad k_{12} = \frac{CL_{D2}}{V_1},$ $k_{21} = \frac{CL_{D2}}{V_2}$	<pre> graph TD Input -- Bolus IV --> 1((1)) 1 -- k10 --> Elimination 1 <--> k12 2((2)) 2 <--> k21 1 </pre>
4	First-order extravascular	2	First order	$C(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-k_{01}t},$ $k_{10} = \frac{CL}{V_1}, \quad k_{12} = \frac{CL_{D2}}{V_1},$ $k_{21} = \frac{CL_{D2}}{V_2}$	<pre> graph LR 0((0)) -- k01 --> 1((1)) 1 -- k10 --> Elimination 1 <--> k12 2((2)) 2 <--> k21 1 </pre>
5	IV bolus	3	First order	$C(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$	<pre> graph TD Input -- Bolus IV --> 1((1)) 1 <--> k12 2((2)) 2 <--> k21 1 1 <--> k31 3((3)) 3 <--> k13 1 </pre>

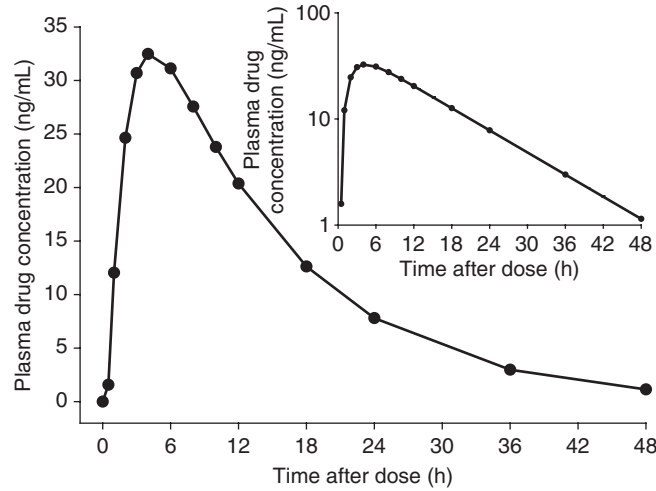


Figure 15.5 Plasma drug concentration–time profile following oral administration of a drug on a linear scale and a semilogarithm scale (inset).

For $t \geq T$,

$$C = \frac{k_0}{k_{10} V_d} [1 - e^{-k_{10}(T-t_0)}] e^{-k_{10}(t-T)} \quad (15.11)$$

where T represents the time at which absorption stops, k_0 the zero-order absorption rate constant, k_{10} the first-order elimination rate constant, V_d the apparent volume of distribution, and t_0 the lag time.

In some cases, plasma concentrations rise quickly to a high level followed with an exponential decrease. The observed data around the peak are underestimated by the first-order absorption. The combination of zero- and first-order input sometimes can give the best fitting to this type of concentration–time profile [7]. Nevertheless, the zero- and first-order absorption processes are mostly observed for many drugs. The Wagner–Nelson (W–N) method, Loo–Riegelman (L–R), deconvolution, nonlinear regression analysis, and several other methods have been used to estimate zero- and first-order absorption kinetics.

15.5.1.1 Wagner–Nelson (W–N) Method. The one-compartment model is based on the assumption that the dose absorbed is distributed into a single compartment and is eliminated from it by first-order process of metabolism and excretion. This is represented schematically in Table 15.3. Following the principle of mass balance, the amount of unchanged drug reaching the fluid of distribution is the amount of drug absorbed at any time, A_t , by the central compartment, the blood stream. The drug absorbed at a given time is equal to the amount of the drug existing in the central compartment plus the amount metabolized and excreted by all pathways and routes. Wagner and Nelson [8,9] derived several absorption equations on the basis of this mass balance for analyzing single-dose one-compartment absorption kinetic data. Since the rates of metabolism and excretion are often found to be directly proportional to the

blood concentration, they proposed that the amount of the drug eliminated by the time t could be estimated by

$$k V_d \int_{t_0}^t C_p dt \quad (15.12)$$

where V_d is the apparent volume of distribution, C_p the plasma concentration of drug at the time when drug is absorbed, and k the first-order rate constant for the elimination of the drug from the body and is equal to the sum of the individual rates of metabolism and excretion. Therefore, integration of the differential mass balance equation over the time range of t_0 and t_∞ results in what has been called *W-N absorption equation*:

$$\left(\frac{A}{V_d} \right)_t = C_p + k \int_{t_0}^t C_p dt \quad (15.13)$$

With Equation 15.13, the amount of the drug absorbed at time t expressed in a concentration unit can be evaluated as being the sum of drug concentration in the systemic circulation and the amount eliminated in a concentration unit. When the time approaches to infinity, the amount of the drug absorbed will asymptotically reach the total amount of the drug absorbed. Since the apparent volume of distribution may not be known without IV PK data, often, Equation 15.13 is represented as the fraction of bioavailable drug absorbed, $(F_{ab})_t$, at time t :

$$(F_{ab})_t = \frac{C_p + k \int_{t_0}^t C_p dt}{k \int_{t_0}^{\infty} C_p dt} = \frac{C_p + k \cdot AUC_{0-t}}{k \cdot AUC_{\infty}} \quad (15.14)$$

To illustrate the procedure for calculating the fraction of bioavailable drug absorbed, a set of concentration–time data following oral administration of a 200-mg dose of drug is listed in Table 15.4 and displayed in Fig. 15.5. The partial AUC_{0-t} value to each time point is calculated by noncompartmental method following the trapezoidal rule. The k value is estimated from the terminal phase of concentration–time profile with a terminal half-life of 8.65 h. As a result of this terminal half-life, the k value is estimated to be 0.0801/h. The product of $k \cdot AUC_{0-t}$ is then calculated and added to the plasma concentration at time t to yield $(A/V_d)_t$. The $AUC_{0-\infty}$ is calculated by the following equation:

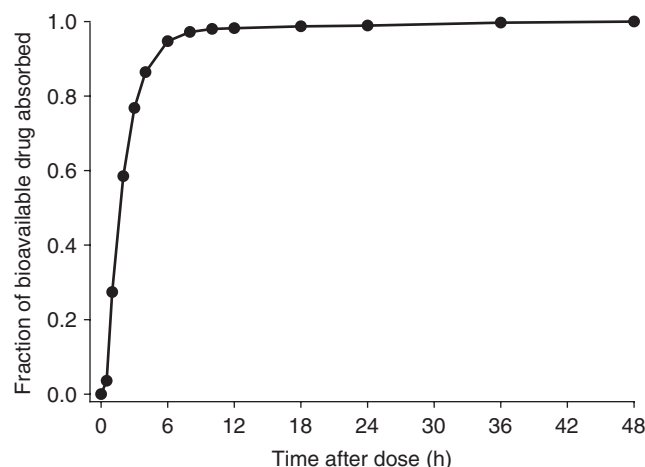
$$AUC_{0-\infty} = AUC_{last} + \frac{C_{last}}{k} \quad (15.15)$$

where C_{last} and AUC_{last} are the concentration of drug at the last PK sampling point and the AUC value from time 0 to the last time point, respectively. With known k and $AUC_{0-\infty}$ values, the product of $k \cdot AUC_{0-\infty}$ is obtained. Finally, the fraction of bioavailable drug absorbed is calculated by Equation 15.14 and is presented in Fig. 15.6.

TABLE 15.4 Calculation of Cumulative Fraction of Drug Absorbed Using W–N Method

Time (h)	C_p (ng/mL)	AUC_{0-t} (ng h/mL)	$k \cdot AUC_{0-t}$ (ng/mL)	$C_p + k \cdot AUC_{0-t}$ (ng/mL)	$(F_{ab})_t$
0	0	0	0	0	0
0.5	1.58	0.396	0.147	1.73	0.0357
1	12.0	3.80	1.41	13.4	0.274
2	24.7	22.1	8.21	32.9	0.585
3	30.7	49.8	18.5	49.2	0.768
4	32.5	81.4	30.2	62.6	0.864
6	31.1	145	53.7	84.9	0.947
8	27.6	204	75.5	103	0.972
10	23.8	255	94.5	118	0.980
12	20.4	299	111	131	0.982
18	12.6	398	148	160	0.987
24	7.81	460	170	178	0.989
36	2.99	524	194	197	0.997
48	1.14	549	203	205	1.000

$k = 0.0801/\text{h}$.

**Figure 15.6** Fraction of bioavailable drug absorbed as function of time after dose.

To utilize W–N method to analyze absorption behavior of a drug, it is not necessary to have IV data available for this drug. The issue with oral PK data alone is inaccuracy in estimating elimination rate constant, k . This is because in some cases, absorption following oral administration is taking place at the same time as drug clearance, tri-exponential drug distribution into the peripheral tissue compartment, or absorption continues well beyond the peak in the concentration–time profile. The mixed distribution and elimination phase frequently shows a longer terminal half-life compared to that following an IV bolus infusion in which the drug is instantly distributed into the blood stream. Therefore, accurate estimate of elimination rate constant has to rely on the PK data following IV bolus infusion.

15.5.1.2 Loo–Riegelman (L–R) Method. When a drug has been administered intravenously first, then extravascularly second, disposition of the drug shows bi- or triexponential. Loo and Riegelman [10] have proposed a mathematically and physiologically more acceptable method to conceive the body to be a two-compartment open system. The scheme and equation for this type of model are presented in Table 15.3.

It is convenient to express all the drug amounts as concentration terms by dividing by V_p , whereupon they become the concentrations in central (C_p) and tissue (C_t) compartments as well as the concentrations of metabolized and excreted drug (C_{me}). Therefore, C_{me} up to time t becomes

$$(C_{me})_{in} = k_{10} V_d \int_{t_0}^t C_p dt \quad (15.16)$$

Integration of the differential equation between the time limits of 0 and t obtains

$$\left(\frac{A}{V_p} \right)_{in} = (C_p)_{in} + k_{10} V_d \int_{t_0}^t C_p dt + (C_t)_{in} \quad (15.17)$$

This is a form of modified W–N equation for the two-compartment model. Taking the similar approach to W–N equation, the fraction of bioavailable drug absorbed, $(F_{ab})_t$, through a first-order process and eliminated through a second-order process at time t becomes

$$(F_{ab})_t = \frac{C_p + C_t + k_{10} \int_{t_0}^t C_p dt}{k_{10} \int_{t_0}^{\infty} C_p dt} = \frac{C_p + C_t + k_{10} \cdot AUC_{0-t}}{k_{10} \cdot AUC_{\infty}} \quad (15.18)$$

The concentration of drug in the central compartment C_p can be determined with plasma samples using a validated bioanalytical method. However, the concentration of the drug in peripheral (tissue) has to be estimated from the distribution rate constants between central and peripheral compartments with an assumption of a linear function on drug concentrations in the central compartment between two mostly closed time points. After approximation, the differential equation of

$$\frac{dC_t}{dt} = -k_{21} C_t + k_{12} C_p \quad (15.19)$$

becomes integratable by Laplace transforms and the second term of the equation can be simplified by a two-term Taylor approximation. Therefore, the concentration of drug in tissue compartment can be estimated by the following equation:

$$(C_t)_{t_n} = \frac{k_{12} \cdot \Delta C_p \cdot \Delta t}{2} + \frac{k_{12}}{k_{21}} (C_p)_{t_{n-1}} \cdot (1 - e^{-k_{21} \cdot \Delta t}) + (C_t)_{t_{n-1}} \cdot e^{-k_{21} \cdot \Delta t} \quad (15.20)$$

where t_n and t_{n-1} are the sample times for samples n and $n - 1$, respectively, $(C_p)_{t_{n-1}}$ is the concentration of drug at the central compartment for samples $n - 1$ and $(C_t)_{t_n}$

and $(C_t)_{n-1}$ are the concentrations of drug at the tissue compartment for samples n and $n - 1$, respectively.

15.5.1.3 Gamma Model of Absorption. Absorption of some drugs that show flat and delayed absorption profiles with a correlation between delay and peak width may not obey the zero- or first-order absorption model described above. A gamma model that describes an asymmetric S-shaped curve with an early flat phase was developed by Debord *et al.* [11] to predict oral cyclosporin PK profile:

$$\frac{dA}{dt} = FD \cdot f(t) \quad (15.21)$$

$$f(t) = \frac{b^a}{\Gamma(a)} t^{a-1} e^{-bt} \quad (15.22)$$

where dA/dt denotes the absorption rate at time t , a the number of sequential compartments connected by an identical input rate constant b (Fig. 15.7), and Γ the gamma function defined by

$$\Gamma(a) = \int_0^{\infty} x^{a-1} e^{-x} dx \quad (15.23)$$

The convolution of the absorption rate at time t dA/dt with the impulse response of a two-compartment model gives the equation for drug blood concentration at time t :

$$I(t) = A_1 e^{-\lambda_1(t)} + A_2 e^{-\lambda_2(t)} \quad (15.24)$$

where $I(t)$ is the concentration at time t following, A_1 and A_2 are the disposition coefficients, and λ_1 and λ_2 are the disposition rate constants.

Figure 15.8 shows an example in which a good fit is obtained with the gamma function, whereas a poor fit is obtained with a classic exponential model with lag time.

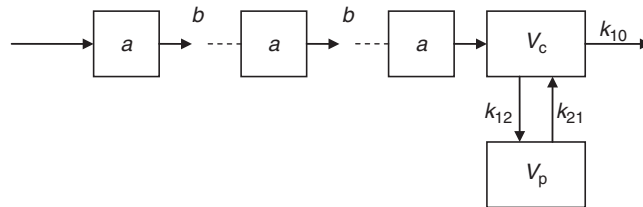


Figure 15.7 Gamma distribution model as a sequence of identical compartments (a) connected by an equal rate constant (b) with an input toward a central compartment followed with a distribution process between the central compartment and peripheral compartment and with a linear elimination process from the central compartment.

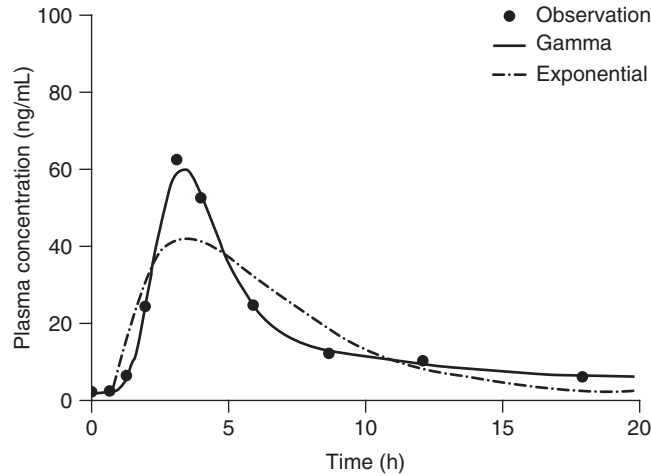


Figure 15.8 Comparison of gamma absorption and a classic exponential with lag-time models.

15.5.1.4 Discontinuous Absorption Model. Delayed gastric emptying of a portion of an orally administered dose can cause double peaks or irregular shape in the absorption and early distribution phases of concentration–time profiles. The factors other than delayed gastric emptying, such as enterohepatic recirculation, storage, and subsequent release of drug from a postabsorptive depot site, may also be responsible for the double peaks. However, the second peak under these circumstances may occur in the distribution phase of the concentration–time profile. A discontinuous absorption model has been developed to describe a drug absorption process along the gastric tract with distinct double peaks in the early phase of concentration–time profile.

The discontinuous absorption model [12] assumes that two absorption sites exist separately in the GI tract. The entire dose is loaded into the first gut compartment with a zero-order input and the drug in the first gut compartment transfers to the second gut compartment, which is the first absorption site. Absorption to the central compartment does not take place in intervening regions between the two absorption sites. When the entire drug moves to the second absorption site, absorption ceases. The amount of drug entering the first absorption site is dependent on the dose and the rate of drug entering the site (k_1). The transition rate (k_t) into the second absorption site is dependent on the efficiency of the first absorption site, the distance between the two absorption sites, and the transition rate in the nonabsorption regions of the gut. All rate constants in the discontinuous model, including transition rate constants in the gut (k_1 and k_t), absorption rate constants (k_{a1} and k_{a2}), and elimination rate constant (k_{10}) are assumed to be first-order rate constants (Fig. 15.9). The equation to describe the fraction of drug in each compartment is solved using Laplace transforms. The drug in central compartment is given by

$$L(A_C) = \frac{[k_{a1} \cdot L(A_2) + k_{a2} \cdot L(A_N)](s + k_{21})}{[(s + k_{21})(s + k_{10} + k_{12})] - k_{21} \cdot k_{12}} \quad (15.25)$$

where $L(A_C)$, $L(A_2)$, and $L(A_N)$ are the Laplace transforms for drug flux in the central compartment and the two absorption compartments, respectively, and s is the Laplace operator.

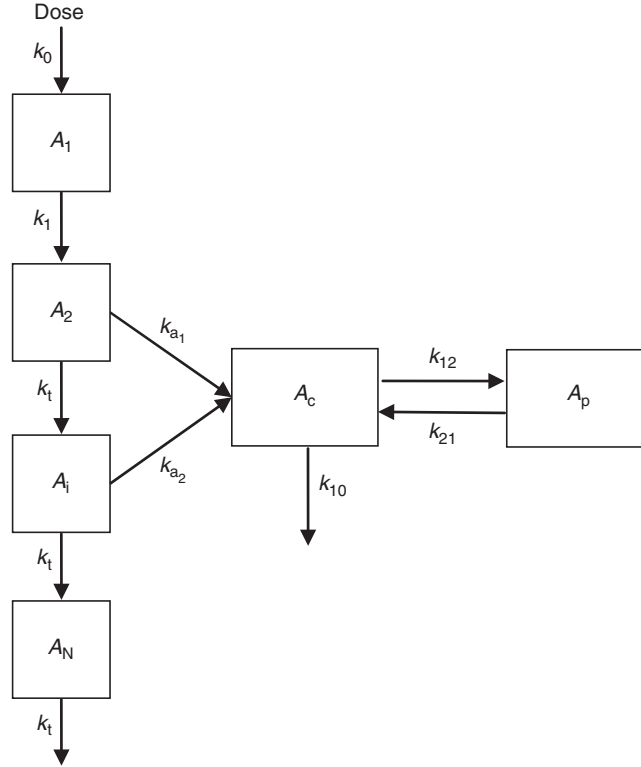


Figure 15.9 Discontinuous oral absorption pharmacokinetic model.

The ratio of the absorption rate constant at one specific site to the sum of all rate constants in this absorption compartment represents the theoretical fraction of drug absorbed at this site. For example, the fractions of drug absorbed at the first and second absorption sites F_1 and F_2 , respectively, are as the follows [12]:

$$F_1 = \frac{k_{a1}}{k_t + k_{a1}} \quad (15.26)$$

$$F_2 = \frac{(1 - F_1)k_{a2}}{k_t + k_{a2}} \quad (15.27)$$

The simulated effect of changes in the number of gut compartments (N), transition rate constant (k_t), and absorption rate constants (k_{a1} and k_{a2}) are plotted in Fig. 15.10. As N increases, the time to reach the second peak is delayed and the concentration in the second compartment decreases. Where the k_t value becomes large, the second peak occurs shortly after the first peak and the fraction of drug absorbed from both the absorption sites is also reduced. The values of k_{a1} and k_{a2} determine the height of these two peaks. The high absorption rate produces a high peak associated with this absorption process.

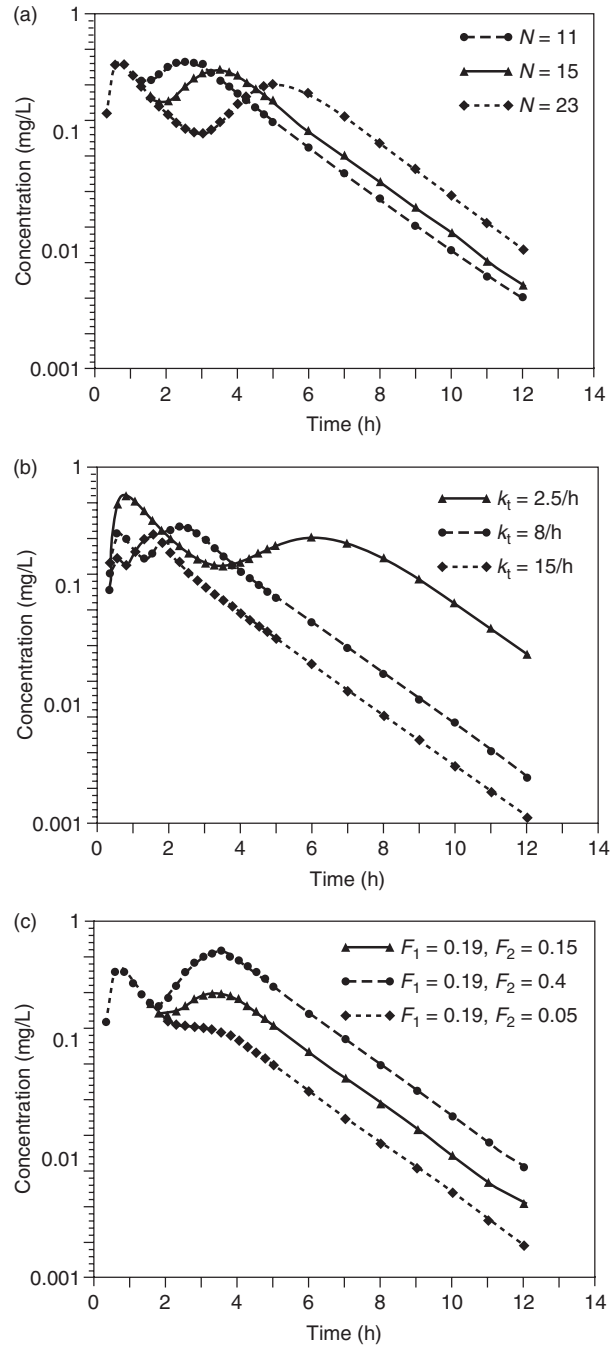


Figure 15.10 Simulated concentration–time profile following oral administration of a hypothetical drug based on a discontinuous gastrointestinal absorption model [12]. (a) Influence of the total number of gut compartments (N). (b) Influence of drug transition rate (k_t) between absorption sites. (c) Influence of the fraction of drug absorbed at the first and second absorption sites.

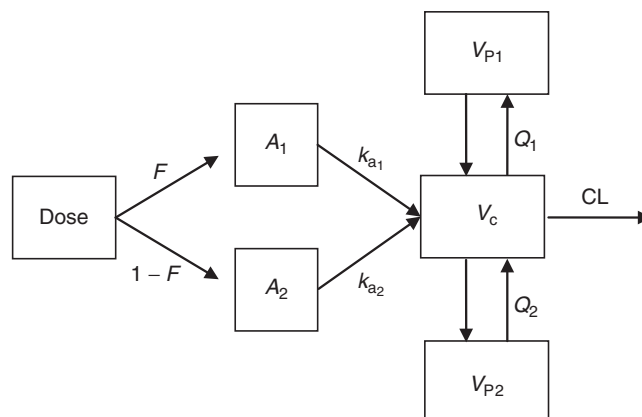


Figure 15.11 Parallel oral absorption pharmacokinetic model.

15.5.1.5 Parallel Absorption Model. When two or more concentration peaks are not distinct in a concentration–time profile, a parallel absorption model can be used to fit these PK data. Figure 15.11 shows the scheme of a dual absorption pathway model with two different first-order absorption rate constants (k_{a1} and k_{a2}) and two different lag times. The drug administered enters two different gut compartments with the different fractions of dose, F and $1 - F$, divided into the rapidly and delayed absorbed subdoses. The drug in the gut compartments is then absorbed into the central compartment in which the drug is either exchanged between the central compartment and two different peripheral compartments or eliminated from the central compartment. A two-compartment model may fit the two-peak concentration–time profiles with less precise parameter estimates due to underparameterization. A three-compartment model is in general superior to the two-compartment model in fitting the slowly declining terminal phase of concentration–time data. The three-compartment model with two parallel absorption processes schematically depicted in Fig. 15.11 was used to describe the PK of piperazine administered with concomitant CV8 [13].

In summary, a delayed peak or multiple peaks that are not so uncommon atypical absorption phenomena after oral drug administration may occur due to the delayed release of drug from its dosage form, cyclical gastric emptying, or influences by intake of food. The clinical implication of these atypical absorption processes should be considered. The compounds with a delayed peak may not be suitable as a medication to treat disorders where rapid $t_{\max}$ and high $C_{\max}$ are important in clinical efficacy. Compounds with multiple peaks may cause some safety issues, especially for those drugs with a narrow therapeutic index.

15.5.2 Evaluation of Distribution Kinetics from Plasma Concentration–Time Data

Drug distribution in the body can be influenced by different processes, including entero-hepatic recirculation, various transport mechanisms, and drug binding to tissue and proteins as well as metabolism. In some cases, the distribution curve of drugs is significantly affected by these processes so that the normal pattern of smooth decreases

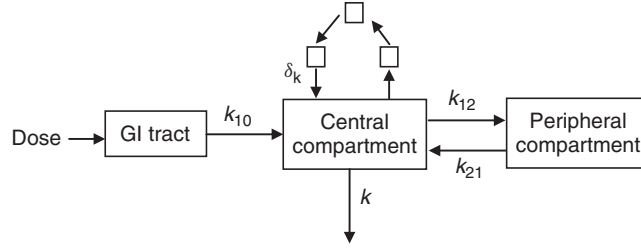


Figure 15.12 The enterohepatic recirculation model consists of a central compartment, a peripheral compartment, and the circulation chain that includes three compartments each of fixed volume.

in plasma concentrations does not exist, resulting in double peaks or multiple peaks when enterohepatic recirculation is the primary process of drug distribution. The typical compartmental models are no longer applied to this type of distribution. More complex PK models are needed to predict drug concentration in circulation that will provide useful information regarding optimal dosing and potential exposure–response correlation.

15.5.2.1 Modeling Enterohepatic Recirculation. The drugs that experience enterohepatic recirculation will show at least two peaks in the plasma concentration–time profile. After oral administration, a dose D reaches the absorption compartment (GI tract). A fraction of the dose is absorbed by a first-order process reaching the central compartment. The drug absorbed is distributed from the central compartment 1 to the peripheral compartment 2 and is eliminated from the central compartment 1 by first-order process if the concentration–time profile can be described by a two-compartment model. Some fraction of the dose absorbed in the central compartment will be added back to the systemic circulation at time τk as a secondary input into the systemic circulation (Fig. 15.12). A two-compartment PK model incorporated with enterohepatic recirculation is as follows [14]:

$$C_j(t) = C_{\text{pred},j}(t) + \delta_1 \cdot C_{\text{pred},j}(t - \tau_1) + \delta_2 \cdot C_{\text{pred},j}(t - \tau_2) + \cdots + \delta_n \cdot C_{\text{pred},j}(t - \tau_n) + \varepsilon_j(t) \quad (15.28)$$

where $C_j(t)$ and $C_{\text{pred},j}(t)$ are the measured and predicted concentrations for the j th subject at time t , respectively, and δ fraction of drug due to enterohepatic recirculation is added back to the systemic circulation at time τk where k is the number of events. An assumption is held for this model that a drug accumulates in a certain compartment or compartments and retains there for some time, then it returns into the circulation system, which eventually results in the second peak of $C_j(t)$. The phenomenon of the second peak in the distribution phase has been observed in species that have a gall bladder, when hepatic recirculation of drug occurs as periodical release of bile into the duodenum with subsequent reabsorption of drug into the circulation system. Random effects, ε , were supplemented to the fraction that will be added back to the circulation and the gall bladder emptying time to account for variability. The model using the nonlinear mixed-effects function provided a good fitting of most individual

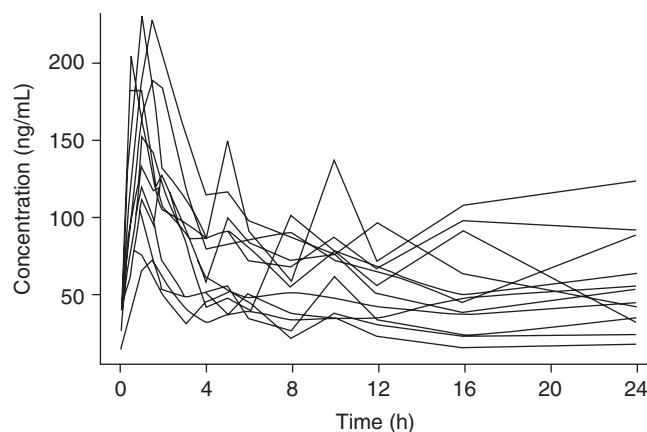


Figure 15.13 Plasma ezetimibe concentration–time profiles with enterohepatic recirculation after multiple-dose administration of ezetimibe [14].

plasma concentration–time profiles for ezetimibe following dose administration [13] and is presented in Fig. 15.13.

15.5.2.2 Modeling Blood–Brain Barrier Transport. Compartmental PK analysis has been applied to characterize the drug concentration–time course in systemic circulation and the brain extracellular fluid (bECF) simultaneously with the advantage over traditional noncompartmental methods where the compartmental analysis quantifies the rate of blood–brain barrier (BBB) transport independent of differences in systemic exposure. This allows comparison of *in vivo* with *in vitro* PK parameters and comparison between compounds that have different systemic PK properties. Furthermore, modeling the bidirectional distribution to and from the brain may reveal the process that determines the time course of drug effect to help design preclinical and clinical studies and interpret pharmacological data from these studies.

A two-compartment model in systemic circulation connected to a one-compartment brain model that is separated by a BBB was proposed to fit unbound plasma and bECF escitalopram concentrations simultaneously in rats [15]. This structure of the model is depicted in Fig. 15.14. Using intracerebral microdialysis, quantitative information about the free drug concentrations at the extracellular target site in the discrete areas of the rodent brain can be obtained. The general differential equations associated with this model are as follows:

$$\frac{dC_1}{dt} = \frac{\text{input}}{V_1} + \frac{k_{21}C_2V_2}{V_1} + \frac{k_{\text{out}}C_{\text{bECF}}V_{\text{bECF}}}{V_1} - (k_{10} + k_{12} + k_{\text{in}})C_1 \quad (15.29)$$

$$\frac{dC_2}{dt} = \frac{k_{12}C_1V_1}{V_2} - k_{21}C_2 \quad (15.30)$$

$$\frac{dC_{\text{bECF}}}{dt} = \frac{k_{\text{in}}C_1V_1}{V_{\text{bECF}}} - k_{\text{out}}C_{\text{bECF}} \quad (15.31)$$

where C_1 and C_2 denote the drug concentrations in the central and peripheral plasma compartments and C_{bECF} is the extracellular concentration in the brain. Input represents

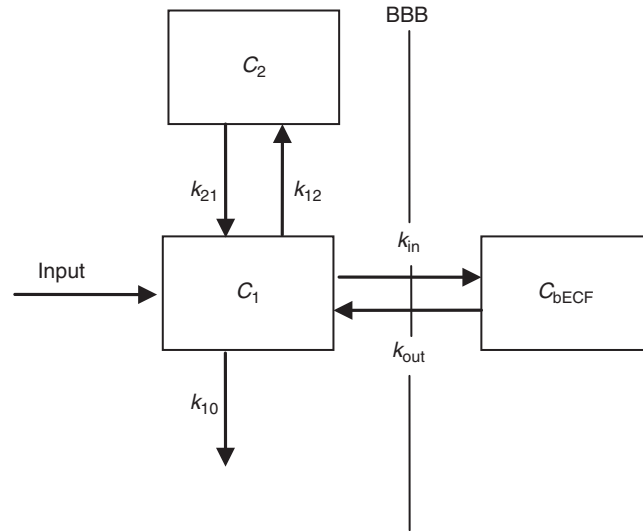


Figure 15.14 Compartmental model of drug transport between plasma and brain extracellular fluid following intravenous injection.

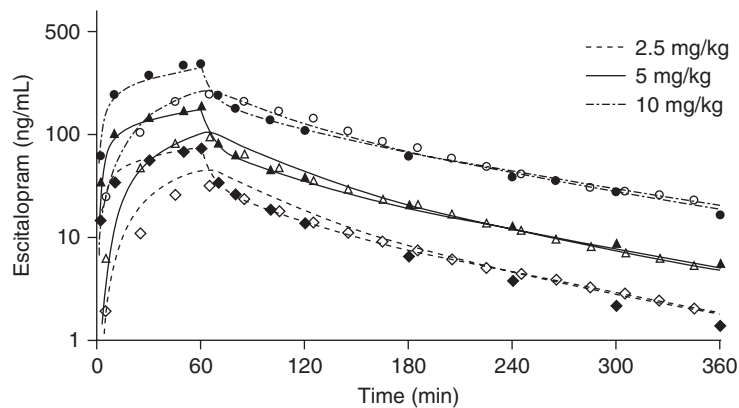


Figure 15.15 Observed (mean) and model-predicted unbound escitalopram concentration–time profiles in plasma (filled symbols) and brain extracellular fluid (open symbols) following intravenous infusion of 2.5-, 5-, and 10-mg/kg escitalopram [15].

the administered dose via IV injection. The k and V signify the rate constant and volume of distribution as detailed in Fig. 15.14. This model was successfully fit to the unbound escitalopram concentration–time profiles in plasma and bECF obtained in an experiment in rats (Fig. 15.15).

Solving the compartmental model provides both the extent and rate of drug transport to brain. The extent of equilibrium across the BBB is influenced by the involvement of active membrane transport mechanisms located at the endothelial cells in the BBB, whereas the rate of drug transport across the BBB is related to physiochemical properties of drug. These PK characteristics will affect the degree, onset, and duration of the

centrally mediated effect. A final BBB model is established on the basis of an iterative analysis of the experimental data using a variety of different models. Both systemic and brain models vary from one- to multiple-compartments and from a linear model to a nonlinear model such as Michaelis–Menten (M–M) model. The goodness-of-fit is employed to select the best model for fitting the experimental data.

15.5.3 Evaluation of Metabolism Kinetics from Plasma Concentration–Time Data

15.5.3.1 Compartmental Analysis of Metabolites. The data analysis of metabolite plasma concentration–time data using a compartmental approach is similar to the analysis for the disposition of parent drug in systemic circulation. Compartmental modeling to include metabolites is more complicated than that for parent drug alone because this type of model need not only characterize parent drug but also requires a link between parent and metabolite as well as possible involvement of nonlinear kinetics. For modeling with parent drug only, the methodical, conceptual, and computational uniformity in modeling various linear biological systems is the dominant characteristic of the linear system approach technology. However, saturation of the metabolic reaction results in nonlinear kinetics.

Figure 15.16 represents an integrated open two-compartment model for a parent drug and an open one-compartment model for the metabolite of the parent drug. Assuming

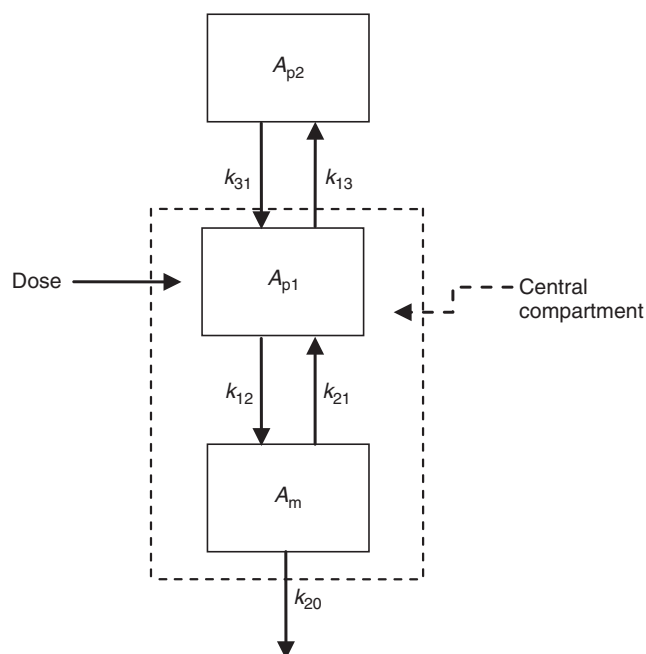


Figure 15.16 A compartment model in which a parent drug is characterized by a two-compartment model and a metabolite is assessed by a one-compartment model. A_{p1} and A_{p2} are the amount of a parent drug in the central compartment and peripheral compartment, respectively. A_m is the amount of the metabolite in the central compartment of the metabolite.

both input and disposition functions are linear, the model can be solved by the Laplace transform with a general fraction theorem. The amount of parent drug (A_{p1}) in the central compartment may be written [16]:

$$A_{p1} = \frac{(k_{20}k_{21} - \alpha)(k_{31} - \alpha)D}{(\beta - \alpha)(\gamma - \alpha)}e^{-\alpha t} + \frac{(k_{20}k_{21} - \beta)(k_{31} - \beta)D}{(\alpha - \beta)(\gamma - \beta)}e^{-\beta t} + \frac{(k_{20}k_{21} - \gamma)(k_{31} - \gamma)D}{(\alpha - \gamma)(\beta - \gamma)}e^{-\gamma t} \quad (15.32)$$

where α , β , and γ are coefficients of the powers, $k_{i,j}$ is the first-order rate constant describing transfer from compartment i to compartment j , t is the time postdose, and D is the dose. The amount of metabolite (A_m) in the central compartment can be expressed as follows:

$$A_m = \frac{k^\circ(k_{20}k_{21} - \alpha)(k_{31} - \alpha)(1 - e^{ab})}{-\alpha(\beta - \alpha)(\gamma - \alpha)}e^{-\alpha t} + \frac{k^\circ(k_{20}k_{21} - \beta)(k_{31} - \beta)(1 - e^{\beta b})}{-\beta(\alpha - \beta)(\gamma - \beta)}e^{-\beta t} + \frac{k^\circ(k_{20}k_{21} - \gamma)(k_{31} - \gamma)(1 - e^{\gamma b})}{-\gamma(\alpha - \gamma)(\beta - \gamma)}e^{-\gamma t} \quad (15.33)$$

where k° is zero-order infusion rate in units of amount per time and b the time when infusion ends. The above equation applies when the infusion begins at a time zero. For continuous infusion, b is equal to t and varies with t . When infusion ends, b becomes constant corresponding to the duration of infusion. In the above model, drug metabolism is described by linear models assuming the concentration of the involved enzyme is high with respect to the drug concentration. However, in many cases, drug concentration in systemic circulation is much higher than enzyme concentration at a metabolic reaction site. This leads to saturation of drug at the enzyme reaction sites, resulting in zero-order kinetics. While drug concentration is at intermediate level, the M–M equation, a nonlinear model, appropriately describes drug disposition, as discussed in Section 15.5.4.3.

A parent–metabolite model was developed to assess the disposition of artesunate (AS) and its active metabolite dihydroartemisinin (DHA) in healthy subjects receiving single or multiple dosing of AS orally [17]. The structure of the model is depicted in Fig. 15.17, which consists of a one-compartment model with first-order absorption and first-order elimination describing the AS concentration–time profile and a two-compartment model with one central and one peripheral compartment for DHA. With this model, the data for both AS and DHA were analyzed simultaneously with a population PK approach nonlinear mixed effects modelling (NONMEM). The model was stable and was able to predict AS and DHA concentration–time data following single and repeat dosing of oral AS equally well.

15.5.3.2 Compartmental Analysis of Drug–Drug Interaction. Concomitantly administered drugs often cause one-direction or mutual impact by increasing or decreasing the systemic exposure of drugs. As a result of the interaction, the probability of causing adverse events may be higher due to increased drug concentration in blood or efficacy of the affected drug may decrease with lowered plasma drug concentration. In human drug–drug interaction (DDI) studies, both parent drug and metabolite

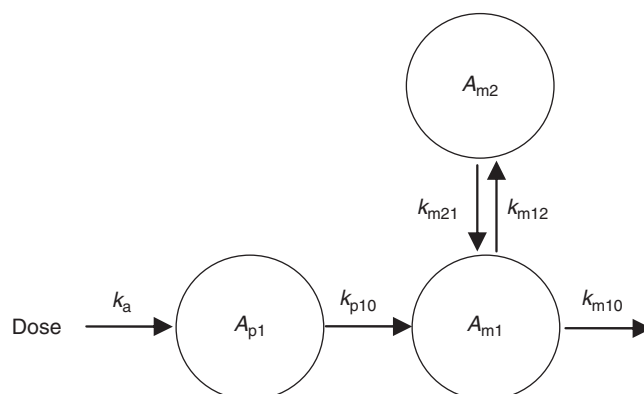


Figure 15.17 Scheme of a parent-metabolite model following oral administration of a parent drug. A_{p1} is the amount of a parent drug in the central compartment. A_{m1} and A_{m2} are the amount of the metabolite in the central and peripheral compartments of the metabolite, respectively. k_a is the absorption constant. k_{p10} and k_{m10} are the elimination rate constants for the parent and the metabolite, respectively. k_{m12} and k_{m21} are the distribution rate constants for the metabolite.

plasma concentration-time profiles may be assessed simultaneously. Evaluation of the PK parameters of the parent drug and its metabolite(s) can provide information on DDI. In an example presented below, the mutual effect of two anticancer agents thiotepa (TT) and cyclophosphamide (CP) is evaluated by a compartment PK model [18].

The prodrug (CP) is metabolized and bioactivated by CYP isoenzymes to form 4-hydroxycyclophosphamide (HCP) that exerts cytotoxicity in the target cells [18]. The resulting metabolite, HCP, is further metabolized to phosphoramidate mustard (PM). These metabolites induce CYP enzymes through a process called *autoinduction*. CP is also metabolized through a noninducible side-chain oxidation, resulting in the formation of 2-dechloroethylcyclophosphamide. On the other hand, TT is metabolized to its main metabolite tepa (T) mediated by CYP2B and 2C. T has pharmacological properties similar to parent TT. When CP is coadministered with TT, the latter inhibits the CYP enzymes responsible for the biotransformation of CP to HCP, resulting in a decrease in HCP concentrations. Meanwhile, CP increases the rate of metabolism of TT to T. These processes represent a mutual DDI between CP and TT and make PK modeling more complicated.

The compartment PK DDI model is schematically pictured in Fig. 15.18. In this model, 2 two-compartment models with first-order elimination from the central compartments of TT and T, respectively, are used to characterize the concentration-time profiles of both TT and T. TT as a perpetrator inhibits CYP enzymes so that bioactivation from CP to HCP is reduced [18]. A two-compartment model and a one-compartment model describe the PK profiles of CP and HCP, respectively. Elimination from the central compartment of CP consists of two pathways, a noninducible route and an inducible route of which the latter leads to the formation of HCP. Both models to describe the time course of CP and TT include two different hypothetical enzyme compartments. In the first hypothetical enzyme compartment, the time course of CP autoinduction is modeled. Biotransformation from CP to HCP is directly proportional to the amount of enzyme in this compartment, whereas TT produces inhibition to

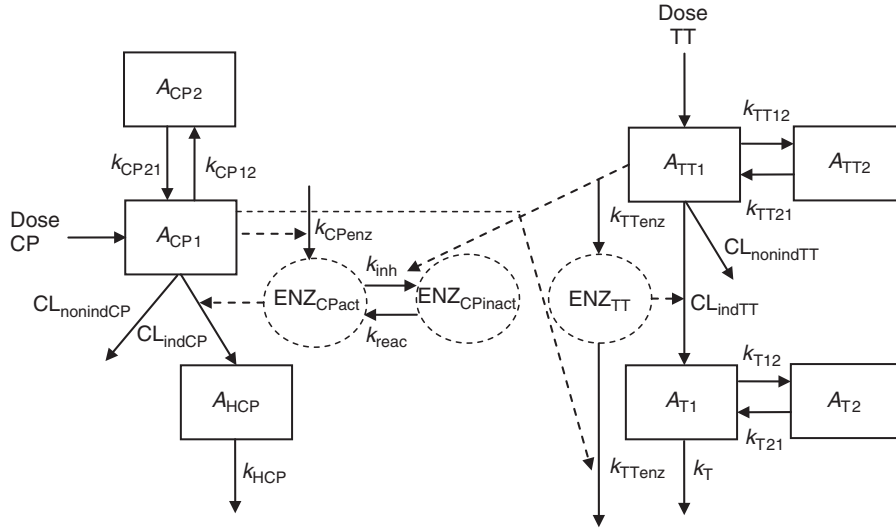


Figure 15.18 Integrated two-compartment models for mutual drug–drug interaction between cyclophosphamide and thiotepa [18].

the metabolism. In the second hypothetical enzyme compartment, an enzyme turnover model, including a zero-order enzyme formation model and a first-order enzyme elimination model, appropriately describes TT and T data. Enzyme elimination is inhibited in the presence of CP with an E_{max} model. When CP infusion starts, the amount of enzyme in the second hypothetical enzyme compartment begins increasing until a maximum amount of enzyme is reached as a result of decreased enzyme elimination by CP. The differential equations describing the mass transport of the different compartments of the model are as follows [18].

Differential equations for the central compartments:

$$\frac{dA_{CP1}}{dt} = R_{CP} - \frac{CL_{nonindCP}A_{CP1}}{V_{CP}} - \frac{CL_{indCP}A_{CP1}A_{ENZCP}}{V_{CP}} - k_{CP12}A_{CP1} + k_{CP21}A_{CP2} \quad (15.34)$$

$$\frac{dA_{TT1}}{dt} = R_{TT} - \frac{CL_{nonindTT}A_{TT1}}{V_{TT}} - \frac{CL_{indTT}A_{TT1}A_{ENZTT}}{V_{TT}} - k_{TT12}A_{TT1} + k_{TT21}A_{TT2} \quad (15.35)$$

$$\frac{dA_{HCP}}{dt} = \frac{CL_{indCP}A_{CP1}A_{ENZCP}}{V_{CP}} - k_{HCP}A_{HCP} \quad (15.36)$$

$$\frac{dA_{T1}}{dt} = \frac{CL_{indTT}A_{TT1}A_{ENZTT}}{V_{TT}} - k_{T12}A_{T1} + k_{T21}A_{T2} - k_TA_{T1} \quad (15.37)$$

Differential equations for the peripheral compartments:

$$\frac{dA_{CP2}}{dt} = k_{CP12}A_{CP1} - k_{CP21}A_{CP2} \quad (15.38)$$

$$\frac{dA_{TT2}}{dt} = k_{TT12}A_{TT1} - k_{TT21}A_{TT2} \quad (15.39)$$

$$\frac{dA_{T2}}{dt} = k_{T12}A_{T1} - k_{T21}A_{T2} \quad (15.40)$$

Differential equations for the enzyme compartments:

$$\frac{dA_{ENZCPact}}{dt} = \frac{k_{CPenz}C_{CP1}}{IC_{50} + C_{CP1}} - k_{inh}A_{TT1}A_{ENZCPact} + k_{reac}A_{ENZCPinact} \quad (15.41)$$

$$\frac{dA_{ENZCPinact}}{dt} = k_{inh}A_{TT1}A_{ENZCPact} - k_{reac}A_{ENZCPinact} \quad (15.42)$$

$$\frac{dA_{ENZTT}}{dt} = k_{TTenz} - k_{TTenz} \left(1 - \frac{E_{max}C_{CP1}}{IC_{50} + C_{CP1}} \right) A_{ENZTT} \quad (15.43)$$

where all the parameters in the differential equations are explained in Fig. 15.18.

This integrated model describes the mutual DDI between CP and TT successfully at the level of enzyme kinetics as shown in Fig. 15.19 where model-predicted concentrations and individual-predicted concentrations of CP, HCP, TT, and T are adequately fitted the corresponding observed concentrations analyzed with a population PK approach with NONMEM. The induction of TT clearance caused by CP, the inhibition of CP activation by TT, and CP autoinduction involving CYP enzymes are adequately characterized with acceptable precision ($RSE\% < 17\%$) and relatively small residual variability ($RSE\% < 79\%$) utilizing this model.

15.5.4 Evaluation of Elimination Kinetics from Plasma Concentration–Time Data

Most drugs are eliminated through hepatic, renal, and/or biliary processes that may follow the first-order or M–M saturable kinetics. Typically, the one-, two-, and three-compartment models are used to describe plasma concentration–time profiles with the elimination of drugs from the central compartment as shown with models and schemes in Table 15.3. Several nontraditional and emerging compartmental elimination PK models are discussed in this chapter.

15.5.4.1 Elimination from a Compartment Other than the Central Compartment.

If either one or two elimination pathways from the central compartment and another peripheral compartment are added to the normal scheme, the elimination rate constants will be overparameterized when solving the three-compartment PK equation. In fact, it is impossible to ascertain from drug concentrations in systemic circulation whether the model should have elimination from one, two, or three compartments. Disposition of dicumarol was modeled with a three-compartment model in which elimination occurred from one of the peripheral compartment only as shown in Fig. 15.20 [19]. The transfer and elimination processes in the three-compartment open system may be represented by the following diffusion equations:

$$\frac{dA_1}{dt} = -(k_{12} + k_{13})A_1 + k_{21}A_2 + k_{31}A_3 \quad (15.44)$$

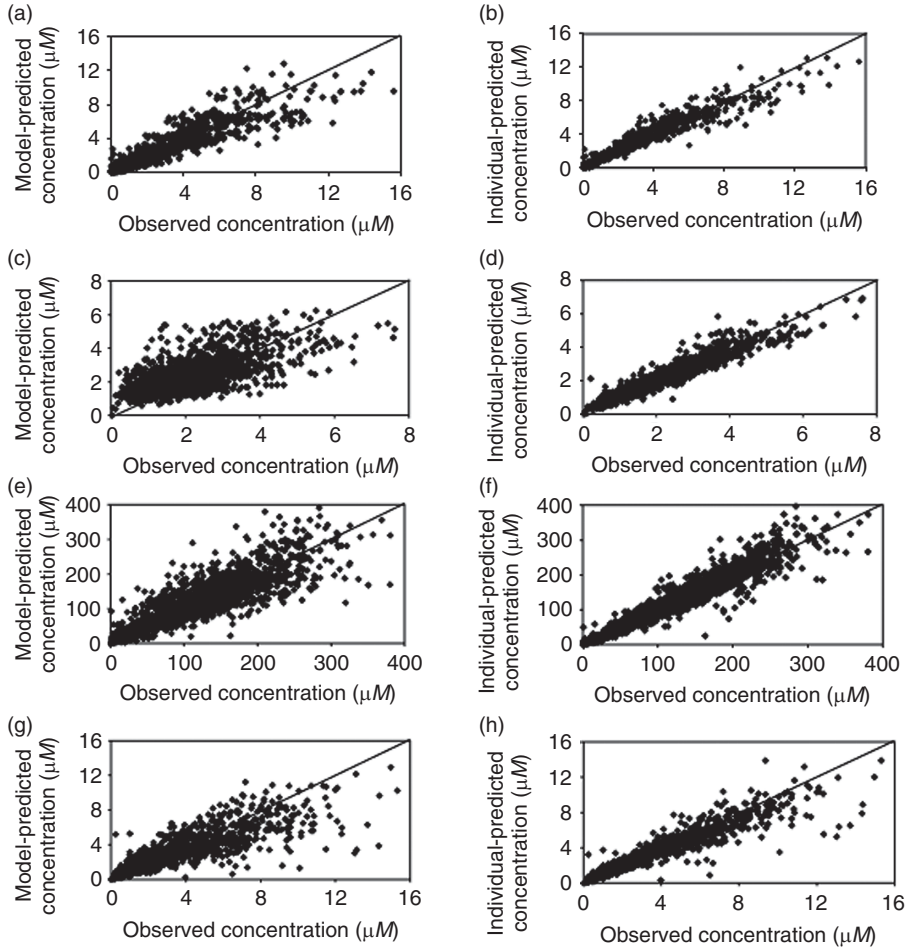


Figure 15.19 Model-predicted and individual-predicted concentrations versus the observed concentrations for thiotepa (a and b), tepa (c and d), cyclophosphamide (e and f), and 4-hydroxycyclophosphamide (g and h) [18].

$$\frac{dA_2}{dt} = -(k_{20} + k_{21})A_2 + k_{12}A_1 \quad (15.45)$$

$$\frac{dA_3}{dt} = -k_{31}A_3 + k_{13}A_1 \quad (15.46)$$

where A is the amount of drug in the designated compartment and k the rate constant.

By assuming that elimination occurred from the more rapidly accessible peripheral compartment, this model was used to describe the plasma concentration–time profile of dicumarol of the apparent first-order elimination rate constant decreased with increasing dose. This phenomenon is explained by the concentration-dependent inhibition of own biotransformation when the high concentrations of the drug are present in the liver and concentration-dependent protein binding of the drug, which was supported by the investigations with isolated perfused rat livers [20].

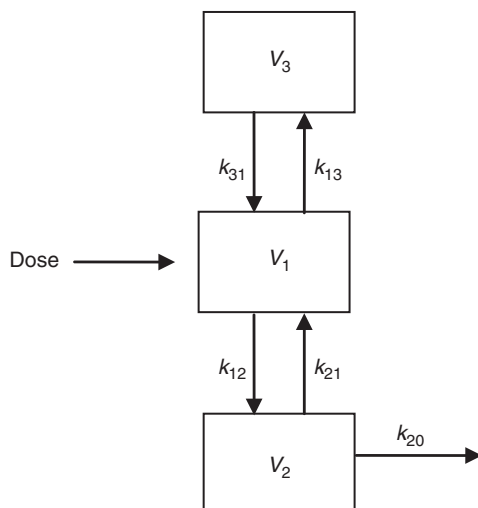


Figure 15.20 A three-compartment PK model for drugs that are eliminated from a peripheral compartment other than the central compartment.

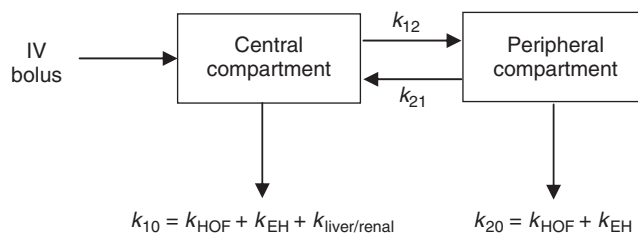


Figure 15.21 Scheme of the nontraditional two-compartment open model that elimination occurs from both the central and peripheral compartments (k_{HOF} = Hofmann elimination rate constant; k_{EH} = ester hydrolysis rate constant).

15.5.4.2 Elimination from Both Central and Peripheral Compartments. When a drug is eliminated through several pathways, including both central and peripheral compartments, traditional compartmental PK models—assuming elimination from a single central compartment—are inaccurate to assess PK data. For example, the V_{ss} value of atracurium besylate, a skeletal muscle relaxant, as underestimated for $\sim 17\text{--}20\%$ [21,22] when a traditional PK model was used. The nontraditional PK model was developed to fit the plasma concentration–time data of cisatracurium besylate, a cis–trans isomerism form of atracurium besylate [23]. The scheme of the nontraditional two-compartment open model is depicted in Fig. 15.21. The model can be solved by the following equations:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (15.47)$$

where

$$V_1 = \frac{\text{Dose}}{(A + B)} \quad (15.48)$$

$$A = \frac{\text{Dose}(k_{20} + k_{21} - \alpha)}{V_1(\beta - \alpha)} \quad (15.49)$$

$$B = \frac{\text{Dose}(k_{20} + k_{21} - \beta)}{V_1(\alpha - \beta)} \quad (15.50)$$

$$\alpha + \beta = k_{10} + k_{12} + k_{20} + k_{21} \quad (15.51)$$

$$\alpha \times \beta = (k_{10} \times k_{20}) + (k_{10} \times k_{21}) + (k_{12} \times k_{20})$$

Input rate:

$$R_{\text{input}} = C_{\text{ss}} \times Cl_{\text{total}} \quad (15.52)$$

Elimination rate:

$$R_{\text{elimination}} = (k_{10} \times A_{1\text{ss}}) + (k_{20} \times A_{2\text{ss}}) \quad (15.53)$$

where $A_{1\text{ss}}$ and $A_{2\text{ss}}$ are the amounts drug in the central and peripheral compartments, respectively, at steady state.

This model has been successfully applied to describe the PK profile of cisatracurium besylate that is eliminated through multiple pathways, including Hofmann elimination, ester hydrolysis, and liver and renal elimination [21,24]. Results from this modeling supported the idea that the major elimination pathway of cisatracurium besylate in patients was Hofmann elimination that is spontaneous degradation in plasma and tissue at normal body pH and temperature. Approximately 77% of CL of cisatracurium besylate was accounted for by Hofmann elimination, while the residual drug (23%) was accounted for by liver and renal elimination.

15.5.4.3 Nonlinear Elimination—Michaelis–Menten and Target-Mediated Drug Disposition. Saturation may occur in all drug disposition processes where enzymes, transporters, or active processes are involved in biotransformation, renal tubular secretion, and active transport and binding. These processes may be specific with respect to the drug and may have limited capacities. For these specialized processes, the use of M–M kinetics or target-mediated drug disposition (TMDD), a specific case of M–M drug disposition, or mixed-order kinetics is usually applicable. For a drug showing M–M PK, capacity limitation of drug disposition/clearance can occur in many processes, including hepatic metabolism and renal and biliary excretion of a drug. For a drug characterized by TMDD first introduced by Levy [25], the saturable disposition/clearance is controlled by the pharmacological target, such as enzymes or receptors, of the drug. The TMDD phenomenon where the temporal profile of plasma or serum drug concentrations of a drug is significantly affected due to its high affinity binding to a biological target with a large amount of drug relative to dose. Since it is difficult to obtain clinical data for target kinetics, M–M kinetics where only free or total drug concentrations in a biological fluid are measured has been applied to the TMDD

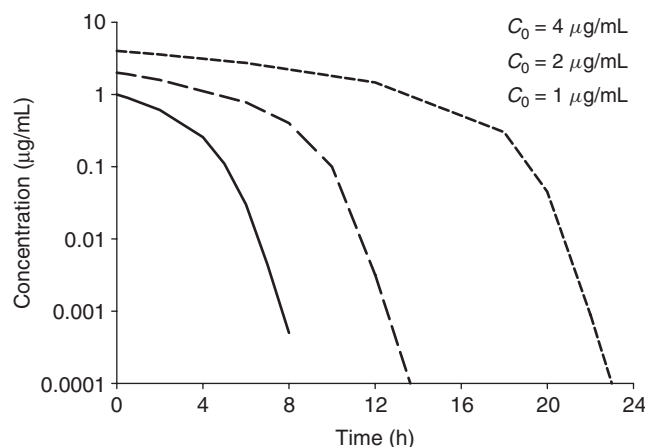


Figure 15.22 Semilogarithmic scale concentration–time profile simulated from Michaelis–Menten model with $V_m = 0.22$, $K_m = 0.1$, and $C_0 = 1, 2$, and $4 \mu\text{g/mL}$.

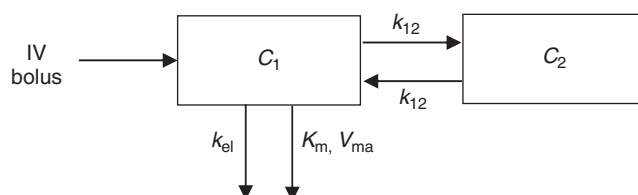


Figure 15.23 Scheme of two-compartment Michaelis–Menten model.

system with less precision. M–M and TMDD models have been used to characterize large molecular weight drugs such as monoclonal antibodies and some small molecular weight drugs such as angiotensin-converting enzyme (ACE) inhibitors [26], an aldose reductase inhibitor imirestat [27], and warfarin [28].

The typical semilogarithmic concentration–time profile of a drug fitting an M–M or TMDD model (Fig. 15.22) appears to be nonlinear with a fast elimination rate at the low dose and a slow clearance rate at the high dose. Depending on the distribution and elimination rates, some drugs with the M–M characteristics may show linear PK or the combination of linear and nonlinear PK initially after administration. The structure of a two-compartment M–M model is shown in Fig. 15.23. For IV bolus injection, drug in the central compartment (C_1) is eliminated from the system by the combination of a first-order rate constant and a saturable process described by the M–M equation. The initial conditions and model equations are as follows:

Initial conditions:

$$C_1(t) = 0, \quad \text{for } t < t_D \quad (15.54)$$

$$C_2(t) = 0, \quad \text{for } t \leq t_D \quad (15.55)$$

$$C_1(t_D) = \frac{D}{V}, \quad \text{for single dose} \quad (15.56)$$

$$C_1(t_{D_1}) = C_1^{(1)}(t_{D_1}) = \frac{D_1}{V}, \quad \text{for the first dose of multiple doses} \quad (15.57)$$

$$C_1(t_{D_n}) = C_1^{(n)}(t_{D_n}) = C_1^{(n-1)} + \frac{D_n}{V}, \quad \text{for multiple doses} \quad (15.58)$$

Model equations:

$$\frac{dC_1}{dt} = -\left(\frac{V_{\max}}{K_m + C_1}\right)C_1 - (k_{12} + k_{el})C_1 + k_{21}C_2 \quad (15.59)$$

$$\frac{dC_2}{dt} = k_{21}C_1 - k_{21}C_2 \quad (15.60)$$

where C_1 and C_2 denote free drug concentrations in the central and peripheral compartments, respectively, V is the volume distribution of the central compartment, k_{12} , k_{21} , and k_{el} are the first-order rate constants, K_m is the M–M constant, and V_m is the maximum elimination rate.

For TMDD, free drug in the central compartment (C_1) binds to its free target (R) at a second-order rate constant (k_{on}), forming a drug–target complex (RC_1), which can either be internalized and degenerated at a first-order rate (k_{int}) or dissociate at a first-order rate (k_{off}). The unbound drug can also be removed from the central compartment by a first-order elimination pathway (k_{el}) and distributed to the peripheral compartment (C_2). The targets are formed with a zero-order rate constant (k_{form}) and are degenerated with a first-order rate constant (k_{deg}). The structure of the general PK model of TMDD is presented in Fig. 15.24 and the equations combined with linear and TMDD elimination and IV bolus infusion are as follows:

$$\frac{dC_1}{dt} = \ln(t) - (k_{el} + k_{12})C_1 + k_{21}C_2 - k_{on}R \cdot C_1 + k_{off}RC_1 \quad (15.61)$$

$$\frac{dC_2}{dt} = k_{21}C_1 - k_{21}C_2 \quad (15.62)$$

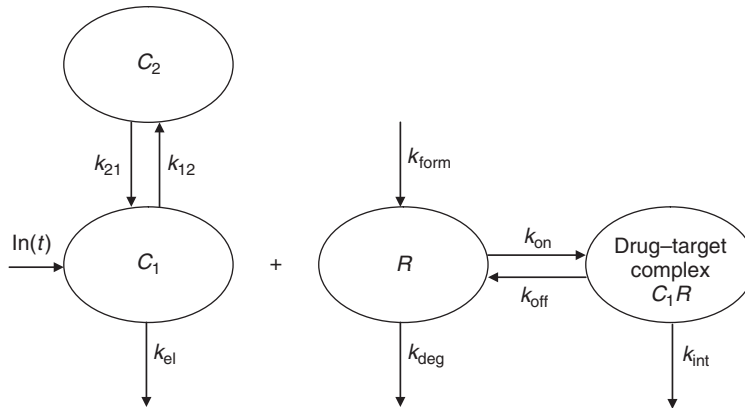


Figure 15.24 Scheme of two-compartment target-mediated drug disposition model.

$$\frac{dR}{dt} = k_{\text{form}} - k_{\text{deg}}R - k_{\text{on}}R \cdot C_1 + k_{\text{off}}RC_1 \quad (15.63)$$

$$\frac{dRC_1}{dt} = k_{\text{on}}R \cdot C_1 - (k_{\text{int}} + k_{\text{off}})RC_1 \quad (15.64)$$

Before applying the TMDD model to a specific drug, one should understand the underlying biological processes of drug binding to the target(s). The model discussed above can only be applied to the simple and specific drug–target binding process of one drug molecule to one target molecule. The drug–target binding occurs only in the central but not in the peripheral compartment. Free target formation and degeneration rates are independent of the drug and unbound target concentrations. Unbound drug distributes to peripheral compartment linearly. After internalizing, the bound drug will release from the drug–target complex so that the targets become free again. Recycling of the free target is not included in the elimination process of the drug–target complex. If a drug–target complex disposition deviates from the processes discussed here, the TMDD model should be modified so that an appropriate model can be used to characterize the PK of the drug.

Drug-binding microconstants (k_{on} and k_{off}) included in the TMDD model are sometimes difficult to estimate from routine clinical studies due to the much larger timescale of the concentration measurements than the timescale of the binding process unless there are extensive data in the transition phase. In this situation, the full TMDD model becomes overparameterized and the fit of the model to the available data becomes unstable. Approximations of the TMDD disposition model such as quasi-equilibrium (QE) [29], quasi-steady-state (QSS) [30], and M–M [29] approximations were further studied by several researchers to help solve the full TMDD model with certain conditions.

When the binding of drug to free receptor and dissociation of the drug–receptor complex are of several orders of magnitude faster than the other drug distribution and elimination processes, equilibrium between the binding and dissociation is reached instantly with unidentifiable k_{on} and k_{off} . As a consequence, QE approximation is suggested based on the following assumption [29]:

$$k_{\text{on}}R \cdot C_1 = k_{\text{off}}RC_1 \quad (15.65)$$

$$\frac{R \cdot C_1}{RC_1} = \frac{k_{\text{off}}}{k_{\text{on}}} \equiv K_D \quad (15.66)$$

where K_D represents the equilibrium dissociation constant. With this additional equation to the four full TMDD equations, four unknowns in the TMDD model will be easily resolved. Furthermore, the free drug and target concentrations in the central compartment are shown as follows:

$$C_1 = \frac{1}{2} \left[(C_{\text{tot}} - R_{\text{tot}} - K_D) + \sqrt{(C_{\text{tot}} - R_{\text{tot}} - K_D)^2 + 4K_D C_{\text{tot}}} \right] \quad (15.67)$$

$$RC_1 = \frac{R_{\text{tot}}C_1}{K_D + C} \quad (15.68)$$

In which, the total concentrations of the drug and target are as follows:

$$C_{\text{tot}} = C + RC_1 \quad (15.69)$$

$$R_{\text{tot}} = R + RC_1 \quad (15.70)$$

When the binding of drug to free receptor and dissociation of the drug–receptor complex are not much faster than drug elimination process, the rate of elimination of the complex is not negligible compared to the dissociation rate. In this case, QE approximation may not be valid. In this case, the QSS assumption that the binding rate is balanced by the sum of the dissociation and internalization rate can be applied to drug–target disposition [29]:

$$k_{\text{on}} R \cdot C_1 = (k_{\text{int}} + k_{\text{off}}) RC_1 \quad (15.71)$$

$$\frac{R \cdot C_1}{RC_1} = \frac{k_{\text{int}} + k_{\text{off}}}{k_{\text{on}}} = K_D + \frac{k_{\text{int}}}{k_{\text{on}}} = K_{\text{SS}} \quad (15.72)$$

$$C_1 = \frac{1}{2} \left[(C_{\text{tot}} - R_{\text{tot}} - K_{\text{SS}}) + \sqrt{(C_{\text{tot}} - R_{\text{tot}} - K_{\text{SS}})^2 + 4K_{\text{SS}}C_{\text{tot}}} \right] \quad (15.73)$$

$$RC_1 = \frac{R_{\text{tot}}C_1}{K_{\text{SS}} + C} \quad (15.74)$$

where K_{SS} denotes the steady-state constant. The QSS approximation can be applied to the drug with fast drug–target association and dissociation as well as fast internalization of the drug–target complex.

When the free drug concentration is large relative to the total target concentration, the time derivatives of the free and total drug concentrations are similar where M–M approximation can be used to solve the full TMDD model [29]. Both M–M and TMDD models are applied to the elimination process with capacity limitation and both systems are saturated at high dose. However, the M–M model exhibits linear parallel clearance at low doses. On the other hand, a very rapid drop and long terminal phase occur in TMDD systems [31]. The rapid decrease in drug concentrations reflects the loss of drug in the central compartment by rapid binding to the targets at low doses. The processes of drug elimination controlled by the rates of k_{12} , k_{21} , K_D , k_{el} , and k_{int} as well as the initial free target concentration attribute to the relatively long terminal phase.

15.6 SUMMARY

The PK of unchanged drug and the pseudo-PK parameters of TRA must be estimated in human ADME studies. With multiple PK samples collected in plasma and excreta, the PK parameters that represent the extent of a drug in or clearance from the circulation system are readily determined using a noncompartment approach that can be executed quickly in routine and traditional PK data analysis without assumption on disposition of drug in the body. Mechanism- or compartment-based PK models contain specific expression to characterize, in a quantitative manner, processes on the causal path from absorption, distribution, and metabolism to elimination. Data analysis using modeling

will provide information on not only the extent of a drug in the body but also the rate of a drug transferring through different parts of the body. Pooling PK data from a human ADME together with the data from the other studies of the same molecule can help scientists build a more robust population PK model, which can be used in interpreting or predicting the underlying physiological or pathological processes that drugs act on and for optimizing therapy with drugs that have a complex disposition.

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16 Physiologically Based Pharmacokinetic (PBPK) Modeling: Usefulness and Applications

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16.1	Summary	1
16.2	Introduction	2
16.3	Basic considerations	3
16.4	Types of models	11
16.5	PBPK models of organs	13
16.6	Whole-body PBPK	23
16.7	Applications of PBPK models	28
16.8	Conclusions	36
	References	37

16.1 SUMMARY

Physiologically based pharmacokinetic (PBPK) models are increasingly being used to describe and define more meaningful parameters that relate to physiology, anatomy, and biochemistry in the prediction of pharmacokinetic (PK) profiles and tissue concentration–time profiles of the parent drug and metabolites and to provide mechanistic insight in drug dynamics. Physiological data (blood flow rates and tissue volumes), physical data (protein binding and tissue partition coefficients), and biochemical data (Michaelis–Menten parameters for transporters and enzymes, $V_{\max}/K_m$) are the information required for building a PBPK model. Recently, single organ PBPK models for the intestine, liver, and kidney were applied to examine the influences of blood flow, protein binding, and activities of transporters and enzymes on the area under the curves (AUCs) and clearances of a drug and its formed metabolite. These in turn are used to build whole-body PBPK models consisting of the major drug elimination organs (i.e., intestine, liver, and kidney), together with

highly perfused, poorly perfused, and adipose tissues. The whole-body PBPK model approach is extremely useful to understand sequential metabolism, the kinetics of metabolites, and examine effects of the transporter and enzyme interplay on the blood and target organ exposures of the drug and its metabolites. The application of PBPK models allows one to make predictions of the exposure in target sites, pharmacological activity, or toxicity and the effects of age, pregnancy, disease states, and drug–drug interactions (DDIs). In this chapter, cases for the application and usefulness of PBPK models are summarized.

16.2 INTRODUCTION

PK models represent mathematical descriptions that relate drug concentration levels to processes of drug absorption, distribution, metabolism, and excretion (ADME) *in vivo*. Various modeling approaches have been developed with varying degrees of complexity for applications in different situations. A popular approach is the classic PK compartmental modeling approach which describes the body as rapidly and/or poorly equilibrating compartments for the distribution of drugs/chemicals [1]. These models are the simplest and therefore the most widely used in the clinical setting to provide information on the extents of drug distribution and elimination. The major disadvantage of these models is that the plasma drug concentrations do not always reflect tissue drug concentrations that relate to drug responses and toxicological effects. Moreover, these approaches show serious limitations since the physiological processes, especially transporter function and the sequential handling of metabolites within metabolite formation organs, are not well described [2]. These limitations pave the way to the development of PBPK approaches.

PBPK models are increasingly being used to describe and define more meaningful parameters that relate to physiology, anatomy, and biochemistry. The approach is based on the assumption that compartments are homogeneous and well-stirred. The earliest description of PBPK models was introduced in the 1930s by Teorell, one of the pioneers of PK who applied mass balance equations on specific tissues and related to tissue volumes and organ perfusion rates on drug absorption, distribution, and elimination [3]. The elegant works of Bischoff and Dedrick during the 1950s–1970s then firmly established the importance of PBPK models in the prediction of drug protein binding, tissue distribution, and the disposition of thiopental and anticancer drugs in animals [4–6]. The usefulness of these models was evident, since animal data, when scaled up, were able to predict the concentration–time profiles in man [7,8]. The concepts are then used toward the description of eliminating organs [9–14].

The PBPK model consists of a number of subcompartments that represent actual tissues and organs of discrete volumes. The differential equations constructed for these models are based on three different classifications of parameters: physiological, thermodynamic/physical, and biochemical. Physiological parameters include tissue volumes (V) and tissue blood flow rates (Q) whose sum provides the cardiac output (Q_{CO}). One basic premise of PBPK models lies with the assumption that there is venous equilibration: the concentration in tissue blood equals that in the emergent blood. Thermodynamic parameters include protein binding (denoted as unbound fractions in plasma, blood, or tissue: f_P , f_B , or f_u , or f_T), the tissue to plasma/blood partition coefficient ($K_{P,T}$ or $K_{P,Tu}$) of the drug, and the transmembrane permeability. Biochemical

parameters exist as the result of metabolic and transport processes and are described by the constants, the $V_{\max}$ or maximum velocity and K_m , the Michaelis–Menten constant. The rate is given by the product of the substrate concentration $[S]$ and the intrinsic clearance, CL_{int} . Under first-order conditions, the CL_{int} is given by $V_{\max}/K_m$. The secretory intrinsic clearance ($CL_{\text{int,sec}}$) is responsible for renal, biliary, or luminal excretion. For transport, the influx (CL_{in}) and efflux (CL_{ef}) clearances are included to represent both the transporter-mediated and passive diffusion processes [15–18].

The prediction of PK profiles of new chemical entities in humans, based on *in vitro* and *in vivo* preclinical data, is an extremely useful technique that provides important information in the drug discovery and development phases to identify drug candidates with desirable, PK properties. These many useful features that appear in PBPK models are particularly pertinent in drug development [19–22] and health risk assessment [23–29]. Another feature rests on the scale-up of PBPK based on animal experiments (mouse, rat, dog, or monkey) to explain drug behaviors in humans [7,8,30–34]. Moreover, PBPK models have been routinely applied to predict tissue concentrations with respect to the route and dose of drug administration, and in the appraisal of how alteration of physiological or biochemical conditions such as in disease states [35–38] or genetic variants in transporters, enzymes, and/or protein binding would affect drug disposition [39,40].

Another area of development rests on single-organ PBPK models such as the intestine, liver, or kidney which furnish mathematical solutions comprising blood flow, protein binding, and transporter and enzymatic activity parameters for the AUC [15,16,18]. The relations among these parameters allow the examination of important determinants on organ clearances, the interplay between transporters and enzymes, and mechanisms of DDI within these frameworks [15,16,18]. These solutions pertain to the single organs and are readily used to compare the kinetics between formed and preformed metabolite.

In this chapter, we review the information needed for building a PBPK model and the general approaches used. We summarize the recently developed single-organ PBPK models for the intestine, liver, and kidney and whole-body PBPK models that are currently applied to gain a mechanistic understanding in drug disposition. The models can predict drug and metabolite concentrations in tissues at any time and provide mechanistic insight in drug dynamics. Currently, model predictions of lipophilic compounds surpass those which require transporter involvement for uptake and efflux or those that exhibit diffusion-limited transport. These PBPK models established for animals may be scaled up to man on the extrapolation of *in vitro* experimental findings to *in vivo* PK parameters.

16.3 BASIC CONSIDERATIONS

A general PBPK model is depicted in Fig. 16.1. The model consists of several tissue compartments that represent anatomic tissue regions in the body where the drug is distributed or metabolized/excreted. Enterohepatic circulation and renal reabsorption, processes that prolong the duration of drug and metabolite in the body, are readily incorporated. Although there is no general rule for the type of organ compartments to be included in the model, organs in which the drug exerts a pharmacologic and/or toxic response should be included. In principle, if all parameters that affect the disposition

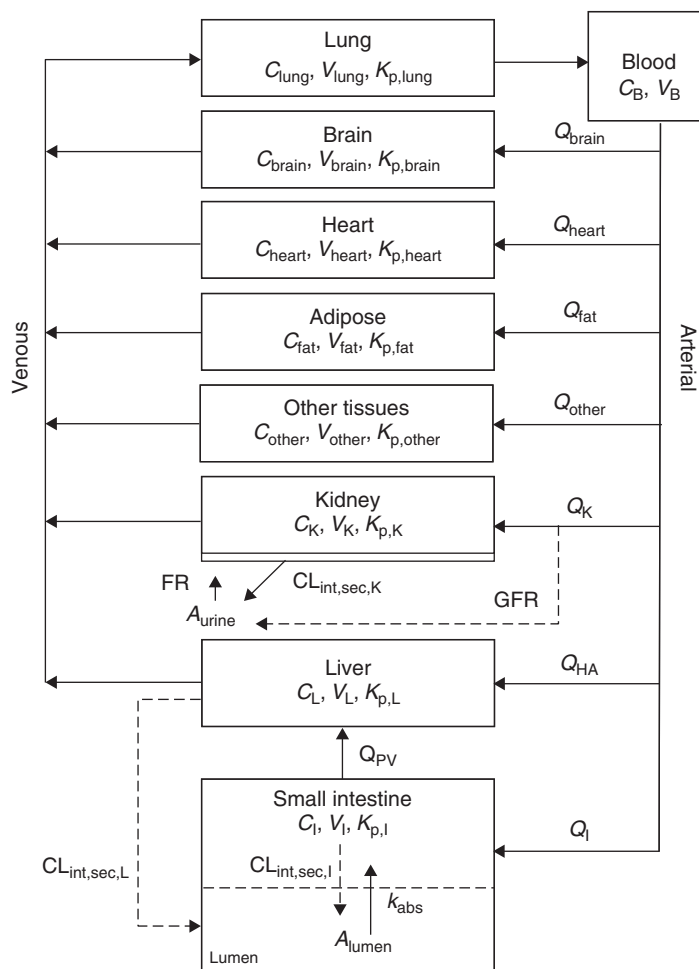


Figure 16.1 A physiologically based pharmacokinetic (PBPK) model of the whole body. Physiological volume (V), blood flow rate (Q), drug concentration (C), and tissue partition coefficient ($K_{p,T}$) in the brain (“brain”), heart (“heart”), lung (“lung”), liver (“L”), kidney (“K”), intestine (“I”), adipose tissue (“fat”), and other tissue (“other”) are some of the needed parameters for modeling; the blood is represented as (“B”). FR is the fraction reabsorbed in the kidney, and GFR is the glomerular filtration rate.

of the drug are known *in vitro*, the model should be able to predict the PK of the drug *in vivo*. However, because of the complexities and differences inherent in the systems, many assumptions are needed to render a sound *in vitro*–*in vivo* extrapolation (IVIVE) before forming the proper mathematical relations to describe drug concentrations in the blood and tissues and physiological processes on transport, secretion, and metabolism within tissue compartments.

16.3.1 Physiological Data: Blood Flow Rates and Tissue Volumes

Blood flow rates and physiological tissue volumes are important parameters of PBPK models. Table 16.1 summarizes these flow parameters that show differences among

TABLE 16.1 Physiological Volumes and Blood Flow Rates in the Human, Rat, and Mouse^a

Tissue	Human (70 kg)		Rat (0.25 kg)		Mouse (~0.02 kg)	
	Volume (mL)	Blood flow rate (mL/min)	Volume (mL)	Blood flow rate (mL/min)	Volume (mL)	Blood flow rate (mL/min)
Blood	5200	—	13.5	—	1.7	—
Brain capillary	1450	700	1.2	1.3	—	—
Brain capillary	—	—	—	—	0.025	0.089
Brain tissue	—	—	—	—	0.226	—
Heart	310	240	1.2	3.9	0.095	0.28
Kidney	280	1240	3.7	9.2	0.34	1.3
Liver	1690	—	19.6	—	1.3	—
Hepatic artery	—	300	—	2.0	—	0.35
Intestine	1650	1100	11.3	7.5	1.5	1.5
Lung	1170	—	2.1	—	0.1	—
Cardiac output (mL/min)	—	5600	—	14.0	—	8.0
GFR (mL/min)	—	125	—	1.31	—	0.28
Urine flow (mL/day)	—	1400	—	50.0	—	1.0

^afrom Refs 43 and 44

species. Blood flow is an important determinant in drug clearance in PBPK modeling. Although the pattern of blood flow, such as bulk flow, plug flow, or dispersive flow, into organs, such as the liver, affects the degree of mixing in the organ and transport and metabolic activities [10,41,42], bulk flow is usually considered. It is noteworthy that the blood flow rate to a specific organ may not reach the tissue site in its entirety. For example, part of the kidney plasma is filtered by glomerular filtration, and only the difference between the plasma flow and the GFR reaches the postglomerular tubular cells [11,12]. In the intestine, the blood flow reaching the enterocytes housing absorptive and secretory apical transporters and enzymes represents a small proportion of the entire intestinal blood flow [14].

16.3.2 Physical Data

16.3.2.1 Plasma Protein Binding. The binding of drugs to plasma protein usually retards the elimination of drugs. The pharmacological activity and toxicity of drugs are generally assumed to be correlated to the unbound drug concentration in the blood or tissue, and only the unbound drug is the species that is metabolized or excreted. Depending on whether the drug is a weak or strong acid or base, or is a neutral compound or zwitterion, it may bind to a single protein or multiple proteins (e.g., serum albumin, α_1 -acid-glycoprotein, or lipoproteins) in blood [10,45]. For poorly extracted drugs, increasing the extent of binding to plasma proteins would decrease the rate of excretion in the kidney and the rate of metabolism in the liver [46,47], but binding exerts less influence on highly extracted drugs, whose elimination rates are more dependent on the blood flow [10,48]. Changes in plasma protein binding from disease states or DDIs may lead to clinical outcomes that require the adjustment of

dosage regimens. Therefore, drug binding to plasma proteins is a key determinant of clearance.

The binding of a drug to plasma proteins is usually a reversible process, denoted by the on (k_{on}) and off (k_{off}) rate constants. The ratio of these constants yields the binding association constant, K_A , and a value of less than $10^4/\text{M}$ suggests poor binding, whereas the one exceeding $10^4/\text{M}$ suggests tighter binding [49]. For the latter case, dramatic changes in the extent of protein binding are evoked on small changes in drug concentration when the binding association constant is high. The unbound plasma concentration, $C_{P,u}$, in plasma can be expressed as follows:

$$C_{P,u} = \frac{-(1 + nK_A P_t - K_A C_P) + \sqrt{(1 + nK_A P_t - K_A C_P)^2 + 4K_A C_P}}{2K_A} \quad (16.1)$$

where P_t is the protein concentration and n represents the number of binding sites within the same class of site. The bound plasma concentration ($C_{P,b}$) is expressed as the difference between the total (C_P) and unbound ($C_{P,u}$) plasma concentrations.

If the binding between a drug and plasma protein involves two classes of sites, one with low affinity and the other with higher affinity, such a binding involving two noncooperative affinities may be formulated as [50]

$$\frac{C_{P,b}}{C_{P,u}} = \frac{n_1 K_{A1} P_t}{1 + K_{A1} C_{P,u}} + \frac{n_2 K_{A2} P_t}{1 + K_{A2} C_{P,u}} \quad (16.2)$$

where K_{A1} and K_{A2} are the binding constants for class I and class II and n_1 and n_2 are the corresponding number of noncompetitive binding sites on the protein molecule. At low drug concentration, protein binding is not saturated and only the high affinity site is involved in the binding. When excess drug is present, the low affinity sites will be further recruited for binding. In such a case, the unbound fraction $C_{P,u}$ is a function of the binding to both the high and low affinity binding sites.

16.3.2.2 Tissue Partition Coefficient. The tissue partition coefficients are important drug-specific input parameters in PBPK modeling. The tissue–plasma (or plasma water) partition coefficient, $K_{P,T}$ (or $K_{P,Tu}$), is defined as the tissue (C_T) to the tissue plasma ($C_{T,P}$) (or plasma water ($C_{T,Pu}$) concentration ratio of the drug. Since the drug concentration in tissue plasma, total or unbound ($C_{T,P}$ or $C_{T,Pu}$), equals that leaving the tissue ($C_{T,P,out}$ or $C_{T,Pu,out}$), the following relations hold at steady state [9,51,52]:

$$K_{P,T} = \frac{C_{T,ss}}{C_{P,ss}} \text{ or } K_{P,Tu} = \frac{C_{T,ss}}{C_{Pu,ss}} \quad (16.3)$$

One may also express this ratio relative to the blood concentration.

The partition coefficient, when multiplied to the volume of the tissue, determines the effective volume of distribution of drug in the tissue ($V_{T,app}$). The sum of all the tissue volumes eventually provides the V_{ss} , or the steady-state volume of distribution, a commonly used parameter to denote the extent of drug distribution, as a plasma or blood equivalent volume [53].

There are several *in vitro* methods to determine $K_{P,T}$ within tissues, some of which are quite time consuming [54–56]. In addition, *in vivo* approaches exist for estimating

$K_{P,T}$ from blood and tissue after constant rate infusion or injection to animals [57–59]. These experimentally determined constants would underestimate the true $K_{P,T}$ (ratio of influx/efflux rate constants or influx/efflux intrinsic clearances) when elimination exists within the organ or tissue, since the denominator of Equation 16.3 is artificially increased due to the presence of the elimination rate constant or elimination intrinsic clearance [60].

Recent approaches have been developed for the prediction of $K_{P,T}$ [58,61,62]. These approaches require some understanding of the physiochemical properties of the drug as well as distribution mechanisms or binding to tissue neutral lipids, phospholipids, and intracellular water. However, most of the models are suitable for small neutral molecules only and will result in significant inaccuracies when applied to compounds that are extensively ionized at physiological pH. To solve this problem, Rodgers and Rowland [63] incorporated drug ionization in both intra- and extracellular water into the approaches developed by Poulin *et al.* [62]. Under physiological condition, basic drugs ($pK_a \geq 7$) such as β -blockers are sufficiently ionized; their electrostatic interaction with acidic phospholipids predominates and accounts for the tissue binding (Eq. 16.4) [63,64]. For acids, very weak bases, neutrals, and zwitterions, their binding to extracellular proteins contributes to the binding and the electrostatic force towards acidic phospholipids is minimal (Eq. 16.5) [63,65].

For basic compounds, K_{pu} is

$$K_{pu} = \left[\left(\frac{1 + X \cdot f_{IW}}{1 + Y} \right) + f_{EW} + \left(\frac{K_{A,AP}[AP]_T \cdot X}{1 + Y} \right) + \left(\frac{P \cdot f_{NL} + (0.3P + 0.7)f_{NP}}{1 + Y} \right) \right] \quad (16.4)$$

For acids, very weak bases, neutrals, and zwitterions, K_{pu} is

$$K_{pu} = \left[\left(\frac{1 + X \cdot f_{IW}}{1 + Y} \right) + f_{EW} + (K_{A,PR}[PR]_T) + \left(\frac{P \cdot f_{NL} + (0.3P + 0.7)f_{NP}}{1 + Y} \right) \right] \quad (16.5)$$

where P is the octanol:water partition coefficient or concentration ratio of the unionized compound in all tissues except the adipose tissue, whose partition coefficient is assessed as the vegetable or olive oil:water partition coefficient or concentration ratio; f is the fractional tissue volume; subscripts IW and EW represent the intracellular and extracellular tissue water, respectively; NP and NL denote the neutral phospholipids and neutral lipids, respectively; $[AP]_T$ is the tissue concentration of acidic phospholipids; and $[PR]_T$ is concentration of extracellular albumin (for acid and weak base) or lipoprotein (for neutral compounds). The terms, X , Y , and Z , are terms that account for the extents of drug ionization as defined in Table 16.2.

$K_{A,AP}$ in Equation 16.4 is the binding association constant for the interaction between basic drugs to acidic phospholipids, and $K_{A,PR}$ in Equation 16.5 is the binding association constant for the interaction between acidic or neutral compounds, weak bases, or zwitterions and extracellular albumin/lipoprotein. For weak bases, the binding association constant in red blood cells (RBCs), $K_{A,RBC}$, may be estimated using

TABLE 16.2 Definition of the Terms X , Y , and Z in Equations 16.4–16.8

	X	Y	Z
Monoprotic base	$10^{pK_a - pH_{IW}}$	$10^{pK_a - pH_p}$	$10^{pK_a - pH_{BC}}$
Diprotic base ^a	$10^{pK_{a2} - pH_{IW}} + 10^{pK_{a1} + pK_{a2} - 2pH_{IW}}$	$10^{pK_{a2} - pH_p} + 10^{pK_{a1} + pK_{a2} - 2pH_p}$	$10^{pK_{a2} - pH_{BC}} + 10^{pK_{a1} + pK_{a2} - 2pH_{BC}}$
Monoprotic acid	$10^{pH_{IW} - pK_a}$	$10^{pH_p - pK_a}$	NA
Diprotic acid ^a	$10^{pH_{IW} - pK_{a1}} + 10^{2pH_{IW} - pK_{a1} - pK_{a2}}$	$10^{pH_p - pK_{a1}} + 10^{2pH_p - pK_{a1} - pK_{a2}}$	NA
Zwitterion	$10^{pK_{a,BASE} - pH_{IW}} + 10^{pH_{IW} - pK_{a,ACID}}$	$10^{pK_{a,BASE} - pH_p} + 10^{pH_p - pK_{a,ACID}}$	$10^{pK_{a,BASE} - pH_{BC}} + 10^{pH_{BC} - pK_{a,ACID}}$
Neutral	0	0	NA

^aIn these expressions $pK_{a1} < pK_{a2}$; pH_p is the pH of plasma

Abbreviation: NA, not available.

Equation 16.6 with the known unbound fractions ($f_{IW,RBC}$, $f_{NL,RBC}$, and $f_{NP,RBC}$), the X , Y , and Z terms from Table 16.2, and $K_{Pu,RBC}$ from Equation 16.7; the $K_{A,RBC}$ so estimated is assumed to equal $K_{A,AP}$, which, in turn, may be applied to estimate K_{pu} in Equation 16.4:

$$K_{A,RBC} = \left[K_{Pu,RBC} - \left(\frac{1+Z}{1+Y} f_{IW,RBC} \right) - \left(\frac{P \cdot f_{NL,RBC} + (0.3P + 0.7)f_{NP,RBC}}{1+Y} \right) \right] \left(\frac{1+Y}{Z[AP]_{RBC}} \right) \quad (16.6)$$

$$K_{Pu,RBC} = \frac{Hct - 1 + \left(\frac{C_B}{C_P} \right)}{f_P Hct} \quad (16.7)$$

Specifically, $K_{Pu,RBC}$ is the ratio of drug concentration in RBCs to that unbound in plasma and represents the binding association constant of drug molecules to RBCs. C_B/C_P is the blood:plasma concentration ratio and Hct is the hematocrit; f_P is the fraction of drug unbound in plasma; and subscripts RBC and P denote the red blood cells and plasma, respectively.

For acidic, neutral, and weakly basic compounds or zwitterions, the binding association constant $K_{A,PR}$ is given by the following equation:

$$K_{A,PR} = \left[\frac{1}{f_P} - 1 - \left(\frac{P f_{NL,P} + (0.3P + 0.7)f_{NP,P}}{1+Y} \right) \right] \left(\frac{1}{[PR]_P} \right) \quad (16.8)$$

After obtaining the K_{Pu} values, the V_{ss} is calculated as

$$V_{ss} = V_P + f_P \sum V_{T,i} K_{Pu,T,i} \quad (16.9)$$

where V_P is the volume of plasma and $V_{T,i}$ is the volume of the i th tissue/organ; the term $V_{T,i} K_{Pu,T,i}$ is $V_{T,app}$ or the apparent volume of the tissue compartment for the i th tissue/organ [63]. Equation 16.9 is a general approach to estimate the steady-state volume of distribution, V_{ss} .

However, PBPK models are very complex and the number of organ/tissue compartments can exceed 15 or more [19,66]. A strategy for simplification is to lump the compartments and reduce the complexity of the PBPK model without losing the predictive power. The underlying strategies are as follows: (i) tissues with same model specification for flow or partition (e.g., both are highly/poorly perfused or of similar K_p) and of same position in the system structure can be lumped together; (ii) serial tissues that equilibrate with one another rapidly should be lumped; and (iii) parallel tissues that have similar rate constants may be lumped [19]. It was suggested that the preliminary estimation of V_{ss} can be based solely on $K_{P,muscle}$ and $K_{P,fat}$ [59,61,63] since drug distribution into muscle and fat accounts for approximately 65–84% of the total V_{ss} ; the $K_{P,muscle}$ may be used for all lean tissues except for the lungs which are represented by the $K_{P,fat}$ [59]. This new idea provides a time-efficient prediction of $K_{P,T}$ for lumped compartments in developing the PBPK models.

16.3.3 Biochemical Data on Enzymes and Transporters

The basic tissue compartment usually represents an individual organ or a group of “lumped” organs/tissues with similar flow parameter or partition characteristics. After giving consideration to the above general concepts, there is the need to consider transporters and enzymes (see Chapters 5–7) as important biochemical parameters in building a PBPK model. Rate equations may then be used to describe the transport and enzymatic processes. For the description of rate processes, the simplest Michaelis–Menten equation is often used, wherein the rate, v , is expressed in terms of the intrinsic clearance for transport, metabolism, and/or secretion (CL_{in} , CL_{ef} , $CL_{int,met}$, or $CL_{int,sec}$):

$$v = \frac{V_{max}}{K_m + [S]}[S] = CL_{int}[S] \quad (16.10)$$

The intrinsic clearance for a particular process is the maximum velocity, V_{max} , divided by the sum of the Michaelis–Menten constant, K_m , and the unbound substrate concentration, $[S]$ or $V_{max}/(K_m + [S])$. For this reason, the binding of the drug to microsomes/cytosol must be examined to better define $[S]$ [67–69]. The V_{max} and K_m values are usually obtained from *in vitro* studies, commonly estimated from the rate plot (v vs $[S]$), the Lineweaver–Burk, Eadie–Hofstee, or Hanes–Wolf plot. Usually, the assumption is that elimination is first order, and CL_{int} is the ratio of V_{max}/K_m . The *in vitro* intrinsic metabolic clearance under linear conditions is given by [70]

$$CL_{int,met,in\ vitro} = \frac{V_{max}}{K_m} \quad (16.11)$$

Metabolic or enzyme functions, estimated from human liver microsomal and cytosolic incubations, supersomes or recombinant expression systems, and the appropriate scaling factors, are converted to per gram liver when the hepatocellularity (cells per gram liver), microsomal protein (mg/g liver), and the overall expression of the cytochrome P450 (CYP) enzyme (pmol/g liver) are involved in converting the *in vitro* data to provide the $CL_{int,met}$ in human liver *in vivo* [43,71–73]. Preference of recombinant systems over human liver microsomes (HLMs) has been expressed, offering improved assay sensitivity compared with HLM for $CL_{int,met}$ determination [74].

The transporter-mediated processes and passive diffusion into and out of the tissue compartment must be summed to yield the influx (CL_{in}) and efflux (CL_{ef}) clearances, respectively. Normally, transporter function may be estimated in cell expression systems, combined with human transporter uptake studies. The influx transport intrinsic clearance (CL_{in}) is usually estimated from *in vitro* hepatic uptake studies. However, quantitative data on how much protein of each of the transporters is expressed within different tissue region is lacking to accurately define the relative proportions or abundancy of sinusoidal transporters for uptake and apical transporters for excretion. Moreover, the localization of the transporter, whether within the cytosol or nucleus or on the membrane surface would greatly affect the transport rate. In order to gain this insight, use of proteomics, with LC-MS/MS technologies to provide the absolute quantification of transporters, must be completed to provide the relative proportions of transporters in the liver, kidney, and blood–brain barrier [75,76]. In absence of these data, hepatocyte uptake information for extrapolation of *in vitro* parameters to *in vivo*,

cell number per unit tissue weight and tissue weight per unit body weight, in addition to the physiological scaling factors, have been used [39,77–79]. At a qualitative level, *in vitro* uptake and biliary excretion data using the rat and human sandwich-cultured hepatocyte systems may be used to assess whether there is species similarity and correlation to the *in vivo* plasma and biliary clearances [80] or DDI in transport; care must be included to describe differences in transporter expression in the system as well as real species differences. Indeed, *in vitro* data have identified marked species differences for the MRPs (multidrug resistance-associated proteins) and BCRP (breast cancer resistance protein) in rat and human hepatocytes; these significant species differences in efflux transporter activities could be integrated into animal data for the prediction of human transport [81]. When there is no significant interspecies difference between rat and man, the scaling factor determined in rats has been used to extrapolate human *in vitro* parameters to those *in vivo*. The transporter-mediated transport clearance should then be considered, in addition to the passive clearance, for the prediction of *in vivo* intrinsic influx and efflux clearances.

16.4 TYPES OF MODELS

Two tissue type models are considered: the membrane-limited model and blood flow-limited model (Fig. 16.2a and b). These PBPK models are also extended to describe the sequential metabolism or the excretion of formed metabolites (Fig. 16.2c). In any one PBPK model, a mixture of these may be used. The membrane-limited subcompartment is used for poorly permeable tissues, whereas for other tissue sites that rapidly equilibrate with blood, the flow-limited compartment may be used.

16.4.1 Membrane-Limited Model

Each tissue compartment basically consists of three well-mixed subcompartments: a vascular space that houses the blood that is perfusing the tissue, an interstitial space, and a cellular space (Fig. 16.2a). The drug enters with the afferent tissue blood flow (Q_T) with arterial concentration (C_A) into the vascular subcompartment, where there is instantaneous equilibrium with drug in the interstitium (C_{TI}). For all intents and purposes, the interstitial space may be combined with the vascular space to yield the extracellular compartment representing the tissue blood subcompartment of concentration, C_{TB} . With venous equilibration, C_{TB} equals the efferent blood concentration ($C_{T,out}$). The rate equations and the clearance terms used to describe the transport and enzymatic processes are the intrinsic clearances (CL_{in} and CL_{ef}) for influx and efflux at the basolateral membrane, respectively, and $CL_{int,sec}$ and $CL_{int,met}$ for excretion at the apical membrane and metabolism within the tissue.

Within the tissue compartment, the drug may become bound to tissue proteins, metabolized by enzymes with metabolic intrinsic clearance, $CL_{int,met}$, or is effluxed out of the cell by transporters and/or passive diffusion (CL_{ef}), or excreted at the apical membrane ($CL_{int,sec}$). The rate equations for the tissue blood (TB) and tissue (T) compartments are

$$V_{TB} \frac{dC_{TB}}{dt} = Q_T C_A - Q_T C_{T,out} + f_T C_T CL_{ef} - f_B C_{TB} CL_{in} \quad (16.12)$$

$$V_T \frac{dC_T}{dt} = f_B C_{TB} CL_{in} - f_T C_T (CL_{ef} + CL_{int}) \quad (16.13)$$

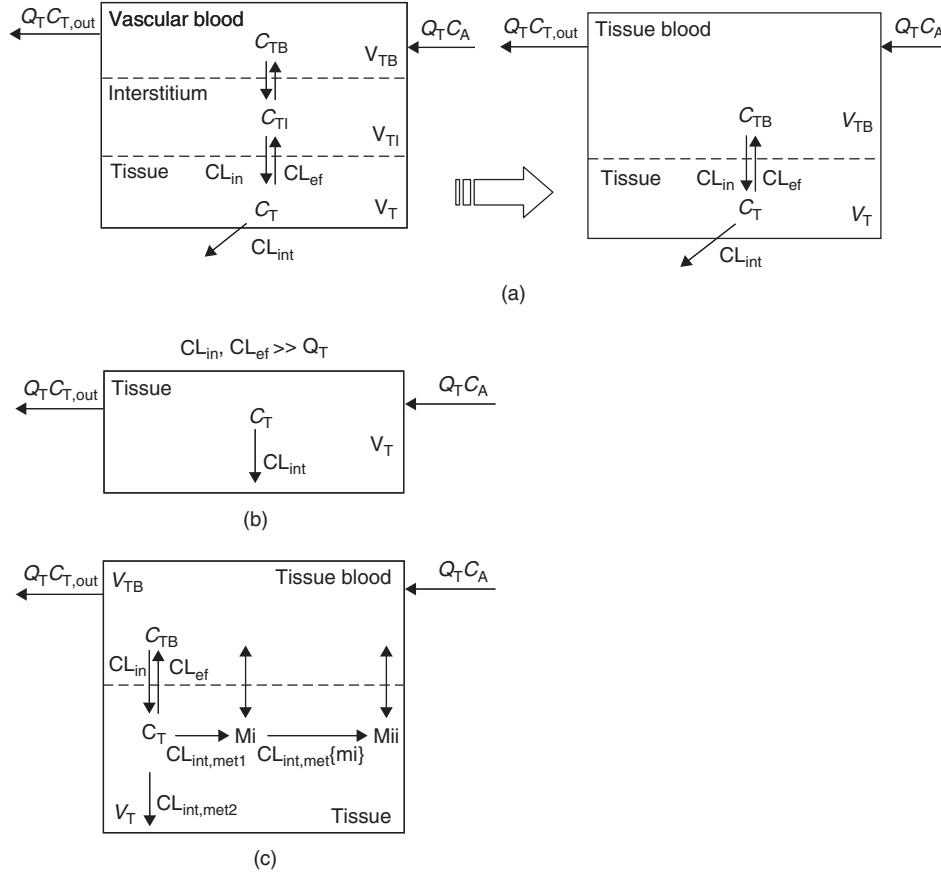


Figure 16.2 Typical PBPK components within an organ. There are two types of tissue models: (a) membrane limited; (b) blood flow limited; and (c) sequential metabolism of the formed primary metabolite (Mi) from parent drug (P) to the secondary metabolite (Mii) may occur within the organ of formation.

where f_B and f_T are the unbound fractions in blood and tissue, respectively, and CL_{int} is the total intrinsic clearance (sum of $CL_{int,met}$ and $CL_{int,sec}$).

16.4.2 Flow-Limited Model

The above relations are simplified with the assumption that the transfer across the membrane is rapid compared to the perfusion rate of the tissue (CL_{in} and $CL_{ef} \gg Q_T$). This means that the vascular, interstitial, and cellular subcompartments are in rapid equilibrium. As a result, these three subcompartments are combined to form one tissue compartment (Fig. 16.2b). The tissue to blood/plasma concentration ratio ($K_{P,T}$ or $C_T/C_{T,out}$) is used in this simplification. The mass balance equation can be written as

$$V_T \frac{dC_T}{dt} = Q_T C_A - Q_T C_{T,out} - f_T C_T CL_{int} = Q_T C_A - Q_T \frac{C_T}{K_{P,T}} - f_T C_T CL_{int} \quad (16.14)$$

where $C_T/K_{P,T}$ replaces $C_{T,out}$.

16.4.3 Model for Sequential Metabolism

The membrane-limited and flow-limited PBPK models are readily extended to describe the formation and sequential handling of the metabolite [82]. The PBPK model serves to describe sequential metabolism very well [15,82], especially when the nascently formed metabolite is immediately metabolized or excreted [83–85]. The rate equations for the tissue blood (TB) and tissue (T) compartments are expressed in Equations 16.15 and 16.16. Note that the set of parameters pertaining to the metabolite are qualified by {mi} for the concentrations, the binding constants, and the transport intrinsic clearances:

$$V_{TB} \frac{dC_{TB}\{mi\}}{dt} = Q_T C_A\{mi\} - Q_T C_{T,out}\{mi\} + f_T\{mi\} C_T\{mi\} CL_{ef}\{mi\} - f_B\{mi\} C_{TB}\{mi\} CL_{in}\{mi\} \quad (16.15)$$

Within the metabolite formation organ, the metabolite is immediately removed by intrinsic clearance, $CL_{int,met}\{mi\}$, and PBPK modeling considers the sequential handling of the formed metabolite [2]:

$$V_T \frac{dC_T\{mi\}}{dt} = f_T C_T CL_{int,met} + f_B\{mi\} C_{TB}\{mi\} CL_{in}\{mi\} - f_T C_T\{mi\} (CL_{ef}\{mi\} + CL_{int,met}\{mi\}) \quad (16.16)$$

PBPK metabolite modeling allows one to compare the kinetics between the formed versus preformed metabolite and brings a new perspective to differences in the kinetics of formed versus preformed metabolite. This is an interesting aspect for safety considerations of metabolites, if the information on the kinetics of the formed metabolite is predicted/gained on administration of a preformed metabolite, as in metabolite-in-safety testing (MIST) [15,86]. Use of the PBPK model will provide more accurate predictions on the kinetics of sequential metabolism for the investigation of risk assessment and toxicity associated with drug metabolite. However, there is a divergence for metabolite handling when metabolites display poor permeability across biological membranes [83,87,88]. The membrane barrier bars the metabolite from entering or leaving the tissue, thereby rendering differences in fates of the formed versus preformed metabolite kinetics.

16.5 PBPK MODELS OF ORGANS

The development of PBPK models, especially on simple organs for drugs and metabolites, has provided solutions for the AUCs and clearances for a drug and its formed metabolite. Rate equations are written for each subcompartment, providing the essential coefficients for matrix inversion and solutions which yield the steady-state concentrations/AUCs of the drug and of the metabolite ($AUC\{mi, P\}$) [15]. The method of matrix inversion, appropriate only under linear kinetic conditions, has been applied to examine whether $AUC\{mi, P\}$ is suitable for estimation of the systemic bioavailability [18].

16.5.1 The Liver

A liver PBPK model that describes membrane-limited transport, with either fast or slow transmembrane transport clearances for summed diffusion-limited and transporter-mediated transport cases, denoted as CL_{in} and CL_{ef} , has been developed [89,90]. The model comprises of four compartments: the reservoir (R, or blood compartment), liver blood (LB), liver tissue (L), and bile compartment (bile) (Fig. 16.3) [15]. The hepatic flow rate and unbound fraction in blood and liver are denoted by Q_H , f_B , and f_L , respectively. The transport processes underlying carrier-mediation and passive diffusion are denoted by the influx and efflux intrinsic clearances (CL_{in}^H and CL_{ef}^H) representing the summed activities of basolateral transporters and passive diffusion (Fig. 16.3). In addition, biliary secretion at the apical membrane and metabolism within hepatocytes are denoted by the biliary intrinsic clearance ($CL_{int,sec,H}$) and metabolic intrinsic clearance ($CL_{int,met,H}$) of phase I and phase II enzymes, respectively (Fig. 16.3). The model also serves to describe hepatic sequential metabolism [2,15,82,84], in which the metabolite formed within the liver tissue is prone to sequential metabolism immediately to form other metabolites ($CL_{int,met,H\{mi\}}$) or is immediately excreted ($CL_{int,sec,H\{mi\}}$).

An advantage of PBPK model is that solutions relating to blood flow, protein binding, and activities of the transporters and enzymes for the elimination of the drug and metabolite may be derived by matrix inversion from the rate equations for the drug and metabolite [15,18,91]. Pang *et al.* [15] had summarized the AUC from time 0 to ∞ for the drug and the metabolite for the liver PBPK model. As shown in Table 16.3, the AUC of the drug is a complex equation that relates to the flow rate (Q_H), f_B , CL_{in}^H , and CL_{ef}^H , and the total hepatic intrinsic clearance ($CL_{int,H}$). In the case of a drug of

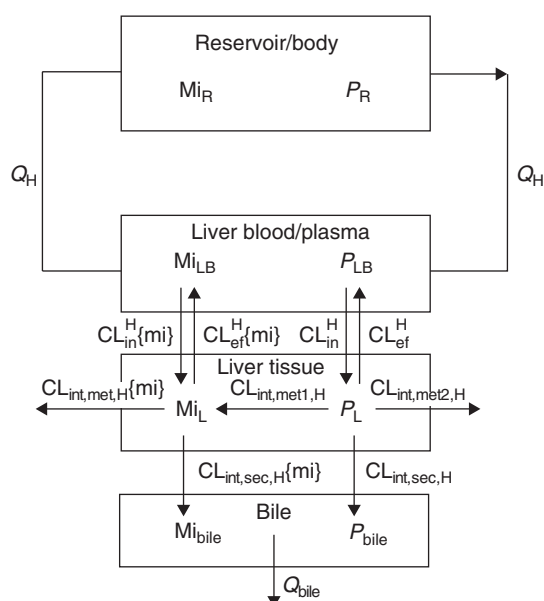


Figure 16.3 Physiologically based pharmacokinetic (PBPK) liver model, with liver as the only elimination organ. See text for description of the model.

flow-limited distribution (i.e., when $CL_{in}^H \cong CL_{ef}^H > Q_H$), these equations are simplified (Table 16.3). The ratio of hepatic metabolic and biliary clearances, $CL_{H,met}/CL_{H,sec}$ equals the ratio of the intrinsic clearances, $CL_{int,met,H}/CL_{int,sec,H}$. From this relation, the unknown parameter, $CL_{int,sec}$ may be obtained with the data from liver perfusion studies or *in vivo* studies [92] when $CL_{int,met}$ is obtained from *in vitro* metabolism studies using 9000g supernatant fractions or liver microsomes. These equations shown in Table 16.3 are useful to allow inferences on mechanisms of DDIs in the liver to be made.

The AUC for the formed metabolite, $AUC_{iv}\{mi,P\}$, after iv dosing of the precursor drug is dependent on the formation (metabolic) intrinsic clearance from the drug, $CL_{int,met1,H}$, and individual components of the $CL_{int,H}$ (or $CL_{int,met1,H} + CL_{int,met2,H} + CL_{int,sec,H}$), as well as binding, transport clearances ($CL_{in}^H\{mi\}$ and $CL_{ef}^H\{mi\}$), and intrinsic clearance, $CL_{int,H}\{mi\}$, of the metabolite [sum of ($CL_{int,met,H}\{mi\}$) and ($CL_{int,sec,H}\{mi\}$)]. In contrast, the AUC obtained from administration of its preformed counterpart that yielded $AUC_{po}\{pmi\}$ and $AUC_{iv}\{pmi\}$ is independent of drug parameters [15,86]. Indeed, solutions for the formed metabolite after oral and intravenous drug dosing ($AUC_{po}\{mi,P\}$ and $AUC_{iv}\{mi,P\}$) have revealed interesting differences from $AUC_{po}\{pmi\}$ and $AUC_{iv}\{pmi\}$ that result from the administration of the preformed counterpart [15,86].

Other aspects that contribute to differences between $AUC_{po}\{mi,P\}$ and $AUC_{iv}\{mi,P\}$ after drug dosing and $AUC_{po}\{pmi\}$ and $AUC_{iv}\{pmi\}$ after preformed metabolite dosing have been attributed to the heterogeneity of transporters and metabolic enzymes. As an improvement, zonal PBPK liver models consisting of three zonal regions: zones 1 (periportal zones), 2 (midzonal zones), and 3 (perivenous zones) or models similar to compartments in series have been developed [92–95]. Another factor is the presence of a basolateral membrane barrier for the metabolite, retarding entry or efflux of preformed metabolite into and out of cells but trapping the formed metabolite within the tissue [83,87,88,96]. Many successful examples of zonal PBPK modeling include enalapril [89,94], morphine [97], estrone sulfate [98], estradiol–17 β -glucuronide [99], and digoxin [95], which were all investigated within rat liver perfusion studies. Furthermore, the role of the efflux transporter, Mrp2, which is responsible for excretion of both the parent drug, estradiol–17 β -glucuronide and its 3-sulfate–17 β -glucuronide metabolite on net desulfation, was elucidated comprehensively within the framework of a liver PBPK model [99,100].

Digoxin, a cardiotonic agent, is an example of a poorly cleared compound whose uptake into hepatocytes is rapid due to passive diffusion and the transporter, Oatp1a4, and whose clearance may be rate-limited by binding to RBCs and albumin [95]. Moreover, for poorly cleared substrates, heterogeneity in the metabolic activities among zones fails to affect the overall clearance when the drug concentration gradient within the sinusoid is shallow [92]. Drugs that are highly extracted exhibit a steep concentration gradient in the sinusoid, and, under this instance, removal rates are greatly modulated by enzyme/transporter heterogeneity. Sequential metabolism in liver could be affected by the zonal localization of metabolic enzymes. For example, different metabolites were observed for salicylamide versus gentisamide, hydroxylated metabolite of salicylamide, administration: due to the close proximity of hydroxylation and glucuronidation enzymes in the sequential metabolism of salicylamide, gentisamide glucuronidation predominates over sulfation; in contrast, gentisamide sulfate conjugates are more abundant when gentisamide is administered [85,101,102].

TABLE 16.3 Equations on the Area Under the Curve (AUC) and Total Hepatic Clearance ($CL_{H, \text{tot}}$)

Parameters	Membrane-Limited Case	Flow-Limited Case
AUC_{iv}	$\frac{\text{Dose}_{iv} [Q_H (CL_{ef}^H + CL_{int, H}) + f_B CL_{in}^H CL_{int, H}]}{f_B CL_{in}^H Q_H CL_{int, H}}$	$\frac{\text{Dose}_{iv} [(Q_H + f_B CL_{int, H})]}{Q_H f_B CL_{int, H}}$
$AUC_{iv} \{mi, P\}$	$\frac{\text{Dose}_{iv} CL_{int, met1, H} CL_{ef}^H \{mi\}}{(CL_{int, met1, H} + CL_{int, met2, H} + CL_{int, sec, H}) f_B \{mi\} CL_{in}^H \{mi\} CL_{int, H} \{mi\}}$	$\frac{\text{Dose}_{iv} CL_{int, met1, H}}{(CL_{int, met1, H} + CL_{int, met2, H} + CL_{int, sec, H}) f_B \{mi\} CL_{int, H} \{mi\}}$
$CL_{H, \text{tot}}$ (total hepatic clearance)	$Q_H \frac{f_B CL_{int, H} CL_{in}^H}{Q_H CL_{ef}^H + CL_{int, H} (Q_H + f_B CL_{in}^H)}$	$\frac{Q_H f_B CL_{int, H}}{[(Q_H + f_B CL_{int, H})]}$
$CL_{H, \text{met1}}$ (formation clearance of Mi)	$Q_H \frac{f_B CL_{int, met1, H} CL_{in}^H}{Q_H CL_{ef}^H + CL_{int, H} (Q_H + f_B CL_{in}^H)}$	$\frac{Q_H f_B CL_{int, met1, H}}{[(Q_H + f_B CL_{int, H})]}$
$CL_{H, \text{met2}}$ (metabolic clearance for formation of other metabolites)	$Q_H \frac{f_B CL_{int, met2, H} CL_{in}^H}{Q_H CL_{ef}^H + CL_{int, H} (Q_H + f_B CL_{in}^H)}$	$\frac{Q_H f_B CL_{int, met2, H}}{[(Q_H + f_B CL_{int, H})]}$
$CL_{H, \text{ex}}$ (biliary clearance of drug)	$Q_H \frac{f_B CL_{int, sec, H} CL_{in}^H}{Q_H CL_{ef}^H + CL_{int, H} (Q_H + f_B CL_{in}^H)}$	$\frac{Q_H f_B CL_{int, sec, H}}{[(Q_H + f_B CL_{int, H})]}$

Source: From Sun and Pang [18] with permission.

16.5.2 The Small Intestine: Traditional Model (TM) and Segregated Flow Model (SFM)

Drugs administered orally are first absorbed, either passively or via active/facilitated transport, across the intestinal luminal membrane to reach the systemic circulation. The various intestinal transport proteins present on the apical membrane are known to participate in the uptake of drugs. Efflux by adenosine triphosphate (ATP)-binding cassette transporters delimits drug absorption in the intestine, whereas absorptive transporters mediate drug absorption [103–105]. Additionally, the intestine possesses the conjugating enzymes, UDP-glucuronosyl transferases) and GST (glutathione S-transferases) [106,107], and CYP3A, accounting for ~80% of the total immunoquantified CYPs [108–112] that play important roles in reducing oral bioavailability. Processes of intestinal absorption, metabolism, and secretion must be considered simultaneously in viewing oral drug bioavailability, and PBPK models have been developed to describe these processes [14,104,113,114].

A traditional, physiologically based model (traditional model, TM), which regards the intestine as a single homogeneous compartment with all of the intestinal blood flow perfusing the tissue, has been developed to account for oral drug bioavailability (Fig. 16.4a) [13]. This model encompasses all the variables of transport, metabolism, efflux, gastrointestinal transit, and absorption. In this model, the intestine is composed of three subcompartments: intestinal blood, tissue, and lumen. The blood or reservoir (R or blood compartment) of volume V_R and intestine compartments are interconnected

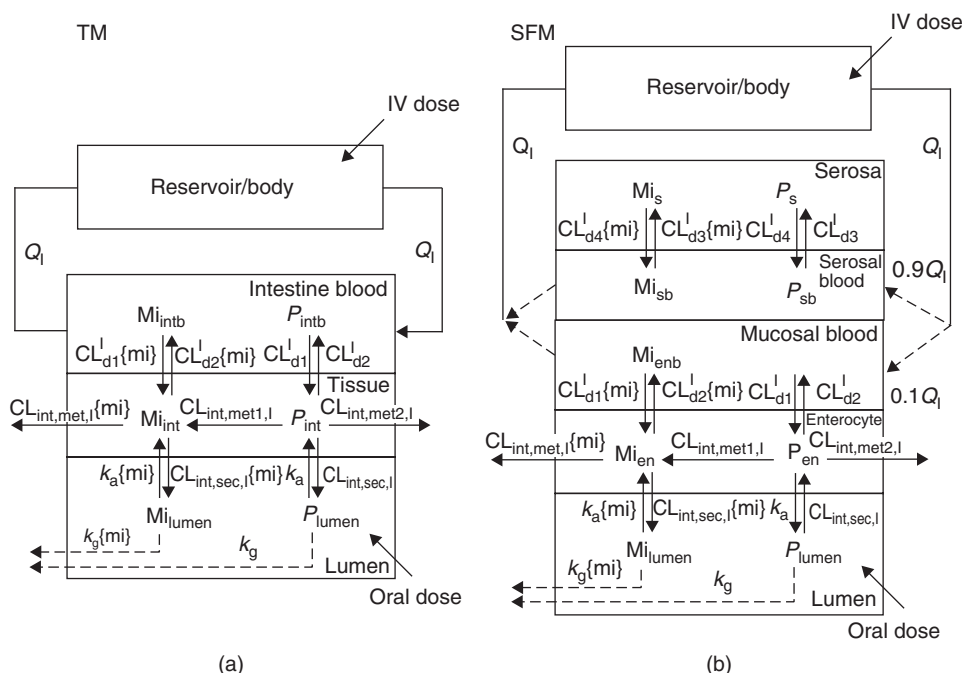


Figure 16.4 Physiologically based pharmacokinetic (PBPK) intestine models: (a) the traditional model or TM and (b) the segregated flow model or SFM, with the intestine as the only elimination tissue. For the SFM, the intestine tissue is divided into the enterocyte (en) region and the serosal (s) region. See text for description of flows, volumes, and assumptions.

by the intestinal or portal venous blood flow, Q_I or Q_{PV} , and V_{intb} , V_{int} , and V_{lumen} represent volumes of the various intestinal subcompartments, intestinal blood, the intestine, and the lumen, respectively. The drug is delivered under constant blood flow (Q_I) into the intestinal blood via the superior mesenteric artery and exits the intestine into the portal vein. The influx of the drug into the intestinal tissue from the blood is characterized by the transport clearance parameter, CL_{d1}^I . Once the drug traverses the basolateral membrane to enter the tissue, it undergoes biotransformation to form metabolites (denoted by the intestinal metabolic clearance, $CL_{int,met1,I}$ and $CL_{int,met2,I}$) or excretion (denoted by intrinsic secretion clearance $CL_{int,sec,I}$) or may be effluxed back to the circulation (denoted by transport clearance CL_{d2}^I). The absorption rate constant of drug and metabolite from the intestinal lumen is denoted by k_a or $k_a\{mi\}$, and their transit or degradation/metabolism rate constants within the lumen, representing net loss in lumen, are denoted by k_g or $k_g\{mi\}$, to denote an ineffective absorption.

Owing to failure of the TM to predict route-dependent intestinal metabolism, namely little or no metabolism occurring after systemic dosing but notable metabolism following oral dosing, the segregated flow model (SFM) has been subsequently developed (Fig. 16.4b). The development was sparked by observations on morphine (M) glucuronidation to the 3-glucuronide (M3G) in the perfused rat intestine preparation: the lack of [3H]M3G formed after systemic [3H]M administration and the abundance of [3H]M3G (13% dose) formed after [3H]M administered intraduodenally support the hypothesis of route-dependent metabolism [13]. According to the SFM, a reduced blood flow rate perfuses the enterocyte layer, reducing the rate of drug delivery to intestinal enzymes or secretory sites and rendering lower values of intestinal clearance [14]. However, during oral absorption, the entire orally administered dose must traverse the enterocyte layer before the substrate enters the circulation. The differential exposure with the route of administration results in different extents of exsorption and metabolism and contributes to the observation of route-dependent metabolism. The SFM is consistent with intestinal blood patterns of about 60–70% of the intestinal flow [115] perfusing the nonabsorptive and nonmetabolizing serosal and submucosal regions, and only a low, partial flow ($\sim 18\%$ [116], 5–7% [117,118], or 10–30% [119,120]) of the intestinal blood perfuses the absorptive and metabolizing enterocyte region where the metabolic enzymes and the transporters are located [14]. The SFM further provides a view that was adopted in commercially available programs such as Simcyp[®] [121]. As shown in Fig. 16.4b, the parent drug and metabolite are delivered under constant blood flow ($0.9Q_I$) to the serosal tissue, which is characterized by the transport clearance parameter, CL_{d3}^I and $CL_{d3}^I\{mi\}$, respectively. The parent drug and metabolite can also be effluxed back into serosal blood (CL_{d4}^I or $CL_{d4}^I\{mi\}$), respectively.

Utilizing these two models, Sun and Pang [18,122] solved the AUC terms for the parent drug and metabolite (Table 16.4). The solutions for AUC_{po} , $AUC_{po}\{mi\}$, and $AUC_{iv}\{mi\}$ are found to be identical for the TM and SFM, whereas for AUC_{iv} , the solution is similar except that Q_I exists in the solution for the TM and Q_{en} for the SFM. The secretory intestinal intrinsic clearance of the drug ($CL_{int,sec,I}$) is found associated with the term $(1 - F_{abs})$, where F_{abs} is the fraction absorbed or $k_a/(k_a + k_g)$. When the absorbed fraction, F_{abs} , is high (~ 1) due to the high k_a relative to k_g , net secretion is effectively reduced to zero. Moreover, AUC_{po} and AUC_{iv} are found to be inversely related to the sum of $(1 - F_{abs})CL_{int,sec,I}$ and $CL_{int,met,I}$, whereas $AUC_{po}\{mi\}$ and $AUC_{iv}\{mi\}$ are further related inversely to the sum of $(1 - F_{abs}\{mi\})CL_{int,sec,I}\{mi\}$ and

TABLE 16.4 Solutions for AUC_{iv} , AUC_{po} , $AUC_{iv}\{\text{mi}\}$, and $AUC_{po}\{\text{mi}\}$ for the TM and SFM, with Intestine as the Only Eliminating Organ^a

AUC Terms	Solutions for the TM and SFM
$AUC_{po}^{TM\&SFM}$	$\frac{F_{abs} \text{dose}_{po} CL_{d2}^1}{CL_{d1}^1 [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met1,I} + CL_{int,met2,I}]}$
$AUC_{po}\{\text{mi},P\}^{TM\&SFM}$	$\frac{\text{Dose}_{po} F_{abs} CL_{int,met1,I} CL_{d2}^1 \{\text{mi}\}}{[(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met1,I} + CL_{int,met2,I}] CL_{d1}^1 \{\text{mi}\} [(1 - F_{abs}\{\text{mi}\}) CL_{int,sec,I}\{\text{mi}\} + CL_{int,met,I}\{\text{mi}\}]}$
AUC_{iv}^{TM} or AUC_{iv}^{SFMb}	$\frac{\text{Dose}_{iv} [CL_{d1}^1 [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met,I}] + Q_I [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met,I} + CL_{d1}^1]]}{Q_I CL_{d1}^1 [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met,I} + CL_{int,met2,I}]}$
$AUC_{iv}\{\text{mi},P\}^{TM\&SFM}$	$\frac{\text{Dose}_{iv} CL_{int,met1,I} CL_{d2}^1 \{\text{mi}\}}{[(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met1,I} + CL_{int,met2,I}] CL_{d1}^1 \{\text{mi}\} [(1 - F_{abs}\{\text{mi}\}) CL_{int,sec,I}\{\text{mi}\} + CL_{int,met,I}\{\text{mi}\}]}$
CL_{iv}^{TM} or CL_{iv}^{SFMb}	$\frac{Q_I CL_{d1}^1 [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met,I} + CL_{int,met2,I}]}{CL_{d1}^1 [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met,I}] + Q_I [(1 - F_{abs}) CL_{int,sec,I} + CL_{int,met,I} + CL_{d1}^1]}$

^aFor the SFM, Q_I is Q_{en} .

^bThe flow rate term is Q_{en} for the SFM.

Source: From Sun and Pang [18] with permission.

$CL_{int,met,I}\{mi\}$. Owing to the effective modulation of absorption on $CL_{int,sec,I}$ by F_{abs} , changes in $CL_{int,met,I}$ would exert a greater effect on drug bioavailability compared to $CL_{int,sec,I}$ [122]. The difference in flow between models imparts a substantial influence on the intestinal clearance of flow-limited substrates. The SFM predicts a markedly higher extent of intestinal metabolism for oral compared to intravenous dosing, and the TM predicts a higher intestinal metabolism compared to that for the SFM.

Owing to the varying segmental distributions of absorptive and secretory transporters and metabolic enzymes along the small intestine, the single intestinal compartments of the TM and SFM have been expanded into three equal segmental compartments, segmental, TM and the segmental, SFM [123]. These advanced PBPK models have been applied to predict the impact of metabolic and transporter activities in different segments of the intestine on the intestinal drug clearance and oral drug availability [123].

16.5.3 The Kidney

The kidney is one of the major excretory organs of drugs and metabolite and exhibits a significant capacity to metabolize drugs [12,124,125]. The isolated kidney perfusion is one technique that allows metabolic and excretory events to be examined simultaneously under controlled conditions and described by PBPK modeling. In the PBPK kidney model, the drug is delivered into renal tubular cells by the slightly reduced renal flow ($Q_K - GFR$) due to removal of plasma water by glomerular filtration (of rate, GFR), and, on the reabsorption of water, the returning flow to the reservoir ($Q_K - Q_U$) is the difference between the renal blood/plasma flow and urinary flow, Q_U (Fig. 16.5). Drug influx and efflux clearances by transporters at the basolateral membrane are described as CL_{in}^b and CL_{ef}^b , respectively. The drug that is reabsorbed or secreted in the urine compartment by transporters at the apical or luminal membrane with influx and efflux clearances denoted by CL_{in}^l and CL_{ef}^l , respectively. The drug enters the renal cell either at the basolateral membrane with influx clearance, CL_{in}^b , or by reabsorption at the apical membrane (with reabsorptive clearance, CL_{in}^l). In the renal tissue, the drug may undergo metabolism denoted by $CL_{int,met,K}$ to form the primary metabolite, Mi , which may undergo transport at the basolateral and apical membranes or further metabolism, denoted by $CL_{in}^b\{mi\}$, $CL_{ef}^b\{mi\}$, $CL_{in}^l\{mi\}$, $CL_{ef}^l\{mi\}$, and $CL_{int,met,K}\{mi\}$, respectively.

The kidney PBPK model has been utilized to explain levels of drugs that undergo glomerular filtration, saturable secretion, and/or reabsorption and effects of inhibitors such as probenecid at the basolateral membrane [126–130]. The change in drug reabsorption due to changes in pH was also applied in this model [128]. PBPK models are used to describe renal metabolite formation and immediate excretion and the existence of a nonconstant renal metabolite clearance denoting the kidney as a metabolite formation organ [12,84].

One advantage of using the PBPK kidney modeling approach is the ability to examine the effects of renal transporters and enzymes on renal clearance, CL_R , of a drug. Table 16.5 shows the AUC and CL_R for the kidney with possible alternate pathways for the drug and its metabolite. The basolateral (CL_{in}^b and CL_{ef}^b) and luminal (CL_{in}^l and CL_{ef}^l) transporters, the GFR , Q_K , and Q_U all do influence the AUC and CL_R of the drug. The ratio of CL_R and $f_p GFR$, or fractional excretion (FE) or excretion ratio (ER), denotes whether reabsorption, net filtration, or secretion takes place by deviation of the ratio from below unity to unity or to above unity. When metabolism is relevant,

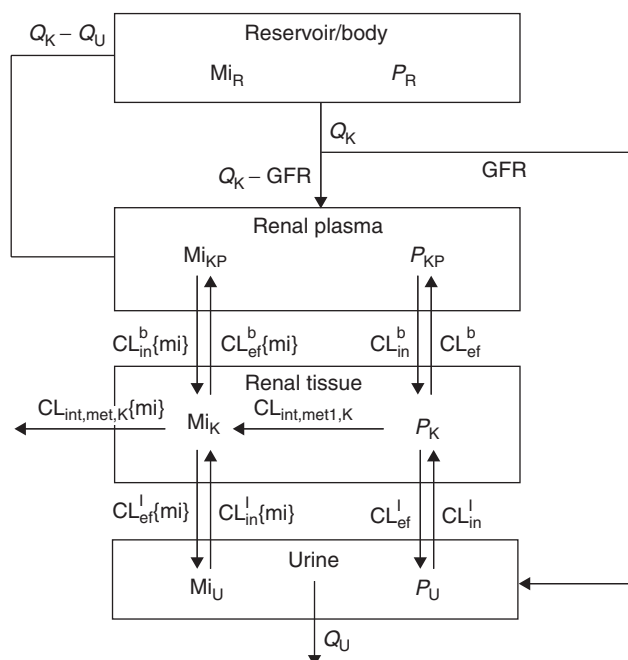


Figure 16.5 Physiologically based pharmacokinetic (PBPK) kidney model, with kidney as the only elimination organ. See text for description of the model.

$CL_{\text{int,met,K}}$ is present in the AUC and CL_R solutions, and the solutions become more complicated (Table 16.5). Indeed, the presence of metabolism as the alternate pathway will affect excretory clearance and further change the FE or ER ratio [91].

Renal PBPK modeling approaches have exploited the concept of drug transport and metabolism to examine the kinetics of a drug and its metabolite(s) in the kidney. In a single-pass kidney perfusion study, the extraction ratio ($E\{\text{mi},P\} = 0.39$) of hippurate (metabolite formed from its precursor drug, benzoate) was found to differ from the extraction ratio when hippurate ($E\{\text{pmi}\} = 0.24$) was administered [131]. The lower extraction ratio of the administered hippurate was explained by the renal PBPK model, which described a rapid hippurate formation within the renal cells and a high secretion activity of hippurate at the apical membrane; in contrast, the preformed hippurate would first undergo filtration before reaching the peritubular cells and partially evade the high secretory sites.

A renal PBPK model has been applied to examine the metabolism and excretion of enalapril, an ACE inhibitor. In both single-pass and recirculating isolated rat kidney perfusion, a higher extraction ratio of enalaprilat, the dicarboxylic acid metabolite, was observed when the precursor, enalapril, was administered compared to that when preformed enalaprilat was administered. The major reason is the presence of a membrane barrier that retards enalaprilat entry into the kidney and back efflux to blood [12,84,89,125,132]. As a result, preformed enalaprilat was mainly eliminated by GFR. For the metabolite enalaprilat that was formed within the kidney, a much higher and time-dependent renal clearance was observed. The time-dependent excretion clearance

TABLE 16.5 Solutions for AUC_{iv} and CL_R with and without Renal Metabolism in the Kidney Only

AUC and CL_R Terms	Solutions
AUC_{iv} (no metabolism)	$AUC_{iv} = Dose_{iv} \frac{\{(\mathcal{Q}_K - \mathcal{Q}_U) [CL_{in}^1 CL_{ef}^b + \mathcal{Q}_U (CL_{ef}^b + CL_{ef}^1)] + \mathcal{Q}_U f_B CL_{in}^b CL_{ef}^1\}}{\mathcal{Q}_U f_B [\mathcal{Q}_K CL_{in}^b CL_{ef}^1 + GFR (\mathcal{Q}_K - \mathcal{Q}_U) (CL_{ef}^b + CL_{ef}^1)]}$
CL_R (no metabolism)	$CL_R = \frac{\mathcal{Q}_U f_B [\mathcal{Q}_K CL_{in}^b CL_{ef}^1 + GFR (\mathcal{Q}_K - \mathcal{Q}_U) (CL_{ef}^b + CL_{ef}^1)]}{(\mathcal{Q}_K - \mathcal{Q}_U) [CL_{in}^1 CL_{ef}^b + \mathcal{Q}_U (CL_{ef}^b + CL_{ef}^1)] + \mathcal{Q}_U f_B CL_{in}^b CL_{ef}^1}$
AUC_{iv} (with metabolism)	$AUC_{iv} = \frac{Dose_{iv} \{(\mathcal{Q}_K - \mathcal{Q}_U) [CL_{in}^1 (CL_{ef}^b + CL_{int,met,K}) + \mathcal{Q}_U (CL_{ef}^b + CL_{ef}^1 + CL_{int,met,K})] + f_B CL_{in}^b [\mathcal{Q}_U CL_{ef}^1 + CL_{int,met,K} (CL_{in}^1 + \mathcal{Q}_U)]\}}{GFR f_B (\mathcal{Q}_K - \mathcal{Q}_U) [CL_{int,met,K} (CL_{in}^1 + \mathcal{Q}_U) + \mathcal{Q}_U (CL_{ef}^b + CL_{ef}^1)] + \mathcal{Q}_K f_B CL_{in}^b [\mathcal{Q}_U CL_{ef}^1 + CL_{int,met,K} (CL_{in}^1 + \mathcal{Q}_U)]}$
CL_R (with metabolism)	$CL_R = \frac{GFR f_B (\mathcal{Q}_K - \mathcal{Q}_U) [CL_{int,met,K} (CL_{in}^1 + \mathcal{Q}_U) + \mathcal{Q}_U (CL_{ef}^b + CL_{ef}^1)] + \mathcal{Q}_K f_B CL_{in}^b [\mathcal{Q}_U CL_{ef}^1 + CL_{int,met,K} (CL_{in}^1 + \mathcal{Q}_U)]}{(\mathcal{Q}_K - \mathcal{Q}_U) [CL_{in}^1 (CL_{ef}^b + CL_{int,met,K}) + \mathcal{Q}_U (CL_{ef}^b + CL_{ef}^1 + CL_{int,met,K})] + f_B CL_{in}^b [\mathcal{Q}_U CL_{ef}^1 + CL_{int,met,K} (CL_{in}^1 + \mathcal{Q}_U)]}$

Source: Taken from Pang [86] with permission.

of formed enalaprilat was partially due to the immediate excretion of the enalaprilat that was formed *in situ* in the kidney (the time-dependent component that is dependent on drug concentration in the kidney) and partially due to filtration of the recirculating metabolite (component reflected by preformed metabolite); the sum contributes to the observed renal clearance of the formed metabolite [12,84,125]. This clearance differs from the conventional estimate of the clearance of the preformed metabolite [12].

16.6 WHOLE-BODY PBPK

Whole-body PBPK models provide an understanding of the behaviors of drugs and metabolites based on physiological parameters. Usually, when the intestine, liver, and kidney, major drug eliminating organs/tissues, are included together with highly perfused, poorly perfused, and adipose tissues (Fig. 16.6), mechanisms governing removal and the rate-limiting step (e.g., metabolism by enzymes, influx by transporters, and secretion via apical transporters) are revealed. In this occasion, the renal clearance may be embedded in the blood compartment to describe the parallel loss of drug from

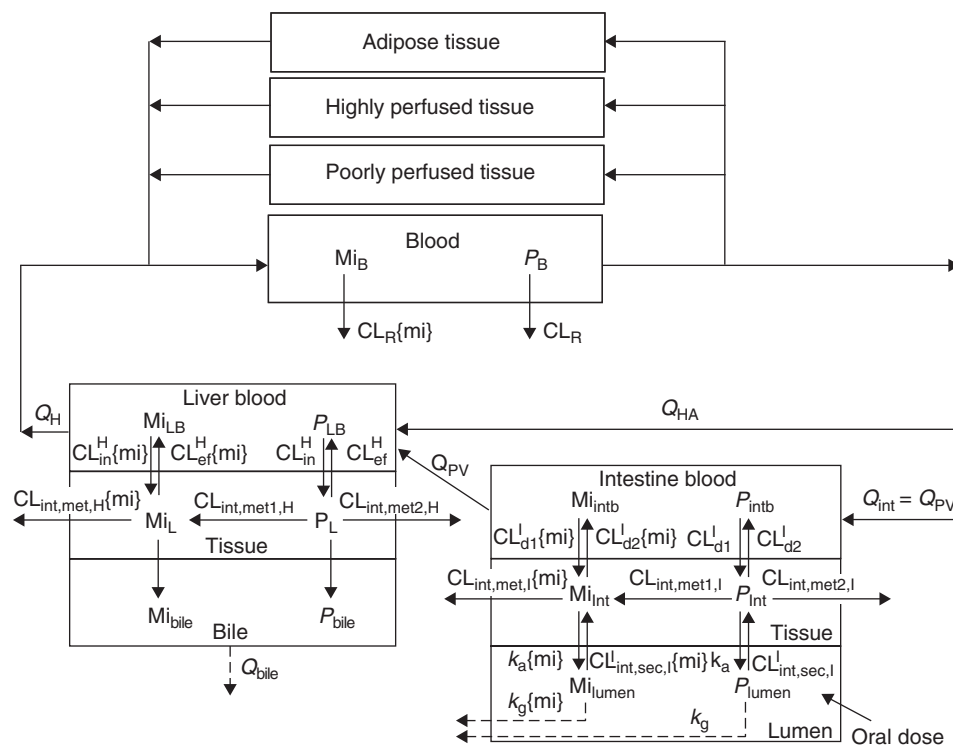


Figure 16.6 Whole-body, physiologically based pharmacokinetic (PBPK) model, with drug and metabolite being excreted by the kidneys with CL_R and $CL_R\{mi\}$, and metabolized either in the intestine or the liver. Sequential metabolism/excretion of the formed metabolite occurs with the organ of formation, and the drug may be further metabolized to other metabolites. Only the intestinal TM is presented here, but the SFM is also considered (Table 16.6). See text for description of the model.

the kidney. The PBPK models provide an account of how the processes of transport and metabolism modulate the kinetics of the drug and its metabolites, and how drug behavior further affects the kinetics of the metabolites (Table 16.6).

The analytical solutions for the AUC and $AUC_{\{mi,P\}}$ solved for the drug and the formed metabolite divulge how the variables affect the exposure and fates of the drug and the formed metabolite within metabolite formation organs and in blood/plasma (Table 16.6). The solutions reveal that the effect of intestinal secretion is nullified when avid absorption takes place (Table 16.6). It may be further discerned that the ratio of $AUC_{po\{mi,P\}}/AUC_{iv\{mi,P\}}$ pursuant to drug oral and intravenous administration, corrected for dose, yields the fraction of dose absorbed, F_{abs} , into the portal circulation when either the intestine or the liver is the only drug metabolizing organ, and the dose-corrected AUC_{po}/AUC_{iv} yields the systemic availability, F_{sys} . On division of AUC_{po}/AUC_{iv} by $AUC_{po\{mi,P\}}/AUC_{iv\{mi,P\}}$, one obtains the intestinal or hepatic availability (Table 16.6) [18].

16.6.1 Sequential Metabolism and Metabolite Behavior

The PBPK model is particularly pertinent to describe the sequential elimination of the formed metabolite, asserting that the metabolite, formed *in situ* within the formation organ, is subject to immediate excretion or sequential metabolism to form secondary or tertiary metabolites. The reason that estimation of the AUC of the metabolite, $AUC_{\{mi,P\}}$, is important because the metabolite may possess active or toxic therapeutics effects [86]. Furthermore, one can take advantage of AUC solutions of the parent drug and the formed metabolites and compare the AUC ratio between intravenous and oral administration to estimate the variables important for drug absorption. Some examples on sequential metabolism are listed in Table 16.7.

The whole-body PBPK model approach clearly explains the influence of transporters and enzymes on the AUCs of the parent drug and its formed metabolites in a route-dependent fashion and that the $AUC_{\{pmi\}}$ differs from the $AUC_{\{mi,P\}}$. Pathways of sequential metabolism of the formed primary metabolite (Mi) can exist either within its organ of formation or in additional eliminating organs. Under this situation, the AUCs and clearances of formed (denoted by subscript, mi) and preformed primary metabolite (denoted by subscript, pmi) differ when there is low permeability of the metabolite, resulting in the delay in the kinetics due to the formation step of the formed metabolite and/or competition for the drug in the same or other organs. These differences have been demonstrated both experimentally and theoretically [15,18,102,122,142–144]. The discrepancy is also caused by differences in enzyme/transporter heterogeneity within the organ involved in formation or further metabolism of Mi [15,18,86,122]. These differences must be borne in mind when the kinetics of the preformed metabolite is used to predict the kinetics of the formed metabolite, especially for MIST.

16.6.2 Examination of Transporter and Enzyme Interplay

Generalized whole-body PBPK models have been developed to describe the impact of transporters and enzymes on the PK of the parent drug and the disposition of formed metabolites. Not until recently, these models lack the appropriate subcompartments within the organs to describe the influence of different transporters and enzymes on the parent drug and metabolites. Sun and Pang [18] described a whole-body PBPK model

TABLE 16.6 Solutions in the Whole-Body PBPK Model

AUC Solutions

For Intestinal Metabolism and Renal Excretion Only

$$\begin{aligned}
 \text{AUC}_{\text{iv}} & \quad \text{AUC}_{\text{iv}} = \text{Dose}_{\text{iv}} \frac{Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} + (Q_{\text{PV}} + \text{CL}_{\text{d1}}^{\text{I}}) [\text{CL}_{\text{int,met1,I}} + \text{CL}_{\text{int,met2,I}} + (1 - F_{\text{abs}}) \text{CL}_{\text{int,sec,I}}]}{\text{CL}_{\text{R}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} + [\text{CL}_{\text{R}} Q_{\text{PV}} + \text{CL}_{\text{d1}}^{\text{I}} (\text{CL}_{\text{R}} + Q_{\text{PV}})] [\text{CL}_{\text{int,met1,I}} + \text{CL}_{\text{int,met2,I}} + (1 - F_{\text{abs}}) \text{CL}_{\text{int,sec,I}}]} \\
 \text{AUC}_{\text{iv}\{\text{mi,P}\}} & \quad \text{AUC}_{\text{iv}\{\text{mi,P}\}} = \text{Dose}_{\text{iv}} \frac{\text{CL}_{\text{int,met1,I}} Q_{\text{PV}} \text{CL}_{\text{d1}}^{\text{I}}}{\text{CL}_{\text{R}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} + [\text{CL}_{\text{R}} Q_{\text{PV}} + \text{CL}_{\text{d1}}^{\text{I}} (\text{CL}_{\text{R}} + Q_{\text{PV}})] [\text{CL}_{\text{int,met1,I}} + \text{CL}_{\text{int,met2,I}} + (1 - F_{\text{abs}}) \text{CL}_{\text{int,sec,I}}]} \times \\
 & \quad \frac{Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} \{\text{mi}\}}{\text{CL}_{\text{R}\{\text{mi}\}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} \{\text{mi}\} + [\text{CL}_{\text{R}\{\text{mi}\}} Q_{\text{PV}} + \text{CL}_{\text{d1}}^{\text{I}} \{\text{mi}\} (\text{CL}_{\text{R}\{\text{mi}\}} + Q_{\text{PV}})] [\text{CL}_{\text{int,met,I}\{\text{mi}\}} + (1 - F_{\text{abs}} \{\text{mi}\}) \text{CL}_{\text{int,sec,I}\{\text{mi}\}}]} \\
 \text{AUC}_{\text{po}} & \quad \text{AUC}_{\text{po}} = \text{Dose}_{\text{po}} \frac{F_{\text{abs}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}}}{\text{CL}_{\text{R}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} + [\text{CL}_{\text{R}} Q_{\text{PV}} + \text{CL}_{\text{d1}}^{\text{I}} (\text{CL}_{\text{R}} + Q_{\text{PV}})] [\text{CL}_{\text{int,met1,I}} + \text{CL}_{\text{int,met2,I}} + (1 - F_{\text{abs}}) \text{CL}_{\text{int,sec,I}}]} \\
 \text{AUC}_{\text{po}\{\text{mi,P}\}} & \quad \text{AUC}_{\text{po}\{\text{mi,P}\}} = \text{Dose}_{\text{po}} \frac{F_{\text{abs}} \text{CL}_{\text{int,met1,I}} [\text{CL}_{\text{d1}}^{\text{I}} (\text{CL}_{\text{R}} + Q_{\text{PV}}) + \text{CL}_{\text{R}} Q_{\text{PV}}]}{\text{CL}_{\text{R}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} + [\text{CL}_{\text{d1}}^{\text{I}} (\text{CL}_{\text{R}} + Q_{\text{PV}}) + \text{CL}_{\text{R}} Q_{\text{PV}}] [\text{CL}_{\text{int,met1,I}} + \text{CL}_{\text{int,met2,I}} + (1 - F_{\text{abs}}) \text{CL}_{\text{int,sec,I}}]} \times \\
 & \quad \frac{Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} \{\text{mi}\}}{\text{CL}_{\text{R}\{\text{mi}\}} Q_{\text{PV}} \text{CL}_{\text{d2}}^{\text{I}} \{\text{mi}\} + [\text{CL}_{\text{R}\{\text{mi}\}} Q_{\text{PV}} + \text{CL}_{\text{d1}}^{\text{I}} \{\text{mi}\} (\text{CL}_{\text{R}\{\text{mi}\}} + Q_{\text{PV}})] [\text{CL}_{\text{int,met,I}\{\text{mi}\}} + (1 - F_{\text{abs}} \{\text{mi}\}) \text{CL}_{\text{int,sec,I}\{\text{mi}\}}]}
 \end{aligned}$$

(continued overleaf)

TABLE 16.6 (Continued)

AUC Solutions

For Hepatic Metabolism ($Q_H = Q_{PV} + Q_{HA}$) and Renal Excretion Only

AUC_{iv}	$AUC_{iv} = Dose_{iv} \frac{[Q_H(CL_{ef}^H + CL_{int,H}) + CL_{in}^H CL_{int,H}]}{CL_R Q_H(CL_{ef}^H + CL_{int,H}) + CL_{in}^H CL_{int,H}(CL_R + Q_H)}$
$AUC_{iv}\{mi,P\}$	$AUC_{iv}\{mi,P\} = Dose_{iv} \frac{Q_H CL_{in}^H CL_{int,met1,H}}{CL_R Q_H(CL_{ef}^H + CL_{int,H}) + CL_{in}^H CL_{int,H}(CL_R + Q_H)} \times$ $\frac{Q_H CL_{ef}^H\{mi\}}{CL_R\{mi\} Q_H(CL_{ef}^H\{mi\} + CL_{int,H}\{mi\}) + CL_{in}^H\{mi\} CL_{int,H}\{mi\}(CL_R\{mi\} + Q_H)}$
AUC_{po}	$AUC_{po} = Dose_{po} \frac{F_{abs} Q_H(CL_{ef}^H + CL_{int,H})}{CL_R Q_H(CL_{ef}^H + CL_{int,H}) + CL_{in}^H CL_{int,H}(CL_R + Q_H)}$
$AUC_{po}\{mi,P\}$	$AUC_{po}\{mi,P\} = Dose_{po} \frac{F_{abs} Q_H CL_{in}^H CL_{int,met1,H}(CL_R + Q_H)}{CL_R Q_H(CL_{ef}^H + CL_{int,H}) + CL_{in}^H CL_{int,H}(CL_R + Q_H)} \times$ $\frac{CL_{ef}^H\{mi\}}{CL_R\{mi\} Q_H(CL_{d2}^H\{mi\} + CL_{int,H}\{mi\}) + CL_{in}^H\{mi\} CL_{int,H}\{mi\}(CL_R\{mi\} + Q_H)}$

Source: From Sun and Pang [18] with permission.

TABLE 16.7 Examples of PBPK Modeling for Sequential Metabolism

Chemicals and Major Metabolic Pathway of Interest	Applications and Features	References
Nicotine → cotinine → polar metabolites	- Studied nicotine disposition in SD rats - Described tissue and plasma kinetics of cotinine, a biomarker for exposure to tobacco smoke - Used <i>in vivo</i> rat nicotine PK study to validate the predictive power of the PBPK model	134
Butadiene → butadiene monoxide → butadiene monoxide glutathione conjugate	- Examined potential carcinogen effect of butadiene, an environmental air pollutant - Validated PBPK model against published observations on butadiene and butadiene monoxide and liver glutathione kinetics in rats and mice <i>in vivo</i> - PBPK model was used to explain species difference in cancer response between mice and rats exposed to butadiene	135,136
Styrene → styrene → styrene-7,8-oxide → styrene-7,8-oxide glutathione conjugate	- Illustrated the carcinogenic potential of styrene due to its active metabolite styrene-7,8-oxide in risk assessment - Included cosubstrate, glutathione, to describe conjugation of styrene-7,8-oxide to its conjugate by glutathione-S-transferase	137
Octamethylcyclotetrasiloxane (D ₄) → short-chain linear siloxanes	PBPK of inhalation kinetics of D₄, a silicon fluid, in humans during rest and exercise	138
Uracil → dihydrouracil → β-ureidopropionate → β-alanine	- Described ¹³CO₂ exhalation after orally administered [2-¹³C]uracil - Reported dihydropyrimidine dehydrogenase as the key enzyme of cytotoxicity of 5-FU - Used [2-¹³C]uracil in identifying patients at risk of 5-FU toxicity	139
Atrazine → chlorotriazines → diaminochlorotriazine → glutathione conjugates	Described oral absorption and oxidative metabolism of atrazine on the blood time course curves of individual chlorotriazines in rat, and the toxic neuroendocrine effects associated with chlorotriazines exposure but not of the nonchlorinated metabolites	140
Dichloromethane (DCM) → formyl chloride → CO	Evaluated two different metabolic hypotheses for DCM toxicity using PBPK modeling for <i>in vivo</i> inhalation gas uptake data exposure in female B6C3F1 mice	141

Source: Adapted from Fan *et al.* 133.

approach that includes both transporters and enzymes and their impact on the AUC and PK of the parent drug and the formed metabolites. This whole-body PBPK model approach clearly explains the influence of transporters and enzymes on the AUCs of the parent drug and its formed metabolites in a route- and time-dependent fashion (Table 16.6).

There are an increasing number of studies with PBPK modeling that examine effects of the interplay of transporters and enzymes on the blood and target organ exposures of the drug and its metabolites [78]. Watanabe *et al.* [79] reported that changes in pravastatin hepatic uptake greatly altered the plasma concentration but had minimal effect on the concentration in liver, whereas alteration of hepatic efflux transporters would greatly affect liver concentration but not plasma concentration. In another study, Rowland Yeo *et al.* [145] found that enzyme inhibition of triazolam by diltiazem and its metabolite, *N*-desmethyldiltiazem, plays a role in affecting the AUC of triazolam.

16.7 APPLICATIONS OF PBPK MODELS

16.7.1 Prediction of Exposure in Tissue Sites

In principle, PBPK models permit the prediction of the concentration of the parent drug and metabolites in any tissue or target organ. Examples are tumor tissue [146], the fetus [147], the testes [28], or other various tissues [4,134,148,149] within the body, providing additional physiological information and mechanistic answers. In addition, PBPK models are superior over traditional PK models in that they can be used to explain the nonlinear uptake of parent drugs from skin into tissue sites [150], membrane-limited uptake of drugs [151], or the disposition of both the parent drug and its metabolite in tissue sites [152]. Table 16.8 lists examples investigating drug exposure at tissue sites which were described by PBPK models.

16.7.2 Prediction of Pharmacological Activity or Toxicity

PBPK/PD models are also useful for predicting the relationship between drug exposure and pharmacological and/or toxicological effects in target organs to explain pharmacological [154,155] and toxicological outcomes [146,156,157]. By providing a link between tissue concentration and pharmacological and/or toxicological effects, PBPK models can be expanded to form mechanistic PK/PD models [158]. When combined with a mechanistic or empirical PD model (PBPK/PD), the therapeutic outcome or adverse events within a certain population could be predicted before clinical studies. The PBPK/PD model is created when the PBPK model is linked to a pharmacodynamic (PD) model by an equation that reflects the researcher's hypothesis of how the drug participates in the initiation of cellular changes leading to measurable outcomes. The temporal change in the dose metric simulated by the PBPK model is linked with mathematical descriptions of the response process associated with the drug.

Table 16.9 summarizes applications of PBPK/PD model to quantify the relationship between the drug and the pharmacologic effect. A semimechanistic PK–PD model, with incorporation of saturable hepatic elimination on the first-pass extraction as well as a

TABLE 16.8 Examples of Whole-Body PBPK Models that Emphasize Drug Exposure at Tissue Sites

Chemicals	Species	Model	References
5-Flourouraacil (5-FU)	Mouse	Flow-limited model to predict accumulation of 5-FU in human tumor tissues	146
Barbiturates	Rat	Flow-limited compartments for lung, liver, kidney, stomach, pancreas, spleen, gut, muscle, adipose, skin, bone, and heart and membrane-limited compartments in brain and testis barbiturates disposition in different tissues	149
Chlordecone	Rat	Flow-limited model to evaluate the effect of age and dosage on percutaneous absorption and tissue disposition of chlordecone	153
Digoxin	Rat	Flow-limited model to predict the concentrations of digoxin in the heart, liver, skeletal muscle, and plasma	148
Everolimus	Rat	Flow-limited model to predict nonlinear binding of everolimus to red blood cells and tissues, and the highest tissue binding in thymus, skin, and muscle	150
Moxalactam	Rat, dog, and human	Membrane-limited model to explain moxalactam epimerization on the warfarin-binding site of serum albumin	151
Nicotine	Rat	Flow-limited model to predict the nonlinear nicotine elimination in the brain, heart, and lung, suggesting that saturable nicotinic binding sites may be important in nicotine disposition	134
<i>p</i> -Phenylbenzoic acid	Rat	Flow-limited model to predict <i>p</i> -phenylbenzoic acid concentration–time profiles in different tissues of the mother and placenta of the fetuses	147
Thiopental	Dog	Flow-limited model to predict thiopental concentrations in blood, viscera, lean tissue, and adipose tissue	4
Tretinoin	Rat, monkey, and human	Flow-limited model to predict tretinoin pharmacokinetics in plasma and different tissues and to extrapolate across species and routes of administration	152

TABLE 16.9 Examples of Whole-Body PBPK/PD Models

Applications and Features		References
<i>Quantified Relationship Between Drug and Effect</i>		
Diisopropylfluorophosphate (DFP)	Effect of DFP on acetylcholinesterase activity	160
Glycyrrhetic acid	Inhibition of 11 β -hydroxysteroid dehydrogenase activity	157
Chlorpyrifos	Evaluated the chlorpyrifos toxicity, pharmacokinetics, and tissue cholinesterase inhibition in neonatal and adult rats	161
Carbofuran	Characterized AChE inhibition from carbofuran exposure	162
<i>Predicted Toxicologic Interactions</i>		
Trichloroethylene and 1,1-dichloroethylene	Predicted the toxicologic interactions and define the interaction threshold	163
Kepon and carbon tetrachloride	Predicted the toxicologic interactions and define the interaction threshold	164
<i>Linked Drug Dosimetry with Biological Response</i>		
Trichloroethylene	Evaluated the organ toxicity from oral exposure and inhalation	165
Chlorpyrifos and diazinon	Determined dosimetry and cholinesterase inhibition	161
Diazinon and its metabolites	Determined dosimetry and cholinesterase inhibition	166
Carbaryl and its metabolites	Predicted the carbaryl-induced inhibition of cholinesterase activity	167
<i>Evaluated Organ Toxicity Resulting from Metabolites</i>		
Trichloroethylene	Evaluated the organ toxicity resulting from metabolites	165
<i>Performed Various Forms of Extrapolation</i>		
Acrylamide	Extrapolated data from rodent to human	168,169
Brivaracetam	Extrapolated data from rodent to human	170

higher intrinsic clearance in female patients, resulted in the best fit to the time course of salivary artemisinin concentrations after repeated dosing and the number of parasites in patients treated with the drug [159]. The effect of diisopropylfluorophosphate (DFP) on acetylcholinesterase (AChE) activity in mouse and rat was studied using a PBPK/PD model, which accurately predicted the AChE activity similar to those found *in vivo* based on the blood concentration of DFP [160].

The PBPK/PD model may also be used to evaluate organ toxicity developed from trichloroethylene (TCE) metabolites [165]. To date, there are over 250 scientific research papers (searched via PubMed, PBPK + toxicity) using similar PBPK modeling approaches to predict and explain the toxicity of drugs, environmental chemicals, or its metabolites in animal or human models [171–173]. Table 16.10 lists examples of PBPK models that were used to predict chemical exposure and toxicity *in vivo*. Doerge *et al.* [168] used a PBPK/PD model to translate the findings from rodent neurotoxicity and cancer risks into estimates of risks in human acrylamide exposure through diet. PBPK/PD models are also useful tools for linking drug dosimetry with biological response and for evaluating the risk associated with a given drug exposure. Barton and Clewell [165] used a PBPK/PD model to evaluate eye malformation, liver effects, immunotoxicity, and kidney toxicity from oral exposure and neurological, liver, and kidney effects by inhalation with dose metrics for TCE.

Other studies had estimated toxicological exposures of chemicals and drugs that ranged from phthalate esters found in drinking water to acetonylacetone (a neurotoxic industrial solvent), trace metals such as iodine and chromium, and therapeutic drugs such as digoxin [28,171] in target organs. Using the appropriate physiological parameters, Sweeney *et al.* [180] were able to relate the incidence of liver adenomas and carcinomas to the liver exposure (AUC) of trichloroacetic acid, the toxic metabolite of perchloroethylene (a solvent used in dry cleaning), in a mouse PBPK model. In another toxicological study, a generalized rat whole-body PBPK model, focusing primarily on the liver, examined the involvement of transporters such as Mrp2 on the liver exposure of pentachlorobipenyl, a carcinogenic environmental pollutant and a substrate of Mrp2, and liver carcinogenicity [179]. Toxicologic interactions have been predicted with PBPK/PD modeling that defined the interaction threshold between TCE and 1,1-dichloroethylene (1,1-DCE) [163] and between kepone and carbon tetrachloride (CCl₄) [164].

16.7.3 Effects of Disease States, Pregnancy, Age, and Polymorphism

PBPK modeling approaches in toxicological studies not only focus on exposure of toxicants to healthy adults but also apply to pregnant women [182], children [183], the elderly [184], and the study of genetic polymorphism [185]. PBPK models enable us to evaluate the drug concentration–time profile for any dose and administration route under various physiological conditions: disease state, pregnancy, age, and polymorphism.

There have only been a few studies that relate to PK of drugs in disease states with PBPK modeling. For instance, a PBPK model describing drug kinetics in interstitial fluid in patients with hemorrhagic shock followed by fluid resuscitation was used to evaluate amoxicillin and clavulanate PK [38]. The predictions fit the data well in most instances and were consistent with observed clinical data. A PBPK model adequately described altered midazolam kinetics in elderly patients undergoing

TABLE 16.10 Examples of Whole-Body PBPK Models that Emphasized Drug Toxicity

Chemicals	Species	Model	References
1,1,1,3,3-Pentafluoropropane	Human	Flow-limited model to explain that exposure of 1,1,1,3,3-pentafluoropropane did not result in detection of the metabolite TFPA in urine, suggesting little or no metabolism	174
Arsenic	Mouse	Flow-limited model to explain lack of change in the apparent volume of distribution and tissue-plasma concentration ratios of dimethylarsonic acid after acute and chronic exposure to arsenic	175
Chloroethane	Rat, mouse, human	Flow-limited model to describe different rates of metabolism of chloroethane by glutathione-S-transferase in different species, consistent with observations on uterine tumors	25
Dibromoacetic acid	Rat	Membrane-limited model for aggregated tissue, kidney, and liver and liver metabolism of dibromoacetic acid in the toxicity or carcinogenicity in liver	176
Doxorubicin	Dog	Flow-limited model to explain increase in doxorubicin exposure in ABCB1 (null) dog that may lead to GI toxicosis	177
Molinate	Rat, human	Flow-limited model to predict molinate concentrations of the rat blood and testes compartment and testicular toxicity	178
Pentachlorobipenyl (PCB126)	Rat	Flow-limited model to relate AUC of PCB126 in liver and incidence of liver toxicity	179
Phthalic acid diesters	Rat	Flow-limited model to predict that plasma monobutyl phthalate kinetics are sensitive to glucuronidation and enterohepatic recirculation	28
Trichloroacetic acid	Mouse	Flow-limited model to assess at trichloroacetic acid liver (AUC) exposure to risk of liver adenomas and carcinomas	180
Trichloroethylene	Mouse	Flow-limited model to relate extents of metabolites formed from trichloroethylene in liver to cause hepatomegaly	181

orthopedic surgery due to increased f_p values and changes in volume of distribution and clearance as a result of blood loss and significant reduction in albumin concentration when compared to normal patients [37]. When PBPK modeling was applied to predict the outcomes of liver cirrhosis, a disease characterized by a decrease in functional hepatocytes, lowered circulating plasma proteins, and altered hepatic blood flow patterns due to the development of portacaval shunts, some success was found [186].

There have also been attempts to apply a PBPK model to describe the effect of renal failure on drug disposition. Tsuji *et al.* [36] developed a PBPK model to predict elevation of plasma and tissue levels of cefalozin, a β -lactam antibiotic, in rabbits with renal failure, and obtained good correlation between predicted and observed tissue concentrations. A PBPK model proposed by Harrison and Gibaldi [35] was used to assess digoxin PK in the dog with renal failure, and a scale-up with the dog model to human was partially successful in predicting digoxin kinetics in patients with renal failure. In renal disease state, a concomitant reduction in hepatic drug metabolizing activities is often found [187,188]. Moreover, changes in transporters [189,190] were found associated with renal [187] and other diseases: inflammatory bowel diseases and inflammation and infection wherein cytokines are elevated [190,191]. These accompanying changes in transporter/enzyme function further add complexities to the quantitative prediction with regard to disease-related factors that may limit use of PBPK models, unless the changes are readily identified [192].

Since drug distribution could be altered in pregnancy [193], PBPK models for pregnancy have been used to predict the concentrations of drugs in maternal tissues, the placenta, and/or the fetus [193–198] (Table 16.11). Gabrielsson and colleagues, in particular, had reported on the effects of pregnancy on methadone [197], theophylline [196], pethidine (meperidine) [198], and morphine [195] kinetics in the rat with a PBPK model. Additional reports of PBPK models on pregnancy are listed in Table 16.11.

The prediction of drug exposure in special populations (different age groups including neonates, infants, children, and adult) is often made with PBPK modeling [199–201]. A general PBPK model has been used for the prediction of the PK of model substrates, such as theophylline for CYP1A2 and CYP2E1 and midazolam for CYP3A4, in neonates to young adults by considering the age-related changes in unbound intrinsic hepatic clearances [199], with predictions that were in good agreement with literature data. Furthermore, Edginton *et al.* [200] and Johnson *et al.* [201] had applied PBPK models to assess the age-related physiological changes in younger children for 5 (paracetamol, alfentanil, morphine, theophylline, and levofloxacin) and 11 drugs (midazolam, caffeine, carbamazepam, cisapride, theophylline, diclofenac, omeprazole, *S*-warfarin, phenytoin, gentamycin, and vancomycin), respectively. The predictions were also in good agreement with observed data and showed that the PBPK models are superior to the method of allometric scaling. Thompson *et al.* [202] further reviewed a physiological database useful for PBPK modeling of the healthy and impaired elderly population.

In addition to the above conditions, PBPK models have also been used to simulate effects of genetic variations [79] and enzyme/transporter heterogeneity [92,95] on drug disposition in eliminating organs and the consequences on pharmacological and toxicological effects [39]. Differences in metabolism due to enzyme abundance (e.g., in the case of CYP2D6 poor metabolizers) or differences in intrinsic enzyme activities (e.g., CYP2C9 variants) could be included in PBPK modeling. Inoue *et al.* [203] had found

TABLE 16.11 Examples for Whole-Body PBPK Model for Pregnancy

Drug	Model	Species	References
Morphine	Flow limited for all tissue; membrane limited (fetus)	Rat	196
Methadone	Flow limited for all tissue; membrane-limited compartments for maternal brain/fetus	Rat, human	197
Pethidine (meperidine)	Flow limited for all tissue; membrane limited for fetus	Rat, human	198
<i>p</i> -Phenylbenzoic acid	Flow limited for some tissue; membrane limited for placenta, brain, gut, spleen, muscle, fat, and skin	Rat	147
Tetracycline	Flow limited for all tissues	Rat	194
Theophylline	Flow limited for all tissue; membrane-limited compartments for maternal brain/fetus	Rat	195

ethnic differences in the *in vivo* clearances of alprazolam, caffeine, chlorzoxazone, cyclosporine, midazolam, omeprazole, sildenafil, tolbutamide, triazolam, *S*-warfarin, and zolpidem with the virtual clinical simulator, Simcyp[®], which contains background information on the abundance of various P450s and different population distributions of their alleles to account for genetic and ethnic differences in metabolism. The interesting phenomenon is well described by Dickinson *et al.* [204] using the Simcyp[®] simulator to predict the impact of genetic polymorphism of CYP2C9 on the PK and PD of *S*-warfarin. Recently, a study on genetic polymorphism of the anionic transporter organic anion transporting polypeptide 1B1 (OATP1B1) examined pravastatin plasma concentrations with PBPK modeling and showed sensitivity of systemic exposure of pravastatin to hepatic uptake [39].

16.7.4 Drug–Drug Interactions

The accurate prediction of potential risks for DDIs is important because DDIs are responsible for reduced efficacy or increased adverse events. DDIs usually result from the induction or inhibition of metabolic enzymes, mostly P450s, or transporters, or from a change in protein binding. Inhibition may involve either simple competitive or time-dependent (mechanism-based) inhibitory kinetics, the latter of which involves destruction of the enzyme/transporter that leads to a reduction in metabolism/transport [17]. Induction of the enzyme/transporter usually involves transcriptional and translational changes that lead to new protein synthesis. Alternately, changes in endogenous substrates, for example, cytokines, or ligands of nuclear receptors can also affect enzyme and transporter levels [190].

A general equation exists for the AUC ratio in the presence of the interactant (or perpetrator) and under control conditions ($AUC_{(I)}/AUC_{(C)}$) to reflect inhibition or

induction of a victim drug:

$$\frac{AUC_{(I)}}{AUC_{(C)}} = \frac{1}{\left(\frac{f_m f_{m,CYP}}{\left(\frac{CL_{int(C)}}{CL_{int(I)}} \right)} \right) + (1 - f_m \cdot f_{m,CYP})} \quad (16.17)$$

where f_m , $f_{m,cyp}$, $CL_{int(C)}$, and $CL_{int(I)}$ are the fraction of the dose metabolized by all P450 isoforms, the fraction of the dose metabolized by each specific P450 enzyme, and the intrinsic clearances under control and inhibited conditions, respectively [205–207].

For reversible metabolism, the ratio of $CL_{int(C)}/CL_{int(I)}$ equals

$$\frac{CL_{int(C)}}{CL_{int(I)}} = 1 + \frac{[I]}{K_I} \quad (16.18)$$

where $[I]$ is the inhibitor concentration and K_I is the inhibition constant.

For time-dependent (mechanism-based) inhibition, the ratio of $CL_{int(C)}/CL_{int(I)}$ equals

$$\frac{CL_{int(C)}}{CL_{int(I)}} = 1 + \frac{k_{inact}}{K_I} \frac{[I]}{k_{deg}} \quad (16.19)$$

where k_{inact} and k_{deg} are the rate constants for inactivation and degradation of the enzyme, respectively [208,209].

For induction, the ratio of $CL_{int(C)}/CL_{int(I)}$ equals

$$\frac{CL_{int(C)}}{CL_{int(I)}} = 1 + \frac{E_{max}[I]}{EC_{50} + [I]} \quad (16.20)$$

where E_{max} and EC_{50} are the maximum induction activity and the concentration of inducer associated with half maximum induction, respectively [210]

These types of static AUC comparisons, however, have been criticized since the inhibitor concentration or $[I]$, taken as C_{max} or unbound C_{max} [211], does not reflect the concentrations of drug and interactant at the site of interaction of the enzyme/transporter. Furthermore, the interaction may lead to changes in protein levels of the enzymes/transporters or consequences on the synthesis and degradation rate. Under the same circumstances, the concentration of the interactant may also be changed.

DDI predictions with the use of PBPK modeling on reversible inhibition afford more accurate predictive ability than conventional methods (Eq. 16.18) that fail to describe the $[I]/K_I$ ratio, given that $[I]$, or the concentration of the inhibitor at the enzyme site, is not static but changes with time and mutual inhibition. Some studies included PBPK models for simulating the changes in drug kinetics with reversible DDIs [211–213]. Furthermore, a whole-body PBPK approach based on a gut compartmental absorption and transit (CAT) model showed improved predictions on DDIs of midazolam, the CYP3A4 substrate, which was coadministered with ketoconazole or verapamil [214]. In comparison, the conventional, static approach with the use of $[I]/K_I$ overpredicted levels of midazolam, whereas predictions by the PBPK model were superior (within

TABLE 16.12 Use of Whole-Body PBPK Models for DDIs

Drug	Combination Drug (Inhibitor)	Species	References
2',3'-Dideoxyinosine	Pentamide	Rat and human	212
Ethoxybenzamide	Salicylamide	Rabbit	215
Midazolam	Clarithromycin	Human	216
Midazolam	Ketoconazole or verapamil	Human	214
Ritonavir, amprenavir, saquinavir, nelfinavir, and indinavir in multiple therapy	Ritonavir, amprenavir, saquinavir, nelfinavir, and indinavir	Rat	217
Tolbutamide	Sulfaphenazole, sulfadimethoxine, and sulfamethoxazole	Rat	40
Triazolam	Erythromycin	Human	211
Warfarin	Bromosulfophthalein	Rat	218

twofold for AUC and $C_{\max}$), regardless of administration route, dosage, and/or coadministration with an inhibitor [214]. These examples are summarized in Table 16.12.

PBPK models that entail dynamic changes in concentration of the perpetrator and victim drug have also been proposed [145,216,217,219]. For example, a simplified PBPK model incorporating two elimination pathways for intestinal and hepatic metabolism of HIV protease inhibitors, including ritonavir, amprenavir, saquinavir, nelfinavir, and indinavir, was constructed to describe multiple HIV therapy and, subsequently, the interaction due to multitherapy with the protease inhibitors [217]. The observed and predicted AUCs according to the PBPK model were in good agreement after oral administration in rats. Zhang *et al.* [219] described changes in intestinal and hepatic P450 protein levels together with changes in activity toward midazolam metabolism due to the mechanism-based inhibition by diltiazem and its major metabolite and predicted the transient and dynamic changes. Quinney *et al.* [216] developed a simple PBPK model, incorporating hepatic and intestinal metabolism by CYP3A and non-CYP3A4 mechanisms, to predict the mechanism-based inhibition of clarithromycin on midazolam metabolism and explained the nonlinear PK of clarithromycin. In another study, Rowland Yeo *et al.* [145] found that the simultaneous competitive and time-dependent enzyme inhibition of triazolam by diltiazem and its metabolite, *N*-desmethyldiltiazem, played a significant role in altering triazolam blood AUC. Clearly, the impact of transporters and enzymes on the exposure of drugs and its metabolite in plasma and target organs are avidly being examined by whole-body PBPK models. Their inclusion, especially under conditions of prolonged inhibition [220], needs to be further investigated.

16.8 CONCLUSIONS

PBPK models are comprehensive models that consider the physiology, anatomy, and biochemistry of the body as well as physicochemical properties of the drug and metabolite. The understanding of absorptive, distribution, metabolic, and excretory

processes through fitting and simulation of concentration–time profiles of plasma and target tissues in generic PBPK models, as discussed in this chapter, allows one to make predictions in target sites and allows proper projection of the effects of age, pregnancy, disease states, and DDIs. Currently, there is an impetus for pharmaceutical industry and FDA to utilize PBPK modeling and commercialized software in drug development and for the prediction of DDIs, genetic polymorphism, and disease states, with animal and *in vitro* studies for the scale-up of transporter and enzyme function to predict transport and metabolic activities in man *in vivo*. These activities have greatly facilitated an understanding of processes affecting the concentration time course, AUC, and clearance of drugs and their metabolites. The increasing use of PBPK modeling in drug development, clinical pharmacology, and risk assessment is a clear testament of the great utility of PBPK modeling in pharmaceutical research.

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17 Pharmacodynamics

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17.1	Summary	1
17.2	Mechanisms of drug action	2
17.3	Drug–receptor interactions	9
17.4	Biomarkers	13
17.5	Mathematical models in pharmacodynamics	19
17.6	Concentration–response relationship	20
17.7	Classification of mathematical models in pharmacodynamics	21
17.8	Tools and softwares commonly used in PK-PD modeling	45
17.9	Conclusions	45
17.10	Future perspectives: systems modeling	45
	Acknowledgments	46
	References	46

17.1 SUMMARY

Pharmacodynamics (PD) is the study of physiological effects of drugs on the body and their mechanisms of actions. It provides the basis for rational therapeutic use of drugs and the design of new and superior therapeutic agents [1]. PD comes from the Greek word for “drug,” “pharmakon,” and dynamics means “variation in intensity.” On the other hand, pharmacokinetics (PK) deals with how the body handles the drug after it is absorbed and involves disposition, that is, distribution, metabolism, and excretion processes. For example, an orally administered anticonvulsant drug has to enter the brain after crossing intestinal, hepatic, and blood–brain barriers. The drug has to reach its site of action (brain) in sufficient concentrations and duration to interact with a specific target (i.e., receptor) to produce the desired effect. The drug concentration measured in plasma is more reflective of drug concentration at the site of action than dose, because the dose–response relationship is confounded by PK variables, including

bioavailability, nature of dose–concentration relationships, and formation of active metabolites.

The effect of the drug can be measured as a clinical end point or using a biomarker. The effect of the drug on clinical end point may require studies of extended duration to manifest, for example, increase in survival time, prevention of stroke, and prevention of bone fracture. In these situations, biomarkers may help track the progress of therapy. For example, in the case of an antihypertensive drug trial, blindness, renal failure, and premature mortality can serve as clinical end points and can be measured after prolonged treatment. A simpler biomarker would be blood pressure changes, which are reduced by treatment with an antihypertensive drug. Adiponectin, blood glucose, or binding of a positron emission tomography (PET) ligand to a specific receptor could also serve as biomarkers to evaluate target modulation by a drug candidate. Other PD measures such as QT interval, liver function tests, and white blood cell count can serve as safety biomarkers.

Quantitation of dose–effect relationship was started in 1910 by Hill [2] when he described the association of hemoglobin and oxygen using a relationship that is more widely known as the *Hill equation*. The $E_{\max}$ model used commonly in all PD models was derived from the Hill equation [3]. This concept was first applied by Clark [4] to evaluate the dose–effect relationship of drugs. The hyperbolic nature of the function describes lack of a dose proportional effect after a given dose. Quantitative analysis of the dose–effect relationship has evolved over the years. A significant advancement in PD modeling was to link the PK and PD models to have a better understanding of the time course of PD effect [5]. Another advancement in PD modeling was a method to account for temporal delay observed in pharmacological response when compared to plasma concentrations [6]. Over the years, many types of mechanistic PD models have led to better understanding of different biomarkers and the importance of measuring the right biomarker to predict the clinical response and aid in the design of better clinical trials.

In this chapter, we describe the basic tenets of pharmacodynamics (drug–receptor interactions and agonists vs antagonists), the relative importance of biomarkers, and provide a summary of basic PD models reported in the literature.

17.2 MECHANISMS OF DRUG ACTION

Pharmacological response is initiated by the interaction between a drug and a binding site on a macromolecule in a tissue. This macromolecule is known as the *drug receptor*. The drug–receptor interaction results in a cascade of different steps within the cell, which ultimately leads to a physiological response:



Receptor is a cellular macromolecule or an assembly of macromolecules, which is concerned directly and specifically with chemical signaling between and within cells [7]. Most receptors are proteins (>90%), except some targets such as DNA. These receptors act as a recognition site and messenger to transfer information from the

outside of the cell to the cytosol. The regulatory effects of a receptor may be exerted directly on its cellular targets, effector proteins, or may be conveyed by intermediary cellular signaling molecules called *transducers*. The receptor, its cellular target, and any intermediary molecules are referred to as *mediators* of the signal-transduction pathway. Frequently, the proximal effector protein is not the ultimate target but rather is an enzyme or transport protein that creates, moves, or degrades a small metabolite (e.g., a cyclic nucleotide) or ion (e.g., Ca^{2+}) known as *second messenger*, which will convey information to a variety of targets. The detailed understanding of the signal-transduction pathways helps us design better molecules that interact with different cellular targets to modify physiological and/or pathological processes. It is very challenging to establish that a certain molecular interaction is indeed the one triggering the particular effect. In this respect, genetically modified models such as receptor knock-out animals are proving increasingly useful. For example, a lack of effect of a drug in mice lacking a particular target can provide strong support that the effects of the drug are mediated by that particular target [8].

The cellular proteins are either human genome-derived proteins or belong to pathogenic organisms. In 2002, after sequencing of the human genome, approximately 8000 targets of pharmaceutical interest were estimated. Only a small fraction of those targets is modulated by approved drugs. In 2006, Imming *et al.* catalogued 218 molecular targets for approved drug substances under different target families: enzymes, G-protein-coupled receptors (GPCRs), nuclear hormone receptors, cytokine receptors, ion channels, transport proteins, and so on. Altogether, it suggests that a very large number of putative drug targets remain to be explored [9,10].

During the process of drug discovery, the discovery teams design potential drug candidates that will have a desired pharmacological effect on the human body. If we look at processes at the molecular level in the human body, we would see multiple simultaneous chemical reactions taking place, keeping the systems healthy and functional. The administered agent on reaching its target site will interfere with these chemical reactions to produce a specific effect. The interaction of a drug with a macromolecular target involves a process known as *binding*. There are usually specific areas in the macromolecule where interactions take place, and they are known as the *binding sites*. The interaction of the drug with its target is mediated through electrostatic or ionic bonds, hydrogen bonds, van der Waals interaction, dipole–dipole interactions, and hydrophobic interactions. None of these bonds is as strong as the covalent bond that makes the structure of the molecule, and so they can form and get dissociated. This means that equilibrium is reached between the bound and unbound drugs and its target. Drugs having a large number of interactions are likely to remain bound longer than those that have only a few. Few drugs form irreversible covalent bonds with their receptor. For example, aspirin binds covalently to cyclooxygenase enzyme and prevents formation of thromboxane. The effect will continue until new cyclooxygenase enzyme is synthesized. Other examples include proton pump inhibitors (e.g., omeprazole) that bind irreversibly to $\text{H}^+ \text{K}^+$ -ATPase pumps and block gastric acid secretion.

When the drug molecule combines with the receptor, there might be a delay in the response depending on the nature of the signal-transduction pathway. Thus, for ion channel opening, the delay is only milliseconds, while for DNA transcription, the delay may be in hours to days. This information is very useful while building the relationship between the concentration and effect for a drug. Cells in different tissues may have multiple receptors, each of which is specific for a particular ligand. For example, on the

myocardial cell, there are β -receptors, muscarinic receptors, and multiple ion channels. Functional groups present in the drug are important in forming intermolecular bonds with the target. However, the carbon skeleton of the drug also plays an important role in binding the drug to its target. Small changes in chemical structure can produce profound changes in potency. For example, codeine, which differs in structure from morphine only by methoxy group instead of a hydroxyl group, is ~ 1000 times less potent than morphine in its action on opioid pathway (Fig. 17.1).

Each drug may interact with multiple receptor subtypes to different extent. Few drugs may be absolutely specific for one receptor or subtype, but most drug candidates will have relative selectivity. Selectivity is the degree to which a drug acts on a given site relative to other sites. At higher concentrations, the same drug may interact with other targets (receptor subtypes or different targets) leading to undesirable effects.

Some of the major drug targets and signal-transduction mechanisms are briefly discussed below.

17.2.1 Ion Channels

Ion channels are membrane proteins, found virtually in all cells that are of crucial physiological importance. They are involved in electrical signaling in the heart and the nervous system, fluid secretions in the lung, gastrointestinal tract, kidney, and a variety of other key processes such as hormone secretions, the immune response, bone remodeling, and tumor cell proliferation [11,12]. Cellular functions in these organs require the passage of ions across the cell membrane. Because of this, drugs targeting ion channels can produce physiological changes in major body functions.

In excitable cells (nerve cells and various types of muscle cells), the orientation of the membrane potential is such that the cell interior is electrically negative against the outside at resting state. The orientation of the membrane potential is reversed for a brief period of time during excitation. This transient reversal is called the *action potential*. Its duration may vary from around 1 ms to several 100 ms depending on the cell type. This action potential rapidly spreads over the entire cell membrane, altering the functional state of the cell. Moreover, excitation of one cell often triggers excitation of neighboring cells by means of electrical or chemical coupling.

Ion channels are generally highly selective for the ions they conduct. More than 300 genes code for subunits of ion channels. Most ion channels can switch between

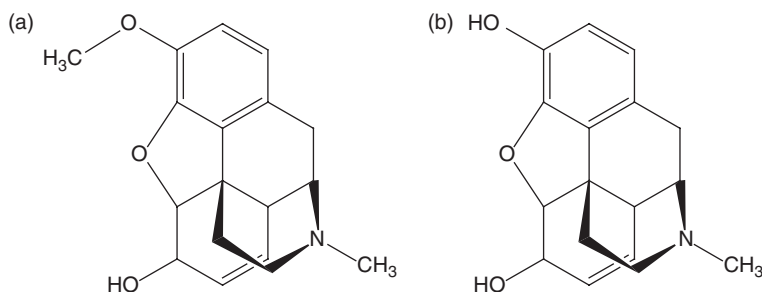


Figure 17.1 Structure of (a) codeine and (b) morphine. Structure determines interaction with the receptor.

open, closed, and inactivated states. Ion channels are classified by the principal ion they carry (sodium, Na^+ ; potassium, K^+ ; calcium, Ca^{2+} ; and chloride, Cl^-) and the mechanism by which they are opened and closed. Ion channels can be categorized as follows:

1. *Ligand-Gated Channels*. These channels either open or close in response to ligand binding. For example, nicotinic acetylcholine receptors allow Na^+ ions into the cell in response to acetylcholine.
2. *Voltage-Gated Channels*. The conductance of these channels is regulated by changes in voltage across the plasma membrane. Local anesthetics block the conductance of Na^+ ions through voltage-gated sodium channels in neurons that transmit pain information from the periphery to the central nervous system and hence the pain perception.

Ion channels are multisubunit proteins, with each subunit predicted to span the plasma membrane multiple times. Symmetrical association of the subunits allows each to form a segment of the channel pore and to cooperatively control channel opening and closing. The ligand-binding domain can be extracellular, within the channel, or intracellular. Agonists may bind to a particular subunit that may be represented more than once in the assembled multimer (e.g., nicotinic acetylcholine receptor) or may be conferred to a single subunit of the assembled channel [e.g., sulfonylurea receptor that associates with the K^+ channels to regulate the ATP-dependent K^+ channels (K_{ATP})]. Sulfonylureas bind to the SUR1 subunits and block the ATP-sensitive potassium channels. Role of K_{ATP} in insulin release is shown in Fig. 17.2. Increase in blood glucose after a meal raises intracellular ATP concentration in the pancreatic β -cell and results in closure of K_{ATP} channels. It results in inhibition of potassium efflux and depolarization of the plasma membrane, thereby leading to increase in influx of calcium through voltage-sensitive calcium channels. Rise in $[\text{Ca}^{2+}]_i$ triggers exocytosis of insulin granules, thereby stimulating insulin secretion [13]. Openers of the same channel (K_{ATP}) may lead to membrane hyperpolarization, resulting in vascular smooth muscle relaxation (e.g., minoxidil).

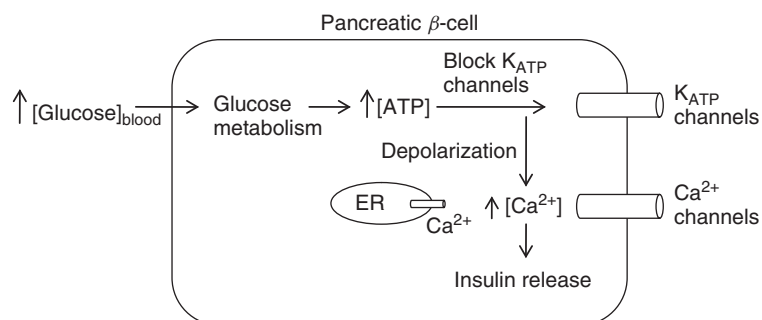


Figure 17.2 ATP-sensitive K^+ (K_{ATP}) channel as a major regulator of insulin secretion. *Source:* Adapted from Ref. 13.

17.2.2 G-Protein-Coupled Receptors

GPCRs are the target for more than 50% of the drugs currently in the market [14]. These proteins are expressed at the extracellular surface of the cell membrane, traverse the membrane, and possess intracellular regions that activate G-proteins. G-proteins are called so due to their interaction with the guanine nucleotides, guanosine triphosphate (GTP), and GDP. GPCRs bind to a wide variety of molecules, including biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions, and proteases. They are involved in diverse biological functions, including the senses of smell, taste, and sight, and regulation of appetite, digestion, blood pressure, reproduction, inflammation, and so on [15,16]. GPCRs can be found in the central nervous system and in the periphery. Their role in different tissues may be different, although the second messengers that result from the initial activation are probably similar (e.g., muscarinic acetylcholine receptors, α - and β -adrenoceptors, serotonin receptors, and histaminergic receptors).

All GPCRs have seven transmembrane regions within a single polypeptide chain. In the resting state, G-proteins exist as attached $\alpha\beta\gamma$ trimer, with GDP occupying the site of the α -subunit. When the GPCR is occupied by an agonist molecule in the extracellular domain, a conformational change occurs and the G-protein binds to cytoplasmic domain of the receptor. Association of the $\alpha\beta\gamma$ trimer with the receptor causes the bound GDP to dissociate and to be replaced with GTP, which in turn causes dissociation of α -GTP unit from the $\beta\gamma$ subunit. The α -GTP is the active form of the G-protein, which diffuses in the membrane and can interact with a number of different effectors. These effectors include adenylyl cyclase, phospholipase C, ion channels, and other classes of proteins. Signals mediated by G-proteins are terminated by the hydrolysis of GTP to GDP through the GTPase activity of the α -subunit. Different G α -protein isoforms have now been identified, each of which have unique effects on their targets. A few of these G-proteins include G-stimulatory (Gs), G-inhibitory (Gi), Gq, G0, and G12/13. The major role of the G-protein is to activate the production of second messengers through different pathways. The key G-protein-coupled effector systems are briefly discussed below.

17.2.2.1 The Adenylate Cyclase/cAMP System. The cyclic 3',5'-adenosine monophosphate (cAMP) is the nucleotide synthesized within the cell from ATP by the action of adenylate cyclase. It is produced continually and inactivated by hydrolysis to 5'-AMP by the action of phosphodiesterases. The cAMP in turn activates various protein kinases. These enzymes catalyze the phosphorylation of serine and threonine residues in different cellular proteins and thereby regulate cell function. The regulatory effects of cAMP on cellular function are diverse (e.g., energy metabolism, cell division, cell differentiation, ion transport, changes in neuronal excitability, and contraction of smooth muscles).

17.2.2.2 Phospholipase C/Inositol Phosphate System. Activation of phospholipase C, a membrane bound enzyme, hydrolyzes a membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to receptors on Ca²⁺ channels in the endoplasmic reticulum, triggering the release of calcium. Calcium can bind to calmodulin and the resulting Ca²⁺-calmodulin complex can further bind to intracellular enzymes such as Ca²⁺-calmodulin-dependent protein kinases. Increase in intracellular Ca²⁺ is the major effector mechanism by which various neurotransmitters, hormones,

and drugs promote contraction of the smooth muscle. The other product, DAG, is hydrophobic, which remains associated with the membrane, activates protein kinase C, and controls phosphorylation of serine and threonine residues of a variety of intracellular proteins. Protein phosphorylation is the key mechanism through which many physiological mediators and drugs produce their effects. For example, release of hormones from many endocrine glands, increase or decrease in the neurotransmitter release and in neuronal excitability, and contraction or relaxation of smooth muscle. The role of GPCRs in regulating enzymes and ion channels is shown in Fig. 17.3.

The receptor-linked G-proteins can also activate phospholipase A₂, which releases arachidonic acid and thus initiates synthesis of prostaglandins and related eicosanoid mediators. The receptor-linked G-proteins are involved in direct regulation of the ion channels. For example, muscarinic acetylcholine receptors are known to enhance K⁺ permeability, thus hyperpolarizing the cells and inhibiting electrical activity.

17.2.3 Tyrosine Kinase and Guanylate-Cyclase-Linked Receptors

There are two classes of tyrosine kinases. Receptor tyrosine kinases (RTKs) are type I transmembrane proteins possessing an N-terminal extracellular domain, which can bind to an activating ligand, a single transmembrane domain, and a C-terminal cytoplasmic domain that includes the catalytic domain. Nonreceptor tyrosine kinases lack a transmembrane domain; most are soluble intracellular proteins and activated in a similar manner as RTKs.

The RTKs catalyze the transfer of phosphate from ATP to hydroxyl groups of tyrosine on target proteins. RTKs play an important role in the control of most fundamental cellular processes, including cell proliferation, cell cycle progression, metabolic homeostasis, transcriptional activation, neural transmission, and aging. They mediate signaling by insulin and a variety of growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PGF), and nerve growth factor. When activated, these receptors dimerize followed by autophosphorylation of tyrosine residues in the cytoplasmic domain. The phosphorylated tyrosine residues serve as the binding site for the SH2 domains of a variety of intracellular proteins, thereby

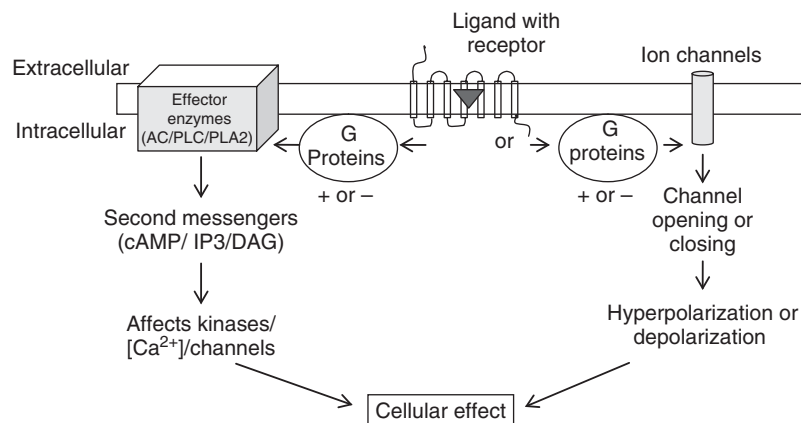


Figure 17.3 G-protein-coupled receptors and main signaling pathways. AC, adenylate cyclase; PLC, phospholipase C; PLA₂, phospholipase A₂; I, inositol triphosphate; DAG, diacylglycerol. Source: Adapted from Ref. 17.

allowing control of many cell functions. Important signaling pathways activated by RTKs include Ras/extracellular signal-regulated kinase mitogen-activated protein (ERK MAP) kinase cascade and PI-3 kinase pathway [18,19]. Examples of tyrosine kinase inhibitors approved recently are imatinib and dasatinib in chronic myeloid leukemia, gefitinib in lung cancer, and lapatinib in breast cancer.

Guanylate cyclase, a membrane bound enzyme, is similar in its regulation to the tyrosine kinase family. Receptors linked to guanylate cyclase, once activated lead to the production of 3',5'-cyclic guanosine monophosphate (cGMP) from GTP. Natriuretic peptide, a hormone secreted from the ventricles in response to volume overload, acts via receptor guanylate cyclase.

17.2.4 Intracellular and Nuclear Receptors

Nuclear receptors that mediate the actions of classical hormones can be divided into two groups. Type I nuclear receptors include the steroid hormone receptors (e.g., androgen receptor, estrogen receptor (ER), glucocorticoid receptor, mineralocorticoid receptor, and progesterone receptor). When the receptor is not bound to a ligand, type I nuclear receptors are located in the cytoplasm associated with molecular chaperons (heat shock proteins) that maintain the receptors in a conformation that allows for ligand binding but not DNA binding. On hormone binding, type I nuclear receptors undergo a conformational change that results in the release of components of the chaperone complex, exposure of a nuclear localization sequence, and translocation to the nucleus where they associate with chromatin and regulate gene transcription [20].

The type II nuclear receptors include thyroid hormone receptor, vitamin D receptor, and retinoic acid receptor (RAR) and retinoid X receptor. In contrast to the type I nuclear receptors, the type II nuclear receptors reside in the nucleus, constitutively bound to DNA, and usually act as transcriptional repressors in the absence of hormone. Another class of nuclear receptors is called *orphan receptors*, for which regulatory ligands are still unknown (e.g., hepatocyte nuclear factor 4, HNF4 α) [20]. Because of the essential role played by nuclear receptors in many aspects of mammalian development, metabolism, and physiology, dysfunction of signaling controlled by these receptors is associated with reproductive, proliferative, and metabolic diseases. For example, tamoxifen used in the treatment of breast cancer acts via ER α , retinoic acid used in acute promyelocytic leukemia acts via RAR α receptors, and thiazolidinediones targeted in type II diabetes act via peroxisome proliferator-activated (PPAR γ) receptors [21].

17.2.5 Desensitization or Tachyphylaxis

Desensitization or tachyphylaxis refers to a spontaneous decline in the response to a continuous or repeated application of agonist [7] and may be elucidated to prevent potential damage to the cell (e.g., high concentrations of calcium may initiate cell death). When repeated administration of a drug results in diminished response, the phenomenon is called *desensitization* or *tachyphylaxis*. This may develop in the course of a few minutes. The term *tolerance* is conventionally used to describe a more gradual decrease in response to a drug, taking days or weeks to develop. The desensitization of receptors may be due to change in receptor morphology, exhaustion of mediators, or loss of receptors (downregulation). Examples include morphine, ephedrine, and amphetamine.

17.2.6 Actions of Drugs Not Mediated by Receptors

Some drugs do not interact with macromolecular receptors to produce effect. For example, antacids such as sodium bicarbonate chemically neutralize gastric acid and reduce the symptoms of heart burn. Mannitol, an osmotic diuretic, is not reabsorbed in the renal tubule and increases the osmolarity of the glomerular filtrate, facilitating excretion of water. Cholestyramine resin, a lipid-lowering drug, sequesters bile acids in the intestine and decreases the absorption of exogenous cholesterol.

17.3 DRUG–RECEPTOR INTERACTIONS

17.3.1 Agonists

Ligands that bind to the receptor and alter the receptor state resulting in a biological response are called *agonists*. When bound by an agonist, a typical receptor is more likely to be in its active conformation. Agonists may act by combining either with the same site as the endogenous agonist (primary or orthosteric site) or, less commonly, with a different region of the receptor macromolecule (allosteric or allotropic site). A full agonist has a strong affinity for its receptor and good efficacy. For example, phenylephrine is an agonist at α_1 -adrenergic receptors. On binding to α_1 -adrenoceptors on the vascular smooth muscle membranes, phenylephrine mobilizes intracellular calcium causing contraction of actin and myosin filaments, thereby decreasing the diameter of the blood vessel. Some agonists (e.g., glutamate) may only be effective in the presence of another ligand (e.g., glycine in the case of glutamate) that binds to a different site on the receptor. In such cases, glutamate is referred to as the *primary agonist* and glycine as a *coagonist* [7]. A typical relationship between response and agonist concentration is shown in Fig. 17.4.

As it can be seen in Fig. 17.4, the response increases with increase in agonist concentration. Up to a certain concentration, the response is directly proportional to the concentration, but at higher concentrations, the curve becomes asymptotic. This is the result of a capacity-limited, saturable process. To accommodate a wide range of concentrations, the relationship between effect and concentration is usually plotted on a semilog scale, which transforms the plot to a sigmoidal shape. When developing concentration–effect relationship, one may consider using free (not protein bound) drug

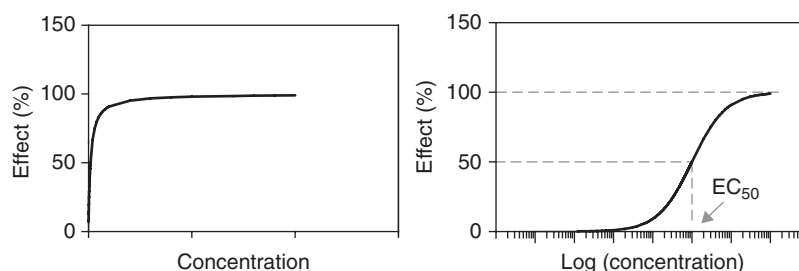


Figure 17.4 Concentration–effect relationship for an agonist.

concentrations since according to the free drug hypothesis, the free drug concentration at the site of action is the species that drives pharmacological activity.

17.3.2 Spare Receptors

Many agonists are able to produce maximal response without fully occupying all the receptors. Thus, not all the receptors in the tissue are required to achieve maximal response with some high efficacy agonists. Agonists may elicit the same maximal response, albeit at different receptor occupancies. This has been demonstrated experimentally by Furchgott (1966) and others that even with irreversible inactivation of some receptors, an agonist is still able to produce maximal response [7,22]. For example, at the skeletal neuromuscular junction, activation of <1% of receptors elicits an action potential and maximal contraction of muscle fibers. Hence, the neurotransmitter acetylcholine produces a maximum response by activating only a small proportion of receptors in the receptor pool.

17.3.3 Partial Agonists

A partial agonist cannot elicit as large an effect (even when applied at high concentrations to occupy all available receptors) as another agonist acting through the same receptors in the same tissue resulting in lower efficacy compared to full agonists (Fig. 17.5 [7]). Inability to produce a maximal response may be due to inability to convert the occupied receptors to an active form. For agents acting on opioid receptors, morphine and fentanyl are full agonists of the opioid receptors capable of inducing strong analgesia, while buprenorphine and pentazocine are partial agonists.

17.3.4 Inverse Agonists

Inverse agonists bind to receptors and reduce the fraction of receptors in active conformation [7]. This can occur when receptors exist both in active (R^*) and inactive

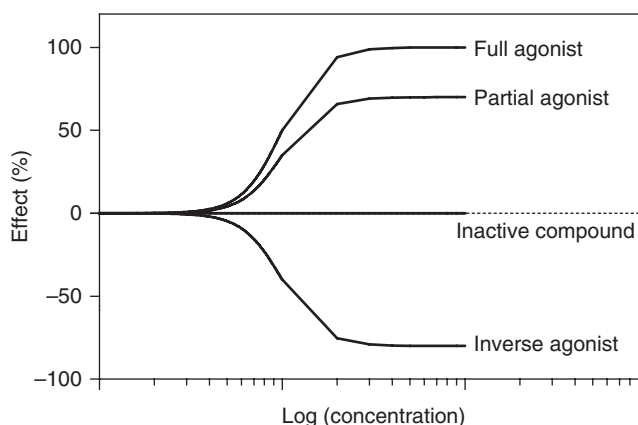
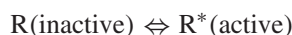


Figure 17.5 Different types of agonist action on receptors.

conformation in the absence of agonists:



An inverse agonist L, combines preferentially with inactive receptors, it will reduce the fraction in the active state:



An inverse agonist may combine either with the same site as a conventional agonist or with a different site on the receptor macromolecule. Conventional agonists increase receptor activity, whereas inverse agonists reduce it. For example, antiallergic H_1 receptor antagonists, cetirizine, loratadine, and epinastine, may act through inverse agonism (i.e., stabilization of inactive confirmation of the histamine H_1 receptor) [23]. This has also been reported for several systems, including benzodiazepine and cannabinoid (CB) receptors.

17.3.5 Affinity

Affinity can be defined as the extent of binding to receptors at any given drug concentration. Drugs that have high affinity require fewer drug molecules to produce a certain degree of binding. However, affinity is not synonymous with efficacy, that is, an antagonist may have affinity but no efficacy. Affinity is usually represented as the reciprocal of the dissociation constant of the ligand–receptor complex (k_{-1}/k_1 , where k_{-1} is the first-order rate constant of dissociation of ligand from the receptor and k_1 is the second-order rate constant of association of the ligand to the receptor). Refer to Section 17.6 for a schematic derivation of a receptor–ligand interaction.

17.3.6 Efficacy and Potency

Efficacy and potency can be determined by graded concentration–effect curves. The ability to produce a response is termed *efficacy* (intrinsic activity). Efficacy is a molecule-related property, and different molecules may have different capabilities to produce a physiological response. It is dependent on the number of drug–receptor complexes formed and the efficiency of subsequent transduction pathways. A drug with greater efficacy may be more therapeutically beneficial than one that is more potent (Fig. 17.6). Potency is an expression of the activity of a drug, in terms of the concentration or amount needed to produce a defined effect (e.g., EC_{50}). EC_{50} can be defined as the concentration of an agonist that produces 50% of its maximal possible effect. $E_{\max}$ is the maximal response produced by the drug. The agonist activities of drugs to induce a response can be categorized by comparing EC_{50} and $E_{\max}$. The maximum response depends on efficacy. If an agonist has high efficacy, it does not necessarily mean that it will display high potency and vice versa.

17.3.7 Antagonist

A drug that reduces the action of another drug, generally an agonist, is referred to as *antagonist*. Antagonism may be competitive or noncompetitive. In competitive antagonism, the antagonist competes with the agonist for the binding site on the receptor.

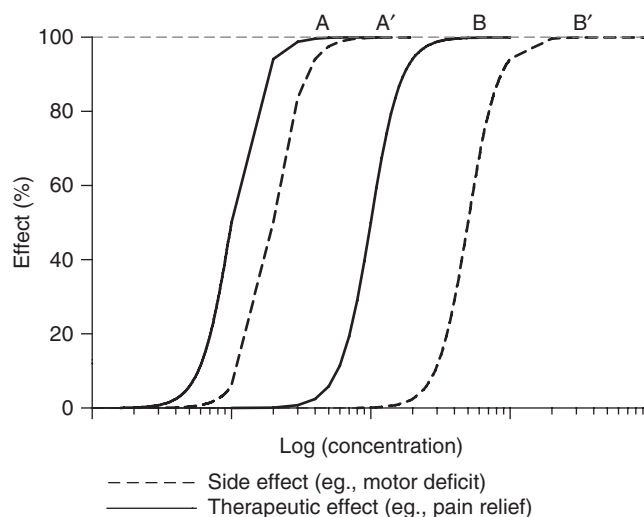


Figure 17.6 Concentration–effect relationship for two agents, A and B. A is more potent than B. A and B are equivalent in efficacy (e.g., pain relief). B is preferable than A due to its separation from concentration–effect relationship for side effect (e.g., motor deficit).

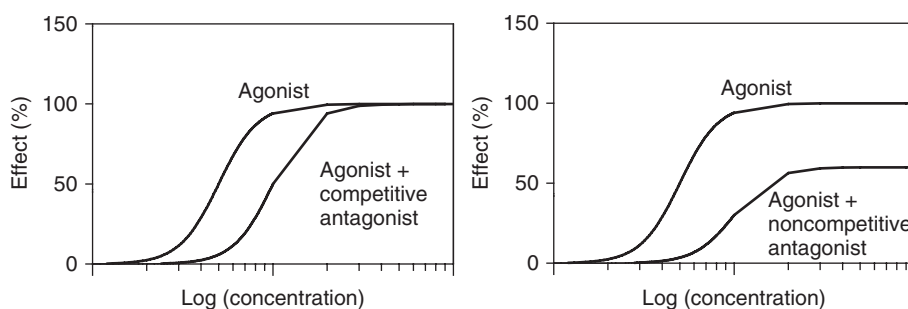


Figure 17.7 Effect of antagonist on agonist concentration–effect relationship.

For example, the antihypertensive drug prazosin competes with the endogenous ligand, norepinephrine at α_1 -adrenergic receptors, thereby reducing blood pressure. Plotting the effect of the competitive antagonist shifts the agonist dose–response curve to the right. Noncompetitive antagonists can bind to either the active site or an allosteric site of the receptor. A characteristic difference between competitive and noncompetitive antagonists is that competitive antagonists reduce agonist potency, whereas noncompetitive antagonists reduce agonist efficacy (Fig. 17.7). For example, cyclothiazide is a noncompetitive antagonist of mGluR1 receptor (metabotropic glutamate receptors) by interacting at an allosteric site of the receptor [24]. In the case of physiological antagonism, the antagonist binds to a different receptor altogether. For example, histamine binds to H_1 receptors on bronchial smooth muscle, causing contraction and narrowing of bronchus, while epinephrine acts as an agonist at β_2 -adrenoceptors on bronchial smooth muscle, causing relaxation.

In the following section, we have discussed how drug effects are measured at the molecular/physiological level. All these receptors, ion channels, and so on are part of systems such as organs. Efforts are being made recently to understand the progression of disease and drug effect on these systems. This understanding helps in identifying suitable entities in the system, which is more representative of the disease and hence more helpful in understanding the effect of a drug.

17.4 BIOMARKERS

The elucidation of the human genome and advances in proteome sciences have led to the identification of numerous potential novel pharmacological targets. Recent statistics in the drug discovery and development space, however, indicate that the failure rate of novel molecular entities (NMEs) is very high, with about 80% attrition occurring in clinical development [25]. The major factor responsible for this attrition is lack of efficacy and/or unacceptable safety. Most novel mechanisms turn out to be pharmacologically not viable as tested in proof-of-concept trials. Also, large epidemiological studies that characterize patient populations with respect to disease and its progression, as well as the role of pharmacologic targets in disease are lagging behind compared to the pace of new target identification at the molecular level. In order for drug discovery/development to be more successful, one needs to have the following:

1. increased probability of success of the unprecedented targets selected,
2. optimized validation of new targets,
3. selecting the best molecules, and
4. accelerating their advancement toward proof of concept in patients.

To stop prosecuting mechanisms or molecules that show insufficient early evidence of biological benefit is equally important, such that resources can be focused on more promising ones.

In a white paper published by the Food and Drug Administration (FDA) in 2004, titled “Innovation or Stagnation,” the need for “a new product development toolkit” was emphasized. In order to improve the translation from laboratory concept to commercialized product, new powerful scientific and technological advances were recommended comprising *in silico* or animal models and biomarkers for safety, efficacy, and new clinical evaluation techniques. Ideally, biomarker research begins during the discovery phase, with strategic application during early clinical development and continued implementation through late stage development.

The Biomarkers Working Group (representation from the FDA, National Institute of Health (NIH), academia, and drug industry) arrived at a consensus definition of a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [26]. Biomarkers may be of greatest value in early stages of the development of a drug to establish proof of concept in both *in vitro* and *in vivo* models.

A clinical end point is defined as a characteristic or variable that reflects how a patient feels, functions, or survives. Clinical end points reflect desired effects of

therapeutic intervention and are the most credible response measurements in clinical studies [26]. “A surrogate end point” is defined as a biomarker that is intended to substitute for a clinical end point. A surrogate is expected to predict clinical benefit (or harm or lack of benefit) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence [26]. The actual clinical outcome has been classically measured as morbidity/mortality parameters or how a patient is functioning and feeling. Relatively few biomarkers have qualified for the evidentiary status of a surrogate end point. The availability of predictive biomarkers is a key factor in translational approaches in drug discovery and development. Their strategic application has utility at several stages, for example,

- demonstrate proof of target engagement in nonclinical species,
- facilitate design and/or selection of lead compounds,
- predict toxicity in nonclinical species,
- selection of first-in-human (FIH) dose,
- design of early clinical trial,
- enable go-no-go decisions toward expensive late phase trials,
- choice of appropriate patient populations,
- demonstrate proof of concept in disease in humans,
- estimation of therapeutic index, and
- regulatory approvals based on surrogate clinical end points.

17.4.1 Types of Biomarkers

17.4.1.1 Proximal and Distal Biomarkers. Biomarkers employed during early clinical development serve to demonstrate target engagement and are termed as *proximal biomarkers*. They inform on the concentration–response relationship between drug PK and modulation of a selected pharmacological response, such as receptor occupancy, *ex vivo* inhibition of an inflammatory marker, kinase inhibition, change in mRNA, and target enzyme activity. Selected examples of proximal biomarkers used in proof-of-pharmacology assessment of novel entities are listed in Table 17.1 [27].

Disease-related biomarkers, termed as *distal biomarkers*, are employed at the same time or somewhat later in the clinical development to demonstrate effect of target modulation by the drug on disease pathophysiology. These biomarkers assess drug’s effect on a disease, are linked to clinical benefits, and thus have the potential of qualifying as surrogate end points. Examples are blood pressure reduction, insulin and blood glucose modulation, cholesterol and HDL modulation, and ACR20 score in rheumatoid arthritis. For additional examples, refer to Table 17.1 [27].

17.4.1.2 Technology-Based Classification of Biomarkers. Depending on the biological target and therapeutic area, a variety of pathway- or disease-related biomarkers have been proposed. Roughly, they can be classified as “-omics”-based (genomic, proteomic, and metabolomic), activity-based, or imaging-based approaches.

TABLE 17.1 Proximal and Distal Biomarkers in Different Disease Areas

Therapeutic Area	Proximal Biomarker	Distal Biomarker	Examples	References
Diabetes	Dipeptidyl peptidase-4 (DPP-4) Glucagon-like peptide-1 (GLP-1) Adiponectin Urinary glucose excretion	Blood glucose HbA1c	Sitagliptin, PPAR agonists, Dapagliflozin	27,35,36
Obesity	Positron emission tomography for targets NYP-5 and CB-1	Body weight	MK-0557 MK-9470	27,37
Cardiovascular: Hypertension and Atherosclerosis	C-reactive protein, HDL, LDL, adiponectin, Serum thromboxane B ₂ levels, Plasma renin activity	Blood pressure	Statins, Aspirin, BAY-10-6734	38–40
Immunology: Rheumatoid arthritis	Tumor necrosis factor α (TNF- α), Multiple cytokines, MCP-1-induced chemotaxis in synovial fluid	ACR20 score	Infliximab	41
Alzheimer's disease	β -Amyloid peptide in CSF and plasma	Cognitive score	LY450139	42
Multiple sclerosis	Lymphocyte count	Aggregate relapse rate and physical disability	Fingolimod	43

17.4.1.2.1 “-omics”-Based Biomarkers. Genomics provides a measure of the transcribed genome (transcriptome). Pharmacogenomics has evolved as one of the main applications of biomarker discovery and has delivered decision-making biomarkers. Examples are provided in Table 17.2. Translatability has been achieved by profiling effects of drug treatment on gene expression in cell/tissue culture or *in vivo* in animals and clustering certain genes/pathways for further validation in human trials. Standardization of gene expression microarrays or gene chip technologies has had a tremendous impact in facilitating the use of this approach in biomarker discovery and development. Genomics is different from genetics in that the latter is an analysis of genome in normal

TABLE 17.2 Examples of Genomic Biomarkers

Drug	Indication	Biomarker
Imatinib mesylate	Stomach cancer	C-KIT expression
Boceprevir	Hepatitis C virus	il28B
Cetuximab	Colorectal cancer	EGFR expression
Cetuximab	Colorectal cancer	KRAS
Maraviroc	HIV	CCR5
Trastuzumab	Breast cancer	Her2/neu expression
Irinotecan	Colon rectum cancer	UGT1A1 variant

or disease state, including identification of genetic variants (insertion, deletion, amplification, single nucleotide polymorphisms, etc.). The correlation of genetic variation in disease state has the potential to provide mechanisms of drug action and select appropriate patient population. One of the earliest examples was the enrollment of breast cancer patients positive for HER2 amplification for treatment with trastuzumab [28]. Another genomic application for patient selection/labeling is that of polymorphic drug metabolizing enzymes CYP2D6, CYP2C9, and CYP2C19. Depending on the CYP allele, patients may be extensive or poor metabolizers of drugs that are primarily metabolized by these enzymes, potentially leading to a significant variability in drug exposure. The US FDA Web site provides a table of pharmacogenomic biomarkers in drug labels and is available at www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics. There is increasing evidence that pharmacogenomics can play an important role in identifying responders and nonresponders to medications, avoiding adverse events, and optimizing drug dose. Drug labels containing information on genomic biomarkers describe the following:

- drug exposure and clinical response variability,
- risk for adverse events,
- genotype-dependent dosing,
- mechanisms of drug action, and
- polymorphic target and disposition genes.

Using gene expression arrays (e.g., Affymetrix) as PD biomarkers has certain limitations [29]. In a rat model of rheumatoid arthritis, methylprednisolone was administered exogenously to normal and diseased rats and cytokine gene expression changes were monitored. Normal rats were shown to exhibit circadian rhythms in gene expression in the liver and muscle that peaked at a time different from the disease rats. After a single dose of methylprednisolone, the gene expression may show a biphasic response and the change in gene expression may not be predictive of that after chronic administration.

Consistency in gene expression across different laboratories is also a challenging issue, so is the influence of environmental factors. Owing to the complexity and variability of gene expression profiles, combination with other biomarkers is often necessary.

Proteomics may be considered a more direct approach to the discovery of mechanistic/functional biomarkers of drug action. The proteome is more complex than the genome and transcriptome and reflects protein abundance, posttranslational modifications, localization in cells, and interaction with other macromolecules. Although this complexity defines the biological homeostasis, it also makes it difficult to identify protein biomarkers and establish robust/reproducible assays that quantify drug-related changes. Defined panels of candidate protein biomarkers can be analyzed via high throughput technologies, such as protein arrays and multiple-reaction monitoring mass spectrometry. ELISA-based protein immunoassays are still considered the gold standard. In Alzheimer's disease, the levels of disease-related CSF biomarkers such as amyloid β -peptide 1-42 (Ab42), tau, and phosphorylated tau (p-tau) may increase diagnostic accuracy. Good correlation is observed in the levels of CSF biomarkers and amyloid imaging by PET.

Metabolomics encompasses the effect of drug treatment on small-molecule endogenous biochemicals, as a result of activation or inhibition of their biosynthetic and/or

disposition pathways. Many of these biomarkers form a part of standard clinical chemistry panels and are applied in the detection of organ-based toxicity. Glucose and cholesterol are examples of metabolomic biomarkers that have been successfully applied in the development and approval of antidiabetic and antiatherosclerotic therapies, respectively. Together, the genetic, genomic, proteomic, and metabolomic approaches constitute “molecular profiling.”

17.4.1.2.2 Activity-Based Biomarkers. Pharmacological target-based activity assays represent the most direct assessment of functional activity of new drugs. Given that plasma remains the most accessible biological fluid during clinical development, *ex vivo* whole-blood or plasma biomarkers are highly desirable. *Ex vivo* whole-blood CD11b, tumor necrosis factor α (TNF- α), or suitable interleukin (IL) assays as inflammatory biomarkers (Table 17.1). Depending on drug distribution, and anticipated site of action, tissue fluids such as CSF and synovial fluid are sampled or tissue biopsies such as liver or tumor are accessed for analysis of biomarkers.

17.4.1.2.3 Imaging-Based Biomarkers. Molecular imaging refers to noninvasive or minimally invasive technologies for visualizing, characterizing, and quantifying anatomical structures and physiological processes at the cellular and subcellular levels, with exquisite spatial, temporal, and biochemical resolution *in situ* and *in vivo* [30]. PET, single photon emission computed tomography (SPECT), computer tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), and optical imaging are used for biomarker measurement. These techniques differ in terms of the mechanisms used to generate images, imaging probe characteristics, and their resolution and sensitivity. The most commonly used imaging approach is nuclear imaging that visualizes radiotracers interacting with protein targets located intracellularly or on the cell surface. PET uses ^{11}C and ^{18}F , while SPECT uses ^{123}I as radionuclides. They are used for tracking the drug itself (small molecule or protein), imaging the pharmacological target, or monitoring key biochemical and physiological processes. There are a few examples in which imaging techniques have been used to support drug registration (oncology, neurology, cardiovascular, and musculoskeletal). There are other examples in which these techniques have been used to make early go-no-go decisions, in the CNS and obesity area from Merck laboratories: for example, CB-1 receptor inverse agonist MK-9470 and neuropeptide receptor Y5 (NPY5) receptor antagonist MK-0557 [27].

In order to select appropriate biomarkers and incorporate them into the design of clinical trials, it is important to be aware of the wide variation in the turnover rates of biomarkers. For example, electrical signals, neurotransmitters, and chemical signals turn over in seconds; hormones, enzymes, and mRNA in several hours; cells and tissues in weeks; organs such as bone in 10 years; and human life span in 100 years.

17.4.2 Biomarker in Different Stages of Drug Development

In early research, the selection of biomarkers to support mechanistic studies in cell-based and animal models should depend on their translation to human samples. Biomarkers to investigate target engagement and its efficacy on disease status should be incorporated in early clinical trials, as it helps assess the possibility of success

in large-scale clinical trials by relating clinical data to preclinical test systems and by determining whether drug is affecting the molecular target and whether it is translating into therapeutic benefit. The use of biomarkers in clinical development can be categorized into four types and show increasing decision-making and regulatory application:

1. *Exploratory*. Supported by *in vitro* and preclinical data but with no consistent information regarding their link to clinical outcome in humans.
2. *Demonstrated (Probable Valid)*. With sufficient preclinical sensitivity and specificity and are linked to a clinical outcome but without reproducible evidence.
3. *Characterized*. Both preclinical and clinical validation, as listed in “1” and “2” above, as well as show reproducibility in more than one prospective clinical study.
4. *Surrogate*. Biomarkers that demonstrate utility as a substitute for a clinical end point. This scheme shows the increasing decision-making and regulatory application.

Biomarker qualification represents the progressive transition from exploratory stage to surrogacy. Biomarkers may also enter qualification stage from general medical practice. For example, low density lipoprotein (LDL) cholesterol was in general medical use before its role in drug development. Biomarkers may also be “disqualified” if evidence does not support their intended use. For example, suppression of intermittent ventricular tachyarrhythmia as a marker of reduction in ventricular arrhythmia and mortality due to myocardial infarction was disqualified as a biomarker because a controlled cardiac arrhythmia suppression trial demonstrated that mortality was increased by antiarrhythmic treatment following an infarction.

The process of biomarker qualification gauges the evidentiary status from exploratory application to prediction of clinical outcome. A consensual framework is required to define surrogacy due to the current lack of consistent evidentiary standards, as identified in FDA’s critical path initiative. Lathia *et al.* [31] have reviewed the types of evidence supporting or not supporting certain surrogate end points. A complication in selecting “a” surrogate end point is that most outcomes are multidimensional, thus crucial information such as safety issues can be missed by employing a single measure. Thus, based on preclinical information, it is better to use multiple biomarkers to evaluate efficacy and safety during development of drug candidates. FDA-NIH consensus conference on biomarkers (1999), FDA Critical Path Initiative (2004) [32], and European Medical Agency Roadmap (EMA, 2010) [33] have advocated different biomarker strategies. The biomarker consortium, a public–private collaboration was launched in 2006. The role of consortia (among regulatory, industrial, academic, and government representatives) in biomarker development and application enables collaborative pooling of (precompetitive) data and benefits all participants by increasing the availability of validated and qualified biomarkers. In order to promote qualification and regulatory acceptance of biomarkers, research data and results are made broadly available to public to help speed disease-specific research.

Successful application of PK-PD modeling in drug discovery and development depends on a thorough understanding of the following:

1. the disposition of the molecular entity being developed, including its Absorption, Distribution, Metabolism, Excretion (ADME) characteristics in preclinical species and human;
2. the pharmacology of target modulation, including assessment of potency and efficacy in *in vitro* binding and functional assays and *in vivo* concentration–response relationships in preclinical species and human; and
3. the disease pathophysiology, including a cause–effect relation between the target and disease and effect of drug on disease or surrogate end points

Therefore, biomarkers play a major role in discovery of target, evaluation of drug candidates in preclinical models, early- and late-phase clinical development, and approval from federal agency with the help of PK-PD modeling incorporated in clinical trial simulations, as exemplified in a recent Pfizer article by Morgan *et al.* [34].

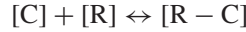
17.5 MATHEMATICAL MODELS IN PHARMACODYNAMICS

PK-PD modeling has been gaining its importance in both drug discovery and development stage in recent years. In one of their reports in 2004, Donald Stanski, the scientific advisor to the Center for Drug Evaluation and Research, FDA, stated that “the latest buzzwords at the FDA are model-based drug development” [44]. The primary objective of PK-PD modeling is to characterize drug-specific parameters such as $E_{\max}$ and EC_{50} , as defined in Section 17.2, as well as system-specific factors such as production (k_{in}) and dissipation (k_{out}) rates of PD responses, k_{e0} , tau (τ), as has been defined in this section. The effect of a drug that depends on its interaction with the receptor is usually known as *primary effect*. The primary effect causes the physiological system and its various components to respond, which translates to clinical response [45]. In preclinical studies, PK-PD modeling is often used to estimate the FIH dose. Another application is the use of physiological modeling and allometry of PK/toxicokinetic data and extrapolation of the results from animals to human. In clinical settings, PK-PD modeling is used to interpret the dose–response data for single ascending dose (SAD) studies and decide doses for the dose escalation studies. This is primarily true where a single dose exhibits change in the PD biomarker. PK-PD modeling also has been used by the FDA to propose dose or dosage regimen(s) that were not originally recommended by the sponsors. A variety of mathematical models have been used to analyze different types of PD responses. There are two kinds of response. A graded or continuous response is a one where the response can be measured as a continuous function of dose, concentration, or time. These types of data have been typically modeled by deterministic models. Examples of such response data would be change in heart rate, temperature, and enzyme activity. The other type of response is known as *dichotomous response* and is either “all” or “none.” Stochastic models have been used to analyze this kind of data. In this section, we describe the different types of models typically used to describe dose–response or exposure–response data and encompass the basic model structure, PK-PD expectations from these types of models, determination of initial estimates of drug-specific and system parameters, and applications reported in

the literature. Additional complexities outside the scope of this discussion provided can be found in the References section.

17.6 CONCENTRATION-RESPONSE RELATIONSHIP

A drug must bind to its target in order to exert its PD effects. As discussed previously, these proteins may be enzymes, transporters, ion channels, or receptors. The most popular and appropriate model for receptor occupancy, proposed by Clark, is based on law of mass action. The law of mass action and small quantity of receptors lead to capacity limitation for most responses (Jusko WJ, State University of New York at Buffalo, New York, personal communication, March 2006). Below is a schematic representation of drug-receptor interaction:



Let us suppose that $[C]$ is the concentration of drug at the site of action, $[R]$ is the available concentration of receptors, R_{TOT} is total concentration of receptors (bound+free), and RC is the concentration of the drug-receptor complex. The primary assumption is that the effect is proportional to the drug-receptor concentration at a given time. At equilibrium, the rate of formation of the drug-receptor complex, governed by a second-order rate constant k_1 is equal to the rate of dissociation of the RC complex, governed by the first-order rate constant k_{-1} . Thus, the equation for the drug-receptor complex is as follows:

$$\frac{d[RC]}{dt} = k_1 \cdot [R] \cdot [C] - k_{-1} \cdot [RC] \quad (17.1)$$

At equilibrium when

$$\frac{d[RC]}{dt} = 0 \quad (17.2)$$

$$k_1 \cdot [R] \cdot [C] = k_{-1} \cdot [RC] \quad (17.3)$$

$$\frac{[R] \cdot [C]}{[RC]} = \frac{k_{-1}}{k_1} = K_D \quad (17.4)$$

where K_D is the equilibrium dissociation constant. A lower value of K_D indicates a poor dissociation of the drug-receptor complex translating into high affinity of the drug for a given receptor.

Solving for $[RC]$ yields

$$\frac{([R_{TOT}] - [RC]) \cdot [C]}{[RC]} = \frac{k_{-1}}{k_1} = K_D \quad (17.5)$$

Rearranging the above equation, one can arrive at

$$\frac{[RC]}{[R_{TOT}]} = \frac{[C]}{[C] + K_D} \quad (17.6)$$

Ariens [46] introduced the concept that effect is directly proportional to the concentration of the drug–receptor complex and the proportionality constant ε was called the *intrinsic activity of the agent*:

$$\varepsilon = \frac{[RC]}{E} \quad (17.7)$$

The maximal drug response occurs when all the receptors are occupied and thus,

$$\varepsilon = \frac{[R_{\text{Tot}}]}{E_{\text{max}}} \quad (17.8)$$

Substituting in Equation 17.6 yields the following equation:

$$E = \frac{E_{\text{max}} \cdot [C]}{[C] + K_D} \quad (17.9)$$

K_D , the affinity for the drug can be replaced by EC_{50} (or the potency) for the agent to yield Equation 17.10. EC_{50} is defined as the concentration of the drug that produces 50% of its maximum effect:

$$E = \frac{E_{\text{max}} \cdot [C]}{EC_{50} + [C]} \quad (17.10)$$

Thus, the binding of a drug to the receptor can translate to the clinical response E . In PD modeling, a drug bound to a receptor may either stimulate (S) or inhibit (I) a process as described by the following equations:

$$S = 1 + \frac{S_{\text{max}} \cdot [C]}{SC_{50} + [C]} \quad (17.11)$$

$$I = 1 + \frac{I_{\text{max}} \cdot [C]}{IC_{50} + [C]} \quad (17.12)$$

17.7 CLASSIFICATION OF MATHEMATICAL MODELS IN PHARMACODYNAMICS

Figure 17.8 depicts the classification of different types of models used to analyze PD data. The models have been described in details in the following sections.

17.7.1 Reversible Effect Models

This category refers to the type of responses that return to the baseline or predose (physiological) levels as the drug is eliminated from the system (e.g., inhibition or stimulation of synthesis or dissipation of response, cell trafficking, and enzyme induction), causing no permanent damage to the system.

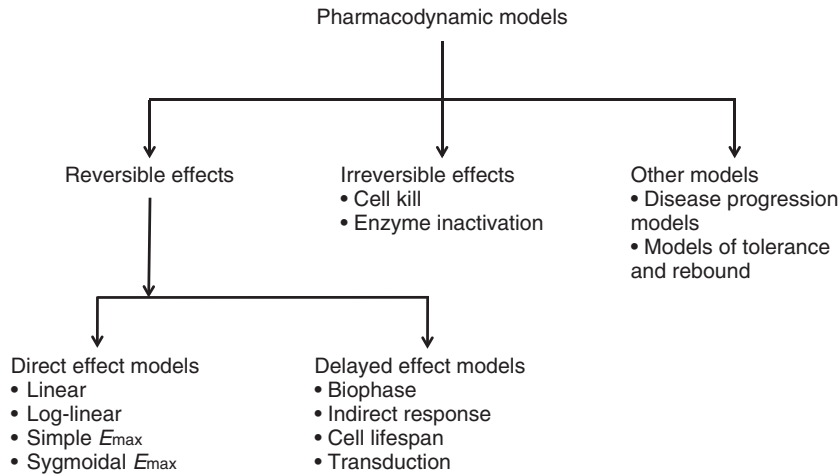


Figure 17.8 Types of pharmacodynamic models.

17.7.1.1 Direct Effect Models. A direct effect is observed when the action of the drug on its target is so rapid that there is no delay observed between the PK and PD profiles, for example, vascular targets or very rapid turnover processes such as muscle activity. The basic expectation of these effects is depicted in Fig. 17.9a and b. Figure 17.9a represents PK (C vs t) and Fig. 17.9b depicts effects versus time. Equation 17.13 represents the PK and Equation 17.14 represents the PD:

$$C_P = \frac{D}{V} \cdot e^{-k_{el} \cdot t} \quad (17.13)$$

$$E = \frac{E_{\max} \cdot C}{EC_{50} + C} \quad (17.14)$$

At $C_{\max}$, the effect reaches a maximum of 100. As the drug is eliminated, the effect diminishes and reaches a minimum at a sufficiently low drug concentration.

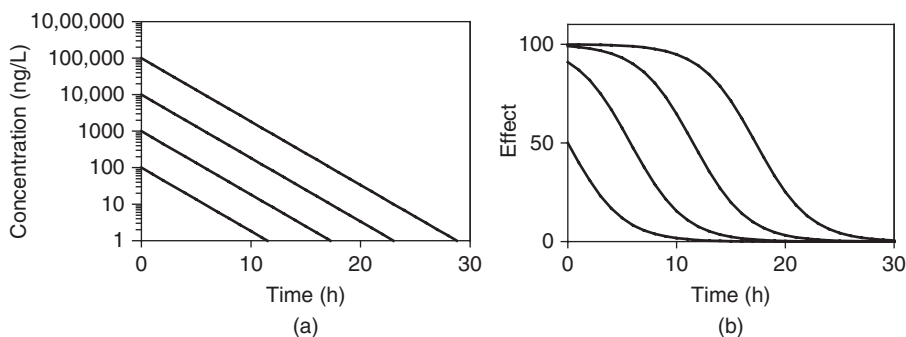


Figure 17.9 PK/PD expectations in case of direct effects. (a) Monoexponential PK and (b) PD. $k_{el} = 0.4$ units, $V = 10$ units, $E_{\max} = 100$, $EC_{50} = 100$.

17.7.1.1.1 Linear Effect Concentration Model. The simplest relationship between effect and concentration is represented by the following equation:

$$E = m \cdot C \quad (17.15)$$

This equation indicates that effect is zero when there is no drug present in the body and it increases with slope “ m ” as the concentration of the drug in the body increases. A situation very similar to this could be the presence of baseline of effect (E_0), which is represented by Equation 17.16. At $C = 0$, the effect is E_0 . As the concentration increases, the effect increases linearly with a slope “ m .” “ m ” can be easily estimated from the slope of the effect versus concentration plot:

$$E = E_0 + m \cdot C \quad (17.16)$$

17.7.1.1.2 Log-Linear Effect Concentration Model. This model finds its application in cases where the concentration varies over a wide range and appears curvilinear when plotted without log transformation. On log transformation, the data appear linear and the slope “ m ” can be estimated easily as described above (Eq. 17.17). The main assumption of this model is that changes in drug concentration and effect are proportional to one another:

$$E = m \cdot \log C + E_0 \quad (17.17)$$

17.7.1.1.3 Simple and Sigmoid $E_{\max}$ Models. A simple $E_{\max}$ and sigmoid $E_{\max}$ model is depicted by Equations 17.18 and 17.19, respectively:

$$E = \frac{E_{\max} \cdot C}{EC_{50} + C} \quad (17.18)$$

$$E = \frac{E_{\max} \cdot C^\gamma}{EC_{50}^\gamma + C^\gamma} \quad (17.19)$$

$E_{\max}$ indicates the maximum response caused by the drug. EC_{50} is defined as the concentration of the drug, which produces half the maximal response. The PK-PD expectation is shown in Fig. 17.9. In sigmoid $E_{\max}$ model, a shape factor is added to the simple $E_{\max}$ to make the slope of the response steeper or shallower. Figure 17.10 depicts how the values for the shape factor, γ , affect the profile. This shape factor provides more flexibility to the function so that the function can orient itself according to the shape of the data.

17.7.1.2 Delayed Effect Models. A drug, after administration, may produce effects which may be delayed from the PK. The maximum effect does not occur at the same time as the maximum plasma concentration. This may be due to distributional delays, turnover of endogenous proteins, or signal transduction. In this type of data if the plasma concentrations from each time point are plotted with the effect at the corresponding time point, they do not produce a hyperbolic profile but produce hysteresis (Greek meaning, the state of being behind or late). Figure 17.11a shows a typical PK-PD expectation for a delayed effect. The PK profile peaks at 1 h and the response peaks

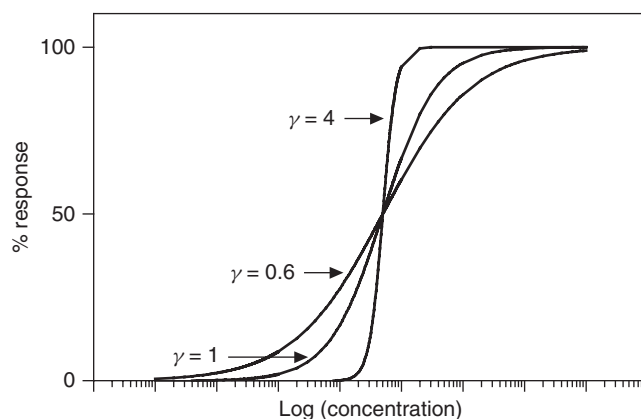


Figure 17.10 Effect of the values of γ on the profiles of a simple $E_{\max}$ model.

at 2 h. Thus, the PD response is delayed by 1 h. Figure 17.11b shows a prototypical hysteresis plot produced when concentration is plotted against effect in case of delayed effects. For the same concentration of 40 units, there are two effects, 95 and 105 units. This profile cannot be analyzed using simple $E_{\max}$ -type model to resolve estimates of drug efficacy/potency. To obtain accurate estimates of efficacy, the PK-PD model needs to incorporate a component that captures the delay. In this section, we have made an effort to identify different types of mechanistic and empirical approaches, which account for these delays in drug response.

17.7.1.2.1 Biophase Model or Link Model. Biophase or link model has been applied to data where drug distribution to the site of action is assumed to be the rate-limiting factor for effect to occur. The term *biophase* was first coined by Furchgott [47]. This modeling approach was proposed and popularized by Sheiner *et al.* [48] for drugs showing delay in response. In their publication, Sheiner *et al.* included a hypothetical delay compartment to account for the disconnection observed in the PK and PD of the compound.

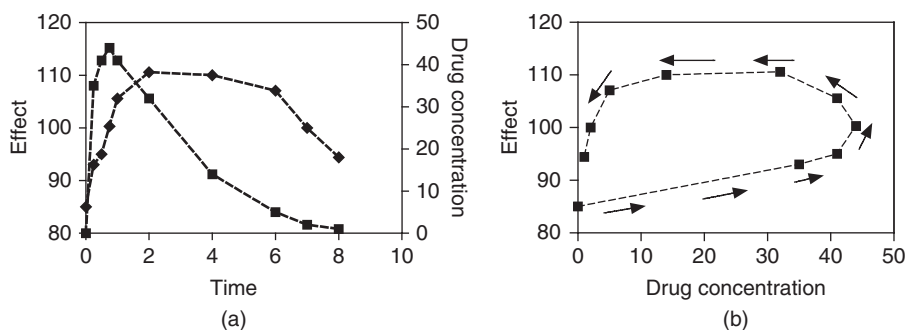


Figure 17.11 (a) Typical PK-PD expectation for a delayed PD effect and (b) hysteresis after plotting concentration with effect.

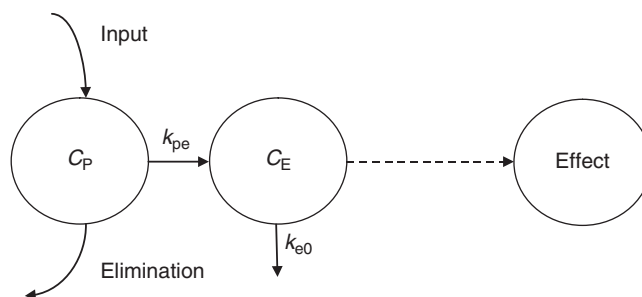


Figure 17.12 Biophase/link model.

The structure of the model is depicted in Fig. 17.12. C_P is the concentration of drug in plasma and C_e is the concentration of drug in the hypothetical effect compartment. Drug enters plasma by “input” and is eliminated by “elimination.” It also distributes to a hypothetical effect compartment by the rate constant k_{pe} and this distribution causes the delay for the observed effect. The basic assumption of this model is that the hypothetical effect compartment does not alter the PK of the drug. The plasma PK parameters are fixed. The rate constants k_{pe} and k_{e0} are assumed to be the same (k_{e0}) due to lack of identifiability. Thus, k_{e0} is defined as the rate constant causing distribution of drug to the hypothetical effect compartment and thus causing delay. An $E_{\max}$ model is then used to describe the effect versus time profile and C_P in the $E_{\max}$ equation is replaced by C_e , as shown in the following equations:

$$\frac{dC_e}{dt} = k_{e0} \cdot C_P - k_{e0} \cdot C_e \quad (17.20)$$

$$E = \frac{E_{\max} \cdot C_e}{EC_{50} + C_e} \quad (17.21)$$

The biophase/link model has been widely used in the literature to model PK-PD relationship of drugs showing delays in effect [49–51]. A nice example of using a biophase model to account for delay of electroencephalography (EEG) effect by midazolam, when using arterial sampling, was demonstrated by Tuk *et al.* [52]. Arterial data showed anticlockwise hysteresis but no hysteresis was observed with the use of venous concentrations.

SIGNATURE PROFILES. Figure 17.13 shows the simulations performed on a typical biophase or link model. The following parameter values were used to perform the simulations:

$$k_{el}=0.4, k_{e0}=0.5, E_{\max}=100, EC_{50}=100, V=1, \text{dose : } 10, 100, 1000, 10,000 \text{ units}$$

As one would expect, the peak effect occurs at a later time than that of $C_{\max}$. $T_{\max}$ of the effect is the same for all doses. Decreases in values of k_{e0} will produce more delay. On the basis of the values of k_{e0} , the effect compartment may produce flip-flop kinetics.

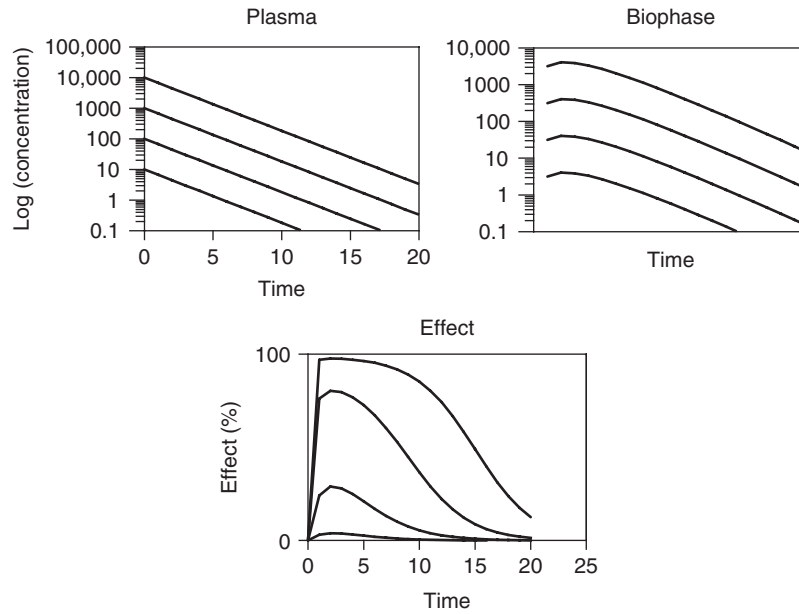


Figure 17.13 Signature profiles of plasma compartment, effect compartment, and response versus time profiles for a biophase model. $k_{el} = 0.4$, $k_{e0} = 0.5$, $E_{max} = 100$, $EC_{50} = 100$, $V = 1$, dose: 10, 100, 1000, and 10,000 units.

EFFECT OF k_{e0} ON SIGNATURE PROFILES. Figure 17.14 depicts the effects of changing k_{e0} on the signature profiles of a biophase model. The dotted line is the plasma concentration and the solid lines depict biophase concentrations at different values of k_{e0} . The value of the elimination rate constant, k_{el} is 0.4 and three profiles of biophase concentrations for k_{e0} values of 0.1, 0.4, and 1 have been plotted. When k_{e0} and k_{el}

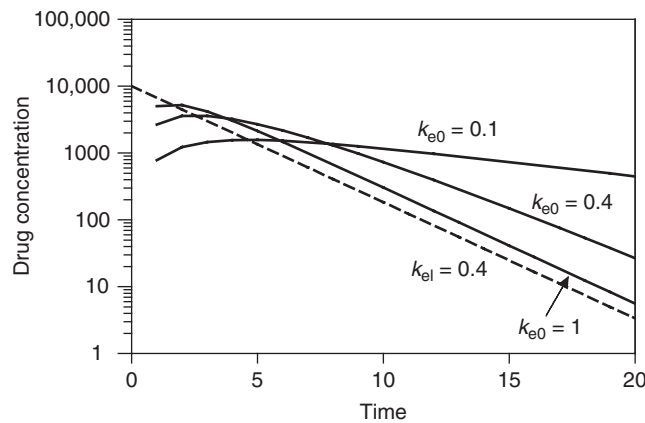


Figure 17.14 Effect of different values of k_{e0} on shapes of hypothetical effect compartment. $k_{e0} = 0.1$, 0.4, and 1. $k_{el} = 0.4$. Dotted line, central compartment; solid lines, hypothetical effect compartment.

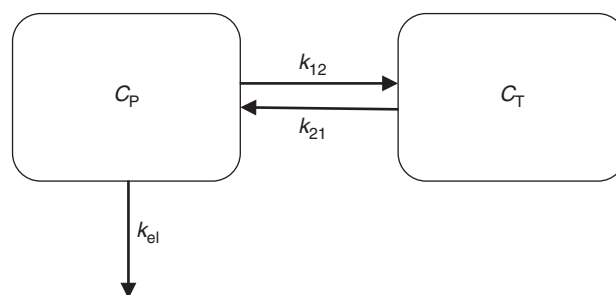


Figure 17.15 Schematic diagram of a mammillary two-compartment model.

are equal ($k_{e0} = k_{el} = 0.4$), the biophase and plasma profiles are not exactly parallel and the biophase profile seems curvilinear. In this case the explicit solution for the biophase compartment is slightly different and is outside the scope of this chapter. The terminal phase of the biophase concentrations is parallel to the plasma concentrations when value of k_{e0} is 1. When k_{e0} is 0.1, the terminal phase of the plasma concentrations and biophase concentrations are not parallel. Thus, this model has a flexibility of capturing PK-PD data where drug leaves the body but the effect still prevails, with values of k_{e0} less than the terminal phase of the PK profile.

DIFFERENCE WITH A MAMMILLARY TWO-COMPARTMENT MODEL. Figure 17.15 depicts a mammillary two-compartment PK model. C_P represents drug concentrations in the central (plasma) compartment and C_T represents drug concentrations in some hypothetical tissue compartment where the drug can distribute to. The first-order rate constant k_{el} represents the elimination rate constant for drug from the central compartment, and k_{12} and k_{21} are first-order rate constants representing distribution from plasma to tissue and tissue to plasma, respectively. Drug is eliminated only from the central compartment. Primarily, there are three disadvantages of using a two-compartment model instead of a biophase model. First, the number of parameters is one more than a biophase model since one has to use k_{12} and k_{21} instead of just k_{e0} . Second, if the plasma kinetics of the drug in monophasic, adding a tissue compartment will always make the plasma concentrations biphasic. In other words, a tissue compartment affects the concentrations of the plasma compartment. But the assumption of a biophase model is that the effect compartment does not alter the plasma concentrations. Third, no matter what value we use for k_{12} and k_{21} , the tissue concentrations will always decline parallel to the plasma concentrations for a two-compartment mammillary model. Whereas for a biophase model, the effect site concentrations do not decline parallel to the plasma concentrations for k_{e0} values less than the elimination rate constant.

INITIAL ESTIMATES. Figure 17.16 describes the method for estimation of k_{e0} . The rationale for taking the difference between time of peak effect and time of peak plasma concentration arises from the belief that the delay observed in effect is due to distribution of the drug to the effect compartment or biophase. If one had biophase concentrations, the time of peak biophase concentrations would be the same as the time of peak effect. Thus, the difference between peak times for effect and plasma concentrations provides the time required for distribution to biophase to be complete. Distribution is a first-order process governed by first-order rate constant k_{e0} . Thus, it

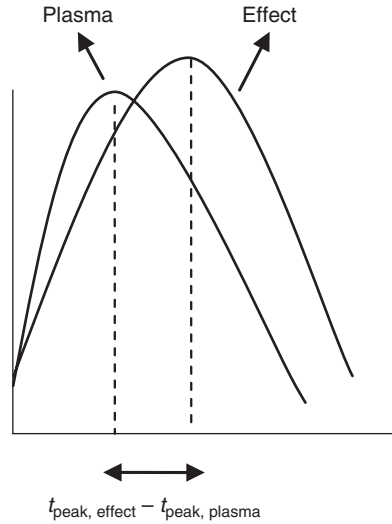


Figure 17.16 Determination of initial estimate of k_{e0} .

would take approximately four $t_{1/2, k_{e0}}$ for the distribution to complete. Hence, k_{e0} is calculated from Equations 17.22 and 17.23. An estimate of $E_{\max}$ can be obtained from the maximal response. EC_{50} estimate can be obtained from concentration at $\frac{1}{2} E_{\max}$ on down-curve of hysteresis arm:

$$t_{1/2, k_{e0}} = \frac{(t_{\text{peak, effect}} - t_{\text{peak, plasma}})}{4} \quad (17.22)$$

$$k_{e0} = \frac{0.693}{t_{1/2, k_{e0}}} \quad (17.23)$$

17.7.1.2.2 Indirect Response Models. “Indirect response” refers to a PD response that is produced when the drug acts on the production or dissipation of the endogenous factors that affect/control the response [53].

The basic equation for the scheme is provided below:

$$\frac{dR}{dt} = k_{\text{in}} - k_{\text{out}} \cdot R \quad (17.24)$$

where k_{in} is the zero-order production rate of response, R , and k_{out} is the first-order rate constant for the loss of response. Usually, R is measured as graded or continuous variable (Section 17.5). R begins at a baseline value and changes with time when a drug is administered and reaches back to its baseline value after the drug is out of the system.

When no drug is present, R has the baseline value R_0 , which is obtained by putting $dR/dt = 0$. Thus, $R_0 = k_{\text{in}}/k_{\text{out}}$.

For the basic indirect response models, four possibilities have been described (Fig. 17.17) [54]. The drug either inhibits or stimulates production or loss of response.

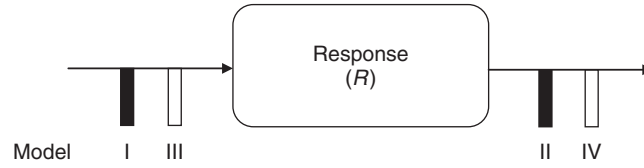


Figure 17.17 Schematic diagram for four basic indirect response models. Black solid bar indicates inhibitory function and white bar indicates stimulatory function.

The effect of the drug on the system can be asserted with the aid of a stimulatory or an inhibitory response. An inhibitory or a stimulatory response can be depicted as $I(t)$ and $S(t)$:

$$I(t) = 1 - \frac{I_{\max} \cdot C}{IC_{50} + C} \quad (17.25)$$

$$S(t) = 1 + \frac{S_{\max} \cdot C}{SC_{50} + C} \quad (17.26)$$

In these equations, C represents the plasma/blood concentrations of the drug. IC_{50}/SC_{50} is the concentration of the drug at half the maximal inhibitory/stimulatory effect. $E_{\max}$ or $S_{\max}$ represents the maximum effect of the drug on the response.

In the inhibitory equation, $I(t)$ assumes that $0 < I_{\max} \leq 1$. If the drug concentration is high enough to produce maximum inhibition of either production or loss of response, $I_{\max}$ is assigned a value of 1. The equation, then, is written in the following form:

$$I(t) = 1 - \frac{C}{IC_{50} + C} \quad (17.27)$$

MODEL I: INHIBITION OF PRODUCTION OF RESPONSE. The turnover equation for this model is as follows:

$$\frac{dR}{dt} = k_{in} \cdot \left[1 - \frac{I_{\max} \cdot C}{IC_{50} + C} \right] - k_{out} \cdot R \quad (17.28)$$

The inhibition of production causes the response to decrease with time to a minimum value and then a gradual return to the predose baseline. Model I has been applied to a variety of drug responses in the literature. Some examples would be reduction of pain [55] and temperature [56] by anti-inflammatory drugs, effect of corticosteroids on cortisol [57], and effect of histamine H_2 -receptor antagonists on the secretion of acid [58].

MODEL II: INHIBITION OF LOSS OF RESPONSE. The turnover equation for this model is as follows:

$$\frac{dR}{dt} = k_{in} - k_{out} \cdot \left[1 - \frac{I_{\max} \cdot C}{IC_{50} + C} \right] \cdot R \quad (17.29)$$

For model II, the signature profile looks opposite of model I. Since the loss of response is inhibited as the response increases with time and returns back to baseline.

Model II has been reported in the literature for cholinesterase inhibition as well as inhibition of water reuptake by diuretics [59]. Inhibition of water reuptake leads to increased diuresis.

MODEL III: STIMULATION OF PRODUCTION OF RESPONSE. The turnover equation for model III is as follows:

$$\frac{dR}{dt} = k_{in} \cdot \left[1 + \frac{S_{max} \cdot C}{SC_{50} + C} \right] - k_{out} \cdot R \quad (17.30)$$

For model III, the drug stimulates the production of the response. Thus, the response increases from baseline with time and then returns back to the baseline. The signature profile is similar to that of model II. In the literature, model III has been applied to bronchodilatory effects of terbutaline [50], growth-hormone-releasing effect of ipamorelin [60], as well as induction of prolactin secretion by a dopamine receptor antagonist [61].

MODEL IV: STIMULATION OF LOSS OF RESPONSE. In model IV, the drug causes stimulation of the loss of response. Thus, the signature profile is similar to that of model I. The response decreases after drug administration and then trends back to the baseline levels as the drug is eliminated from the system.

The turnover equation of this system is as follows:

$$\frac{dR}{dt} = k_{in} - k_{out} \cdot \left[1 + \frac{S_{max} \cdot C}{SC_{50} + C} \right] \cdot R \quad (17.31)$$

Model IV has been applied to terbutaline's effect on lowering plasma potassium levels [59,62]. As the plasma concentrations for terbutaline increase, the plasma potassium concentrations decrease, the peak of decrease in potassium concentrations is delayed by approximately half an hour.

DIFFERENCE BETWEEN BIOPHASE/LINK MODEL AND INDIRECT RESPONSE MODELS. The movement of drug in and out of the hypothetical distribution compartment might apparently look similar to the processes k_{in} and k_{out} . For a data set that should be fitted with an indirect response model if fitted with a biophase/link model would perform equally well for a single dose. The fits, however, would become worse with simultaneous fitting of more than one dose levels to obtain a common set of parameters. One of the primary reasons lies in the basic expectations from the two models. For biophase/link models, the maximum effect is expected to occur at the same time, whereas for the indirect response models, the peak effect is further delayed with increasing doses [54]. Data simulated with indirect response models when fitted with a biophase model produces different values of parameters with data for different dose levels [63–65].

SIGNATURE PROFILES. Figure 17.18 shows signature profiles for the indirect response models. A pattern of decrease and return to baseline is observed with models I and IV. An opposite pattern, increase in response and return back to baseline, is observed with models II and III. With an increase in dose, all the parameters, maximum response, initial slope, and $T_{max, effect}$ also increase. The peak response is delayed with increase in dose [66,67].

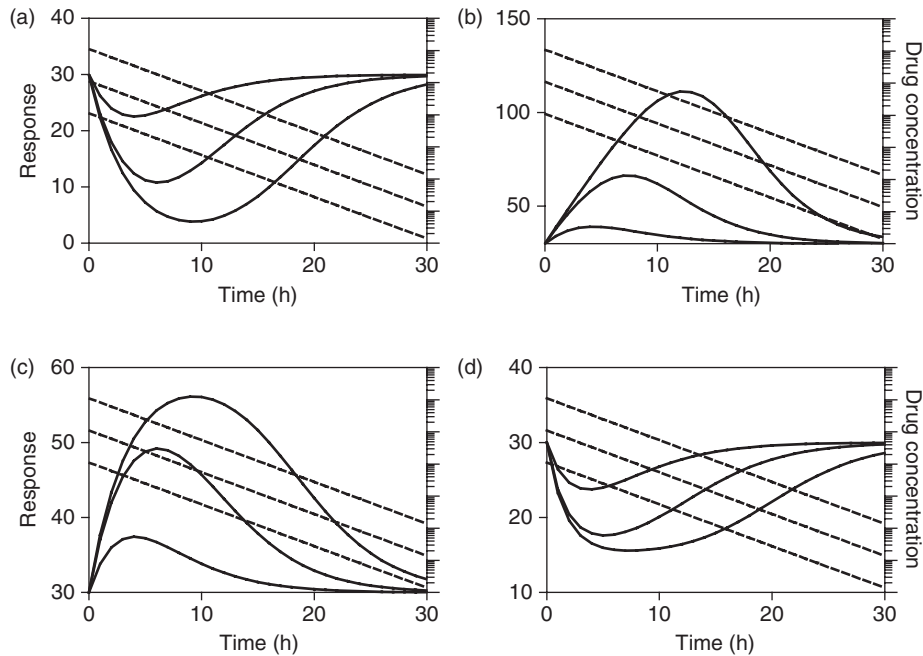


Figure 17.18 Signature profiles for four basic indirect response models. $V = 90$, $k_{cl} = 0.3$, $I_{\max} = 1$, $S_{\max} = 1$, $SC_{50} = 100$, $IC_{50} = 100$, $k_{in} = 9$, $k_{out} = 0.3$, $R_0 = 30$. Doses: 10–1000 units. (a) Model I, (b) model II, (c) model III, and (d) model IV. *Source*: Parameters obtained from Ref. 67.

TABLE 17.3 Initial Parameter Estimates for Basic Indirect Response Models

Parameter	Model I	Model II	Model III	Model IV
$I_{\max}/S_{\max}$	$\frac{(R_0 - R_{\max})}{R_0}$	$\frac{(R_{\max} - R_0)}{R_0}$	$\frac{(R_{\max} - R_0)}{R_0}$	$\frac{(R_0 - R_{\max})}{R_0}$
k_{in}	$-\frac{S_I}{I_{\max}}$	$\frac{S_I}{I_{\max}}$	$\frac{S_I}{S_{\max}}$	$-\frac{S_I}{S_{\max}}$
k_{out}	$\frac{k_{in}}{R_0}$	$\frac{k_{in}}{R_0}$	$\frac{k_{in}}{R_0}$	$\frac{k_{in}}{R_0}$
IC_{50}/SC_{50}	See Ref. 58 for the initial estimates for IC_{50}/SC_{50}			

INITIAL PARAMETER ESTIMATES. The details of initial parameter estimates have been provided by Sharma and Jusko [66]. Table 17.3 contains the equations to calculate the initial estimates.

17.7.1.2.3 Cell Life Span Models. This type of model has mainly found its application in the effect of substances, which stimulate the growth of cells in the body like the hematopoietic cells (RBC, etc.). Cell life span is defined as the time a cell spends in a population. Cell life span models assume that the loss of response (population of cells)

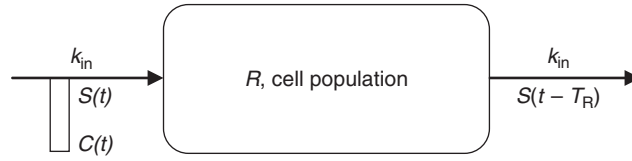


Figure 17.19 Basic one-pool cell model.

does not follow first-order processes but occurs due to natural senescence. A population of cells, R is controlled by two processes, a production rate and a loss rate [68]. Production can be due to physiological processes such as cell division, differentiation, and maturation, whereas cell loss may be due to senescence and random conversion to another type, such as hematopoietic cells. The following is the basic equation for the turnover of a cell population:

$$\frac{dR}{dt} = \text{production}(t) - \text{loss}(t) \quad (17.32)$$

Figure 17.19 shows a basic one-pool cell model. As mentioned previously, the loss process is assumed to be dependent on either natural senescence or conversion to another type of cells. Each cell lives for a specific period of time (T_R) and then dies or becomes another cell. The number of cells disappearing in a given time interval is equal to the number of cells generated. Or, the production rate is equal to that of the loss rate but delayed by T_R :

$$\text{Loss rate} = \text{production rate } (t - T_R)$$

The drug stimulates the production of response, which here is the cell population with a stimulatory function $S(t)$:

$$S(t) = \frac{S_{\max} \cdot C^\gamma}{SC_{50}^\gamma + C^\gamma} \quad (17.33)$$

where C is the concentration of the drug in the cell compartment, $S_{\max}$ is the maximum extent to which the production of cells can be achieved, and SC_{50} corresponds to the concentration at which the drug exhibits 50% stimulation of cell population. A Hill coefficient, γ , may be required to adjust for the shape of the response or can be set equal to one.

In this system, the production rate is described by the following equation:

$$\text{Production}(t) = k_{\text{in}} \cdot S(t) \quad (17.34)$$

Since loss rate is delayed by the life span of the cell (T_R), the equation for loss rate would be as follows:

$$\text{Loss}(t) = k_{\text{in}} \cdot S(t - T_R) \quad (17.35)$$

Thus, the basic one-pool cell model can be described by Equation 17.36:

$$\frac{dR}{dt} = \frac{k_{in} \cdot S_{max} \cdot C(t)^\gamma}{SC_{50}^\gamma + C(t)^\gamma} - \frac{k_{in} \cdot S_{max} \cdot C(t - T_R)^\gamma}{SC_{50}^\gamma + C(t - T_R)^\gamma} \quad (17.36)$$

$k_{in} \cdot S_{max}$ may be written as S_m and represents the maximum rate of cell production by the drug.

BASELINE (R_0). In the one-pool cell model depicted above, the production rate is equal to the loss rate and only delayed by the life span of the cell. The cells are produced by a zero-order rate constant k_{in} , which indicates that the number of cells born does not depend on the pool, R . In this scenario, the baseline value would be the same and not depend on any system parameters.

Thus, the value for the baseline was derived by Krzyzanski *et al.* [68] by assuming the production of cells was shut down. The equation thus obtained for the baseline is then expressed as follows:

$$R_0 = k_{in} \cdot T_R \quad (17.37)$$

PRESENCE OF AN ENDOGENOUS ACTIVE SUBSTANCE. If an endogenous compound stimulates the cell population, the baseline equation does not remain the same. Assuming a concentration of the endogenous compound to be C_0 , the baseline equation will now be basally stimulated as follows:

$$R_0 = k_{in} \cdot T_R \cdot \left(1 + \frac{S_{max} C_0^\gamma}{EC_{50}^\gamma + C_0^\gamma} \right) \quad (17.38)$$

SIGNATURE PROFILES. Figure 17.20a depicts the PK profiles of an agent which stimulates the production of cell population. PK was assumed to be one compartment with first order elimination with IV bolus dosing. Figure 17.20b depicts signature profiles for one-pool cell life span model for three dose levels. The rising part of the curves is entirely dependent on the production rate in an apparent zero-order manner. The profile reaches the peak exactly at the life span of the population [68]. After reaching its peak, the loss rate takes over and the response reaches the baseline. Figure 17.20c depicts the profiles at different values of T_R . As the values of T_R increase, the peak appears later as expected since the model predicts the peak to appear at T_R .

INITIAL ESTIMATES.

$$\begin{aligned} T_R &= T_{max} \\ k_{in} &= \frac{R_0}{T_R} \\ S_{max} &= \frac{R_{max}}{R_0} - 1 \\ S_I &= k_{in} \cdot S_{max} \end{aligned}$$

The estimate of S_{max} is reliable only at large doses since at smaller doses, true maximum effect is not achieved. S_I is the maximal initial slope.

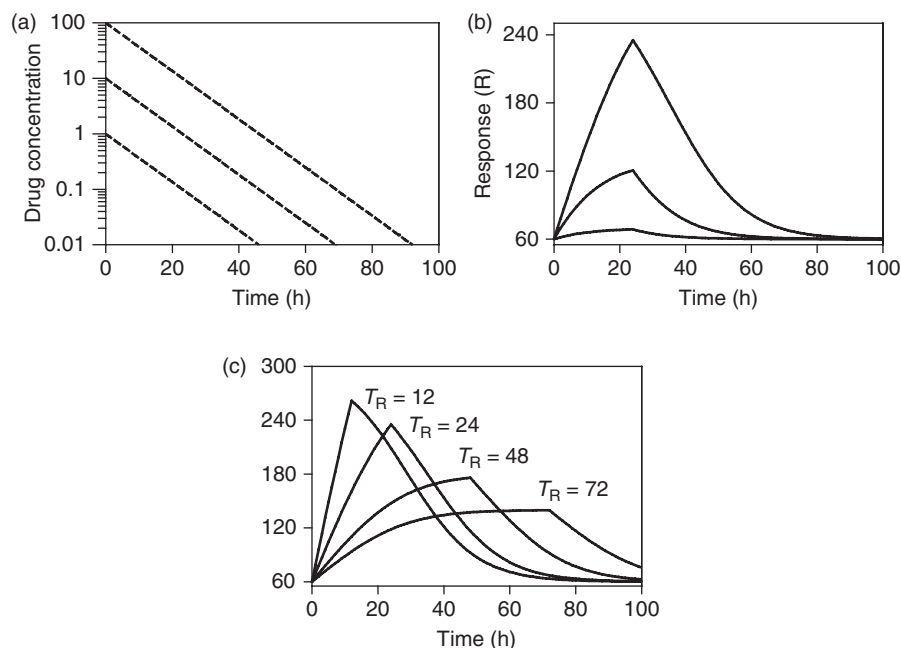


Figure 17.20 Signature profiles for one-pool cell life span model. $k_{el} = 0.1$; $V = 1$; dose = 1, 10, and 100; $T_R = 24$; $S_{max} = 4$; $SC_{50} = 10$; and $\gamma = 1$. *Source*: Parameters obtained from Ref. 69.

APPLICATION OF CELL LIFE SPAN MODELS. Cell life span models have been applied mainly to drugs that alter the production of hematopoietic cells [69–76]. Woo *et al.* [74] studied the effects of recombinant human erythropoietin (rHuEPO) on reticulocytes, RBC, hemoglobin, and mean corpuscular hemoglobin (MCH) in rats. The four-pool cell life span model appropriately described the change in the PD parameters and generated life span values for two precursor compartments as well as for reticulocytes. To date, cell life span concepts have been only applied in case of rHuEPO and in one case for thrombopoietin (TPO) and recombinant human granulocyte colony stimulating factor (rHuG-CSF) [68].

17.7.1.2.4 Time-Dependent Transduction Models. Time-dependent transduction models have been used to describe the underlying mechanism for a delayed PD response as signal transduction or some unknown mechanism. Signal transduction is the process by which a cell converts an extracellular signal into a response. A typical signal-transduction process is shown in Fig. 17.21. The signal “s” enters the cell and binds to the receptor R. This signal–receptor complex can cause an immediate direct effect. On the other hand, the signal–receptor complex can also enter the nucleus and bind to the gene response element to cause a gene-mediated delayed effect. If the intermediate steps are not measured, the data could be modeled empirically with a transduction model. Ligands for nuclear hormone receptors, described in Section 17.2, cause signal-transduction cascade and can produce long delays in response. GPCRs and ion channels, on the other hand, do not produce this kind of long delays once a ligand binds to them. Figure 17.22 depicts typical PK-PD expectation for a time-dependent transduction model [77].

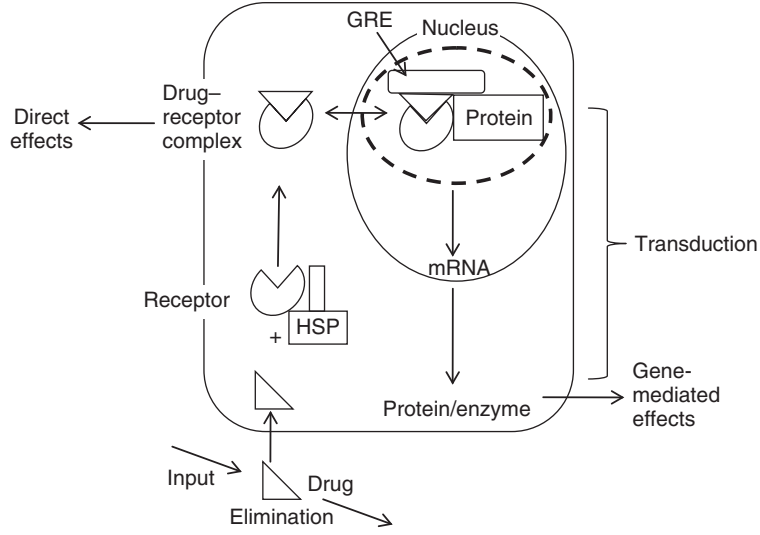


Figure 17.21 Schematic diagram for a signal-transduction process. *Source:* Adapted from Ref. 77.

The dotted line shows the PK profile and the solid line shows the PD profile. It is curious to note that the drug may be almost out of the system, but the PD marker is still at its baseline. In the case of biophase, indirect response (IDR), and cell life span models, the peak of the PD response appears later than the PK peak. However, in the case of transduction, one can see a significant delay, as depicted in (Fig. 17.22). Mathematically, the transit compartment technique is primarily used to model this delay between the PK and the PD response [78,79]. Figure 17.23 depicts a typical transit compartment model. $T_1, T_2, \dots, T_N$ are the transit compartments. Mathematically, the transit compartment model contains a series of differential equations (Eqs. 17.39–17.42), which describe a cascade of events between target occupancy and the PD effect. τ is defined as the mean transit time of signal from one compartment to the other and is assumed to be the same for all the compartments. γ is the power coefficient, which amplifies or dampens the signal of the transducer and is analogous to the Hill coefficient.

INITIAL ESTIMATES. The number of transit compartments (N), mean transit time (τ), and the power coefficient (γ) should be determined by fitting the model to the data:

$$\frac{dR}{dt} = \frac{1}{\tau} \cdot \left(1 - \frac{I_{\max} \cdot C}{IC_{50} + C} - R \right) \quad (17.39)$$

$$\frac{dT_1}{dt} = \frac{1}{\tau} \cdot (R - T_1) \quad (17.40)$$

$$\frac{dT_2}{dt} = \frac{1}{\tau} \cdot (T_1^\gamma - T_2) \quad (17.41)$$

$$\frac{dT_n}{dt} = \frac{1}{\tau} \cdot (T_{n-1} - T_n) \quad (17.42)$$



Figure 17.22 PK-PD expectation for a time-dependent transduction process. Dotted line, pharmacokinetics; solid line, pharmacodynamic expectation.

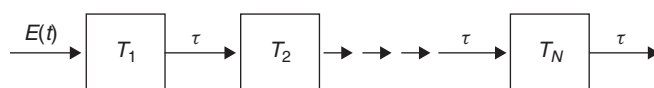


Figure 17.23 Transit compartment model for describing time-dependent transduction system.

SIGNATURE PROFILES. Figure 17.24 depicts the signature profiles for a single-step transduction process at different dose levels. The initial slope of these profiles is dose-independent. The time to peak effect is dose-dependent unlike biophase/link model. For medium to high dose levels, decline in response is parallel and inflection point represents the EC_{50} . The profiles mimic IDR model III (stimulation of k_{in}) for single-step processes but would not in case where multiple transduction processes exist. Figure 17.25a depicts the effect of increase in τ on the signature profiles for a transduction system. As τ increases, the profiles shift to later times and the maximum effect is diminished. Similarly, Fig. 17.25b depicts the effect of increase in the number of transduction compartments. The effect is similar to that of increase in τ . With an increase in the number of compartments, the profiles shift to later times and the maximum effect diminishes.

APPLICATIONS. Application of transduction models have been found in the literature in multiple therapeutic areas. These models have been applied to the cardiovascular area by Perlstein *et al.* [80]. A signal-transduction model with three delay compartments and a shape factor on the second compartment was used to capture the delay of the PD

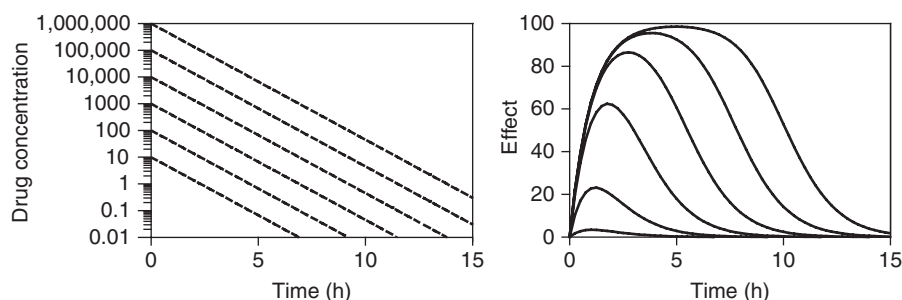


Figure 17.24 Signature profiles for single-step transduction process. $V = 0.1$; $k_{el} = 1$; $E_{max} = 100$; $EC_{50} = 100$; $\tau = 1$.

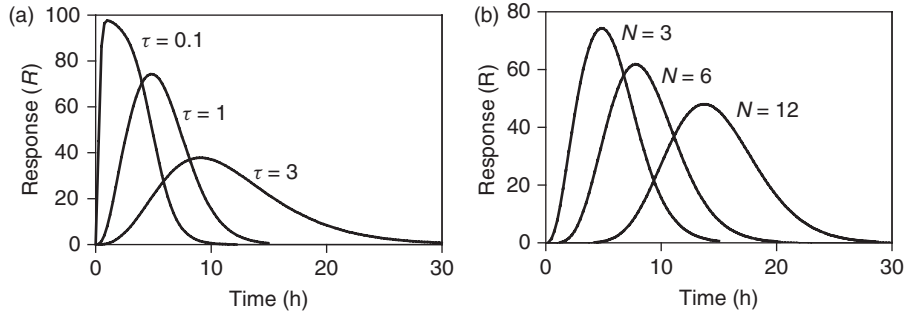


Figure 17.25 The effect of τ and N on the signature profiles for a transduction system. (a) Effect of increase in the values of τ and (b) effect of increase in the number of delay compartments (N).

response. Delays in tumor growth have also been described extensively in the oncology area [81–85]. Lobo and Balthasar [81] modeled the delay in tumor growth using the killing rate constant with transit compartments. Simeoni *et al.* modeled the delay by dividing the tumor compartment into four transit compartments and called them *injured tumor cells*. The sum of all the injured cell compartments and the tumor compartment will produce the final response that is invariably a decrease in tumor weight [82].

17.7.2 Irreversible Effects

Effects such as cell killing and enzyme inactivation are known as *irreversible effects*. The response returns to the baseline not due to turnover of the response but due to growth of cells or synthesis of new enzymes. These agents generally covalently bind to the target, which promotes the destruction of cells/enzymes.

17.7.2.1 Cell Kill Models. This type of model was first applied by Jusko [86] in 1971. Figure 17.26 depicts the basic structure of the model.

R is the PD response, which is the number of cells in a population. The growth rate of the cells (describe by kilograms) is a first-order process and depends on the number of cells present in the population. The cells have their natural death governed by the first-order rate constant k . C represents the concentrations of the chemotherapeutic

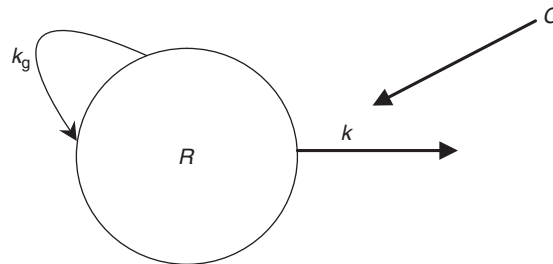


Figure 17.26 Basic structure of a cell killing model.

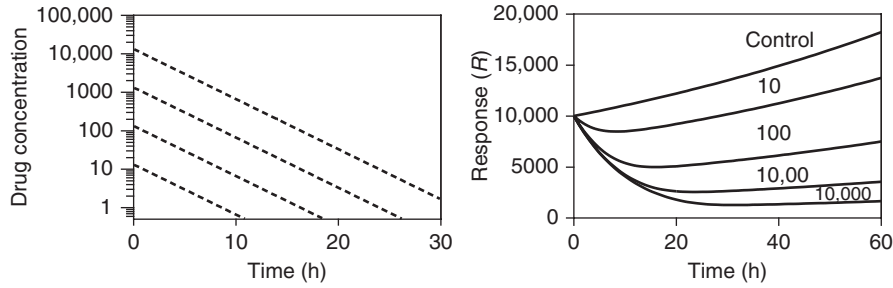


Figure 17.27 Signature profiles for a cell killing model with nonlinear killing function. $V = 0.75$, $k_{el} = 0.3$, $k_g = 0.01$, $K_{max} = 0.1$, $KC_{50} = 10$. Dose = 0, 10, 100, 1000, and 10,000 units.

agent that stimulates cell death. The equation for this type of system is provided below:

$$\frac{dR}{dt} = k_g \cdot R - k \cdot C \cdot R \quad (17.43)$$

Integration of the above equation gives

$$R = R_0 \cdot e^{k_g t} \cdot e^{-k \cdot AUC_0^t} \quad (17.44)$$

Looking at the above equation, one might mistakenly think that killing of the cells is independent of time, but the time component is hidden in the AUC term.

Instead of a linear cell kill constant, a nonlinear cell kill constant can be used as well. The rate constant for killing, k , will be modified to the following equation:

$$k = \frac{K_{max} \cdot C}{KC_{50} + C} \quad (17.45)$$

Signature profiles for the cell killing model with a nonlinear kill rate function are depicted in (Fig. 17.27). Monoexponential decline of PK was assumed. The PD profiles show decline in response and the response returns beyond the baseline as one would expect since the response in proliferating cells grows when there is no drug in the system. The control curve, on the other hand, only grows since there is no drug in the system to kill the cells.

17.7.2.1.1 Initial Estimates. Figure 17.28 shows the method of determination of initial estimates from the model.

In Fig. 17.28, plotted on the Y-axis is the log of survival fraction (R/R_0) and plotted on the X-axis is time. The initial estimates for k_g and k are obtained from the following equations, where t_D is the doubling time of the cells and can be obtained from observed data:

$$k = -\frac{\ln S_F}{AUC_0^t} \quad (17.46)$$

$$k_g = \frac{0.693}{t_D} \quad (17.47)$$

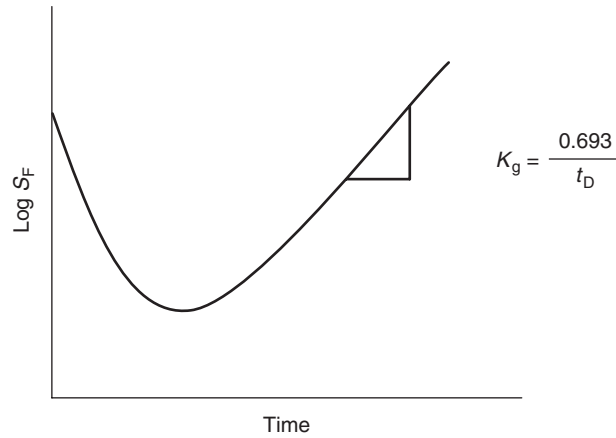


Figure 17.28 Method for obtaining initial estimates for a basic cell kill model.

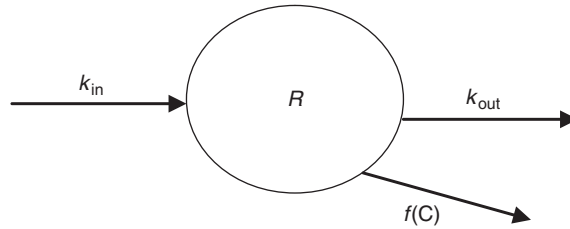


Figure 17.29 An irreversible enzyme inactivation model.

17.7.2.1.2 Applications. These models have been applied [87] to effect of piperacillin in studies of neutropenic mice infected with *Pseudomonas aeruginosa*. For all the doses, the survival fraction goes down, and as the drug is eliminated, the bacteria continues to grow again.

17.7.2.2 Model for Enzyme Inactivation. The structure of a typical enzyme inactivation model is depicted in Fig. 17.29.

R represents the enzyme concentrations. The enzyme is produced by a zero-order rate constant k_{in} and a first-order rate constant k_{out} . $f(C)$ represents the irreversible effect of the drug on the turnover of the enzyme. In absence of drug C , the model is similar to a turnover model, where $R_0 = k_{in}/k_{out}$. Equation 17.48 shows the turnover of the enzyme in presence of the drug. The effect of the drug can either be linear or represented by an E_{max} function. Equations 17.49 and 17.50 describe the two types of drug effect:

$$\frac{dR}{dt} = k_{in} - k_{out} \cdot R - f(C) \cdot R \quad (17.48)$$

$$f(C) = k \cdot C \quad (17.49)$$

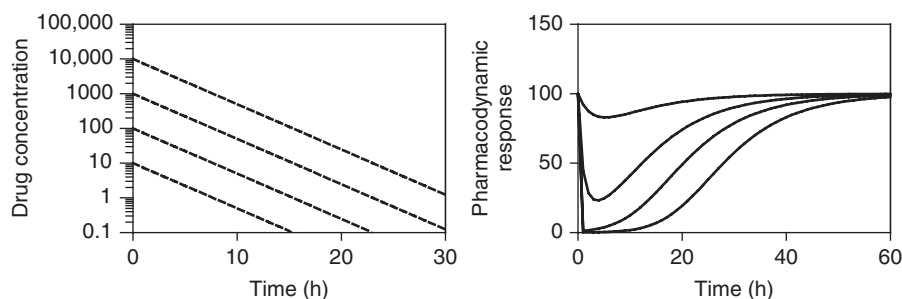


Figure 17.30 Signature profiles for an irreversible enzyme inactivation model. $k_{el} = 0.3$, $V = 1$, $k_{in} = 10$, $k_{out} = 0.1$, $k = 0.01$. Dose = 10, 100, and 1000 units.

$$f(C) = \frac{K_{max} \cdot C}{KC_{50} + C} \quad (17.50)$$

17.7.2.2.1 Signature Profiles. Figure 17.30 depicts the signature profiles for enzyme inactivation model at a range of doses. The response declines from baseline to a minimum and returns back to the baseline with time. There is a dose-dependent shift in the maximal effect; however, the peak effect occurs earlier unlike indirect response models, where peak effect occurs later at higher doses [88].

17.7.2.2.2 Applications. The basic turnover-irreversible model has been applied to inhibition of gastric acid secretion of pantoprazole [89]. This model was further used to simulate reduction in acid secretion at 40-mg dose to validate the model. The model was further validated by integration of clinical data, which showed reduction in time to maximal effects with an increase in concentrations or dose as well as higher infusion rates. This kind of model has also been applied to antiplatelet effects of aspirin [90].

17.7.3 Other Models

17.7.3.1 Disease Progression Models. Clinical pharmacology is a combination of how a disease progresses and the effect of drug on the progression of disease. It was first indicated by Holford and Sheiner in 1981 that the baseline in patients, which we use in an E_{max} model (E_0), is not a constant number. The change in E_0 over time should be taken into consideration while analyzing the clinical data [91]. The progression of disease refers to time course or trajectory of a biomarker or a PD or clinical end point, which is a measure of the clinical status of the patient. This trajectory of the disease progression can be linear, nonlinear, or cyclical. Thus, a model for progression of a disease should incorporate these factors with mathematical function to describe the time course of disease progression [92]. Also, there may be situations where the disease conditions change the underlying PK and PD. These changes should also be incorporated during analysis of clinical data and clinical trial simulations.

17.7.3.1.1 Effects of Drugs in Disease Progression. The effect of a drug on the disease progression may be of three types [93]. Figure 17.31 depicts the three types of drug effects on disease progression.

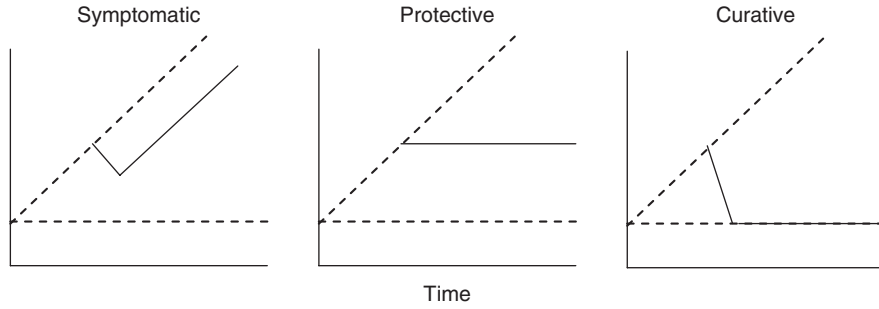


Figure 17.31 Types of effects of different drugs on disease progression.

SYMPTOMATIC. The effect of a drug is named symptomatic if it has a beneficial effect on the disease but does not change the underlying process of the disease or the trajectory of the disease. The drug delays the progression of the disease, and as the drug exits the system, the disease follows its normal path. This type of drug effect has been seen with tacrine in the treatment of Alzheimer's disease [94]. The drug delays the deterioration of the patient by six months, and as soon as the therapy is stopped, the deterioration follows its natural path.

DISEASE MODIFYING OR PROTECTIVE. A drug is said to be protective or disease modifying if it interferes with the natural history or trajectory of the disease and causes a permanent change. Thus, the effect remains even after the drug has been eliminated or therapy has been stopped. Protective effects may be of two types, one which alters the time course of progression of the disease and the other which changes the maximal status of the disease.

CURATIVE. The effect of a drug is said to be curative if the therapy completely arrests the progression of the disease. Again, removal of drug from the system or stopping of therapy keeps the patient in the state before the disease started [92]. Chemotherapeutic agents such as antibacterial agents and some anticancer agents exhibit curative effects.

17.7.3.1.2 Models of Disease Progression.

LINEAR MODELS. The linear model of disease progression is the simplest model used to describe the time course of progression of several diseases. The model assumes that the patient has a baseline disease condition S_0 and a slope α , which is responsible for the change in the disease condition with time. Equation 17.51 shows the disease model without the effect of the drug:

$$S(t) = S_0 + \alpha \cdot t \quad (17.51)$$

A disease progression model is built in two steps. First, the model for the disease is built and then the effect of the drug is added to it. Equation 17.52 shows the effect of the drug $[f(C)]$:

$$S(t) = S_0 + \alpha \cdot t + f(C) \quad (17.52)$$

Equation 17.52 shows a symptomatic drug effect on the disease. The drug does not act on α . Once the therapy is stopped or the drug goes out of the system, the same slope α resumes. Equation 17.53, on the other hand, shows the protective effect of the drug. In this case, the drug acts on the slope α , and at the end of therapy, the value of α changes to a different value, α' :

$$S(t) = S_0 + (\alpha + f(C)) \cdot t \quad (17.53)$$

ASYMPTOTIC MODELS. The linear models have their own disadvantages. They are useful for time frames that are mainly linear. Also, the biological systems, more often than not, change nonlinearly with time. Thus, asymptotic models are often used to describe a variety of diseases. There are different kinds of asymptotic models used in the literature to describe different disease processes. These models are named so since they are associated with some kind of plateau of the disease biomarker. Table 17.4 depicts different types of disease models and the effect of drugs on the disease.

Apart from the examples shown in Table 17.4, other asymptotic models can be used such as inverse Bateman function [92] and cyclical modification of inverse Bateman function [92].

GROWTH MODELS. Apart from all the linear and asymptotic models, different types of growth models have also been used to describe disease progression. A Gompertz-type growth function has been used to describe the time course of Parkinson's disease [92], as depicted in the following equation:

$$\frac{dR}{dt} = \beta \cdot R \cdot (\beta_{\max} - R) - (k_{\text{death}} \cdot R) \quad (17.54)$$

In addition to these models, transit compartment models have also been used to describe a time course of disease [92].

TABLE 17.4 Asymptotic Models of Disease Progression and Effect of Drug on Disease

Model	Equation	Symptomatic Drug Effect	Disease Modifying or Protective Drug Effect	References
Exponential	$S(t) = S_0 \cdot e^{-k_{\text{PROG}} \cdot t}$	$S(t) = S_0 \cdot e^{-k_{\text{PROG}} \cdot t - f(C)}$	$S(t) = S_0 \cdot e^{-[k_{\text{PROG}} + f(C)]t}$	87
E _{max} function	$S(t) = S_0 + \frac{S_{\max} \cdot t}{SC_{50} + t}$	$S(t) = S_0 + \frac{S_{\max} \cdot t}{SC_{50} + t} \pm f(C)$	$S(t) = S_0 + \frac{S_{\max} \cdot [1 + f(C)] \cdot t}{SC_{50} \cdot [1 + f(C)] + t}$	88,89
Nonzero asymptotic function	$S(t) = S_0 \cdot e^{-k_{\text{PROG}} \cdot t} + S_{\text{ss}} \cdot (1 - e^{-k_{\text{PROG}} \cdot t})$	$S(t) = S_0 \cdot e^{-k_{\text{PROG}} \cdot t} + S_{\text{ss}} \cdot (1 - e^{-k_{\text{PROG}} \cdot t}) \pm f(C)$	$S(t) = S_0 \cdot e^{-k_{\text{PROG}} \cdot [1 + f(C)] \cdot t} + S_{\text{ss}} \cdot (1 - e^{-k_{\text{PROG}} \cdot [1 + f(C)] \cdot t})$	84

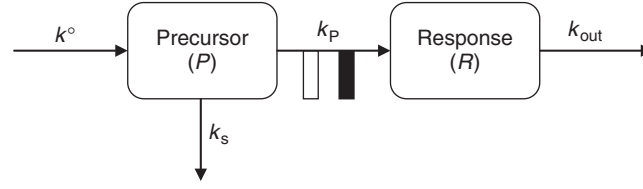


Figure 17.32 Schematic diagram of a precursor-dependent indirect response model.

17.7.3.2 Models of Tolerance and Rebound. As defined in Section 17.2, tolerance is referred to as a *phenomenon where the response to the drug exposure is diminished due to repeated administration of the drug*. This phenomenon might be observed in the clinic as well as in preclinical species and has been described by the following PD models.

17.7.3.2.1 Precursor-Dependent Indirect Response Model. This model describes the phenomenon of tolerance and rebound in case of indirect PD responses. The model in general shows how a drug can affect a precursor pool by stimulation or inhibition of the elimination of the precursor. The structure of the model is depicted in (Fig. 17.32). Precursor P is synthesized by a zero-order rate k_0 and lost by the first-order rate k_s . Precursor is converted to response R by a first-order rate k_p . The response, R , is dissipated by a first-order rate k_{out} [95]. The drug either stimulates or inhibits the conversion of the precursor to the response by inhibitory $I(t)$ or stimulatory $S(t)$ functions as follows. The rate of change of response and the precursor can be described by the following set of differential equations:

$$\frac{dP}{dt} = k^\circ - k_p(1 \pm H(t)) \cdot P - k_s \cdot P \quad (17.55)$$

$$\frac{dR}{dt} = k_p(1 \pm H(t)) \cdot P - k_{out} \cdot R \quad (17.56)$$

$H(t)$ represents either stimulatory or inhibitory Hill functions, as described by the following equations:

$$S(t) = 1 + \frac{S_{max} \cdot C}{SC_{50} + C} \quad (17.57)$$

$$I(t) = 1 - \frac{I_{max} \cdot C}{IC_{50} + C} \quad (17.58)$$

The signature profiles for the stimulation model after four doses starting from 10 units to 10,000 units are shown in Fig. 17.33. In precursor-dependent IDRs, rebound is mainly due to depletion (stimulation model) or accumulation (inhibition model) in the amount of precursor with time, flowing into the drug compartment. The maximal effect increases with increase in dose. The effect reaches the baseline and further goes down producing rebound. The peak of the rebound shifts toward later time points with dose. Figure 17.34 depicts the effect of multiple dosing on the signature profiles of a

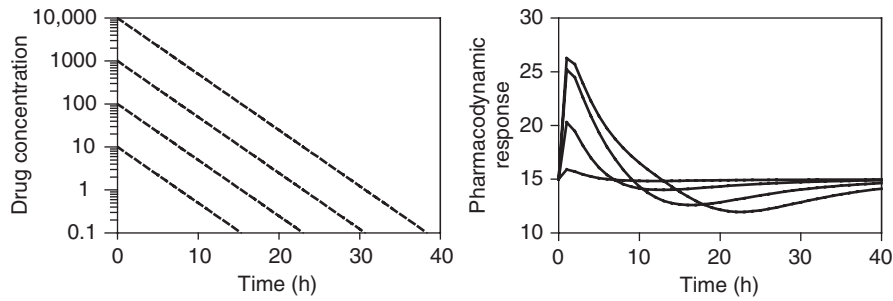


Figure 17.33 Signature profiles from a precursor-dependent indirect response model. $k_{el} = 0.3$, $V = 1$, $R_0 = 15$, $P_0 = 300$, $k_p = 0.1$, $k_s = 0$, $k_{out} = 2$, $E_{max} = 1$, $EC_{50} = 100$.

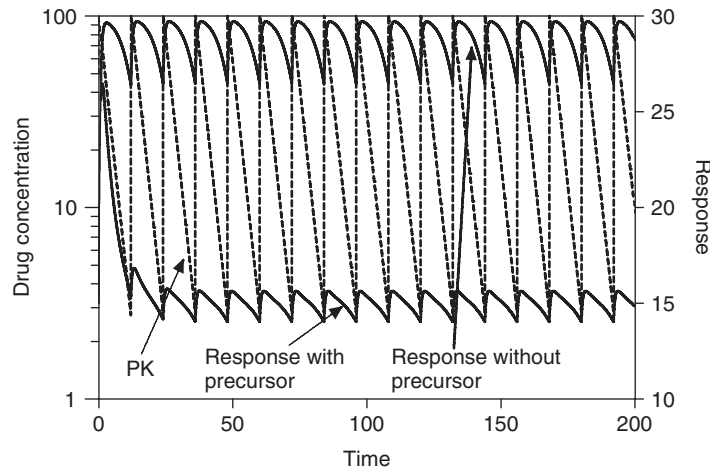


Figure 17.34 Development of tolerance during multiple dosing for a precursor-dependent IDR. Dose of 100 units given at an interval of 12 h. Dotted line, multiple dose pharmacokinetics; solid lines, multiple dose pharmacodynamics for models with and without precursor.

precursor-dependent indirect response model. Plotted in the same graph is the mono-exponential PK profiles and two PD profiles, one with the precursor compartment and the other without precursor compartment. The profile without the precursor compartment increases with multiple dose and reaches steady state as the PK reaches steady state with a slight delay in the peak times. When a precursor compartment is added, the PD response decreases with the first three doses and then achieves a steady state with response values much less than that achieved by the model without a precursor compartment. Thus, the model with a precursor compartment exhibits tolerance with multiple doses.

17.7.3.2.2 Counterregulatory Model. This type of model relies on the opposing effect acting on the response. The formation of the modulator is dependent on the response and a first-order rate constant, k_1 , and dissipates with the rate constant k_2 .

The turnover of the modulator is provided by the following equation:

$$\frac{dM}{dt} = k_1 \cdot R - k_2 \cdot M \quad (17.59)$$

The modulator compartment is then introduced into the mechanistic PD model as an opposing effect. The following equation shows such a modulator effect on an indirect response model:

$$\frac{dR}{dt} = k_{in} - k_{out} \cdot \left[1 - \frac{I_{max} \cdot C}{IC_{50} + C} \right] \cdot R \cdot (1 + M) \quad (17.60)$$

Counterregulatory models in their various forms have been used a multiple times in the literature [96,97]. Bauer and Fung developed a counterregulatory model to account for the effect and generation of tolerance by nitroglycerine in a rat model [97]. Counterregulatory mechanisms have been introduced in the indirect response model of diuresis to account for tolerance development by furosemide [97].

17.8 TOOLS AND SOFTWARES COMMONLY USED IN PK-PD MODELING

PK-PD modeling requires a computer to undergo iterative procedure to generate PK and PD parameters. Various softwares have been used from the inception of this kind of data analysis. Various softwares used are WinNonlin, ADAPT II and V, Matlab (extensively used in chemical engineering as well), SAAM II, Phoenix, and SAS for individual analysis. For population modeling, more popular softwares are NONMEM, S-ADAPT, R, S-PLUS, SAS, WinNonmix, ADAPT V, and Phoenix. For simulations, Berkeley Madonna is extensively used. Simulations for this chapter were performed with WinNonlin [98], ADAPT V [99], and Berkeley Madonna [100]. GraphPad Prism [101] was used for plotting the simulated data.

17.9 CONCLUSIONS

In order to perform and apply PK-PD modeling successfully, one requires a sound understanding of various disciplines such as biology, pharmacology, and mathematical and statistical sciences. Knowledge of biology and pharmacology helps in understanding a disease and therefore choosing the right biomarker for the development of a disease model. Modeling has diverse applications in both drug discovery and development, starting from understanding of efficacy animal models through dose selection for FIH studies, and throughout drug development.

17.10 FUTURE PERSPECTIVES: SYSTEMS MODELING

A recent interest in the area of PK-PD modeling is the development and application of systems modeling in drug discovery and development. Systems modeling refer to modeling a system such as heart or lung using same mathematical principles as used

in classical PK-PD modeling. Modeling has been thriving on applied mathematics to understand the quantitative relationship between kinetics of drug in the body and its effect [102]. Systems model mainly refer to the development of physiologically based disease progression models to which a drug effect can be incorporated. Modeling efforts in physiology have resulted in organ models such as the human lung model [103] and the lumped heart model [104]. Building similar systems with progression of disease is the latest challenge in PK-PD modeling. Once such a model has been built, drug effect can easily be incorporated. Some initial efforts have been made in this area recently by Landersdorfer and Jusko [105] and Danhof and colleagues [106]; however, the area is still in its evolution stage. A recent white paper from NIH states that, “quantitative systems pharmacology will create understanding of disease mechanisms and therapeutic effects that span biochemistry and structural studies, cell and animal-based experiments and clinical studies in human patients” [107].

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18

Translational Drug Discovery Research: Integration of Medicinal Chemistry, Computational Modeling, Pharmacology, ADME, and Toxicology

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18.1	Introduction	1
18.2	Drug discovery process	6
18.3	Role of translational research and biomarkers in drug discovery and development	41
18.4	Summary	45
	References	46

18.1 INTRODUCTION

The drug discovery and development process involves several sequential steps, from target identification, computational modeling, medicinal chemistry, biological screening, lead generation and optimization, preclinical and clinical studies to final registration of a drug. The past two decades of drug discovery research and development witnessed an explosion of technological advances but with little success in increasing the approvals of new drugs reaching the market each year. The cutting-edge technologies developed in the past two decades include miniaturized high throughput screening assays, genetically modified animal models, ultra-sensitive liquid chromatography (LC)–mass spectrometry (MS)/MS systems enabled with structure

elucidation tools, advanced imaging systems, nano-technology, siRNA technology, OMICS (genomics, proteomics, and metabonomics), and many more. However, these technological advances have not been meaningfully translated for success of new drugs reaching the market. The number of new drugs approved now is no greater than 50 years ago, that is, between 20 and 30 drugs, although mid 1990s have seen approvals of up to 50 new chemical entities (NCEs). Alongside, the cost of new drug development has been escalating 13.4% annually since 1950s and recently has been estimated between US\$1.3 and 1.7 billion, although these estimates have been debated [1]. Therefore, in retrospect, these facts warrant a critical review of factors that impede the success of new drugs reaching the market.

A review of the reasons of failures of drug approval for human use in the past three decades indicates that the major reason from the 1960s through the 1980s was unfavorable pharmacokinetics (PKs; 39%) in phase 1 clinical trials [2], while failures due to lack of efficacy and safety were estimated to be 30% and 10%, respectively (Fig. 18.1a). Early assessment of PK liabilities in drug discovery using *in vitro* and *in vivo* approaches led to reduced number of failures (14%) in phase 1 clinical trials (Fig. 18.1b) [3]. Nevertheless, during late 1990s to early 2000, failures due to safety issues escalated to 43% with minimal change in efficacy failures (36%), which can be attributed to enhanced drug exposures due to improved PK profiles of the drug candidates. In 2010, several drugs failed in phase 3 trials (Table 18.1) due to lack of efficacy, although the results until phase 2 trials were promising. During 2007–2010 [4], almost 90% of failed phase 3 trials were either due to lack of efficacy (66%) or safety issues (21%; Fig. 18.1c). Among these failures, about 28% drugs were for oncology targets and 18% for neurological disorders (Table 18.2). This is a very important factor to consider as many pharmaceutical companies ignored the complexity of biology in rational drug design and discovery. As Arrowsmith stated, “propensity to assume that success in one disease will translate into success in new and significantly different disease. This is particularly apparent in oncology, for which success in one tumor type has been assumed to translate into success in variety of other tumors, without firm evidence that the mechanism of action remains relevant” [5]. For example, sunitinib (Sutent, Pfizer) was approved in 2006 for renal cell carcinoma and imatinib-resistant gastrointestinal (GI) stromal cell carcinoma and subsequently for rare pancreatic neuroendocrine cancers [6]. However, between April 2009 and May 2011, Pfizer has reported unsuccessful late-stage trials with sunitinib in breast cancer, metastatic colorectal cancer, advanced nonsmall-cell lung cancer, and castration-resistant prostate cancer. These failures clearly indicate that mechanisms of tumor development are different from one organ to another. Forcing drug candidates into expensive late-stage clinical trials for additional multiple indications than the one initially sought after can contribute to poor success of drug approval, due to lack of mechanistic mode of action of the drug candidate. Another point to consider is that predictive value of proof-of-concept (POC) trials in animal models is inadequate and in certain cases may not translate to desired clinical outcome [7]. Frye *et al.* [8] points out that about 49% of known targets have not been validated for efficacy in humans, thus widening the gap for discovering novel drugs for unmet medical needs. With the availability of complete human genome sequence data, along with understanding their functions, the number of potential drug targets are expected to increase from the 500 or so that underlie our current pharmacopeia to potentially tens of thousands [9]. Thus, in the future, more preclinically and clinically unvalidated targets will be added to new drug discovery research.

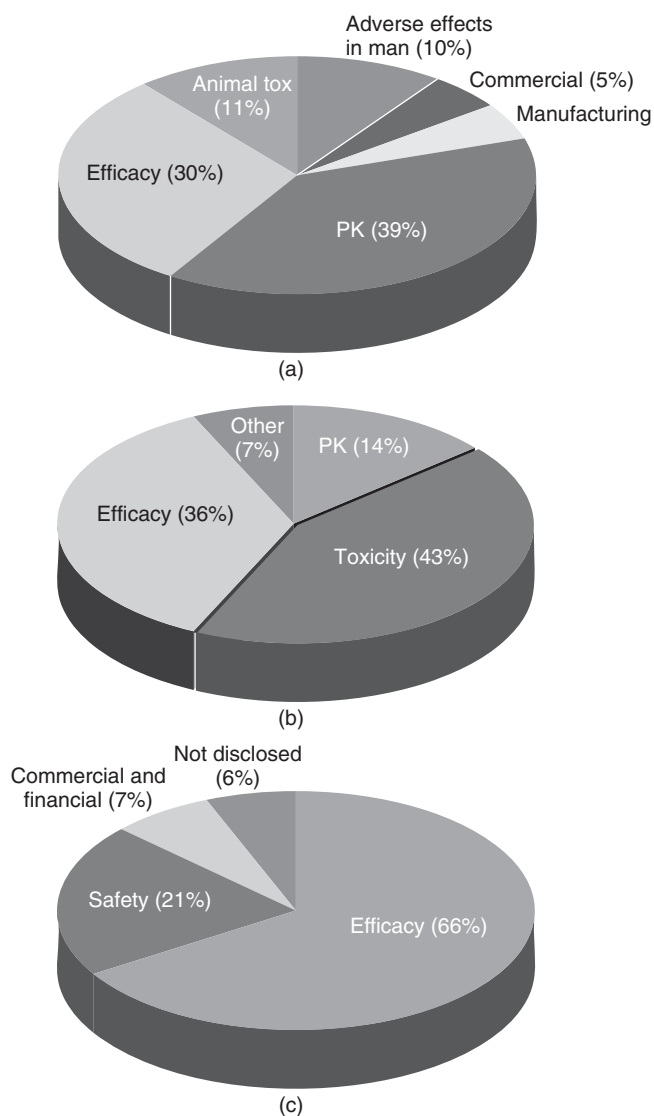


Figure 18.1 Pie-charts representing (a) pharmacokinetics contributing to (39%) drug failures, while failures due to lack of efficacy and safety were estimated to be 30% and 10%, respectively, (b) PK liabilities in drug discovery using *in vitro* and *in vivo* approaches led to reduced number of failures (14%) in phase 1 clinical trials, and (c) almost 90% of failed phase 3 trials were either due to lack of efficacy (66%) or safety issues (21%). (See color insert.)

Translational research offers promise to close the gaps between efficacy and safety outcomes from animal disease models and those in patients through rational drug design [9]. Rational drug discovery requires an early assessment of all factors affecting the likely success of a drug candidate in the subsequent preclinical and clinical phases of drug development. These factors include pharmacology, physico-chemical properties, drug permeability, transport, absorption, drug metabolism (DM), and toxicology.

TABLE 18.1 Top 10 Phase III Failures During 2010

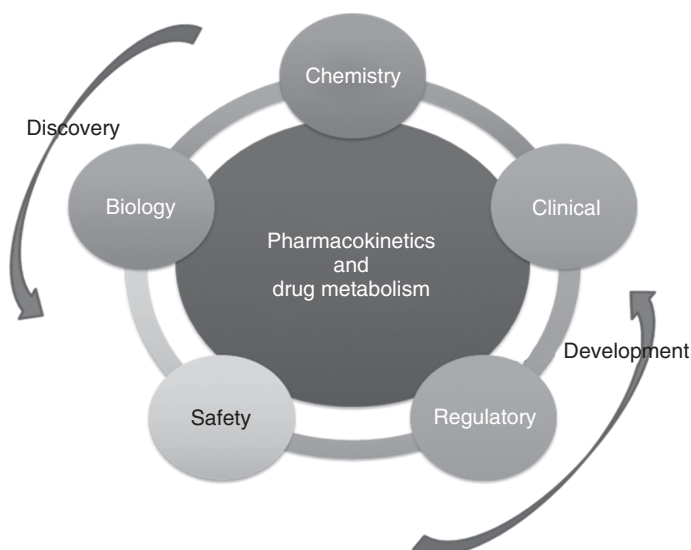
Drug Name	Chemical Class	Company	New Indication	Unmet Need?	Reason for Failure
Dimebon (latrepirdine)	Small molecule	Pfizer/Medivation	Alzheimer's, Huntington	Yes	No efficacy
Ocrelizumab	Monoclonal antibody	Roche	Rheumatoid arthritis	Yes	No efficacy
Taspoglutide	GLP1 analog ^a	Roche	Type 1 diabetes	Yes	Serious side effects
Semagacestat	Small molecule	Eli Lilly	Alzheimer's disease	Yes	No efficacy
ASA404 (vadimazen)	Small molecule	Novartis/Antisoma	Nonsmall cell lung cancer	Yes	No efficacy
NOV-002	Glutathione disulfide mimetic	Novelos	Oncology in combination with chemotherapy	—	No efficacy
Zibotentan	Small molecule	Astra Zeneca	Nonmetastatic prostate cancer	—	No efficacy
Vicriviroc	Small molecule	Schering/Merck	HIV-1	Novel target (CCR5)	No efficacy
Recentin (cediranib)	Small molecule	Astra Zeneca	Metastatic colorectal cancer	—	Poor efficacy compared with avastin
NV1FGF	pCOR ^b DNA plasmid	Sanofi aventis	Critical limb ischemia (PAD) ^c	—	No efficacy

^aGlucagon-like peptide 1 analog.^bpCOR, conditional origin replication.^cPeripheral arterial disease.

The use of mechanistic PK–PD models early in the drug discovery process along with novel biomarker monitoring could potentially reduce attritions during late-stage drug development. The advances in automation of analytical technologies has dramatically improved our ability to dissect out the fundamentals of absorption, distribution, metabolism, excretion, and toxicology (ADMET) liabilities through the development of powerful *in silico* and high throughput screening methods. This is fueling a shift away from the conventional nature of ADMET assessment toward a more rational, *in cerebro* approach to drug design. On the other hand, PD models have also considerably shifted from traditional approaches to molecular biology approaches in dissecting the cell signaling pathways, humanized animal models, and imaging tools to select appropriate and selective biomarkers that play causal role (not casual) in assessing drug efficacy and safety. These advances in technologies are being increasingly employed in mechanistic

TABLE 18.2 Phase III and Submission Failures According to Therapeutic Area During 2007–2010

Therapeutic Area (Total $n = 83$ Compounds)	Failures (%)
Anticancer (23)	28
Nervous system (15) ^a	18
Alimentary and/or metabolism (11) ^b	13
Anti-infectives (11)	13
Cardiovascular (7)	8
Other (16)	19

^aIncludes neurodegenerative diseases.^bIncludes diabetes and obesity.**Figure 18.2** Provides an overview of the key areas in the drug discovery and development and highlights the central role of pharmacokinetics and drug metabolism in the process. (See color insert.)

PK–PD studies during early drug discovery to select promising candidates for drug development.

This chapter provides an overview of the drug discovery process with special emphasis on integrating medicinal chemistry with pharmacology, DMPK, and toxicology (Fig. 18.2). Key PK–PD concepts combined with applications of recent advances in DM, drug transport (DT), exploratory toxicology, and biomarker tools allow accurate prediction of clinical efficacy and safety during preclinical evaluation. The strategic use of translational PK–PD approaches starting from early drug discovery through late-stage clinical development for a successful realization of drug product with minimal failures are discussed in the following sections.

18.2 DRUG DISCOVERY PROCESS

Current state of the art in drug discovery involves at least three phases (Fig. 18.3). During the *first* phase, computational modeling, which is also popularly known as *in silico modeling* or *virtual screening*, is used. Software have been developed in the past two decades (Table 18.3) using proprietary and nonproprietary databases that include physico-chemical, enzyme/receptor docking, and ADMET properties. These databases help a computational modeling scientist to design “drug-like” chemical structures for a particular biological target of interest.

During the *second* phase of drug discovery, computational scientists feed these data to medicinal chemists who then attempt to synthesize the “drug-like” molecules of interest. It is also imperative that patent liabilities be considered before synthesis is initiated. These molecules then are screened in various pharmacological assays to identify molecules with “drug-like” properties.

During the *third* phase of drug discovery process, which is known as *lead optimization stage*, the medicinal chemists handover the molecules to ADMET scientists who conduct various *in vitro* and *in vivo* ADMET evaluations to generate “leads” with drug-like properties with minimal ADMET liabilities. These leads are then advanced by the regulatory scientists who will initiate investigational new drug (IND), enabling studies for submission to regulatory authorities.

The three phases of drug discovery may be iterative as a cyclical process until there is consensus among computational scientist, medicinal chemist, pharmacologist, and ADMET scientist for the quality of leads generated. At an early phase, it is essential that the pharmacokineticist, pharmacologist, and toxicologist collaborate on building PK–PD and toxicokinetics (TKs)–toxicodynamics relationships that can predict efficacious clinical dose and appropriate desired clinical response with broad therapeutic index.

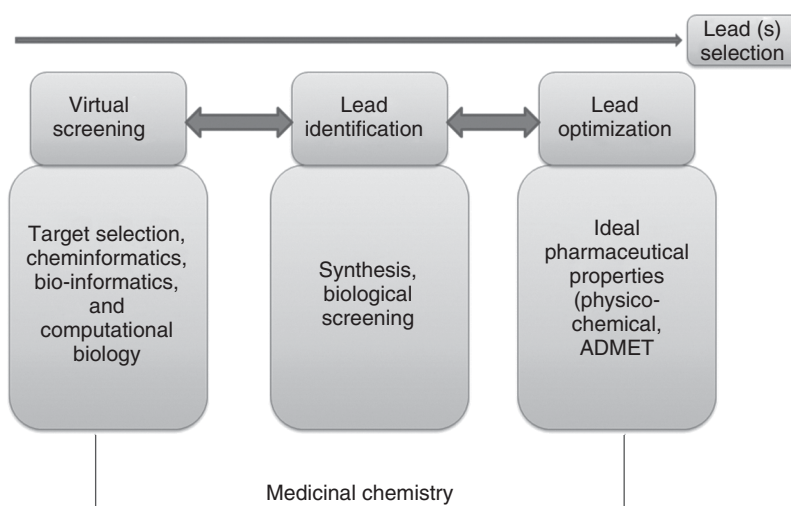


Figure 18.3 Gives an overview of the different stages of drug discovery process and highlights the contributions of virtual screening and computational biology in the lead identification and optimization process.

TABLE 18.3 *In Silico* Software Used in Drug Discovery

Software	Application	License
Accelrys Discovery Studio	Molecular modeling, docking	Commercial
ACD Labs	ADMET, cheminformatics, data management	Commercial
Autodock, iGemDock, Vina, PyRx	Docking, virtual screening	Open source
Bioclipse/OpenTox	Cheminformatics, QSAR, toxicity, ADMET	Open source
ChemOffice	Cheminformatics, ADMET, data management	Commercial
DEREK, TOPKAT, MultiCASE	Toxicity prediction	Commercial
FAF drugs	ADMET	Open source
GastroPlus, SimCYP	Absorption and metabolism modeling	Commercial
Inteligand	Pharmacophore discovery, docking	Commercial
Knime	Workflow development, data analytics, model building, automation, cheminformatics, bioinformatics	Dual
Meteor	Metabolism prediction	Commercial
MetaPrint2D	Metabolism prediction	Open source
OECD Toolbox	Toxicity prediction	Open source
OpenBabel	Command line small molecule manipulation	Open source
PADEL	2D and 3D chemical descriptor calculation	Open source
Pipeline Pilot	Workflow development, data analytics, model building, automation, cheminformatics, bioinformatics	Commercial
Pymol	Macromolecular visualization	Dual
R Bioconductor	Microarray, proteomics, statistical data analysis	Open source
RDKit, CDK, Indigo	Cheminformatics programming libraries	Open source
Taverna	Workflow development, data analytics, model building, automation, cheminformatics, bioinformatics	Open source
Toxtree	Toxicity prediction	Open source
Tripos Simulations Plus	ADMET	Commercial
Weka	Data mining, model building	Open source

18.2.1 Computational Modeling and Simulations in Drug Discovery

Computational analyses play a big role in drug target identification and validation and also in lead identification and optimization. A lead compound is one that is believed to have potential to treat disease without having serious life-threatening adverse effects.

Microarray analysis for example is often used to profile gene expression and pick genes that are differentially expressed and may be potential drug targets or biomarkers for the underlying pathology [10,11]. More extensive experimentation and time series analysis of the gene expression signatures is becoming routine due to the widespread availability and affordability of standardized microarrays. Proteomic technologies hold out a similar promise and protein microarrays are gaining wider acceptance [12]. However, these technologies generate a humongous amount of data that need to be effectively stored, cleaned, transformed, and finally analyzed using statistical and data-mining tools. Depending on the desired endpoint, this can result in discovery of new drug targets or validation of existing ones. It can also help in profiling toxicity-related gene signatures for a given molecule [13]. The initial data are in the

form of images that need to be analyzed using imaging software to estimate the signal strength of the genes. Later, this signal or expression data are analyzed using unsupervised clustering methods such as principal component analysis, expectation minimization, statistical methods (analysis of variance and analysis of covariance), and machine learning algorithms such as support vector machines. Visualization techniques are also frequently employed [14]. While there are several commercial software for genomic analysis, the predominant current platform is “R.” This is an open-source statistical language and analysis platform with extensive modules for data mining of genomics and proteomics [15]. Several companies, including Amazon (EC2) and revolution analytics (<http://www.revolutionanalytics.com>), offer “R” on the “cloud” to enable distributed scalable life science data analysis. Academic research centers such as the European Bioinformatics Institute also offer cloud-based “R” analysis for microarray data (<http://www.ebi.ac.uk/Tools/rccloud/>).

18.2.1.1 Pharmacophore Modeling Tools. Assuming that the protein drug target is known and its three-dimensional (3D) structure is available, molecular modeling tools such as PyMol (PyMOL Molecular Graphics System 2002) [16] and Chimera, are used to probe its structure and locate the binding sites [17]. In case of enzymes, these may be substrate or cofactor/coenzyme sites or other allosteric sites. Since the idea is to find small molecules that bind to these sites, open-source software such as AutoDock4 [18] and Autodock Vina [19,20] may be used to simulate the binding energies. Small molecule ligands with good docking scores and correct binding mode may indicate potential leads.

The ZINC database together with PubChem are good sources of small molecule ligand libraries. One can also construct custom combinatorial libraries using small molecule fragment/reagent databases from companies along with library generation tools. Available chemicals directory (Accelrys Inc.) (ACD) and comprehensive medicinal chemistry database (Accelrys Inc.) (CMC) are some of the fragment-like databases frequently used to this end.

It is important to note that results from such docking simulations using different tools, both commercial and open source, may differ widely, depending on the size and flexibility of the protein and the ligand. This approach is referred to as *virtual screening* or virtual high throughput screening (*vHTS*) and is the computational equivalent of high throughput screening. Most top bioinformatics vendors have well-established tools for docking and protein modeling. There are several open-source software that facilitate distributed vHTS [21].

Irrespective of whether the protein structure is available, if a ligand, say an inhibitor, cofactor, or substrate is known, it is often fruitful to use ligand-based similarity approaches for lead identification [22]. These include 2D similarity searching using fingerprints, functional groups, and atom-neighborhood descriptors. Many of these approaches are available as part of the Open Babel and Mayachemtools software. For scientists not familiar with command line tools, there are software such as Knime™ and Taverna, which enable development of workflows for fingerprint calculation, similarity searching, and many other tasks.

Leads may also be discovered by 3D similarity searching based on shape and molecular electrostatic potential as exemplified by OpenEye tools and open-source alternatives such as Shaep [23]. Some software, such as Inte:Ligand LigandScout, allow forming a negative image of the binding site as a pharmacophore for further shape searching.

Although the terms *chemoinformatics* and *computational chemistry* are sometimes used interchangeably, there are key differences between the two approaches. Computational chemistry-based approaches are generally based on 3D information, energy minimization, and forcefields and cheminformatics approaches focus on occurrence of particular patterns of chemical substructures based on graph theory. Both approaches are complementary and should be used in tandem for lead identification wherever applicable.

18.2.1.2 ADMET Modeling Tools. The chemical structure so obtained needs to satisfy several criteria befitting a “good” lead. Lipinski’s “rule of five” is in widespread use for filtering chemical libraries for good absorption, distribution, metabolism, and elimination (ADME) properties. Recent attempts have led to the “Pan Assay Interference Compounds (PAINS)” filter, useful to identify and remove promiscuous, reactive, unstable, and potentially toxic molecules [24]. Absorption and metabolism properties may be modeled using Gastroplus or Simcyp software. Toxicity prediction algorithms may also be used at this stage to rank the compounds. Most of the toxicity predictions can be conducted using commercial software such as TopKat and DEREK™. Computational toxicity prediction includes quantitative structure property relationship (QSPR) models, for example, TopKat, case-based reasoning (MultiCASE), and rule-based expert systems (DEREK).

Once a few leads are obtained, and if the patent and synthetic landscape is clear, attention shifts to changing the lead(s) so as to preserve activity but optimize ADMET properties. Products from companies such as ACDlabs allow one to filter fragments/substructures based on properties and replace certain parts of the lead so as to optimize them. Although it allows one to explore a large chemical space, multifactorial optimization is inherently difficult and not always achievable computationally where considerable input is necessary from medicinal chemists and ADMET scientists. As an example, aqueous solubility of the lead can be computationally modulated to attain better intrinsic solubility or $\log S$. Similarly, other substructure modifications may reduce the $\log P$ or increase the microsomal stability [25].

Leads can also be optimized computationally for cytochrome P450 (CYP) inhibition [assessing for potential drug–drug interactions (DDIs)], human ether-a-go-go-related gene (hERG) inhibition (assessing for potential cardiac arrhythmias), and other ADMET properties. It is important to note that computational optimization has to be constrained by experimental data wherever available. Most models for physicochemical properties such as $\log S$, $\log P$, $\log D$, and pK_a are based on a limited training set and the predictions may not be very useful for molecules with diverse chemistry.

Knime is a useful bioinformatics and cheminformatics “aware” workflow development software, which has plugins for descriptor, fingerprint, calculation, substructure searches, integration of high content screening data, “R” analytics plugins for microarray data, and more [26]. Bioclipse is an open-source cheminformatics software, based on the Eclipse™ Integrated Development Environment platform. It has plugins for QSAR, Phase I metabolic hotspot prediction and Carcinogenicity and Mutagenicity alerts. Several other plugins for toxicity prediction are available in the OpenTox version of Bioclipse. The OpenTox Bioclipse version also includes Caco-2 models. Toxtree is another useful software for toxicity predictions. Of late, regulatory agencies such as US food and drug administration (FDA) and organisation for economic co-operation and development (OECD) have taken considerable interest in computational methodologies and their toolbox has an impressive array of models and cheminformatic capabilities.

Table 18.3 lists the applicable license and the task for which they can be applied. Starting with drug target identification and validation, computational approaches facilitate the discovery of novel biologically active small molecules with a good ADMET profile (lead identification), which can then be optimized (lead optimization). These methodologies raise flags that may identify significant issues with the molecules and simultaneously expand the chemical space that can be explored by orders of magnitude. The virtual and potentially virtuous methods can help prioritize drug discovery efforts by guiding, but not replacing, human intuition.

18.2.2 Lead Identification, Optimization, and Selection Process in Drug Discovery

The objective of this step is to ensure the lead has optimal pharmaceutical properties assuming these properties will lead to successful clinical outcome to market the NCE as a drug. This process involves a multidisciplinary approach involving a medicinal chemist, biologist/pharmacologist, ADMET scientist, and clinician with sound understanding about the target patient population and at times involves contributions from marketing, formulation, and regulatory representatives. This team meets frequently to understand and discuss the action plans for the gaps in science, regulatory, and commercial challenges that may be encountered and clinical value the drug candidate offers to the patient.

This process is crucial for nominating a compound with ideal pharmaceutical properties for successful preclinical and clinical development. It is therefore critical to optimize pharmaceutical properties of the drug candidate at an early stage of drug discovery with limited data. Table 18.4 lists ideal pharmaceutical properties to help determine whether a candidate compound has potential to be developed as a drug. Failure to comply with these properties may have adverse consequences, including ADMET liabilities and lack of efficacy in advance stages of drug development.

18.2.2.1 Pharmacokinetics or Absorption, Distribution, Metabolism, and Excretion.

PKs (pharmaco-drug and kinetic-movement) is the quantitative study of the time course of drug ADME processes. PK is the science that describes the movement of drugs in the body [27]. A basic tenet of PK is that the magnitude of both the desired therapeutic response and/or toxicity of a drug are a function of its concentration in the body [27,28]. Successful pharmacotherapy is often dependent on maintaining the drug concentrations within a defined range, which is efficacious while being safe or has a favorable therapeutic index. PKs is one of the primary considerations, in the selection of a drug candidate, during early drug discovery phase. This is discussed extensively in the chapters titled *Pharmacokinetics and Toxicokinetics in Drug Discovery and Development* and *Pharmacokinetic Data Analysis and Modeling of ADME Processes* of this volume. Many virtual pharmaceutical companies, due to limited funding, skip *in vitro* permeability assays and directly proceed for *in vivo* PK studies in mice or rat. The argument is that if a compound is sufficiently bioavailable, it must pass through biomembranes. These *in vivo* PK methods can be applied only for a limited number of compounds and may not be useful for screening numerous compounds. Some companies conduct cassette dosing where 5–6 compounds are simultaneously dosed in animals, but these models usually provide erroneous PK data primarily due to DDIs [29–31]. However, cassette dosing can help reduce the number of animals and samples

TABLE 18.4 Ideal “Drug-Like” Properties of Lead Candidates in Drug Discovery

Property	Ideal Range	Comment
<i>Physico-chemical</i>		
Molecular weight (Lipinski’s rule of five)	150–500 (CMC mean 349)	Exception: macrolide antibiotics with MW >500
Aqueous solubility	1–3 (CMC mean 2.5); a score >3 is considered as poorly soluble compound	1 = > 1 g/L; 2 = 0.1–1 g/L; 3 = 0.01–0.1 g/L; 4 = 0.001–0.1 g/L; 5 = < 0.001 g/L
Polar surface area	20–130 (CMC mean 75)	—
log <i>P</i> (Lipinski’s rule of five)	–0.7 to 5.0 (CMC mean 2.5)	—
Nonrotatable bonds	0–10 (CMC mean 5.56)	—
Number of hydrogen bond donors (Lipinski’s rule of five)	<5 (includes nitrogen or oxygen atoms with one or more hydrogen atoms)	—
Number of hydrogen bond acceptors (Lipinski’s rule of five)	<10 (includes nitrogen or oxygen atoms)	—
p <i>K</i> _a	Nonionized	Ionized compounds taken up via uptake transporter mechanisms may be accepted
Permeability (Caco-2)	100 nm/s	Some substrates of efflux transporters (e.g., p-glycoprotein) are poorly permeable that may require further investigation
<i>In vitro metabolism</i>		
Metabolic stability	100% stable	Variable and sometimes with several inactive metabolites and occasionally with active metabolites
CYP phenotyping	Identify polymorphic metabolism	Genetic screening in patients with appropriate dose adjustments may be required in some patients
<i>In vitro</i> and <i>in vivo</i> species comparison	No qualitative and quantitative differences in metabolism across species	This is often a major issue where significant species differences in metabolism may be found. MIST guidance document from FDA addresses on how to overcome these issues

(continued overleaf)

TABLE 18.4 (continued)

Property	Ideal Range	Comment
<i>In vitro</i> drug–drug interactions:		
CYP inhibition (competitive)	<1 μM	—
CYP inhibition (mechanism-based)	Not Applicable (NA)	—
CYP induction	Negative	Occasionally CYP induction may result in autoinduction of metabolism with loss of pharmacological activity. Dose adjustments may be required
Protein binding	<50%	10–99.9%
<i>In vitro</i> toxicology		
hERG channel inhibition	Negative	Cardiovascular toxicity due to drug–drug interactions with fatal arrhythmias (QT prolongation) is often an issue with poly pharmacy in geriatric patients
Ames mutagenicity	Negative	No threshold
<i>in vivo</i> bone marrow micronucleus	Negative	No threshold
Tolerability toxicology	Negative	Reversible toxicity is accepted if it is manageable without fatal consequences. Toxicity at high doses with wide therapeutic margin with relative to therapeutic dose
<i>in vivo</i> PK–PD		
Bioavailability	40–100%	—
PK–PD	Excellent efficacy in animal models with good correlation to pharmacokinetic properties	Many times, no acceptable animal models are available for measuring PK–PD correlations. Humanized animal models with genetic manipulation are increasingly becoming popular. A molecular biomarker that can relate to target engagement by potential drug candidate are also used in PK–PD modeling

for bioanalysis (cassette analysis of all compounds in the same sample) and increase the throughput in screening several compounds.

18.2.2.2 Physico-Chemical Properties. Several articles in the literature have reviewed physico-chemical properties of drugs that are favorable and provide optimal biological activity with minimal safety liabilities. Lipinski's "rule of five" has been very popular and considered as a guide by many medicinal chemists to synthesize molecules in drug discovery phase [32] and discussed elsewhere in this book in another chapter.

Ritchie and Macdonald [33] compared the aromatic ring count along with other drug-like properties (aqueous solubility, lipophilicity, serum albumin binding, and CYP and hERG inhibition) for their potential for developability as drugs. On the basis of their analysis, they concluded that three or less aromatic rings are better for developability of NCEs as drugs. Recent data by Ritchie *et al.* [34] indicate that increasing ring counts have detrimental effects on developability in the order of carboaromatics > heteroaromatics > carboaliphatics > heteroaliphatics (except for heteroaliphatics exerting a beneficial effect in certain cases). Increasing aromatic ring count also influence several developability parameters such as lipophilicity and molecular weight and contribute to poor oral bioavailability. They also suggested that fused aromatic systems may have a beneficial effect relative to their nonfused counterparts. Their analysis revealed that newer drugs have increased heteroaromatic rings than nonfused aromatic rings.

The physico-chemical properties that are considered most important during drug discovery process include solubility, polar surface area (PSA), ionization constant (pK_a), and lipophilicity ($\log P$).

18.2.2.2.1 Solubility. Although an ever-increasing number of NCEs exhibit poor aqueous solubility, the classical approach involves use of cellular and noncellular *in vitro* models that are deficient in their ability to accurately model the absorption of compounds. In many instances, poorly soluble drugs necessitate the inclusion of excipients to facilitate efficient delivery and enhance their bioavailability. Thus, there exists an increasing demand for *in vitro* models that can effectively evaluate the effects of excipients on permeability of NCEs [27,35,36]. Herein, we provide an overview of those solubility models currently in use and discuss their associated benefits and drawbacks. Furthermore, we review the challenges encountered in assaying the permeability of poorly soluble drugs and critically assess those experimental modifications and solutions employed thus far in terms of their capacity to generate results with accuracy and precision.

Methods for measurement of solubility in early discovery phase include kinetic and thermodynamic solubility. Kinetic solubility determination is carried out by spectrophotometry, turbidimetry, or nephelometry. Solubilities in simulated gastric fluid, simulated intestinal fluid (SIF) and fasting state SIF can be determined for selected compounds from the early discovery phase to assess its solubility in biological fluids *ex vivo*.

18.2.2.2.2 Ionization Constants (pK_a). During the discovery phase for NCEs as potential drug candidates, the determination of pK_a values can be valuable in the selection process [37–40]. Determination of dissociation constants for compounds capable of ionizing at various pH ranges can influence its solubility and absorption across the

GI tract. In humans, the pH of stomach is close to 2 and in the small intestine around 6 that can be affected by food [27].

Classically ionization constant (pK_a) is expressed using Henderson–Hasselbach equations, which can provide the extent of ionized versus unionized compound at a particular pH.

For acidic compounds: $pH = pK_a + \log([\text{ionized compound}]/[\text{unionized compound}])$

For basic compounds: $pH = pK_a + \log([\text{unionized compound}]/[\text{ionized compound}])$

The major problem for pK_a determination in drug discovery is poor solubility of compounds. Two different methods have been published in the recent years that employ cosolvents or surfactants. With the cosolvent approach, the solubility of some unionized molecules can be enhanced by mixing solvents such as methanol, dioxane, or acetonitrile with water (MDM). However, it has been observed that not all compounds dissolve in any one cosolvent–water mixture. Völgyi [37] investigated the four-component cosolvent system and showed that MDM dissolves the lipophilic compounds sufficiently and it can be used for compounds that are not soluble in methanol–water or other single organic cosolvent mixtures (e.g., 2-propanol, DMF, DMSO, and acetone). However, MDM also dissolves polar compounds so it can be considered as an efficient cosolvent for pK_a determination in drug research. Ravichandran *et al.* [38] argued that the cosolvent approach may result in erroneous pK_a determinations due to existence of two liquid boundaries, and the change in dielectric constant may lead to variation in the ionization behavior of the NCE. These investigators used nonionic surfactants such as Tween 80, CremophoreEL, or Labrasol to determine pK_a values of poorly soluble compounds. The solubilization occurs by entrapment of NCE molecule into micellar structure formed by the surfactant, which can entrap ionic, hydrophobic, and amphiphilic molecules.

pK_a determination using GLpKa:GLpKa is an automated instrument developed by Sirius analytical UK to determine the pK_a compounds [39]. The advantages of this instrument are that it requires very low amount of compound and consumes less time. pK_a determination by GLpKa can be performed by two methods, pH-metric method and spectrophotometric titration, for compounds with chromophore.

18.2.2.2.3 Polar Surface Area. The PSA of a molecule is an important physico-chemical parameter to predict its permeability and absorption into the body. It is defined as the surface area of nitrogen and oxygen atoms in a molecule plus the surface of the hydrogen atoms attached to these hetero-atoms. It has been established that a PSA value of over $140 \text{ \AA}^2$ usually yields molecules that are poorly absorbed from stomach and GI tract, whereas values below $60\text{--}70 \text{ \AA}^2$ suggest potential penetration of the blood–brain barrier, especially for central nervous system (CNS)-active drugs. PSAs can be calculated using several software, discussion on which is out of the scope for this chapter and is covered in another review [40].

18.2.2.2.4 Lipophilicity ($\log P$ and $\log D$). Compounds to permeate across biological membranes require certain balance of lipophilicity to hydrophilicity. Lipophilicity is inversely related to solubility, that is, the higher the lipophilicity, the lower the aqueous solubility. The balance between these two properties is very critical for compounds

TABLE 18.5 $\log D$ Values and Their Significance in Drug Permeability and Absorption for Orally Active Drugs

$\log D$ at pH 7.4	Significance for Drug Development
<0	Poor absorption across the GI tract and blood brain barrier. Susceptible for rapid renal clearance
0–1	A good balance between solubility and permeability. Good oral absorption but poor CNS permeability
1–3	Optimum range for oral absorption and CNS permeability. Low metabolic liabilities
3–5	Lower solubilities. High metabolic turnover
>5	Poor solubility and poor absorption across the GI tract. High metabolic turnover

to be absorbed from the GI tract. Relationship between $\log D$ and permeability across biological membranes is, however, nonlinear, permeation decreasing in both low and high end of $\log D$ values, and often a $\log D$ value of 5 is considered as an upper limit of desired lipophilicity (Table 18.5).

Frequently, NCEs synthesized in drug discovery are amphoteric molecules with both acidic and basic functional groups. Normal amphoteric molecules can undergo change from anion–neutral–cation depending on the pH. If specific ion-pair partitioning does not occur, only the neutral species of the three ionic states dissolves into the organic (octanol) phase. For such type of ampholytes, the relationship between $\log P$ and $\log P_{\text{app}}$ is given by the following equation:

$$\log P = \log P_{\text{app}} + \log(1 + 10^{\text{pH} - \text{p}K_{\text{a}}^1} + 10^{\text{p}K_{\text{a}}^2 - \text{pH}})$$

where $\text{p}K_{\text{a}}^1$ and $\text{p}K_{\text{a}}^2$ are the macro or stepwise protonation constants.

However, for amphoteric molecules with zwitterions coexisting in solution, the partition behavior is more complex. GLp K_{a} is an automated instrument developed by Sirius analytical UK to determine the $\log P$ or $\log D$ compounds [39]. This method is more advantageous and accurate as compared with the conventional shake-flask method.

18.2.2.3 Permeability and Drug Transport. For oral absorption, drugs from the GI tract should permeate the intestinal barriers and enter the portal blood circulation. Biomembranes comprise a lipid bilayer, with lipid layer interiorized and the hydrophilic end forming the surface. Interactions of membrane proteins at contact surface between single cells form tight junctions that form small aqueous pores. The number of these junctions depends on the cell or tissue type; for the small intestine, the area of aqueous pores amounts to about 0.01% of the whole surface. Compounds can cross biological membrane by two passive processes: transcellular (through the membrane) and paracellular (via the pore). As described in the chapter titled *Oral Absorption: from Physiology to Regulatory* in this volume, most compounds can be absorbed by the paracellular route, but the process is invariably slower than the transcellular; lipophilic compounds are rapidly absorbed by the transcellular route and hydrogen bonding acceptor–donor properties can affect permeation of drug candidates across the bilayer [41]. In addition,

many efflux and uptake drug transporters present in the intestinal tract influence the absorption of the drugs.

The assay tools routinely used in drug discovery for assessing permeability of NCEs include parallel artificial membrane permeability assay (PAMPA), human colon carcinoma cells (Caco-2) [42], Madin–Darby canine kidney (MDCK) cells, or LLC-PK1 cell line (derived from porcine renal epithelial cells). In early drug discovery, PAMPA and Caco-2 cells are more routinely employed [43]. If efflux of the NCE by P-glycoprotein (P-gp), BCRP, or MRP2 is suspected, then bidirectional permeability assays in Caco-2 cells or LLC-PK cells are performed. Alternatively, individually transfected MDCK cell lines with MDR1, BCRP, and MRP2 can be used to assess if any of these transporters are involved in the efflux of the NCEs.

18.2.2.4 Drug Metabolism and Drug–Drug Interactions. DM plays an important role in elimination/clearance of drugs, which in certain cases leads to generation of pharmacologically active metabolites. In certain cases, DM contributes to adverse events (AEs) that may be induced by reactive metabolites [44]. Drugs coadministered or food may lead to drug–drug/food interactions affecting the PK–PD and safety [45].

Other chapters in this Volume discuss chemical/biochemical interactions of various phase I and II enzymes that play a role in DM pathways. In the following sections, the impact of DDIs and reactive metabolites on drug discovery and development is discussed [46]. In addition, the evolution of drug metabolite identification using LC-MS/MS methods in the past decade is also described briefly.

18.2.2.4.1 Key Enzymes Involved in Drug Metabolism. Liver is the primary site of DM. However, extrahepatic sites of DM such as intestine, lungs, kidneys, bone marrow, and brain may be involved. The biological function of metabolic transformation is to increase the aqueous solubility of a drug to enable its excretion such that the drug does not cause toxicity to the body. Metabolism usually occurs via phase I and II pathways. The phase I metabolism occurs by introduction of polar groups via hydroxylation, dealkylation, and hetero-atom oxidation. If these phase I metabolites have adequate aqueous solubility, they can be excreted via bile and urine. The phase II metabolism occurs via conjugation reactions such as glucuronidation, sulfation, or glutathione conjugation. These conjugated metabolites have high aqueous solubility and are thus rapidly excreted. In certain cases, reactive drug metabolites form glutathione conjugates that are further processed to mercapturic acids before excretion [47].

CYP superfamily are a major class of drug-metabolizing enzymes that are involved in the metabolism of >90% of the drugs that are used clinically. CYPs are membrane bound enzymes and are primarily present in the endoplasmatic reticulum within the cell. The major human CYP enzymes involved in DM are CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. CYP2D6 and CYP3A4 contribute to the metabolism of ~20% and 50% of drugs currently in the market [48]. Microsomes also contain another phase I enzyme family known as *flavin mono-oxygenases* that predominantly catalyze the formation of sulfoxides and nitro-oxides [49]. Nonmicrosomal phase I enzymes that are involved in DM include monoamine oxidase (MAO), aldehyde oxidase, and xanthine oxidase [50]. MAOs are present in the mitochondria and are involved in oxidation of amines, while carbonyl reductases and alcohol and aldehyde dehydrogenases are cytosolic enzymes being involved in the oxidation of alcohols and aldehydes and in the

reduction of aldehydes and ketones. Aldehyde oxidase and xanthine oxidase, also known as *molybdo-flavor proteins*, primarily catalyze the oxidation of heterocyclic nitrogen-containing compounds, α -carbon adjacent to nitrogen atom.

Uridine diphosphoglucuronosyl transferases (UGTs) are the most prominent phase II enzymes present in microsomes [51]. Human UGT1A1, UGT1A6, and UGT2B7 are responsible for glucuronidation of majority of compounds prescribed. UGTs can catalyze the formation of *O*-, *N*-, and *S*-glucuronides and glucuronidation of carboxylic acids can result in highly reactive acyl-glucuronides.

Other important drug-metabolizing enzymes are peroxidases [52], including myeloperoxidase, eosinophil peroxidase, prostaglandin-H synthase [53], microsomal and soluble epoxide hydrolases, sulfotransferases, glutathione *S*-transferases, *N*-acetyltransferases, and methyltransferases. Peroxidases [52] can catalyze the formation of reactive free radical intermediates and have been implicated in bone marrow [54] and urinary bladder toxicities [55].

Genetic polymorphisms of many drug-metabolizing enzymes can contribute to interindividual variability in metabolism [56]. In some cases, the genetic polymorphism in CYPs resulted in toxicities in individuals with poor metabolism. The most widely studied enzyme with regard to polymorphisms is CYP2D6 followed by CYP3A4/5, CYP2C9, and CYP2C19. In selected cases, UGT polymorphisms have also been considered to be important for interindividual variability in metabolism and toxicity [57].

Another important matrix for the metabolism of drugs is blood compartment [58], of which serum esterases have been found to be the major contributors. These include cholinesterase, serum arylesterase, carboxylesterase, and red blood cell (RBC) esterases. These enzymes play a role in both activation of prodrugs and deactivation of drugs. Recent examples of marketed prodrugs are adefovir, tenofovir, valganciclovir, olmesartan, parecoxib, tamiflu, famciclovir, and ximelagatran [59].

18.2.2.4.2 Identification of Key Enzymes Involved in Drug Metabolism. *In vitro* studies on the metabolism of drugs are usually performed using liver preparations such as isolated perfused livers, liver slices, liver homogenates, isolated hepatocytes, subcellular liver fractions (S9, cytosol, and microsomes), or recombinant metabolizing enzymes overexpressed on nonexpressing cell systems, particularly CYP enzymes. Previously, identification of CYP enzymes was performed using three different methods, including use of (i) individual recombinant CYP enzymes, (ii) microsomes with specific CYP chemical inhibitors, and (iii) correlation analysis using at least 15 different individual microsomes characterized for individual CYP activities using specific probe substrates. The combination of all three methods was recommended for accurate identification of different CYP enzymes involved in DM [60,61]. FDA guidance, however, recommends using two methods in particular use of recombinant CYPs and chemical inhibition in human liver microsomes. Identification of key metabolizing enzymes can be performed by the use of human CYP enzymes derived from CYP transfected insect cells (supersomes and baculosomes) or bacteria (bactosomes) [62]. Use of individual recombinant enzymes may result in incomplete and inaccurate identification of CYP enzymes involved in DM for the following reasons:

1. Recombinant CYP enzymes in microsomes do not have the same micro-environment as in human liver microsomes and contain varying levels of redox partners such as CYP reductase, cytochrome b5, and phosphatidylcholine. Thus,

the catalytic properties of recombinant CYP enzymes do not exactly mimic the properties of CYP enzymes in human liver microsomes [63].

2. Many drugs are metabolized by more than one CYP enzyme. In such cases, the relative roles of CYP enzymes in DM can depend on the affinity (K_m) and capacity of individual CYP enzyme. For example, metabolism of drugs by CYP3A4 may occur by low affinity–high capacity, whereas CYP2D6-dependent metabolism of drugs may occur with high affinity–low capacity. Using individual recombinant enzymes thus lacks the competition between different enzymes and forces the metabolism of drugs yielding inaccurate results.
3. Recombinant enzymes are generated through deletion/modification of terminal sequence of individual CYP genes. This may also alter the catalytic properties of these enzymes relative to native enzymes present in human liver microsomes.

The chemical inhibition method of CYP phenotyping utilizes pooled (typically, 10–50 subjects) human liver microsomes in the presence of specific chemical inhibitors. For example, ketoconazole and quinidine are specific inhibitors of CYP3A4 and CYP2D6, respectively, at concentrations as low as 1 μM . Determination of the kinetic constants (K_m and V_{max}) for each metabolite is necessary as CYP enzymes have varying affinities and capacities to form drug metabolites. Thus, metabolite identification along with structural elucidation is required before carrying out chemical inhibition studies on NCEs. In addition to the chemical inhibitors, use of specific antibodies that block various CYP functions has been explored [64].

Some perplexing issues with CYP enzymes are their ability to catalyze atypical kinetic reactions that follow non-Michaelis–Menten kinetics [65] and include the following:

1. *Sigmoidal autoactivation*, in which the drug itself can activate its own metabolism as the concentrations are increased. Typical profile of these substrates show initial lag period in metabolism, but addition of more substrate increases metabolism rapidly in more than dose-proportional manner. Sigmoidal activation may also occur with the presence of other drugs in a concentration-dependent manner (hetero-tropic activation). Sigmoidal autoactivation should not be confused with CYP induction where the latter is dependent on drug-induced transcriptional activation of CYP genes (Section 18.2.2.4.4).
2. *Substrate inhibition* profile that is characterized by a decrease in velocity of metabolism as the concentration of drug is increased. It has been described by a two-site model in which one binding site is productive, whereas the other site is inhibitory and operable at high substrate concentrations, resulting in decreased velocity with increasing concentrations.
3. *Biphasic kinetic* profile that is characterized by an initial Michaelis–Menten-like increase in velocity with increasing substrate concentration. However, the profile does not become asymptotic, with the reaction profile eventually becoming linear with increasing substrate concentration. This results in the inability to predict an apparent V_{max} and thus apparent K_m [66].

18.2.2.4.3 Cytochrome P450 Inhibition and Drug–Drug Interactions. It is now well known that a drug can affect the metabolism of another drug, a concept known as

metabolic DDIs. Cases of this are plentiful and are discussed in detail elsewhere in this Volume. Since drug interactions often result in AEs, sometimes with fatal outcome, it is essential to screen NCEs in early drug discovery for their ability to inhibit, *in vitro*, the major CYP enzymes using human liver microsomes or human hepatocytes. Despite many CYP enzymes present in humans, five major CYPs (1A1/2, 2C9, 2C19, 2D6, and 3A4) account for the metabolism-related DDI issues for >90% of marketed drugs. Hence, the inhibition of the above five major CYP enzymes should be measured during early drug discovery process. Sometimes, one CYP enzyme may metabolize NCEs to metabolite(s), which may inhibit another CYP enzyme(s). Additionally, metabolism may also produce reactive metabolites that can irreversibly inactivate the CYP enzyme (e.g., troleandomycin inactivation of CYP3A4). This type of inhibition is known as *time-dependent* or *mechanism-based CYP inhibition*.

Current industry practice to assess CYP inhibition by NCEs is incubation of human liver microsomes with specific CYP probe substrates using LC-MS/MS. One tends to probe the metabolism-related DDIs of drug candidates by monitoring the impact of the test compounds on CYP metabolic activity using a known substrate. This is based on the assumption that whenever DDI occurs, regardless of the mechanism(s) involved (e.g., competitive, noncompetitive, or uncompetitive), the compound will interfere with the performance of a coadministered drug that is metabolized by the same enzyme [67,68]. Probe substrate inhibition assays for five of the major CYP enzymes [phenacetin for CYP1A2, diclofenac for CYP2C9, (*S*)-mephenytoin for CYP2C19, dextromethorphan for CYP2D6, and midazolam and testosterone for CYP3A4] in human liver microsomes are being used and metabolites of probe substrates are analyzed with rapid LC-MS/MS methods. The data generated can be plugged into a physiologically based pharmacokinetic (PBPK) model such as Simcyp (Section 18.2.4.2) and help in drug design in early discovery.

18.2.2.4.4 Cytochrome P450 Induction and Drug–Drug Interactions. Some CYP enzymes are regulated via soluble (cytosolic) or nuclear receptor activation. These receptors include Ah receptor for CYP1A1/2, pregnane X receptor (PXR) for CYP2C9, 2C19 & 3A4/5 and constitutive androstane receptor (CAR) for CYP2B and CYP2C induction. There are several excellent articles on this subject and thus this section is limited to highlight some of the concerns associated with CYP induction [69] which in certain cases may lead to reduction in therapeutic efficacy of co-medications. Some examples include: (i) rifampicin-mediated induction of CYP3A4 and increased clearance of oral contraceptives; (ii) carbamazepine induced CYP3A4 and increased clearance of benzodiazepines. Sometimes induction process may result in autoinduction of the test DM itself resulting in drug-resistance, following multiple doses of the same drug every day for two weeks. In some other cases autoinduction of metabolism may result in the increased formation of reactive metabolites [70]. Typically CYP1A1, 1A2, 2B6, 2C9, 2C19 are prone to induction in response to their gene activation by certain drugs. Interestingly, there are no reports on CYP2D6 induction to date, while phase-II enzymes like uridine diphosphate glucuronide (UDPG) have been reported to be induced by drugs.

The inductive effect of any drug on CYP enzymes can be evaluated using primary human hepatocytes. In the human liver, CYP3A4 is the most highly expressed CYP enzyme and is highly inducible by a wide variety of drugs. Induction of CYP3A4 can also have serious toxicological consequences as a result of increased DM that

contributes to DDI may lead to bioactivation of drugs to carcinogenic or toxic metabolites. Treatment of patients with prototypical CYP3A4 inducer rifampicin can lead to reduced plasma levels of the antidiabetic drug pioglitazone, leading to poor glycemic control in patient in which these medications are prescribed simultaneously. Upregulation of CYP3A4 can also exaggerate toxic responses to drugs as with rifampicin and acetaminophen coadministration.

The discovery of a family of nuclear receptors such as PXR [71], CAR and glucocorticoid receptor (GR) gives insight into the molecular explanation of CYP3A induction by xenobiotics. A stable hepatoma cell line expressing the human PXR and the CYP3A4 distal and proximal promoters plus the luciferase reporter gene can be used to assess the ability of a drug to induce CYP3A4 and CYP2B6. The mechanism of CYP3A4 induction is typically via activation of the PXR [72], resulting in activation of Retinoic X Receptor and formation of the hetero-dimer PXR–RXR complex. This hetero-dimer complex then binds to a response element in the CYP3A4 gene called the *PXR response element* [73,74].

18.2.2.4.5 Mechanism-Based Inhibition. Mechanism (or time) based enzyme inhibition is associated with irreversible or quasi-irreversible loss of enzyme function, requiring synthesis of new enzyme before activity is restored. The consequences of mechanism-based inhibition (MBI) are (i) auto-inhibition of the metabolic clearance of the inactivator itself and (ii) prolonged inhibition of the clearance of other drugs that share the same enzyme.

There may also be serious immuno-toxicological consequences if a reactive intermediate is covalently bound to the enzyme. The prediction of the *in vivo* consequences of mechanism-based enzyme inhibition requires estimates of the maximum inactivation rate constant (K_{inact}), the inactivator concentration associated with half K_{inact} (K_I), the concentration of the inactivator at the active site (I), and the first-order rate constant of the *in vivo* degradation of the enzyme (k_{deg}). Values of k_{inact} and K_I can be determined from appropriately designed *in vitro* studies.

MBI is a time-dependent as well as nicotinamide adenine dinucleotide phosphate (NADPH)- and concentration-dependent process, and in certain cases, drugs are converted by CYPs to reactive metabolites. The inactivation of CYP3A4 can be due to chemical modification of the heme, the protein, or both by covalent binding of modified heme to the protein [75]. Compared with reversible inhibition, MBI of CYP3A4 more frequently causes unfavorable DDI, as the inactivated CYP3A4 has to be replaced by newly synthesized CYP3A4 protein. DDI can lead to higher exposure of coadministered drugs, sometimes leading to toxicity. For these reasons, DM groups within pharmaceutical companies need a well-established screening assay to assess mechanism-based inactivation of major human CYP enzymes by new chemical substances that are being developed by the company [75].

The irreversible MBI of CYP3A4 refers to the inactivation of the enzyme via the formation of a metabolic intermediate that binds tightly and irreversibly or is covalently attached to the enzyme. At least three different mechanisms may lead to the inactivation of CYP during the metabolism of drug. The intermediate species can react irreversibly with nucleophilic amino acids in the active site or with the heme nitrogen atoms. A metabolite intermediate can also bind strongly to the heme iron to form a metabolite intermediate complex that does not actually destroy the CYP enzyme but renders it catalytically inactive. This is often described as quasi-irreversible inhibition. It is

believed that mechanism-based inhibitors may prove to be much more enzyme specific than reversible inhibitors [76].

Some of the criteria that are routinely used to determine whether a substrate is a mechanism-based inhibitor are as follows: (i) Loss of enzyme activity during preincubation must be time dependent. The enzyme activities, corrected for any loss of activity in the absence of an inhibitor, remaining after the preincubation should have a linear relationship to preincubation time. (ii) The inhibition requires that a cofactor, NADPH, is present and that metabolism is occurring due to NADPH dependence for electron transfer in the catalytic cycle of CYPs. (iii) The degree of inhibition should be concentration dependent with saturation kinetics. (iv) Inhibition should be irreversible and activity should not return on dialysis or gel filtration since the mechanism-based inhibitor should be covalently bound to the enzyme. Microsomes prepared from different cells overexpressing single human CYPs are now routinely used to investigate MBI of specific CYPs. These *in vitro* systems are commercially available and can be used to characterize time-, NADPH-, and concentration-dependent inhibition as well as to determine the enzyme kinetics parameters [77].

18.2.2.4.6 Concurrent CYP Inhibition and Induction: A Clinical Dilemma for Predicting DDI from *In vitro* Studies. The prediction of DDIs based on reversible CYP inhibition has been a standard part of preclinical programs in the pharmaceutical industry for many years [78]. This common practice has led to underprediction of the magnitude of DDI when the underlying inhibitory mechanism, including time-dependent inactivation (e.g., erythromycin and verapamil). Interestingly, some of these CYP inactivators are also potent competitive inhibitors and inducers of CYP3A4, further complicating the value of predictive tools. Although recent efforts have yielded methods whereby *in vitro* data can be used to provide accurate predictions of DDI arising via a time-dependent inactivation mechanism, it is important to acknowledge discrepancies that have been observed with some of the drugs tested, for example, cyclosporin, erythromycin, ethinyl estradiol, indinavir, mibefradil, oleandomycin, rifampicin, and verapamil [78]. These discrepancies may represent the mixed nature of the underlying mechanisms by which the precipitants interact with CYP3A4. These types of mixed inhibition–induction of the same CYP isoform can present challenges in the design of clinical DDI studies, which should be considered in early drug discovery process. In the recent years, *in vitro* hepatocyte models where both gene expression profiling using mRNA quantitation alongside CYP activity profiling are increasingly being used to understand the mixed inhibition–induction models in greater details.

18.2.3 Regulatory Considerations of Drug Metabolism and Drug Interaction Studies

Until the late 1990s, metabolic studies conducted by pharmaceutical industry were mainly restricted to use of radiolabeled NCEs for determining their ADME properties. There was lack of uniformity in conduct of metabolism studies by different pharmaceutical industries. In the late 1990s, FDA issued regulatory guidances that defined the role and practice of contemporary DM [79,80]. Since the publication of this guidance, DM has become integral part of drug discovery and development process [81]. In 2008, FDA published guidance entitled “Safety Testing of Drug Metabolites” [82]. This guideline gives an in-depth understanding of the metabolites to be analyzed in

preclinical and clinical studies. Table 18.6 gives a compiled list of regulatory guidance relevant to PK, DM and toxicity studies.

18.2.3.1 Safety of Metabolites. Conventionally, parent drug plasma concentrations have been used to assess systemic exposure to drugs in animals and humans. Adequate safety margin can be established based on the fold difference in exposure of parent in toxicological species compared with humans. Nonetheless, the main problem during drug discovery process is that no human data are available on species differences in DM and DT as related to their safety. *In vitro* studies using human liver microsomes or human hepatocytes can shed some light on potential human metabolites. However, when metabolites play a role in toxicity and if such metabolites are (i) present in humans (unique metabolite) but absent in toxicological species and (ii) if these metabolites have higher exposure levels in humans than in the toxicological species, then the safety of the metabolite(s) may not be adequately assessed in toxicity studies in animals. Pharmaceutical Research and Manufactures of America published a document entitled “Metabolites in Safety Testing” (MIST) [83]. This document provides a guideline to the pharmaceutical industry on the use of metabolite information in safety testing. The FDA guidance [82] emphasized that identification of unique human metabolite later in development could affect the progress of the molecule to registration.

LC-MS/MS advances have certainly become very useful in identifying potential human metabolites during drug discovery process. *In vitro* species comparison of metabolism in liver microsomes and hepatocytes including of human are very useful. Further studies *in vivo* in animal species can help address if there are any potential unique human metabolites that can contribute to safety issues later during clinical trials.

Some pharmaceutical companies are now conducting radiolabeled ADME studies early in drug discovery to avoid finding a unique human metabolite later in the development stage. FDA published guidance on the use of radiolabeled drugs in 2005 [84]. This guideline enlists the objectives of the use of radioactive drugs in human subjects to obtain basic information regarding DM. The purpose of such studies is not to determine safety and effectiveness of the drug. Tissue distribution studies in rats can provide the data to calculate the doses for use of radioactive drugs in human subjects.

18.2.3.2 Drug Interaction Studies. FDA’s guidance “Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies *In vitro*” was issued in 1997 [80]. This regulatory document, for the first time, clearly defined the role of DM in the drug development. This document emphasizes the investigation of NCE metabolites together with its interaction with likely coadministered drugs during drug development. The primary site of metabolism, primary metabolic pathway, and the proportion of total clearance that each primary pathway contributes should be identified and elucidated. The FDA guidance for industry “Bioanalytical Method Validation” [85] provides guidelines for validation of LC-MS/MS assays to ensure uniformity across the scientific community. FDA suggests that decisions pertaining to selection of metabolites for testing for pharmacological activity or monitoring in humans should be made by the industry in consultation with regulatory agencies.

18.2.3.3 Drug Distribution. Distribution is the process by which a drug is transported in body fluids from the blood stream to the tissues of the body. A drug can be administered through a variety of routes, including intramuscular, intravenously,

TABLE 18.6 Regulatory Guidances on Drug Metabolism, Drug Interactions, Pharmacokinetics, and Safety Evaluations

No.	Title	Type	Date	Category
1	Drug metabolism/drug interaction studies in the drug development process: studies <i>in vitro</i>	Final	04/01/1997	Clinical pharmacology
2	Pharmacokinetics in patients with impaired renal function	Final	05/14/1998	Clinical pharmacology
3	General considerations for pediatric pharmacokinetic studies for drugs and biological products	Final	11/01/1998	Clinical pharmacology
4	Population pharmacokinetics	Final	02/01/1999	Clinical pharmacology
5	<i>in vivo</i> drug metabolism/drug interaction studies—study design, data analysis, and recommendations for dosing and labeling	Final	11/24/1999	Clinical pharmacology
6	Bioanalytical method validation	Final	05/01/2001	Biopharmaceutics
7	Pharmacokinetics in patients with impaired hepatic function: study design, data analysis, and impact on dosing and labeling	Final	05/30/2003	Clinical pharmacology
8	Exposure–response relationships: study design, data analysis, and regulatory applications	Final	05/05/2003	Clinical pharmacology
9	Pharmacokinetics in pregnancy: study design, data analysis, and impact on dosing and labeling	Draft	11/01/2004	Clinical pharmacology
10	Drug interaction studies: study design, data analysis, and implications for dosing and labeling	Draft	09/12/2006	Clinical pharmacology
11	Pharmacokinetics in patients with impaired renal function: study design, data analysis, and impact on dosing and labeling	Draft	03/22/2010	Clinical pharmacology
12	Clinical pharmacogenomics: premarketing evaluation in early phase clinical studies	Draft	02/17/2011	Clinical pharmacology
13	S2A-specific aspects of regulatory genotoxicity tests for pharmaceuticals	Final	04/01/1996	ICH-safety
14	S2B genotoxicity: a standard battery for genotoxicity testing of pharmaceuticals	Final	11/21/1997	ICH-safety
15	S2(R1) genotoxicity testing and data interpretation for pharmaceuticals intended for human safety	Draft	03/24/2008	ICH-safety
16	S3A toxicokinetics: the assessment of systemic exposure in toxicity studies	Final	03/01/1995	ICH-safety
17	S3B pharmacokinetics: guidance for repeated dose tissue distribution studies	Final	03/01/1995	ICH-safety
18	S7A safety pharmacology studies for human pharmaceuticals	Final	07/01/01	ICH-safety
19	S7B nonclinical evaluation of the potential for delayed ventricular repolarization (IQT interval prolongation) by human pharmaceuticals	Final	10/19/2005	ICH-safety
20	S8 immunotoxicity studies for human pharmaceuticals	Final	04/12/2006	ICH-safety
21	S9 nonclinical evaluation for anticancer pharmaceuticals	Final	04/12/2006	ICH-safety
	E14 clinical evaluation of QT/QTc interval prolongation and proarrhythmic potential for nonantiarrhythmic drugs questions and answers	Final	11/18/2008	ICH-efficacy

^aAbbreviation: ICH, International Conference on Harmonization.

subcutaneously, orally, or topically (via dermal, nasal passages, ear, eye, etc.). After administration, a drug will distribute itself into all of the body's compartments and tissues that it is able to. The time it takes for this to occur is called the *distribution phase* and is usually rapid. A drug is said to be distributed into a theoretical volume, called the *volume of distribution* (V_d). The volume is usually measured in liter per kilogram (L/kg) and is considered theoretical because it is based on sampling drug concentrations immediately after dosing with the assumption that the drug is uniformly distributed throughout the body. Given that most drug targets are not in vascular space, access to target organs commonly relies on drug distribution. For example, for a drug to be active in the central nervous system, it must penetrate the blood–brain barrier.

The barriers for drug distribution to extravascular target organs include protein binding in systemic circulation as well as tissue uptake. Of course, metabolism and transport processes involved in hepatic elimination also represent a major barrier in tissue distribution. Since metabolism and transport have been discussed in other parts of this chapter, we will briefly discuss on the role of protein binding in drug disposition.

18.2.3.4 Plasma Protein Binding and Drug Disposition. The binding of a drug to plasma proteins enables the transport of drugs via the blood to target sites of action throughout the body. Only the fraction of drug unbound from plasma proteins is available to diffuse from the vascular system and accumulate in tissues, thereby enabling interaction with therapeutic targets and with drug clearance pathways. Therefore, the extent of drug binding to plasma proteins can have a significant impact on PK parameters such as clearance rates and volume of distribution. As the unbound drug is the fraction of the drug available to interact with therapeutic targets, the extent of protein binding can have significant effects on the pharmacodynamic (PD) properties of a compound. Among plasma proteins, human serum albumin and α -(1)-acid glycoprotein are the two most important proteins for binding of many drugs [86]. The static and dynamic topology of drug binding sites on these proteins, investigated by various spectroscopic techniques, X-ray crystallography, quantitative structure–activity relationships (QSARs), molecular modeling, photo affinity labeling, site-directed mutagenesis, and so on, has been reviewed elsewhere and not discussed here [87,88].

The physiological significance of *in vitro* plasma protein binding studies has always been debated. Several endogenous molecules, for example, free fatty acids, blood urea nitrogen, and bilirubin also bind to plasma proteins and compete with drug binding sites on plasma proteins. Changes in the endogenous molecule concentrations during pathological conditions, such as liver and kidney failure or various inflammation diseases, could significantly vary from person to person and may cause interindividual variation in protein binding of drugs [89]. However, the free fraction of the drug is usually assumed to be available for tissue distribution and in equilibrium with plasma compartment. Additionally, sometimes, drugs may partition into RBCs [90], which may complicate the interpretation of PK data.

Experimentally, drug plasma protein binding can be determined using equilibrium dialysis, ultrafiltration, or ultracentrifugation method [91]. Currently, more widely acceptable method to determine the plasma protein binding is considered to be equilibrium dialysis. Rapid Equilibrium Dialysis (RED) device (Thermo Scientific Pierce) and HTD 96 (Micro Equilibrium Dialysis from HTDialysis LLC) are the two, commercially available, high throughput 96-well formats used to determine the protein binding of drugs at discovery stage.

RED devices are more popular because of their unique advantage of achieving faster equilibrium. The RED device consists of disposable inserts and a base plate formatted to a standard microplate footprint [92]. Each single-use, disposable insert is made of two side-by-side chambers separated by a vertical cylinder of dialysis membrane (8K, 12K, or 25K molecular-weight cutoffs (MWCO)) validated for minimal nonspecific binding. This format requires no extensive assembly steps or specialized equipment, and each chamber or well is easily accessible from the top of the insert after insertion in the base plate. Additionally, the high surface-to-volume ratio of the membrane compartment allows rapid dialysis, where equilibrium can be reached in 4 h with high levels of reproducibility and accuracy. In many cases, experiments can be completed in >100 min. The RED device has been extensively validated for plasma-binding assays producing results consistent with those reported in the literature. The RED device offers significant improvements in automation, time requirements, versatility, and product reliability compared with other commercially available systems.

The HTD 96 device from HTDialysis has very easy setup. It can be assembled in minutes, easily cleaned, and is reusable. This also offers a wide selection of dialysis membranes. It is compatible with standard 96-well laboratory equipment and supplies. It requires 30–150 μL working volumes and has automation friendly design. A number of drugs have been analyzed using this device giving reproducible protein binding data [93].

18.2.4 Recent Advances in ADME Concepts and Related Technologies

During the past two decades, scientists from academia and pharmaceutical industry made several advances in understanding the drug absorption and dispositional characteristics. Especially, understanding the mechanisms of DM and DT made a major impact expanding into translational research for modern drug discovery.

18.2.4.1 Recent Advances in Clearance Concepts. The systemic clearance is representative of the elimination of the drug from the body. It is one of the important PK parameters that can help understand how the drug is cleared from the body. Physiologically, it represents the rate of elimination of a drug relative to the plasma concentration. The concept of clearance was originally proposed by Rowland and has been extremely useful for dosage adjustment because at the typical therapeutic doses, the systemic clearance of a drug is usually constant over the interval of plasma concentrations of clinical importance. This parameter is the one of the most relevant parameters in human efficacious and safe dose calculation from preclinical PK studies.

Clearance of the drug from the body depends on the intrinsic ability of the organs such as the liver and kidneys to metabolize or excrete. Also, clearance is a function of the blood flow rate (Q) to these organs. Clearance by definition represents the volume of blood cleared through an organ in unit time. To estimate clearance, the drug needs to give intravenously. Clearance cannot be estimated after an oral dose since the total dose does not reach the systemic circulation. The total clearance is evaluated from the PK data as shown in the following equation:

$$\text{CL} = \text{dose}/\text{AUC}$$

For certain drugs that get metabolized completely, renal clearance is negligible. In this case, systemic clearance can be approximated to hepatic clearance (CL_H) by liver. Hepatic clearance is given by

$$CL_H = Q_H * ER_H$$

where Q_H is the hepatic blood flow rate and ER_H is the hepatic extraction ratio. Hepatic clearance can be estimated approximately from *in vitro* intrinsic clearance (CL_{int}) data as shown in the following equation:

$$CL_{int} = V_{max}/K_m$$

where V_{max} is the maximum rate of metabolism and K_m the substrate concentration at half the maximum velocity (V_{max}).

With the recognition of the importance of drug transporters during the past decade, Kusuvara and Sugiyama [94] have developed a methodology to evaluate hepatic clearance measures with the addition of transporters, defined as follows:

$$CL_{int} = CL_{int,met} + PS_{int,bile}$$

where $PS_{int,bile}$ is the intrinsic efflux clearance of unbound drug into the bile, while $CL_{int,met}$ is the intrinsic metabolic clearance of unbound drug in the liver. Now the intrinsic organ clearance ($CL_{int,org}$) is given by

$$CL_{int,org} = \left[\frac{PS_{inf}}{PS_{eff} + CL_{int}} \right] \cdot CL_{int}$$

where PS_{inf} and PS_{eff} represent the intrinsic clearances for cellular uptake (influx) and efflux into the systemic circulation, respectively.

At the boundary condition where $CL_{int} \gg PS_{eff}$, that is, where the great majority of drug taken up by the hepatocytes is metabolized or excreted into bile, then $CL_{int,org}$ in the previous equation can be approximated by

$$CL_{int,org} = PS_{inf}$$

Above-mentioned equation seems to be consistent with the data presented for atorvastatin using isolated rat liver perfusion [95] and *in vivo* in rats [96] and humans [97,98], the results published using (i) multiple indicator dilution perfusion, (ii) *in vitro* rat hepatocyte uptake, (iii) *in vitro* rat liver microsome clearance data, and (iv) *in vivo* CL_{int} measurements demonstrated good *in vitro*–*in vivo* clearance correlations. They further predict *in vivo* human hepatic uptake clearance and suggest that these values approximate overall human intrinsic clearance measurements for statin drugs, including atorvastatin, pravastatin, pitavastatin, and fluvastatin. Benet further suggested that incorporating efflux transporter and metabolism inhibition data as well as studies where protein binding is changed should also be taken into equations above proposed by Kusuvara and Sugiyama [94]. For example, the $CL_{int,met}$ portion of Equation 18.8 proposed by Kusuvara and Sugiyama [94] is assumed to be independent of transport

processes and in all other treatments of intrinsic metabolic clearance. This was emphasized earlier in Equation 18.3 as proposed by Rowland *et al.* [99], where the partition coefficient between unbound concentration in the measured systemic circulation and that in the eliminating organ is assumed to be constant. However, inhibition, induction, or activation of transporters could potentially change this ratio so that in essence what we now call intrinsic metabolic clearance is not intrinsic, but rather varies with transporter changes. Studies with atorvastatin in humans [96] not only showed inhibition by rifampin of atorvastatin uptake (mediated by OATP1B) into the liver but also caused a change in the ratio of the atorvastatin lactone to the atorvastatin acid, thereby changing the amount of substrate available to the enzymes in the liver, since metabolism of atorvastatin by CYP3A4 occurs via the lactone, not the acid [100]. Thus, as we understand the biochemical and molecular mechanisms of drug disposition, the clearance concepts and equations need to be updated as we move along in the future.

18.2.4.2 Use of Simcyp in Predicting Human ADME. The simulation and prediction of human PK/PD using *in vitro* data from human tissues and animal models based on previous knowledge is becoming very important in preclinical drug development [101]. Even regulatory bodies, notably the US-FDA has a Critical Path Initiative that emphasizes the need for quantitative description of PK/PD for understanding the efficacy, safety, and dosing regimen design for any given drug candidate [102,103]. The success rate in drug development can be enhanced and also the cost incurred in this process can be reduced by the use of modeling and simulation tools. Simcyp (Simcyp Limited, Sheffield, UK) is one such commercially available tool.

ADME simulator [104], the Simcyp drug interaction prediction model, is a population-based PBPK model and considers the interplay among demographic (age, weight, height, sex, race, and disease), genetic, anatomical (organ size), physiological (blood flow, enzymes, transporters, plasma proteins, hematocrit, transit time, and pH), and drug-specific factors (molecular-weight (MW), pK_a , $\log P$, solubility, PSA, K_m , V_{max} , and f_u). Simcyp time-based models can be used to predict mechanism-based drug interactions of CYP3A [105]. The simulator combines the experimental ADME data generated routinely during preclinical drug discovery and development from *in vitro* enzyme and cellular systems and relevant physiochemical parameters of the compounds and dosage form with demographic, physiological, and genetic information on different patient populations to predict *in vivo* PK parameters and profiles. It predicts outcomes not just as “average” but across populations, allowing the characteristics of individuals at the extremes risk to be evaluated. Population-based simulator has also been used for identification of CYP enzymes involved in the metabolism of drugs (e.g., paroxetine) in humans [106]. Simcyp simulator can also be used for selection of alternative CYP3A4 probe substrates for clinical drug interaction studies using *in vitro* data and *in vivo* simulation [107]. PK modeling using Simcyp has also been utilized in understanding the role of UGT1A4 under CYP3A inhibited conditions in the metabolism of midazolam in samples from human clinical studies [108].

18.2.4.3 Biopharmaceutical Drug Disposition Classification System. Intestinal cells also contain drug metabolizing enzymes which can result in poor absorption of drug and metabolites entering the systemic circulation. Benet [109] introduced Biopharmaceutical drug disposition classification system (BDDCS) (Fig. 18.4) where he proposed that understanding the interplay between transporters and metabolism in

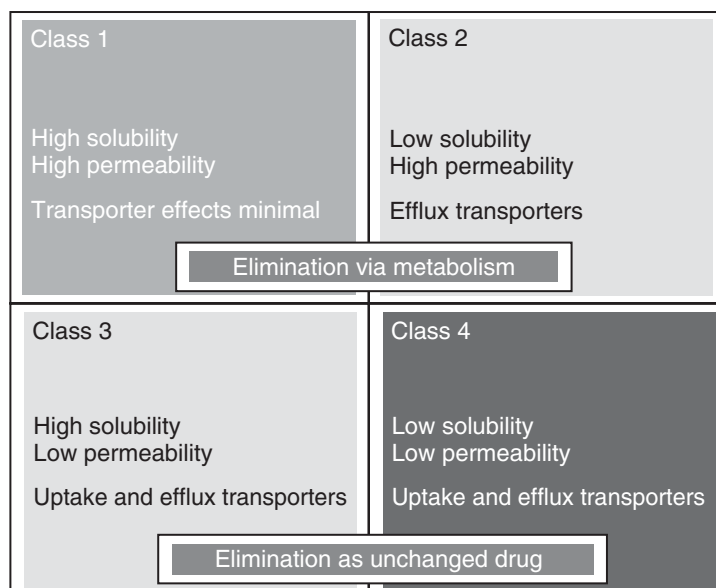


Figure 18.4 Biopharmaceutical drug disposition classification system (BDDCS), represents classification of the drugs into a four classes, based on drug solubility, permeability, metabolism, and drug transporters involved in the process of ADME. (See color insert.)

the intestine and liver is a very important determinant of drug disposition characteristics. According to this classification system, for highly soluble and highly permeable *class 1* compounds, metabolism is the major route of elimination. Transporters play a minor role in the intestine and liver. In contrast, for the poorly permeable *class 3 and 4* compounds, uptake transporters and perhaps efflux transporters could be important for drug elimination. Metabolism plays a minor role in drug elimination for class 3 and 4 drugs. Highly permeable, poorly soluble, and extensively metabolized *class 2* compounds present the most complicated relationship in defining the impact of transporters on drug disposition due to marked transporter–enzyme interplay. Uptake transporters are not important for bioavailability of class 2 drugs but can play a major role in hepatic and renal elimination. Efflux transporters have major effects on drug bioavailability, absorption, metabolism, and elimination of class 2 drugs. It is difficult to accurately characterize drugs in terms of the high permeability, that is, $\geq 90\%$ absorbed. The extensive metabolism may substitute for the high permeability characteristic. Thus, BDDCS may provide predictability in characterizing drug disposition profiles for all classes of compounds.

18.2.4.4 LC-MS/MS Advances in Pharmacokinetics and Drug Metabolism. In drug discovery, a quick assessment of drug concentrations require the development of rapid and accurate bioanalytical methods. Typically, bioanalytical groups support PK and TK studies, but in drug discovery, they also support a wide variety of *in vitro* studies, including *in vitro* metabolic stability, Caco-2 permeability, DDIs, and protein binding studies. Therefore, development of methods with higher throughput is very demanding in drug discovery programs [110,111]. Science of chromatography and

mass spectroscopy has rapidly advanced with detection levels of drugs and metabolites at femtomole and attomole levels. The advances in technology led to the development of LC/MS-based techniques in bioanalysis [112–116]. The elements of LC/MS applications ultimately owes the credit to the power of high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) to resolve the drugs and their metabolites using shorter run times (1–5 min) combined with the ability of MS to identify the drug molecules in the biological milieu at femtomole and attomole quantities. This integration of LC and MS effectively to quantitate the drugs at less than picogram levels has expedited the ADMET programs in the drug discovery process.

18.2.4.4.1 Quantitative Bioanalysis. The quantitation of drugs in biological matrices also requires efficient clean-up methods to exclude proteins and other endogenous molecules before analyzing the processed samples. This clean-up process should be such that 80% or higher levels of drug are recovered. Before analyzing drug concentrations in biological matrices, a quick and reliable bioanalytical method is needed with a calibration curve ranging from low nanomolar to high micromolar concentrations. This is one of the major problems because there is no prior biological data to decide on concentration ranges for calibration curve. Choosing an internal standard close to the structure without interfering multiple reaction monitoring (MRM) transitions is also required. The LC-MS-based *in vivo* PK screening approaches allow analysis of large number of samples with a throughput of up to 7000–10,000/month/instrument. In earlier times, a conventional set-up comprised single auto-sampler/HPLC column and MS systems in which one sample was injected at a time. The technological advances have allowed higher throughputs with rapid chromatography using UPLC, automated data processing, and sample pooling strategies (cassette dosing, dried blood spot analysis, etc.). The use of two auto samplers on two columns in parallel with a single triple quadrupole mass spectrometer, have led to reduction in HPLC/UPLC column equilibration time and common hold-ups associated with sample loading.

In early stages of drug discovery research, the bioanalysis allows rapid data generation so as to understand the PK properties of the NCE (along with its metabolites). Thus, in drug discovery, a bioanalyst develops a “fit-to-purpose” research method. In brief, the bioanalyst after receiving biological samples processes the samples quickly by protein precipitation and the supernatant is injected into LC-MS/MS system. The high resolving power of LCs and reverse phase columns and highly sensitive mass analyzers provide quick data that enable a pharmacokineticist to assess whether the NCE under test possesses favorable PK properties. Thus, LC-MS/MS techniques proved to be a cardinal in regular bioanalysis [117].

18.2.4.4.2 Metabolite Identification. Identification of metabolites and metabolic pathways (biotransformation) is essential during early drug discovery process to understand potential liabilities and risks associated with metabolites. Most common phase I and II metabolites screened for identification are shown in Table 18.7. Objectives of metabolite identification during drug discovery include the following:

1. Identification of metabolic soft and hot spots in an NCE and recommend medicinal chemists to design metabolically stable compounds. The soft spots include inactive polar metabolites that may be rapidly eliminated from the body. The hot spots include reactive intermediates (electrophiles and free radicals), which may

TABLE 18.7 List of Common Phase I and II Metabolites

Biotransformation	Mass Change (Da)
<i>Phase I metabolites</i>	
Hydroxylation	+15.9949
Epoxidation	+15.9949
N/S oxidation	+15.9949
Dihydroxylation	+31.9898
Demethylation	−14.0157
Oxidative displacement of chlorine	−17.9662
Oxidative displacement of fluorine	−1.9957
Dehydrogenation	−2.0157
Desethylation	−28.0312
Nitro reduction	−29.9742
Hydrogenation	+2.0157
Dehydration	−18.0106
Decarboxylation	−43.9898
Reductive displacement of chlorine	−33.9611
Reductive displacement of fluorine	−17.9906
Loss of nitro group	−44.9851
Alcohol to carboxylic acid	+13.9792
Ketone formation	+13.9792
Hydration	+18.0106
Methyl to carboxylic acid	+29.9741
<i>Phase II metabolites</i>	
Glucuronide conjugation	+176.0321
Sulfate conjugation	+79.9568
Methylation	+14.0157
Acetylation	+42.0106
Glycine conjugation	+57.0215
Taurine conjugation	+107.0041
Cysteine conjugation	+119.0041
Glutathione conjugation	+305.0682
N-Acetylcysteine conjugation	+161.0147

covalently bind to tissue macromolecules (proteins/DNA) and/or cause oxidative stress resulting in toxicity or genotoxicity.

2. Identification of potential metabolites that have pharmacological activity. In such an event, a possibility of developing metabolite itself as a new drug can be explored.
3. Identification of species differences in metabolite profiles so as to explore occurrence of possible human metabolites and estimate their exposure in preclinical species. Recent FDA guidance [82] recommends identifying potential human metabolites as early as possible so that expensive and time-consuming toxicology studies with metabolites can be avoided if the metabolite exposures are covered in toxicology studies. *In vitro* studies using human liver microsomes and hepatocytes are often employed to identify potential human metabolites, preclinically.

Elucidation of the structure of many metabolites is not straightforward, especially when complex rearrangement leading to a unique metabolite and/or the formation of metabolites with isobaric molecular ions [118–122]. Recent advances in the LC-MS/MS technology allow detection of metabolites with high sensitivity—as low as femtomoles. LC with diode array detector is widely used to separate the mixture of metabolites and drugs, while MS serves as an ideal detector to identify the structure of metabolites [118]. In most cases, chromatogram obtained from diode array detector complements the mass chromatogram. Mass spectrometric detectors are more sensitive and robust compared with other detectors such as diode array detectors and nuclear magnetic resonance (NMR) spectroscopy. However, final confirmation of metabolite structure may need to be established using NMR spectroscopy. Different types of ion sources such as electrospray ionization (ESI), nano-ESI, and atmospheric pressure chemical ionization serves as interface between LC and MS and assist in the ionization of molecules.

18.2.4.4.3 Tandem Mass Spectrometers and Ion Trap Instruments in Metabolite Identification. Development of triple quadrupole mass spectrometers (tandem mass spectrometers) coupled with LC has made a significant impact in the metabolite identification research. Triple quadrupole instruments providing MS² fragmentation patterns are necessary to identify the metabolic soft spots [123,124]. To date, MRM mode remains to be the most sensitive method to confirm trace levels of circulating metabolites in biological fluids derived from animal or human ADME studies.

Recent technology such as triple quadrupole ion trap mass spectrometers where trap technology is integrated with triple quadrupole mass spectrometers is both sensitive and qualitative [125,126]. Triple quadrupole ion trap mass spectrometer coupled with LC is one of the ideal equipment to characterize the structures of metabolites. These instruments are more sensitive and provide MS² and MS³ fragmentation spectra of metabolites formed even in femtomole quantities and help characterize their structures. Apart from MS² and MS³ fragmentation spectra, neutral loss (NL) scans, precursor ion (PI) scans, and enhanced resolution (ER) scans from the trap instruments are characteristic to detect the metabolites. For example, NL scan of m/z 176 is used to identify phase II metabolites such as glucuronide conjugates. Other important and routinely screened glutathione adducts are identified by NL scan of m/z 129 and negative PI scans of m/z 272. ER scans are helpful in identifying the isotopic pattern of the metabolite structures. Apart from the above, combination of different scan modes such as enhanced mass-enhanced product ion (EMS-EPI), EMS-NL, EMS-PI, ER-EPI, and MRM-EPI scans with information-dependent acquisition have been effective tools in identifying the structure of metabolites. Recently, we have reported the importance of different scan modes in identifying the structure of metabolites using triple quadrupole ion trap mass spectrometer (API4000 Q-trap) coupled with LC. As an example, use of different scan modes such as EMS-EPI, NL-EPI, and PI-EPI scans has been successfully employed to confirm new metabolite of glyburide [119].

Accurate mass measurements (high resolution) in metabolite identification experiments can provide information that can distinguish isobaric molecular ions and correct fragmentation pattern. Higher versions of ion trap instruments such as orbitrap are capable of providing high resolution/high mass accuracy measurements of precursors and product ions regardless of relative ion abundance [127,128]. Orbitrap instruments also have the MS^{*n*} capabilities (i.e., acquisition of MS¹⁰ spectrum is possible, which are

highly useful in assigning the metabolic softspot in the molecule). Lim and coworkers have evaluated linear ion trap/orbitrap to identify the metabolites of carvedilol [118]. A total of 58 *in vitro* metabolites of carvedilol have been identified, which includes several glutathione conjugates. In this study, high resolving power and mass accuracies of instrument were utilized to obtain the chemical formula and structures of metabolites of carvedilol, including isobaric species with greater confidence. Mass accuracy of linear ion trap/orbitrap has been reported up to fifth decimal and this advantage has enabled differentiation of isobaric hydroxylated metabolites with greater confidence [118]. Orbitrap mass spectrometers are equipped to identify and characterize the structure of metabolites. Metabolites of nefazodone have been characterized using both quadrupole ion trap and linear ion trap/orbitrap. One research article describes the use of linear ion trap (LTQ)-Orbitrap for detection of nefazodone metabolites that allowed detection of isotopic pattern in which 28 metabolites of nefazodone were confirmed, while quadrupole ion trap could detect all the metabolites but had reduced quality of isotopic patterns that hindered metabolite confirmation [128].

18.2.4.4.4 Time-of-Flight Mass Spectrometers in Identification of Metabolites. Triple quadrupole-time-of-flight mass spectrometers (Q-TOF) with LC have been employed successfully for metabolite identification. In triple Q-TOF mass spectrometers, the ions of interest are mass selected in first quadrupole, followed by fragmentation in a second quadrupole or hexapole and analyzed by TOF detector. TOF detectors have high resolution and the ions of interest can be separated from isobaric molecular ions, thus facilitating the measurement of mass to charge (m/z) value of ions with an accuracy of 2 ppm [129]. For example, when a drug with ether functional group ($R-CH_2-O-CH_3$) is metabolized (i.e., O-demethylation followed by oxidation of alcohol) to acid ($R-COOH$), the molecular weight of the drug remains the same and if the fragmentation pattern of drug and metabolite is similar, the quadrupole resolution is not enough to differentiate them. In such cases, accurate mass measurements by TOF analyzers can differentiate the drug from metabolite and structures can be assigned. TOF analyzers can differentiate masses up to fourth decimal with precision and provides high mass accuracy for identification of the metabolites.

Rapidly developing mass spectrometric imaging technology has also been employed for metabolite identification directly in biological tissues [130–132]. Ion trap instruments with ionization sources such as matrix-assisted laser desorption ionization, desorption ESI, and direct analysis in real time coupled to LC have also been employed successfully to image the drug and metabolites.

18.2.5 Drug–Target Interactions and Pharmacodynamics

PD is the quantitative relationship between (observed) plasma and tissue concentrations of the active moiety and the magnitude of the (observed) pharmacological effect. A PK–PD model is a mathematical description of these relationships; the model parameters provide information about intrinsic drug properties. The knowledge of the combined PK–PD model and the parameter estimates allows prediction of concentration versus time ($c-t$) profiles and effect versus time ($E-t$) profiles for different dosing regimens. To understand the clinical significance of PK/PD modeling, it is important to realize that the observed $E-t$ profile of an individual patient depends on three main determinants: drug input, that is, dose, dose rate, and route of administration,

and intrinsic PK and intrinsic PD properties of the NCE. PK–PD modeling allows the scientist to simulate the concentration (or exposure) of NCE to the observed efficacy parameters that are clinically relevant. PK–PD evaluations in preclinical animal models help in obtaining a meaningful prediction of these parameters for human studies and to predict efficacious human dose. In addition, preclinical safety studies in animals can help determine the therapeutic margin between the safe dose and efficacious dose.

Generally, a biological “target” may be considered as the naturally existing cellular or molecular structure involved in the pathology of interest that the new drug to be discovered is meant to interact with and provide therapeutic action. Following administration of drug, it must reach the target tissue where the PD effect is anticipated. The target can be any biological molecule, including proteins (enzymes and receptors), nucleic acids (DNA, RNA, and gene), and ion channels. Many of the targets are macromolecules that specifically bind to the NCE and trigger the PD effect. The target compartment is assumed to be kinetically homogeneous, that is, concentration of the NCE is homogeneous and the drug–target interaction is instantaneous. The drug–target interaction is governed by the law of mass action. Hill [133] was the first to develop explicit mathematical model of simulated drug action to account for the time courses and concentration–effect curves obtained when nicotine was used to provoke contraction of the frog *rectus abdominis* muscle. According to the occupancy theory, the drug effect is a function of two processes:

1. Binding of drug to the receptor and drug-mediated activation of the target
2. Propagation of this initial target activation with a cascade of biomolecular events to the desired therapeutic effect, where the intensity of the therapeutic effect is proportional to the number of target sites occupied by drug

Depending on the disease indication, PD responses are measured as follows:

1. Clinical endpoints, for example, reduction in disease activity, survival, or cure
2. Surrogate endpoints, for example, blood pressure and progression free survival
3. Biomarkers, for example, cytokines, chemokines, inflammatory mediators, growth factors, transcriptional factors, and endogenous metabolites

These PD endpoints are currently being widely employed for understanding the PK–PD relationships early in drug discovery and development stages. As discussed earlier, despite extensive preclinical efficacy evaluations, the failures due to lack of efficacy in clinical phase II and III development is still a major problem. Several factors can be attributed to these failures, including

1. Lack of mechanistic understanding of the target and its contribution to disease pathogenesis
2. Species differences related to the target and associated biological effects
3. Inadequate understanding of PK–PD relationships at preclinical stages leading to errors in human efficacious and safe dose selection

Selection of preclinical animal model for efficacy evaluation has always been challenging. In majority of cases, only rodents (mouse and rat) are employed. Where species

differences in targets are noted, genetic manipulation of these animals is performed so as to knock-off the mouse target and knock-in the human target. However, if the biological effect triggered by target activation is different from humans, then translation of preclinical efficacy to clinical situation will be erroneous.

Interestingly, the regulatory guidelines for preclinical safety evaluation recommend use of a rodent and a nonrodent species. Among the nonrodent species, large animals such as dogs and nonhuman primates are most widely employed for safety assessment. In contrast, large animals such as dogs and nonhuman primates are rarely utilized for preclinical efficacy evaluation. Unfortunately, very little effort has ever gone into development of disease models in large animals, which can be attributed to one or more of the following reasons:

1. Difficulties in developing the disease models in larger animals.
2. Concerns regarding animal ethics in using large animals for research purposes.
3. The high costs involved in large animal disease modeling.

An interesting fact is that during drug discovery and development, >60% cost goes into clinical development. Thus, if a drug fails during phase II or III trials due to lack of efficacy, all the efforts put in developing the drug in terms of time and cost are futile. These facts warrant for a greater understanding of the pharmacology of NCEs at the early preclinical development stages and perhaps utilize more than one species, in particular large animals. In future, whether drug companies divest more efforts and resources at the preclinical development stages to avoid failures in clinical stages of drug development that too at the cost of huge losses remains to be seen. Table 18.8 shows all the preclinical DMPK and toxicology studies that are conducted before initiating first in man studies. Figure 18.5 proposes a rational and stepwise evaluation of efficacy and safety at drug discovery stages, using two or more different animal models and includes at least one large animal model, so as to reduce the late stage failures due to efficacy or safety issues.

18.2.6 Exploratory Toxicology During Drug Discovery

In the process of drug discovery, it is very important to evaluate the potential toxicological risks of a drug candidate as early as possible. The preclinical safety programs are aimed at understanding safety liabilities early in drug discovery and help in optimal design of good laboratory practice (GLP) toxicity studies so as to minimize the high rates of compound attrition during development.

Regulatory guidelines prescribe the investigation of safety in preclinical species for any NCE before it can be administered to man. These animal tests are intended to reveal potential toxicity associated with the candidate NCE and are conducted as a tiered program. The first studies conducted are referred to as *pilot studies*, preliminary studies, known as *dose range finding* studies, and these are used to select doses for subsequent regulatory studies. However, such studies require synthesis of several hundred grams of NCE and consume time. These studies are typically conducted as regulatory submission packages. However, by the time a toxicologist detects a problem or safety issue in a regulatory study, several months of time is lost in synthesizing sufficient quantities of the drug, conducting the regulatory study with a negative outcome. Thus, most pharmaceutical companies prefer integration of safety assessment measures into earlier

TABLE 18.8 List of Different DMPK and Toxicity Studies Conducted Before First-in-Man Studies

Hit	Hit to Lead	Lead Optimization	Preclinical Evaluation
Hit Identification	Tier 1 DMPK Assays (96- or 384-well assays)	Tier 2 DMPK-TOX Assays	Tier 3 DMPK-TOX Assays
Target identification	$\log D$, pK_a	Caco-2/MDCK transporters	PK evaluation using stable
Computational modeling	Kinetic solubility	Microsomal CYP inhibition (ic_{50} and K_i)	Polymorph/salts (mouse, rat, and dog)
Synthesis of NCE	PAMPA/Caco-2 assay	Microsomal time-based CYP inhibition (K_{inact})	CYP inhibition (K_i and K_{inact})
Lipinsky rule	Microsomal/RhCYP stability	CYP phenotyping (K_m/V_{max})	K_m/V_{max}
SAR	RhCYP inhibition (%)	CYP induction (activity assays)	Radiolabeled studies
Deuterated standards	Time-based RhCYP inhibition	Human hepatocytes	GLP genetic toxicology
Metabolite synthesis	RhCYP phenotyping	Metabolite ID	GLP safety pharmacology
Evaluation of biological potency and activity	Plasma protein binding	Reactive metabolite ID	MTD toxicity testing
Selection of hits	CYP induction (mRNA)	hERG channel assay	GLP two- to four-week toxicity testing
	Mouse, rat, dog, and monkey PK	Screening Ames assay	Allometric scaling
	Allometric scaling for predicting human PK	Screening micronucleus assay	Preclinical PK/PD
	<i>In vitro/in vivo</i> Pharmacological model POC	Toleration toxicity (one to two weeks)	First-in-human dose selection
	Preliminary PK–PD	Phospholipidosis Steatosis Cholestasis Cytotoxicity	

phases of drug discovery, which are called *tolerability toxicity studies*. These studies can also be part of investigative toxicology studies where a mechanistic basis of toxicity may be understood. The application of the FDA's Critical Path Initiative, a 2004 FDA white paper, *Innovation or Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products* encourages pharmaceutical companies to conduct more investigative and mechanistic toxicology during drug discovery phase before it is too late.

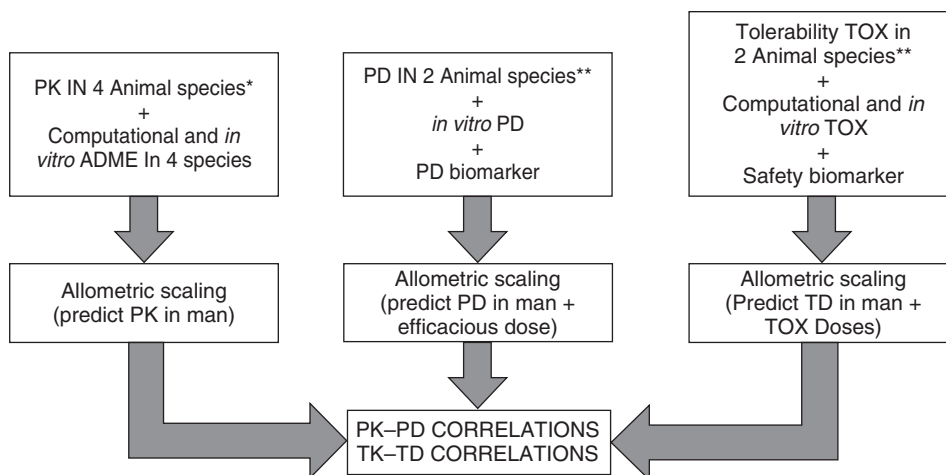


Figure 18.5 Integration of pharmacodynamics, DMPK, and exploratory toxicology during early drug discovery. *Mouse, rat, monkey and dog and **rodent and large animal.

Exploratory toxicology is performed on NCE candidates with proven efficacy in animal models to assess any potential safety risks. Exploratory toxicology is one of the main phases of nonclinical toxicology assessment in which the NCE has to undergo a battery of *in vitro* and *in vivo* testing. The exploratory toxicity studies are typically not required to be reported to the regulatory agencies and need not be conducted according to the GLP guidelines. These studies allow early selection or rejection of compounds during early drug discovery stages, thereby saving time, effort, and expenditures. This process will help a medicinal chemist to synthesize better lead candidates devoid of potential safety liabilities. A variety of approaches are used, including *in silico* computational tools, applications of OMICS technologies (toxicogenomics), and some limited *in vitro* and *in vivo* tolerability toxicology studies. Some mechanistic data derived from toxicogenomics and other studies may help identifying potential safety biomarkers, which may be of clinical use during clinical trials.

18.2.6.1 Computational Toxicity Testing. As described earlier in Section 18.2.1, computational tools (Table 18.3) may be utilized early in drug discovery stages to design molecules that are devoid of any potential toxicity. *In silico* techniques for the prediction of toxicity have been developed since the late 1970s. These methods use computational approaches and are based on the structure of a compound. They are based on the principle that “structure defines activity and that the reaction of a chemical with a biological system is driven by its molecular structure.” *In silico* methods have the advantage that they can make fast predictions for a large set of compounds in a high throughput mode, predict even before the compound has been synthesized, and can therefore be used at a very early stage in the drug development process. There are two *in silico* methods used for the prediction of toxicity: QSAR and expert computer systems (e.g., DEREK, MultiCASE, and TOPKAT). QSAR means the quantitative relationship between a chemical structure and its biological/toxicological activity. QSAR models calculate the relationship between a chemical structure and their biological activity with the help of chemical descriptors that are generated from the

molecular structure. The expert computer systems predict the toxicity using different databases containing diverse chemical structures and their observed toxicity [134].

18.2.6.2 Tolerability Toxicity Studies. Tolerability studies are among these first tiered studies conducted in rodents and perhaps in dogs during lead optimization process of drug discovery. These tests are conducted to understand whether the drug has any specific safety issues near the pharmacological doses. These studies are often conducted with small number of animals with multiple doses for five to seven days. Such studies provide early indication of whether pharmacological doses are well tolerated. Any safety issues as an extension of pharmacology can be identified in these early studies. A safety issue related to pharmacological extension is usually a “class effect” where inhibiting or modulating a specific therapeutic target can result in serious adverse or toxic events. Such targets have very low potential for discovering therapeutically useful drugs where risks outweigh benefits.

Many times early investigative tolerability studies are limited to physical observations only. In some cases, these studies may include TKs, clinical chemistry, and histopathology assessment as well. These studies may also utilize novel technologies such as toxicogenomics and metabolomics for a mechanistic understanding of toxicity.

18.2.6.3 Toxicogenomics. Use of genomics to predict toxicity of NCEs is based on the principle that drugs induce a multitude of complex molecular perturbations involving differential gene expression at the transcriptional and translational level, leading to toxic outcomes. The data for changes in gene expression caused by a NCE under test can be used to identify its potential to cause toxicity at lower doses or at earlier time points. The application of this gene-expression profiling technology to toxicology is called *toxicogenomics* [135]. The advantages of the toxicogenomic approach include increased sensitivity, low variability, reduced number of animals, and lower compound requirements. The limitations of these tools are (i) variability of gene expression from tissue to tissue, (ii) necessity of relevant databases, and (iii) systems that are expensive to run and maintain [136,137]. Another disadvantage of this tool is the generation of large amounts of data that can be cumbersome to analyze and interpret.

18.2.6.4 Genotoxicity Assays. The genotoxicity assays are used to determine the potential of a NCE to cause DNA damage that may lead to an increased incidence of tumors or birth defects through heritable effects in the germ cells. Genetic toxicology is generally positioned as part of the drug safety or toxicology function during preclinical stages of drug discovery. Assessment of genetic toxicity involves subjecting the test NCE to a defined battery of genotoxicity tests. This battery of tests generally includes a bacterial mutagenesis assay (Ames test), an *in vitro* cytogenetics assay, and an *in vivo* cytogenetics assay (micronucleus assay).

These genotoxicity assays for any test compound are carried out according to the guidelines prescribed by International Conference of Harmonization (ICH), as well as GLP guidelines and generally require multigram quantities of compound. Genotoxicity testing was historically relegated to the later stages of drug development. However, the need for earlier testing was apparent to drug companies that experienced termination of a drug's development when unacceptable genotoxicity was discovered during the regulatory submission stage, up to which point huge monetary, personnel, and,

most importantly, time resources had been expended. Today, it is possible to measure standard mutagenic and clastogenic endpoints with as little as 5–30 mg of compound, allowing genotoxicity screening to be conducted much earlier in drug discovery stages.

Among the more widely used scaled-down genotoxicity screening assays are a miniaturized version of the Ames bacterial mutagenesis assay and an *in vitro* micronucleus (IVMN) assay.

18.2.6.4.1 Mini (Screening) Ames Genotoxicity Assay. The bacterial reverse mutation assay (Ames test) with *Salmonella typhimurium* is a well-established assay to identify the chemicals that can produce gene mutations [138]. The Ames test typically uses five strains of *Salmonella* with preexisting mutations that render the bacteria unable to synthesize the essential amino acid histidine and are unable to grow in histidine-free medium. The chemicals that induce mutations on these particular genes restore gene function and allow the cells to synthesize histidine and therefore grow in its absence, therefore called *reverse mutation assay*. A disadvantage of this test is that it is time consuming, expensive, and requires higher quantities of compound. Flammann *et al.* [139] miniaturized the Ames test for the screening of pharmaceuticals and required smaller quantities of the test compound as compared with the conventional Ames test. The mini-Ames is performed in microwell plates using two *Salmonella* strains, TA98 and TA100. TA98 detects frameshifts and TA100 detects base substitutions leading to missense mutations. Whereas the original Ames test is carried out by plating bacteria onto selective agar plates, the mini-Ames is carried out in liquid culture using 96-well plates. The bacterial plates are incubated with the test compounds for 72 h, after which bacterial growth is measured spectrophotometrically using a pH indicator that changes color in response to the acidification of the media due to bacterial growth. Compounds can be tested in both bacterial strains with and without S9, at different concentrations along with reference compounds. The advantages of mini Ames includes small amount of compound requirement and rapid turnaround time. The major limitation of these miniaturized versions of the Ames test lies in the impossibility to work with all the five strains used in the regular Ames test [140].

18.2.6.4.2 In vitro Micronucleus Assay. The IVMN assay is a widely used screening test, which shows a high level of concordance with regulatory chromosomal aberration assays [141]. Micronuclei are chromatin-containing bodies that represent fragments or even whole chromosomes that were not incorporated into a daughter cell nucleus at mitosis. The purpose of the assay is to detect agents that induce chromosome damage leading to the induction of micronuclei in interphase cells. The agents that induce micronuclei include clastogens (through interaction with the DNA) or aneugens (through interference with the spindle apparatus). The major mechanisms responsible for micronucleus induction are double DNA strand breaks leading to micronuclei with acentric fragments [142].

The IVMN assay is a simple and rapid method routinely used as a prescreening test for the quick assessment of *in vitro* chromosomal aberration, which is part of the recommended regulatory battery of tests for genotoxicity testing. It can also detect aneuploid-inducing agents, which are difficult to detect with the *in vitro* chromosomal aberration assay.

The IVMN assay is conducted in chinese hamster ovary (CHO-K1) cells seeded in multiwell plates and treated with the test compounds for 24 h without S9 and for 3 h

with S9. Cytochalasin B is added after 24 h and the cells are incubated for an additional 24 h, after which the cells are fixed and scored for micronuclei under the microscope.

18.2.6.5 hERG Binding Assay. The hERG encodes the inward rectifying voltage gated potassium channel in the heart (IKr), which is involved in cardiac repolarization. Inhibition of the hERG current causes QT interval prolongation resulting in potentially fatal ventricular tachyarrhythmia called *torsade de pointes*. The hERG potassium channel is expressed in the mammalian heart and is crucial for repolarization and relaxation of cardiac muscle during every heartbeat. Human mutations of this gene increase susceptibility to QT-interval prolongation on an electrocardiogram (ECG). This prolonged interval can lead to lethal ventricular arrhythmias. A diversity of drugs from widely differing chemical scaffolds block this channel.

Much attention recently has been focused on identifying the drugs that prolong the QT interval. It is imperative to investigate any NCE for its potential to adversely prolong repolarization before its first use in man. To reduce the risk of failure of a drug candidate in preclinical safety studies due to QT prolongation, it is important to screen compounds for activity on hERG channels early in the lead optimization process. A number of *in vitro* and *in vivo* hERG assays are available. There are four broad categories of *in vitro* models: overexpression of human hERG channel on human embryonic kidney cells (HEK293), mouse fibroblasts and Chinese hamster ovary cells, isolated tissues such as canine mid-myocardium and Purkinje fibers, and the isolated intact (Langendorff-perfused guinea pig or rabbit) hearts [143]. Multilead ECG recordings in conscious or anesthetized guinea pigs, rabbits, dogs, or pigs comprise some *in vivo* models for testing effects of the candidate drug on QT prolongation [144].

Current regulatory guidelines consider hERG assays a major component of an integrated cardiac risk assessment that takes into account chemical class of the compound, assay sensitivity and specificity, and potency of test compound in repolarization assays in comparison to reference compounds [145].

18.2.6.6 Phospholipidosis. Phospholipidosis is a lysosomal storage disorder characterized by excessive accumulation of intracellular phospholipids in tissues such as liver, kidney, and lung [146]. The drugs that induce phospholipidosis are known to contain both hydrophobic and hydrophilic cationic domains called *cationic amphiphilic drugs*. These include antibacterials, antipsychotics, antidepressants, antianginals, anti-malarials, antiarrhythmics, and cholesterol-lowering agents [147]. Several mechanisms have been proposed for drug-induced phospholipidosis, including inhibition of the enzymatic activity of phospholipidases and binding of the drug to the phospholipid to form drug–phospholipid complexes that cannot be broken down by phospholipidases [148].

Phospholipidosis does not necessarily constitute toxicity, and can resolve by itself, but it is predictive of drug or metabolite accumulation in affected tissues that have led to liver, kidney, or respiratory failure. Depending on the type and number of tissues affected, phospholipidosis occurrence in test animals can raise safety issues, which may be critical for the risk assessment. Consequently, phospholipidosis is of concern to the FDA, which in 2004 formed the FDA Phospholipidosis Working Group to study the problem and recommend strategies for phospholipidosis screening studies [149]. Safety profiling of potential phospholipidosis-inducing drugs early in the drug

discovery phases can help in impediments in the progress of the candidate drug to the later phases of drug development.

To detect the phospholipidosis induction in drug discovery phase, various *in vitro* methods have been developed and used as phospholipidosis screening assays. These include use of fluorescent dyes (e.g., Nile red) or fluorescently labeled phospholipids (e.g., 1-acyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino-dodecanoyl]-sn-glycerol-3-phosphocholine) in cell culture.

18.2.6.7 Cytotoxicity Assays. Cytotoxicity is simply the quality of any NCE for being toxic to the cells, regardless of the mechanism. Cytotoxic compounds can be either developed as a therapeutic that targets rapidly dividing cancer cells, or in case of a different therapeutic indication, cytotoxic compounds can be identified and excluded during initial high throughput screens for unwanted cytotoxic effects before they are selected for further development. Several mechanisms may be involved in cytotoxicity of a given NCE. These mechanisms include inhibition of mitochondrial pore transition [148], covalent binding to cellular macromolecules [149], induction of oxidative stress via free radicals [150], or alteration of cell cycle phases [151]. Many of these cell-damaging events may lead to apoptotic or necrotic cell death.

It is interesting that cellular glutathione (GSH) plays a vital role in protecting the cell from reactive metabolite damage. In many cases, the reactive metabolites covalently bind to proteins, but cellular GSH can prevent such damage and protect the cells. Alternatively, cellular GSH may also protect from free radical-mediated damage by scavenging the free radical metabolites of drugs. However, in this process, thiyl radicals of GSH may induce formation of oxygen radical and increase in cytotoxic events [152].

There are various types of *in vitro* assays used to detect the cytotoxicity. Examples of a couple of widely used methods are described below.

Cell death causes damage to the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells. Lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant on damage of the plasma membrane [153]. Adenosine triphosphate (ATP) that is present in all metabolically active cells can be determined in a bioluminescent measurement [154,155].

Another method is based on colorimetric principle that relies on the metabolic activity of viable cells, which reduces tetrazolium salts [e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, commonly known as *MTT*] to a blue formazan or alamar blue that converts to resorufin [156].

18.2.6.8 Other *In vitro* Mechanism-Based Toxicity Assays. These include use of *in vitro* models to examine the mechanisms underlying actions of chemicals on organs or tissues observed *in vivo*. The *in vitro* models are designed to elucidate possible mechanisms of drug toxicity and their relevance to the target organs. The test NCE is delivered directly to the cells and the changes in viability or other functional parameters measured. Table 18.9 gives examples of test systems and their potential to predict their potential to cause toxicity.

Thus, current trend of including toxicology earlier in the discovery phase permits identification of unsafe compounds in the initial preclinical phase and thus allows for early withdrawal of bad actors and provides the opportunity to turn a potentially harmful compound into a successful one. Exploratory toxicology improves the quality

TABLE 18.9 *In vitro* Test Systems and Their Potential to Predict Different Toxicities

Test System, Example	Prediction
Primary hepatocyte culture	Hepatotoxicity including phospholipidosis and cholestasis
Tissue slices/organ components liver or kidney	Hepatotoxicity/nephrotoxicity
Bone marrow cell cultures	Hematopoietic toxicities
Whole embryo culture	Predict teratogenicity
Fertilized chicken eggs	Corrosion and irritancy
hERG assay	Cardiotoxicity
Langherhan's cells	Immunotoxicity
Ames and micronucleus assays	Genotoxicity

and quantity of drugs entering clinical studies. It enhances the safety and efficacy of new compounds and allows compounds to be prioritized early in the development process.

18.3 ROLE OF TRANSLATIONAL RESEARCH AND BIOMARKERS IN DRUG DISCOVERY AND DEVELOPMENT

Translational medicine refers to the meaningful transition of a NCE found to be effective preclinically, for use as a therapeutic in humans [157]. Translational PK–PD modeling involves integration of *in silico*, *in vitro*, and *in vivo* preclinical data with mechanism-based models to anticipate the effects of new drugs in humans. Translational models hold promise to facilitate design and/or selection of lead compounds, selection of first-in-human dose early clinical trial design, and POC studies of NCEs. Mechanism-based PK–PD modeling aims at the prediction of exposure–response relationships in humans, including their inherent intra- and interindividual variability. Exposure is expressed as time course of drug concentration in plasma, whereas response is expressed as the time course of drug-effect intensity. Mechanism-based PK–PD models contain specific expressions to quantitatively characterize processes on the causal path between plasma concentration and effect. Prediction of efficacy in humans based on the efficacy data in the animal models is challenging and there is need for mechanism-based biomarkers that allow building POC [158]. In addition, predicting safety challenges in clinic makes use of information from genomics, proteomics, and metabonomic data sources [13,159]. Biomarkers can be defined as quantifiable physiological or biochemical marker, which is sensitive to intervention or drug treatment. A safety biomarker is a treatment-emergent finding that substitutes for or translates into a clinically relevant adverse outcome. In case of cardiovascular safety evaluation, the two biomarkers that have been used for any drug candidate are assessment of (i) left ventricular ejection fraction by either echocardiography or multi-gated acquisition scan and (ii) electrophysiological measurement of QT/QTc duration, assessed by ECG, for predicting risk of a potentially fatal arrhythmia called *torsades de pointes* [160]. Biomarkers are represented by diverse parameters such as (i) serum parameters (e.g., the primary product of an enzyme, which is inhibited by the drug), (ii) disease severity (e.g., C-reactive protein (CRP) and other markers in inflammatory processes), (iii) imaging (e.g., plaque morphology in drugs affecting atherosclerosis,

liver fat content as safety biomarker, or positron emission tomography) [156,159]. The successful use of PK–PD modeling and biomarker data is dependent on (i) selection of mechanism-based biomarkers and its link with clinical endpoint, (ii) quantification of drug/metabolites in biological fluids under GLP conditions, (iii) GLP-like assay methods for biomarkers, (iv) mechanism-based PK–PD modeling and validation [161]. Unless a biomarker is validated, it is not acceptable to regulatory agencies as surrogate endpoint for determination of drug's efficacy. Biomarker selection and validation are important processes that ensure the successful application of the biomarkers in drug discovery and development.

18.3.1 Success Stories of PK–PD Evaluation in Humans from Preclinical Studies

PD studies in animal models of disease allow extensive evaluation of a number of disease parameters and biomarkers. These data from preclinical studies together with PK evaluation allow establishment of meaningful PK–PD relationships. The preclinical PK–PD data are instrumental in validating chosen biomarker that can then be employed in POC studies in humans. A few examples of application of the preclinical PK–PD data, which has successfully allowed meaningful PK–PD evaluation in humans, are discussed below.

Efavirenz (Sustiva), an anti-HIV drug, was found to cause necrosis of the renal proximal tubular epithelium in rats after an oral dose of 700 mg/kg. This renal toxicity was not observed when efavirenz was dosed in cynomolgus monkeys or humans. The nephrotoxicity in rats was attributed to the formation of a glutathione adduct that was further found to be a mixture of cysteine–glycine and cysteine adducts [162]. Mechanistic studies with labeled efavirenz and coadministration with acivicin, a g-glutamyltranspeptidase inhibitor, confirmed that the formation of the glutathione conjugate and its processing finally to the cysteine conjugate was necessary for eliciting the nephrotoxicity in rats. This glutathione conjugation pathway was missing in cynomolgus monkey or in humans. This species-specific pathway and its absence in humans, followed by detailed mechanistic studies, helped place this issue in the proper perspective in terms of risk to patients. It was concluded that the nephrotoxicity in rat observed at high doses was not a safety concern for humans.

The relationship between PK and PD can also be utilized for optimal dose selection. Efalizumab, a recombinant humanized monoclonal IgG1 antibody, was found to be efficacious for the treatment of moderate to severe chronic plaque psoriasis. Efalizumab, a targeted inhibitor of T cell interactions, binds to the CD11a subunit of lymphocyte function-associated antigen 1 (LFA-1), thereby preventing LFA-1 binding to intercellular adhesion molecule 1. The PK and PD data from the efalizumab clinical development program were utilized to select of the optimal weekly subcutaneous dose of efalizumab (1.0 mg/kg) in adults [163].

Katz and coworkers proposed the use of LXR-623, a novel liver X-receptor agonist in atherosclerosis. They proposed that enhancing reverse cholesterol transport via the upregulation of cholesterol transporters such as the ATP-binding cassettes ABCA1 and ABCG1 could inhibit the progression of atherosclerosis. The authors found that LXR activation resulted in a dose-dependent increase in ABCA1 and ABCG1 expression [164].

Angus and coworkers made an attempt to rationalize the dosage of artesunate in patients with acute falciparum malaria. An inhibitory sigmoidal maximum effect ($E_{\max}$)

PD model typical of a dose–response curve was fitted to the relationship between dose and shortening of parasite clearance time (PCT). The $E_{\max}$ was estimated to be 28.6 mg/kg as an oral dose, and the 50% effective concentration was 1.6 mg/kg of body weight. They concluded that there was no reduction in PCT with the use of single doses of artesunate higher than 2 mg/kg, and therefore reflects the average lower limit of the maximally effective dose. This PK–PD relationship prevented the need to conduct highly expensive and long clinical studies [165].

Olsen *et al.* [166] used PK–PD modeling as a tool to predict the therapeutically effective plasma levels of antipsychotics. They predicted therapeutically effective steady-state plasma levels by means of conditioned avoidance response behavior in rodents and correlated it with clinically relevant plasma exposure for the classical antipsychotic drug haloperidol and four second-generation antipsychotics: sertindole, clozapine, risperidone, and olanzapine. The authors established the PK–PD relationship by modeling the time–response and time–plasma concentration data. Overall, a good correlation was observed between the rat dopamine D_2 receptor occupancy levels providing 50% response in the conditioned avoidance response test and the dopamine D_2 receptor occupancy levels reported from responding schizophrenic patients treated with antipsychotics. The proposed PK–PD model could act as a guide for determining effective plasma concentrations of potential antipsychotics in the clinical setting. This would hence accelerate the overall drug development process.

18.3.2 Current Gaps in Predicting PK–PD in Humans from Preclinical Studies

There are numerous challenges in the conduct and implementation of PK–PD data. The first major concern is that plasma concentrations may not necessarily reflect the concentrations of a drug at the site of action. This leads to lack of correlation between plasma concentrations and the drug effect leading to hysteresis or proteresis loops that can be resolved using indirect link models. To precisely describe the concentration–effect relationships, complicated PK–PD models have been proposed that require a thorough understanding of multiple receptors, presence of active metabolites, partial agonists, agonist/antagonist relationships, presence of endogenous agonists or antagonists, baseline effects, and baseline characterization. There is always a risk of development of tolerance that may be due to downregulation of receptors or change in the sensitivity of receptors. This may lead to tachyphylaxis and is an example of proteresis loop. This kind of tolerance has been observed with morphine. On the contrary, there may be sensitization or upregulation of receptors as that seen with cortisol and adrenocortico tropic hormone (ACTH) that would require more sophisticated modeling techniques to build a PK–PD correlation [167]. Other factors that affect the PD evaluation include status or disease state and genetic and environmental differences, which render establishment of a PK–PD correlation difficult. The most challenging aspect of PK–PD modeling is the correlation of PK–PD model with safety or efficacy outcomes. This involves a detailed knowledge of pathophysiology of disease and its progression. Data for disease progression in case of chronic diseases come from epidemiological studies that may not cover the underlying factors that play a role in the disease pathophysiology. For instance, in cardiovascular disease, the mortality outcomes have been correlated to a decrease in cholesterol levels or decrease in systolic and diastolic blood pressure. In contrast, both these endpoints are favorable outcomes and cannot be correlated to death in patients as an endpoint.

Likewise, in case of anti-HIV agents, some correlation has been observed between viral load and efficacy [168]. Such gaps that remain represent challenges to effective use of PK–PD correlations and their successful application in drug development.

18.3.3 Impact of OMICS and Other Novel Technologies on Future of PK–PD Assessment in Preclinical and Clinical Trials

The disciplines evolving from the sequencing of the human genome (genomics), transcriptomics [169], proteomics [170], and metabonomics [171,172] have the potential to facilitate drug discovery, but the greatest value lies in the integration of these diverse technologies. “Metabonomics” refers to the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification. It does not measure the metabolism of the compound itself but rather looks at the pattern of change in hundreds or thousands of endogenous biomolecules, which forms the “fingerprint” of efficacy or toxicity. Metabolomics is used for the metabolic regulation and fluxes in individual cells or cell types and the measurement thereof. Metabonomics data compliment gene expression and proteomic data. Metabonomics can provide information on a whole organism’s functional integrity over time after drug exposure. Metabonomics data can facilitate early *in vivo* toxicological testing, lead compound selection, prelead optimization, and *in vivo* toxicological testing. During development, the data can find new preclinical and clinical safety biomarkers and mechanisms and allow for metabotyping and the validation of animal models against human disease profiles [173]. Advances in “omics” technologies (genomics, transcriptomics, proteomics, and metabonomics) were touted as having the potential to revolutionize our approach to disease diagnosis, prognostication, and development of novel therapeutics. However, whether the “omics” can hold the promise of rapid advances in medicine “from the lab bench to the bedside” remains to be seen [174]. Although great progress has been made in this area, understanding processes such as disease progression (or drug response) requires systematic insight into dynamic (and temporal) differences in gene regulation, interaction, and function. Thus, an integration of these technologies with the PK and PD would fasten the path of drug development. In fact, the number of new drugs (NCEs) or biologics submissions to the FDA and other regulatory agencies worldwide has decreased significantly over the past decade. Current costs of taking an NCE to market has increased to nearly \$1 billion [175], which represents a barrier to investment in innovative therapeutics, particularly those with limited market value or diseases, which affect third-world or economically challenged populations. Today, the identification of disease-related genes and pathways is fundamental to drug-discovery programs. Although the sequence of the human genome is known, we have names/functions for only 15–20% of the ca. 32,000 genes. Till date, almost all the genes indicated for human hereditary (monogenic) diseases have been identified; however, the greater challenge will be to unravel the polygenic disease phenotypes and/or multifactorial diseases. Bioinformatics represents a novel way to expand our knowledge of diseases, link genotypes to phenotypes, unravel polygenetic disease causality, and identify new therapeutic targets. Application of these technologies should provide insight into the mechanistic basis for the disease pathobiology. The fine structure mapping aided by single nucleotide polymorphisms (SNPs) can provide a more detailed analysis. It is because of their dense distribution across the genome that SNPs are viewed as ideal markers for large-scale genome-wide

association studies to discover genes in a number of common complex diseases. High throughput SNP analysis technologies, which can analyze thousands of SNPs in large sample sets, provide an excellent tool for use in genetic association studies. Another area where SNP technology has a great impact is in pharmacogenomics, where genetic markers that predict response to therapeutic agents are sought. Pharmacogenetic approaches can aid the designing of strategies for reducing side effects of drugs, particularly for a percentage of the population prone to AEs. SNPs have also contributed to resolve safety issues as in the case of the HIV drug Abacavir that had exhibited hypersensitivity reactions in ~4% of the population receiving the drug post marketing. In case of HIV disease management, viral load and resistance genotyping has helped in adopting appropriate treatment regimens for the patients [176]. The application of genomics can contribute throughout the drug development process from initial discovery all the way through life-cycle management. The current most widely used technique for the study of gene-expression patterns on a genomic scale is DNA microarray. Microarrays allow for the simultaneous measurement of changes in the levels of thousands of messenger RNAs within a single experiment.

Systems biology is the analysis of relationships between a biological system and its response to genetic or environmental stimuli. Technically, systems biology involves modeling and simulating the complex dynamic interactions between genes, transcripts, proteins, metabolites, and cells using integrated systems-based approaches. It encompasses many of the “omic” technologies described above and uses computational and mathematical models to analyze and simulate networks or pathways. It also takes into account spatial and temporal relationships that give rise to cause and effect in biological systems. One approach is the development of “therapeutic engineering,” which is the application of the principles of mathematics, engineering, physics, chemistry, and biology to better understand human pharmacology, physiology, toxicology, and pathophysiology. Computational therapeutics or *in silico* pharmacology is concerned with the development of techniques for using software to collect, manipulate, and link biological and medical data. In current times, there is a costly, but bridgeable, gap in understanding processes such as disease progression (or drug response), which requires insight into dynamic (and temporal) differences in gene regulation, interaction, and function. There is a need to have fusion between mathematical modeling, informatics, and disease understanding to make the leap to effective acute and preventative care (effective total disease management). Use of the “omic” technologies can help facilitate the detection, diagnosis, and management of therapy. Therefore, the application of these technologies provides hope to allow accurate disease monitoring and paves way for improving treatment strategies so as to reduce patient morbidity and mortality in the coming future.

18.4 SUMMARY

Drug development is a sequential process from discovery to preclinical development, through clinical drug development and beyond. PK assessment involves *in vitro* testing during discovery stage, followed by preclinical development with substantial animal work, next clinical phase I in healthy volunteers, followed by phase II and III in patients with target disease. The role of PK, PD, and metabolism from an industry perspective is to allow rational selection of candidate compounds that can be taken forward for development as a therapeutic that can be used to treat patients through desired

route of administration. Modern drug research strategy for early discovery to late-stage development is to develop and utilize validated *in vitro* assay methodologies and *in vivo* animal models that are predictive of human ADME, efficacy, and safety. PK/PD analysis, modeling, and simulation is becoming increasingly important in drug labeling due to its potential for predicting drug behavior in populations that may be difficult to study in adequate numbers during drug development and its value in optimizing clinical trial designs. The ability to correlate drug effects and model these during the drug development value chain, from mechanistic studies in preclinical models, biomarkers in phase I, surrogate markers in phase II, and clinical endpoints in phase III are key to the success of any new drug research program.

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19 ADME of Herbal Dietary Supplements

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19.1	Summary	1
19.2	Introduction	3
19.3	Regulatory perspectives	4
19.4	General issues with assessment of herb–drug interactions	15
19.5	Underlying mechanisms for herb–drug interactions	17
19.6	Commonly used herbs in the United States of America	20
19.7	Conclusions and future perspectives	33
	Abbreviations	34
	References	34

19.1 SUMMARY

Herbal dietary supplements have been used and studied over 5000 years ago by ancient civilizations such as the Chinese, Indian, Greek, Roman, and Egyptian, among others, for the prevention and treatment of various ailments. Today, the most popular traditional healing systems that utilize plant extracts are Chinese herbal medicine (zhōngyào and pinyin), Indian (ayurveda and siddha), traditional African medicine, Graeco-Arabic medicine (unani tibb), Native American medicine, classical Greek and Roman herbal medicine, Japanese (kampo), and so on. In spite of modern medical advances, herbal dietary supplements continue to be widely used for health maintenance, disease prevention, and even disease treatment. Sales of herbal dietary supplements in the United States have increased from ~\$4 billion in 1999 to ~\$5 billion in 2009.

New herbal products get to the market much faster than pharmaceuticals, as in the United States, these are considered “dietary supplements” and are subject to the regulation specified in the Dietary Supplement Health and Education (DSHE) Act of 1994, which provides a very different framework for the regulation of herbal products

when compared with pharmaceutical drugs in terms of establishing efficacy, safety, and postmarketing surveillance. Herbal products may contain a single herb or combinations of several different herbs and are defined as such by both FDA (United States Food and Drug Administration) and EMEA (European Medicines Agency) regulatory agencies. Herbal dietary supplement manufacturers, while responsible for ensuring the safety of the herbal product, are not required to submit evidence of safety or efficacy to the FDA before commercialization. In addition, these regulations do not require that manufacturers report adverse events to the FDA and nor is there a guideline on how safety should be established. Hence, there is no impetus for manufacturers of herbal products to characterize the ADME properties of an herbal formulation or assess the potential for herb–drug interactions.

Approximately 15 million adults in the United States use herbal dietary supplements for the treatment of health conditions or for health maintenance; however, only one third inform their physician of this use. One misconception among the general population is that these agents lack adverse effects since they are obtained from a natural source. As a result, these herbal dietary supplements are often coadministered with prescription drugs (e.g., anticancer, anticoagulants, cardiovascular, and antidepressants), which increases the potential of pharmacokinetic and/or pharmacodynamic herb–drug interactions that are not apparent until there is a clinical manifestation. St. John’s wort (SJW), a popular herb used in the treatment of depression, has been implicated in a number of clinically significant interactions with pharmaceuticals. Coadministration of SJW with cyclosporine, an immunosuppressant that has a narrow therapeutic index has been fatal; patients who had undergone heart and kidney transplants and stabilized with cyclosporine experienced organ rejection following administration of SJW.

Herb–drug interactions, like drug–drug interactions, can occur via a number of mechanisms. A classical approach to the study of herb–drug interactions involves the assessment of the effect of the herbal dietary supplement on the absorption, metabolism, distribution, and excretion (ADME) of the drug versus the effect of drug on the biodisposition of the herb. The ADME mechanisms for the known herb–drug interactions involve, in most cases, inhibition or induction of hepatic and intestinal drug-metabolizing enzymes of the cytochrome P450 (CYP) family and/or drug transporters such as P-glycoprotein (P-gp). With the increased use of herbal supplements, the incidences of herb–drug interactions are increasing due to several reasons. These include easy availability of the herbs with no requirements for a prescription, lack of awareness of safety issues combined with the general perception that the natural origin of these herbs makes them safe for use, and sometimes the limitations of modern medicine in improving the quality of life which drives the patient to self-medicate themselves with herbal dietary supplements. Therefore, assessment of the safety, efficacy, and potential for herb–drug interactions is warranted during the development of pharmaceuticals. However, unlike evaluation of drug–drug interactions, determining the potential for herb–drug interactions can be quite challenging for various reasons, which has been covered later in this chapter.

This chapter will provide a review of the potential for herb–drug interactions based on the literature reports for those herbs that are most popularly used in the United States. The herbs covered are aloe vera, American and Asian ginseng, bitter orange, black cohosh, cranberry, curcumin, danshen, dong quai, echinacea, evening primrose oil, garlic, ginger, ginkgo, green tea, kava, licorice, milk thistle, saw palmetto, Siberian ginseng, SJW, and valerian. Clinical studies and relevant human *in vitro* studies that

have evaluated metabolism- or absorption-based mechanisms to understand the interactions between these herbs and commonly used drugs are reviewed.

19.2 INTRODUCTION

The study and the use of herbal dietary supplements dates back over 5000 years. Herbal products have been used by ancient civilizations such as the Chinese, Indian, Greek, Roman, and Egyptian, among others, for the prevention and treatment of various ailments. At present, the most popular traditional healing systems that utilize plant extracts include traditional Chinese medicine (TCM), traditional Indian medicine (TIM) (consisting of ayurveda and siddha), traditional African medicine, Unnani Tibb, Native American medicine, and classical herbal medicine (Greek and Roman), and so on. Among the traditional healing systems, TCM and ayurveda herbs are being evaluated for efficacy and safety profiles with acceptable science-based approaches [1,2]. Recently, danshen dripping pill, a TCM, passed US FDA phase II clinical trials for a cardiovascular indication and is currently posed to undergo phase III clinical trials [3]. Benefits of curcumin, an ingredient of turmeric, an ayurvedic herb, and a common ingredient in Indian cuisine are being assessed systematically and data from a few phase I trials support the subsequent phase II trials for a few different indications such as Alzheimer's disease [4] and colorectal cancer [5]. The principles of TCM are based on the harmony of two opposite forces, namely Yin and Yang. When Yin and Yang are in disharmony, it results in disease. Harmony is maintained through acupuncture, massages (e.g., Shiatsu), diet, and herbal medicines [6]. Ayurveda recognizes the connection between the body, mind, and soul and believes that a balance of these three aspects is required for optimal health [1], which is achieved through diet, herbs, exercise (yogasana for the body and the mind), massage, and meditation.

In the United States, dietary supplements are defined by the 1994 Dietary Supplement Health and Education Act (DSHEA) [7,8], as products that are not used exclusively as food but are intended to be consumed in addition to an individual's diet. The law states that dietary supplements are taken by mouth and contain one or more dietary ingredients. Examples of dietary ingredients include vitamins, minerals, herbs, or other biological material, amino acids, and enzymes. Dietary supplements are required to be clearly labeled and may be sold in the form of tablets, capsules, powders, liquids, extracts, or teas. These products are often labeled as not having been approved by the FDA or that the product is not to be used to treat or diagnose a disease; yet, they are commonly used for a wide range of purposes including treatment of a disease state. Some of the common reasons for consumption of dietary supplements in the United States include supplementing a necessary substance not found in large enough quantities in the diet (e.g., vitamins), as a prophylactic for a disease or condition, for boosting the immune system, or as a tonic for improving general health (physical state such as stimulating weight loss etc., or mental state such as sharpening memory, etc.), and sometimes also for the treatment of a disease state. Herbs are defined as a part of the plant such as roots, stems, tree bark, leaves, flowers, fruits, or seeds or a product prepared from these plant parts. Herbal products may contain a single herb or a combination of multiple herbs.

The 20 top-selling herbal dietary supplements in the United States in 2009 include cranberry, soy, saw palmetto, garlic, echinacea, ginkgo, milk thistle, SJW, ginseng,

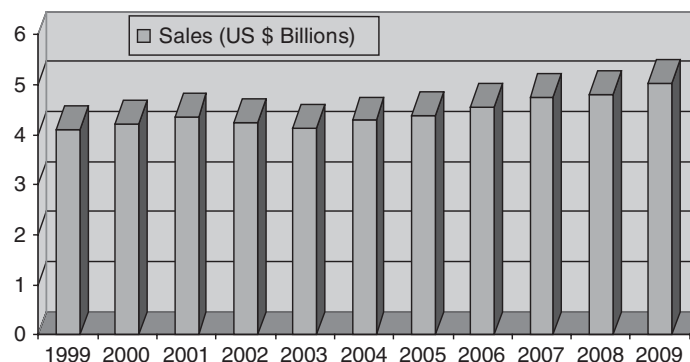


Figure 19.1 Herbal sales in United States from 1999 to 2009. *Source:* Adapted from *Herbal Gram*. 2010;86:62–65.

black cohosh, green tea, evening primrose, valerian, horny goat weed, bilberry, elderberry, grape seed, ginger, aloe vera, and horse chestnut [9]. Sales of herbal products has been on an upward trend from 2003 onward and increased from ~\$4.1 billion in 1999 to \$5.03 billion in 2009 as shown in Fig. 19.1.

Information presented in this chapter is limited to the commonly used herbal medicinal products that are commercially available in the United States. These will be referred to as *herbal dietary supplements* and do not include nutraceuticals such as vitamins and other dietary constituents. A list of the herbs covered herein for which absorption and/or metabolism-related interactions are reported with other commonly used drugs in humans, either *in vitro* or based on clinical data, are presented in Table 19.1.

19.3 REGULATORY PERSPECTIVES

Herbs are perceived as dietary supplements in the United States. A general outlook regarding herbs is that they are safe because they originate from a natural source. Hence, congressional action directed that herbs in the United States will be considered to be “dietary supplements” and will be regulated by the DSHEA of 1994, which was an amendment of the Federal Food, Drug, and Cosmetic (FDC) Act to establish standards with respect to dietary supplements and for other purposes [10]. Frankos *et al.* [11] have presented a concise review on FDA regulations pertaining to dietary supplements. It is interesting and important to understand the evolution of the federal regulations as it pertains to drugs, food, food additives, and dietary supplements and its role in ensuring public safety. Milestones in the history of the development of the current-day regulations are summarized below:

1. The history of the US federal regulation of foods and drugs began with the signing of the Pure Food and Drugs Act in 1906. This act sought to explicitly prohibit adulteration in foods, drinks, and drugs. In addition, this act provided regulations to ensure accurate labeling of products [12].
2. In 1938, “The Food, Drug, and Cosmetic Act” (FD&C Act) replaced the Pure Food and Drugs Act of 1906. The FD&C Act was the first law worldwide by

TABLE 19.1 List of Herbs with Absorption-Mediated or Metabolism-Mediated Effect of Herb on Commonly Used Drugs

Herb	Test System	Probe Drug	CYP/P-gp	Outcome	Mechanism	References
Asian ginseng (<i>Panax ginseng</i>)	<i>In vivo</i>	Debrisoquine	CYP2D6	Change in metabolite/parent ratio	Inhibition	Gurley <i>et al.</i> (2005) [67,79]
Black cohosh (<i>Actaea racemosa</i>)	<i>In vivo</i>	Debrisoquine	CYP2D6	Change in metabolite/parent ratio	Weak inhibition	Gurley <i>et al.</i> (2005) [67,79]
Curcumin (<i>Curcuma longa</i>)	<i>In vivo</i>	Caffeine	CYP1A2	Decreased metabolite formation	Inhibition	Chen <i>et al.</i> (2010) [97]
		Caffeine	CYP2A6	Increased urinary metabolite formation	Induction	
	<i>In vitro</i> (recombinant human CYP)	Benzyloxyresorufin	CYP2B6	Decreased metabolite formation	Inhibition	Appiah-Opong <i>et al.</i> (2007) [95]
		Diclofenac Benzyloxyresorufin, dibenzylfluorecein	CYP2C9 CYP3A4		Inhibition Inhibition	
	<i>In vitro</i> (human liver microsomes, human liver cytosol for SULT and LS180 cells for SULT and UGT)	Phenacetin	CYP1A2	Decreased metabolite formation in the presence of curcumin, curcuminoid mixture, and curcuminoid extract	Weak inhibition	Volak <i>et al.</i> (2008) [96]

(continued overleaf)

TABLE 19.1 (Continued)

Herb	Test System	Probe Drug	CYP/P-gp	Outcome	Mechanism	References
Danshen (<i>Salvia miltiorrhiza</i>)	<i>In vivo</i>	Bupropion	CYP2B6		Strong inhibition	Izzat <i>et al.</i> (1998) [99]
		Flurbiprofen	CYP2C9		Moderate inhibition	
		S-mephenytoin	CYP2C19		Strong inhibition	
		Dextromethorphan	CYP2D6		Weak inhibition	
		Triazolam	CYP3A4		Moderate inhibition	
		Acetaminophen	SULT		Strong inhibition	
Dong quai (<i>Radix angelica</i>)	<i>In vitro</i> (human liver microsomes)	Acetaminophen	UGT		Strong inhibition	Qui <i>et al.</i> (2010) [100]
		Warfarin	CYP2C9	Enhanced anticoagulation	Inhibition	
		Midazolam	CYP3A4	Increased oral clearance	Induction	
		Phenacetin	CYP1A2	Decreased metabolite formation	Inhibition	
Echinacea (<i>Echinacea purpurea</i>)	<i>In vivo</i>	Bupropion	CYP2B6	Decreased metabolite formation	Inhibition	Sevior <i>et al.</i> (2010) [101]
		Omeprazole	CYP2C19			Sevior <i>et al.</i> (2010) [101]
Garlic (<i>Allium sativum</i>)	<i>In vivo</i>	Caffeine	CYP1A2	Change in metabolite/parent ratio	Inhibition	Gorski <i>et al.</i> (2004) [104]
		Midazolam	CYP3A4		Induction	Gurley <i>et al.</i> (2005) [67,79]
		Chlorzoxazone	CYP2E1	Change in metabolite/parent ratio	Inhibition	

Ginkgo (<i>Ginkgo biloba</i>)	<i>In vitro</i> (recombinant human CYP)	7-Benzoyloxyresorufin	CYP3A4P-gp	Decrease in metabolite formation (CYP3A) and stimulation of ATPase assay (P-gp)	Inhibition	Foster <i>et al.</i> (2001) [123]
	<i>In vivo</i>	Nifedipine	CYP3A4	Increase parent plasma concentration	Inhibition	Smith <i>et al.</i> (2001) [66]
		Midazolam	CYP3A4	Decreased AUC and $C_{\max}$ for parent	Induction	Robertson <i>et al.</i> (2008) [168]
		Fexofenadine	P-gp	Reduction in $T_{\max}$	Induction	Fan <i>et al.</i> (2009) [171]
		Talinolol	CYP3A4	Increased AUC, $C_{\max}$, and $t_{1/2}$ for parent	Inhibition	
		Valproic acid and phenytoin	CYP2C19	Increased parent plasma concentrations	Inhibition	Kupiec <i>et al.</i> (2005) [162]
		Omeprazole	CYP2C19	Decreased parent/metabolite ratio	Induction	Yin <i>et al.</i> (2004) [163]
		Midazolam	CYP3A	Increased AUC for parent	Inhibition	Uchida <i>et al.</i> (2006) [169]
		Tolbutamide	CYP2C9	Decreased AUC for parent	Induction	Smith <i>et al.</i> (2001) [66]
		Nifedipine	CYP3A4	Increased $C_{\max}$	Inhibition	

(continued overleaf)

TABLE 19.1 (Continued)

Herb	Test System	Probe Drug	CYP/P-gp	Outcome	Mechanism	References
Green tea	<i>In vitro</i> (recombinant human CYP)	7-Ethoxy-3-cyanocoumarin	CYP1A2	Decreased metabolite formation	Strong inhibition	Zou <i>et al.</i> (2002) [154]
		7-Methoxy-4-trifluoromethylcoumarin	CYP2C9		Strong inhibition	
		7-Hydroxy-3-cyanocoumarin	CYP2C19		Strong inhibition	
	<i>In vitro</i> (human liver microsomes)	Paclitaxel	CYP2C8	Decreased metabolite formation	Moderate inhibition	Etheridge <i>et al.</i> (2007) [155]
		S-warfarin	CYP2C9		Moderate inhibition	Mohutsky <i>et al.</i> (2006) [156]
		Testosterone	CYP3A4		Moderate inhibition	He and Edeki (2004) [157]
	<i>In vitro</i> (human liver microsomes)	Irinotecan	CYP3A4	Decreased metabolite formation	Inhibition	Mirkov <i>et al.</i> (2007) [195]
		Tolbutamide	UGT1A1 CYP2C9	Decreased metabolite formation	Inhibition	Nishikawa <i>et al.</i> (2004) [196]
Kava	<i>In vivo</i>	Bufuralol Testosterone Chlorzoxazone	CYP2D6 CYP3A4 CYP2E1	Decreased serum ratio of metabolite/parent	Inhibition	Gurley <i>et al.</i> (2005) [67,79]

Licorice (<i>Glycyrrhiza glabra</i>)	<i>In vitro</i> (recombinant human CYP)	7-Ethoxy-4-trifluoromethyl coumarin	CYP2B6	Time- and concentration-dependent inactivation	Inhibition and inactivation	Kent <i>et al.</i> (2002) [209]
		7-Benzoyloxy-trifluoromethyl coumarin	CYP3A4			
		7-Ethoxy-4-trifluoromethyl coumarin	CYP2C9			
	<i>In vitro</i> (human liver microsomes)	Benzoyloxyresorufin	CYP3A	Decreased metabolite formation	Inhibition	Budzinski <i>et al.</i> (2000) [210]
	<i>In vivo</i>	Metronidazole	P-gp	Decreased serum concentration and AUC of metronidazole	Induction	Rajnarayana <i>et al.</i> (2004) [226]
		7-Benzoyloxy-trifluoromethyl coumarin	CYP3A4	Mechanism-based inhibition	Inhibition	Sridar <i>et al.</i> (2004) [234]
	<i>In vitro</i> (recombinant human CYP)	7-Ethoxy-4-trifluoromethyl coumarin	CYP2C9	Decreased metabolite formation	Inhibition	Venkataramanan <i>et al.</i> (2000) [233]
		Testosterone	CYP3A4			
		4-Methyl umbelliferone	UGT1A6/9			
	<i>In vitro</i> (human hepatocytes)	Testosterone	CYP3A4	Decreased metabolite formation	Inhibition	Venkataramanan <i>et al.</i> (2000) [233]

(continued overleaf)

TABLE 19.1 (Continued)

Herb	Test System	Probe Drug	CYP/P-gp	Outcome	Mechanism	References
Saw palmetto (<i>Serenoa repens</i>)	<i>In vitro</i> (recombinant human CYP)	7-Methoxy-4-trifluoromethyl coumarin	CYP2C9	Decreased metabolite formation	Inhibition	Yale <i>et al.</i> (2005) [241]
		3-[2-(<i>N, N</i> -diethyl- <i>N</i> -methyl amino)ethyl]-7-methoxy-4-methyl coumarin	CYP2D6			
		7-Benzoyloxy trifluoromethyl coumarin	CYP3A4			
St. John's wort (SJW)	<i>In vivo</i>	Chlorzoxazone	CYP2E1	Decrease in ratio of metabolite/parent	Induction	Gurley <i>et al.</i> (2002) [118,180]
		Midazolam	CYP3A4	Decrease in ratio of metabolite/parent	Induction	Rengelshausen <i>et al.</i> (2005) [252]
		Voriconazole	CYP2C19			
		Digoxin	P-gp	Change in apparent permeability	Induction	Gurley <i>et al.</i> (2008) [80,106,200,230]; Johne <i>et al.</i> (1999) [47]; Mueller <i>et al.</i> (2004) [264]
		Fexofenadine	P-gp	Change in apparent permeability	Induction	Dresser <i>et al.</i> (2003) [265]; Wang <i>et al.</i> (2002) [48,266]

Talinolol	P-gp	Change in apparent permeability	Induction	Schwarz <i>et al.</i> (2007) [267]
Tacrolimus	CYP3A4 and P-gp	Increased clearance of parent drug	Induction	Mai <i>et al.</i> (2003) [269]
Imatinib	CYP3A4 and P-gp	Increased clearance of parent drug	Induction	Frye <i>et al.</i> (2004) [270]; Smith <i>et al.</i> (2004) [271]
Irinotecan	CYP3A4	Increased clearance of the active metabolite	Induction	Mathijssen <i>et al.</i> (2002) [272]
Indinavir	CYP3A4	Decreased plasma concentration of parent	Induction	Piscitelli <i>et al.</i> (2000) [273]
Nevirapine	CYP3A4	Increased oral clearance	Induction	Erickson <i>et al.</i> (1999) [274]
Nifedipine	CYP3A4	Increased oral clearance	Induction	Wang <i>et al.</i> (2009) [276]
Verapamil	CYP3A4	Increased oral clearance	Induction	Tannergren <i>et al.</i> (2004) [277]
Atorvastatin	CYP3A4	Increased oral clearance	Induction	Andre'n <i>et al.</i> (2007) [278]
Simvastatin	CYP3A4	Increased oral clearance	Induction	Sugimoto <i>et al.</i> (2001) [279]
Norethindrone and ethinylestradiol	CYP3A4	Increased oral clearance	Induction	Hall <i>et al.</i> (2003) [281]
Norethindrone and ethinylestradiol	CYP3A4	Increased oral clearance	Induction	Murphy <i>et al.</i> (2005) [283]

(continued overleaf)

TABLE 19.1 (Continued)

Herb	Test System	Probe Drug	CYP/P-gp	Outcome	Mechanism	References
		Ketodesogestrel and ethinylestradiol	CYP3A4	Increased oral clearance	Induction	Pfrunder <i>et al.</i> (2003) [282]
		Fexofenadine	P-gp	Decreased plasma concentrations	Induction	Dresser <i>et al.</i> (2003) [265]; Wang <i>et al.</i> (2002) [48,266]
		Omeprazole	CYP3A4	Increased formation of CYP3A4-mediated sulfoxidation and decreased concentration of parent	Induction	Wang <i>et al.</i> (2004) [286]
			CYP2C19	Increased formation of CYP2C19-mediated hydroxylation and decreased concentration of parent	Induction	

	<i>In vitro</i> (recombinant human CYP)	7-Benzoyloxy trifluoromethyl- coumarin	CYP3A4	Decreased metabolite formation	Inhibition	Zou <i>et al.</i> (2002) [154]
		3-[2-(<i>N</i> , <i>N</i> -di-ethyl- <i>N</i> -methyl amino) ethyl]-7-methoxy- 4-methylcoumarin	CYP2D6			
		7-Ethoxy-3- cyanocoumarin	CYP1A2			
		7-Methoxy-4- trifluoromethyl coumarin	CYP2C9			
		7-Ethoxy-3- cyanocoumarin	CYP2C19			
Valerian	<i>In vitro</i>	Dibenzylfluorescein	CYP3A4	Decreased metabolite formation	Inhibition	Lefebvre <i>et al.</i> (2004) [290]

the FDA to require scientific safety testing before a drug could be approved for marketing and it placed the burden of proof on the manufacturer to do so [13]. This act was instituted in response to a 1937 tragedy when a Tennessee drug company marketed a new pediatric formulation of sulfanilamide in which diethylene glycol (DEG) was added as a solubilizer, which also served to make its taste appealing to the pediatric patients [14]. DEG had never been tested for safety before its use in this product and was responsible for over 100 deaths. In addition, the FD&C act established a category of foods for special dietary use and required that the labeling include information on the vitamin, mineral, or other dietary content.

3. Following the enactment of the FD&C Act of 1938, FDA attempted to regulate dietary supplements [15]. One approach undertaken was to classify these products as drugs based on health claims made on the label, while another was to publish regulations which required that the dietary supplement products be declared as drugs if containing potencies exceeding 150% of the US recommended daily allowance. This was followed by the vitamin and mineral legislation (the Rogers/Proxmire amendment) in 1976, prohibiting the FDA from limiting the potency or nutrient combination of vitamin and mineral supplements and from classifying them as drugs based solely on their potency or combination.
4. Another landmark in the legislative history of dietary supplements is the 1990 "Nutrition Labeling and Education Act" (NLEA) [16]. It required that the labels of all packaged foods contain nutrient information and allowed the FDA to establish the appropriate scientific evaluation and approval standards needed. The FDA chose to ensure that standards set for dietary supplements would be similar to that needed for drug approvals (Dietary Supplement Act of 1992). As required by this act, the FDA put forth "A Notice of Proposed Rule" (ANPR) in 1993. The guidelines within this act regulated certain dietary supplements as drugs. The ANPR elicited considerable protest from the public and the dietary supplement industry because FDA appeared to be reproposing regulatory provisions withdrawn or struck down by court actions in previous years. The ANPR was a significant motivating factor in industry and congressional effort to develop and pass the DSHEA of 1994.
5. The purpose of DSHEA was to allow the public increased access to these products that would otherwise have been curtailed if they had been subject to the same regulations as the pharmaceuticals, while still allowing the FDA to intervene if there are safety issues, false claims, or adulteration. These regulations provide a very different platform compared to the pharmaceuticals for establishing efficacy and safety and for conducting postmarketing surveillance. Under this Act, manufacturers of herbal products are responsible for ensuring the safety of the herbal product; however, they are not required to provide proof of safety and efficacy to make certain limited claims regarding the use of the herb. Also, these regulations do not require that manufacturers report adverse events to the FDA, and they do not specify how safety should be established. Hence, there is no impetus for manufacturers of herbal products to characterize the potential for herb-drug interactions.

Key features of this act include the specific categorization of dietary supplements as food; clarification that dietary supplements are not food additives and

hence not subject to premarket safety testing and FDA approval, ensures the current good manufacturing practice (cGMP) regulations for dietary supplements and allows certain statements of well-being claims without obtaining premarket authorization from the FDA. The dietary supplement and nonprescription drug consumer protection act of 2006 required dietary supplement companies to report adverse events to the FDA and since 2008 to list an address or phone number on dietary supplement product labels that customers can use to report serious adverse events and report to the FDA.

The DSHEA defines a dietary supplement as “a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: (A) a vitamin; (B) a mineral; (C) an herb or other botanical; (D) an amino acid; (E) a dietary substance for use by man to supplement the diet by increasing the total dietary intake; or (F) a concentrate, metabolite, constituent, extract, or combination of any ingredient described in clause (A), (B), (C), (D), or (E).” A dietary supplement is further defined as “a product that is intended for ingestion in the form of capsule, powder, soft gel, gel cap or other form, if not represented for use as a conventional food item or as a sole item of a meal or a diet, and contains one or more dietary ingredients and is labeled as a “dietary supplement”.”

6. To address the deficiencies within the DSHEA, Gershwin *et al.* [14] have put forth a proposal to have dietary supplement manufacturers submit human safety data for premarket approval by the FDA. Their proposal has a number of benefits which include allowing the FDA to fulfill its mission of consumer protection for dietary supplements, to address concerns of the mainstream medical community, to minimize toxicities, to resolve consumer confusion, and to restore consumer confidence. In addition, Collins *et al.* [17] reported differences between dietary supplement containing ω -3 fatty acid and those ω -3-fatty acid formulations that are available by prescription, thereby further emphasizing the need for dietary supplements to be regulated in a manner similar to pharmaceutical drugs.

19.4 GENERAL ISSUES WITH ASSESSMENT OF HERB-DRUG INTERACTIONS

It has been a regulatory requirement to conduct different *in vitro* and, if necessary, clinical studies to assess a drug's potential for causing interactions with other drugs that are typically coadministered. This information is presented in the label of the drug product. However, a quick review of the drug product labels for products entering market in the past five years demonstrates that information regarding the potential for adverse effects or lack of efficacy due to interaction of the drug with an herbal dietary supplement arising from their concomitant use is lacking. Bent and Ko [18] have reviewed the contributing factors for the challenges associated with obtaining improved confidence with herbal products.

When approaching the study of herb-drug interaction, there are two main questions. The first is what does the drug do to the biological fate of the herb? Given the extent of information currently available, this question is rather difficult to answer, as the biodisposition of the herb including the pharmacokinetic (PK) characteristics is often not very well characterized. The second question is what does the herb do to the

biodisposition of the drug? Theoretically, this question can be addressed, albeit not without significant challenges (presented below) to interpreting the data in a clinically relevant manner and these are listed below.

Most herbal dietary supplements are isolated from a natural source such as roots, tree bark, stem, leaves, flowers, fruits, or seeds by one or more processes of extraction. As a result, they contain multiple chemical constituents that may include alkaloids, glycosides, flavonoids, sterols, saponins, tannins, and terpenes [19]. In most cases, more than one compound contributes to the therapeutic activity. Some herbal products also contain multiple herbs as it is believed that they exert complementary effects. In most cases, the active ingredient(s) is not known. Hence, assessment of herb–drug interaction is not quite relevant to the clinical scenario if performed with just a single compound that is known to be the lead compound for eliciting pharmacological effect. Therefore, a mixture of all the chemical constituents that is present in the final herbal dietary supplement needs to be utilized in the assessment of interaction potential to be representative of the clinical scenario. However, it is not an easy task to maintain consistency with respect to the type of chemical constituents and their concentrations between batches.

1. *Interbatch Consistency for Potency:* Several environmental factors, such as the location of the plant, altitude, the quality of the soil, variation in temperature, extent of rainfall, shade, dew, humidity, frost, as well as chemical treatment (fertilizers, pesticides, antimicrobials, etc.) can influence the chemical composition that is found in the plant extract. Hence, it is very common to have batch-to-batch variation with respect to both the types of compounds present in the extract as well as their concentrations. For example, analysis of 25 ginseng products that were commercially available demonstrated a 15- to 200-fold variation in the concentration of the two ingredients that are believed to be active [20]. Some manufacturers utilize the process of standardization, whereby several batches of the herb containing differing amounts of the active ingredient are combined in an effort to obtain the desired concentration of the active ingredient in the product. While such an approach provides for minimal interbatch variation in the dose of the active ingredient, the rest of the presumed nonactive components remain highly variable and the risk for alteration in the biodisposition and safety of the coadministered drug still exists, if the rest of the compounds in the herbal product contribute to an herb–drug interaction.
2. *Contaminants:* Contamination and adulteration of herbal products have been reported to be an issue after chemical analysis of some TCMs and ayurvedic herbal products [21–26]. In particular, these preparations were found to contain lead, mercury, and arsenic at levels that would result in serious health problems if taken at recommended doses. The contamination of herbals with pesticides, bacteria, molds, fungi, and mycotoxins can also result in health issues [27]. These contaminants present the potential for alteration of the true potential of the herbal product to interact with the drug-metabolizing enzyme being assessed and should be considered in the interpretation of data.
3. Most clinical trials that have been conducted with herbal products offer limited utility due to small sample size, less rigorous study design, and most importantly use of products where the composition is not very well defined [28].

4. The percentage of patients who self-medicate with herbal products and volunteer the information to physicians is alarmingly low presumably due to the perception that they are taking something perceived to be “safe” [29,30]. Hence, it is very likely that a clinically reported adverse event that may have been caused due to alteration of the drug PK by the herb would not be investigated as an herb–drug interaction due to lack of accurate patient history.
5. The regulatory status that herbs are assigned in the United States, that is, as dietary supplements, does not require manufacturers of herbal products to evaluate the potential for herb–drug interactions.
6. Assessing the PK of herbal drugs is fairly complex due to multiple bioactive compounds in the product as well as due to the dose of a single compound being in the lower milligram range due to which the plasma concentrations are often very low (picogram to nanogram per liter). Hence, the bioanalytical methods need to be very sensitive to assess the PK of the major constituents of the herb.

These facts make evaluation of herb–drug interaction relatively challenging when compared to the typical assessment of drug–drug interaction(s) during the development process of a pharmaceutical drug product.

19.5 UNDERLYING MECHANISMS FOR HERB–DRUG INTERACTIONS

For most herb–drug interactions, the potential for such an interaction is not known until it manifests clinically in the form of adverse effects or lack of pharmacology of either the drug or the herb. Theoretically, both PK and pharmacodynamic (PD) mechanisms may be implicated; however, this chapter focuses on the PK-based mechanisms only. Alteration in the PK of the drug due to interaction with the herb can occur at the level of ADME. This chapter focuses on the herb–drug interactions that arise out of interaction of the herb with a transporter for which the drug is also a substrate or those which are metabolism mediated. The PK profile of many drugs is known to be altered by coadministration of herbal products which is mediated by the herb by either altering the absorption of the drug or by inhibiting or inducing the drug-metabolizing enzymes, most commonly the CYP enzyme(s), which contribute to the metabolism of the drug. Butterweck and Derendorf [31] have presented a review article on this subject.

19.5.1 Absorption-Based: Herb–Drug Interactions due to Association with Transporters

Clinically reported interactions of herbal dietary supplements with concomitant administration with drugs have been reported for several drugs. Examples include cyclosporine, tacrolimus, imatinib, irinotecan, digoxine, fexofenadine, simvastatin, saquinavir, and indinavir, which are known to be substrates for P-gp, also known as *multidrug resistance protein* (MDR1) or ABCB1, a well-researched drug transporter protein [32] that is described in the chapter titled *ABC Drug Transporters and their Impact on Drug Disposition/Drug Sensitivity and Resistance*. P-gp mediates the ATP-dependent efflux of drugs from cells, is expressed in a number of tissues, and is responsible for the transport of an extremely large number of substrates [33]. P-gp presents a high transport capacity and broad substrate specificity: a number of

clinically relevant drugs with structurally different features and belonging to different classes (examples include digoxin, loperamide, berberine, irinotecan, doxorubicin, vinblastine, paclitaxel, and fexofenadine) can be effluxed by P-gp [34]. Substrates of P-gp are generally hydrophobic molecules either cationic or neutral [33–35].

Some P-gp drug substrates are able to inhibit P-gp-mediated transport of other substrates leading to potential drug–drug interactions [35–38]. A number of herbal supplements used by cancer patients in combination with cancer drugs have been reported to modulate P-gp expression and/or activity [39]. Piperine from black pepper and silymarin from milk thistle were reported to inhibit P-gp activity *in vitro* [39]. Curcumin from turmeric and several catechins from green tea were shown to reduce P-gp expression and activity *in vitro* [40]. Using the *in vitro* caco-2 cell model, Hou *et al.* reported that 30 μ M concentration of curcumin increased the accumulation of rhodamine 123 (a P-gp substrate) by approximately twofold in the receiver cell similar to verapamil, a known P-gp inhibitor [41]. In this study, curcumin inhibited P-gp expression and rhodamine efflux by about 30% and the authors proposed that curcumin inhibits the AP-1 (activator protein-1) transcriptional factor and NF- κ B (nuclear factor- κ B), which regulates MDR1 expression. Terhaag and colleagues conducted a clinical study with a randomized, open-labeled design using 12 healthy volunteers and a wash out period of one week between the administration of a single oral dose of 50 mg talinolol and the concomitant administration of curcumin (300 mg/day for six days) and a single oral dose of 50 mg on day 7 [42]. The effect of curcumin administration on talinolol PK was a 53% increase in oral clearance and decreases in both area under the concentration curve (AUC) and $C_{\max}$ (by 33 and 28%) compared to administration of talinolol alone. The authors attributed the observed decrease in talinolol AUC and $C_{\max}$ to low intraluminal concentration of curcumin or to upregulation of ATP-binding cassette transporters such as MRP2. One of the challenges with predicting absorption-related interactions is sometimes the lack of *in vitro* to *in vivo* correlation as demonstrated by the talinolol—curcumin example. Han and colleagues [43] conducted a clinical study to investigate the effect of concomitantly administered silymarin on the PK of talinolol in healthy Chinese volunteers and its association with a *MDR1* C3435T genetic polymorphism. Eighteen healthy adult men (six *MDR1* 3435CC homozygotes, six *MDR1* 3435CT heterozygotes, and six *MDR1* 3435TT homozygotes) were recruited in a two-phase, randomized, single-blind, crossover design. The PK of talinolol was measured after coadministration of placebo or 140-mg silymarin capsules three times daily for 14 days. The peak plasma concentration ($C_{\max}$) of talinolol was significantly higher after silymarin administration as compared with placebo ($p = 0.007$). The area under the plasma concentration-time curve of talinolol from 0 to 36 h (AUC_{0-36h}) and when extrapolated to infinity ($AUC_{0-\infty}$) was increased by $36.2 \pm 33.2\%$ and $36.5 \pm 37.9\%$, respectively, by silymarin coadministration. The oral clearance (CL/F) of talinolol was decreased by $23.1 \pm 16.6\%$ ($p < 0.001$) during the silymarin-treated phase. No change in the time to peak concentration ($t_{\max}$) and the blood elimination half-life ($t_{1/2}$) of talinolol was observed between the placebo and silymarin-treated phases.

Studies indicate that P-gp expression, similar to CYP3A, is inducible. CYP enzymes and P-gp are regulated via the pregnane X-receptor (PXR), which controls the transcription of their genes (P-gp encoded by *MDR1*). Herbal dietary supplements can modify the first-pass effect for drugs through changes in the PXR activity or by direct competitive or allosteric interference at the active substrate-binding regions of CYP enzymes and transport proteins [44].

SJW induces the activity of intestinal CYP3A4 and P-gp via the common regulator PXR [45]. In addition, some SJW constituents inhibit CYP isoenzymes (1A2, 2C9, 2C19, 2D6, and 3A4) [46]. Therefore, an initial inhibition of human CYP3A4 by SJW leads at first to an increased bioavailability of CYP3A4/P-gp substrates, such as digoxin or fexofenadine [47,48], but is then followed by a decrease in bioavailability due to enzyme induction. Hyperforin has been identified as the constituent in SJW responsible for PXR-mediated stimulation of CYP and P-gp. Guggul (*Commiphora mukul*), a resin, from the mukul tree is approved as a cholesterol-lowering drug in India and is known to induce the expression CYP3A4 and P-gp to a similar extent to SJW [49].

Cell lines such as caco-2 that express P-gp and inside-out membrane vesicles prepared from these cell lines can be used to determine whether a drug is a P-gp substrate or inhibitor. Mice deficient in Mdr1a or Mdr1a/b are also used for assessing the role of P-gp *in vivo*. Knockout mice may be used as a reference for complete inhibition of P-gp [50]. The clinical significance of a P-gp inhibitor can be investigated in humans by assessing P-gp-mediated clearance or exposure using digoxin as a model probe substrate [51].

Mardin–Darby canine kidney (MDCK) cell lines stably expressing organic anion transporters (OATs) such as OAT1, OAT3, and OAT4 have been used to assess *in vitro* the potential for renal transport of anionic drugs [52]. Mouse knockout models (OATP1b) for *in vivo* experiments have also been developed and reflect more closely activity associated with OATP1B1 and OATP1B3, respectively [53]. Aristolochic acids present in some TCM preparations have been shown to inhibit human OAT1, OAT3, and OAT4 [54].

In vitro cell-based and *in vivo* mouse-based knockout mice are used to assess substrates and inhibitors of the breast cancer resistance protein (BCRP) transporter [53]. Tamaki *et al.* [55] evaluated the inhibitory effects of nine herbal extracts on BCRP-mediated transport using *in vitro* membrane vesicles isolated from mammalian cells expressing wild-type BCRP (SB-BCRP-M-VT) and methotrexate as a BCRP-specific probe substrate. Extracts of soybean, black cohosh, rutin, and passion flower almost completely inhibited BCRP-mediated transport of methotrexate at 100 μ M and chlorella, milk thistle, and Siberian ginseng inhibited BCRP-mediated transport of methotrexate by 22, 45, and 36%, respectively [55].

19.5.2 Metabolism-Based: Herb–Drug Interactions due to CYP Inhibition

There have been several clinical reports of herb–drug interactions arising due to inhibition of one or more of the CYPs commonly responsible for drug metabolism. Typically, an increase in side effect(s) will be expected if an herb has the potential to inhibit metabolism of a drug, thereby decreasing metabolic clearance of the drug and increasing plasma drug concentration. The commonly used *in vitro* and *in vivo* methods of assessing the potential for drug–drug interaction [56] can also be employed to evaluate the potential for herb–drug interaction with the exception of *in silico* methods. Since the utility of *in silico* methods to predict the binding properties of ligands to mammalian CYPs lies in having knowledge of the chemical structure of the bioactive molecule (drug/herb), the identity of the pharmacologically active molecule in the herb needs to be known. Since most herbs contain multiple bioactive compounds,

application of *in silico* methods and data interpretation to predict clinical herb–drug potential is not as straightforward.

19.5.3 Metabolism-Based: Herb–Drug Interactions due to CYP Induction

If an herb causes induction of a CYP that is responsible for the metabolism of the drug, it will result in increased metabolic clearance of the drug. Typically, this results in reduced pharmacological effect or lack of therapeutic effect of the drug, since, with a few exceptions, most metabolites are usually less pharmacologically active than the parent drug. Although the commonly employed experimental methods can be used to assess herb–drug interactions, the limitations posed due to the inherent nature of the herbal products applies here as well, as discussed above in Section 19.3. Examples of drug interactions that occur due to induction of CYP enzymes after administration of herbal products, such as SJW, are reported on the FDA website. The warning reported on the FDA web site for SJW is “This herb is considered an inducer of liver enzymes, which means it can reduce the concentration of medications in the blood. SJW can reduce the blood level of medications such as Lanoxin, the cholesterol-lowering drugs Mevacor and Altocor (lovastatin), and the erectile dysfunction drug Viagra (sildenafil)” [57].

19.6 COMMONLY USED HERBS IN THE UNITED STATES OF AMERICA

The 20 top-selling dietary supplements in the United States according to information resources incorporated in 2009 include cranberry, soy, saw palmetto, garlic, Echinacea, ginkgo, milk thistle, SJW, ginseng, black cohosh, green tea, evening primrose, valerian, horny goat weed, bilberry, elderberry, grape seed, ginger, aloe vera, and horse chestnut [9]. The market information firm SPINS of Schaumburg, Illinois, has a slightly different list of top 20 selling dietary supplements for 2009 and include aloe vera, flaxseed, wheatgrass, acai, turmeric, milk thistle, stevia, elderberry, saw palmetto, Echinacea, garlic, Echinacea with goldenseal combination, oregano oil, valerian, ginkgo, chlorophyll/chlorella, black cohosh, cranberry, evening primrose, and green tea [9]. In this chapter, we have covered a number of the top 20 selling herbal dietary supplements from both sources and in addition covered popular traditional Chinese herbals, dan-shen and dong quai. A more recent comprehensive review of clinical evidence for drug–herb interactions has been presented in a review article by Kennedy and Seely [58], while Izzo and Ernst [59] have reviewed preclinical data to determine the potential for herb–drug interaction in addition to the clinical data. Relevant herb–drug interactions reported in the literature in humans, *in vitro* and clinically, are summarized in Table 19.1.

19.6.1 Aloe (*Aloe vera*, Family Asphodelaceae)

The succulent leaf of *Aloe vera* contains clear gel which has skin healing and soothing properties in addition to moisturizing when used topically for minor cuts and burns including sunburns [60,61]. *A. vera* juice or capsules are also being used orally in the treatment of diabetes, asthma, epilepsy, and osteoarthritis because of its anti-inflammatory effect [62,63]. This herb is very widely used orally as an adjunct treatment

of diabetes II; there are no published clinical reports of ADME-based interactions with other drugs.

19.6.2 American and Asian Ginseng (*Ginseng* sp., Family Araliaceae)

The root of ginseng is believed to have a number of different therapeutic uses and is most often used in dried form, either whole or sliced. Ginseng leaf is sometimes used, also in the dried form. The Asian ginseng (*Panax ginseng*) and the American ginseng (*Panax quinquefolius*) are taken orally and used as nourishing stimulants (such as in energy drinks), anti-inflammatory, for prevention of cancer, and in the treatment of erectile dysfunction in men due to their vasodilating effect [64]. The active ingredients are ginsenosides.

In vitro assessment of some of the active ingredients of ginseng in cDNA-expressed CYP system showed that ginsenoside Rd caused weak inhibitory activity against CYP3A4 (with IC₅₀ values of 58 and 74 μ M for two different substrates) and CYP2D6 (IC₅₀ of 76 μ M) and even weaker inhibition of CYP2C19 and CYP2C9 activities with IC₅₀ values over 100 μ M, while ginsenoside Rc increased the activity of CYP3A4 (~1.5-fold) and CYP2C9 (~1.7-fold), although assay artifact was not completely ruled out [65]. Clinical studies have shown that ginseng does not have the potential to cause CYP- or P-gp-based drug interactions [66–71] except for some slight changes observed in metabolite-to-parent ratio when using debrisoquine as a CYP2D6 probe after pretreatment with *P. ginseng* in a study reported by Gurley *et al.* [67]. It has been reported clinically that concomitant use of *P. ginseng* and phenelzine resulted in mania for which the mechanistic basis is not known, however, thought to be pharmacological [72,73]. A few clinical cases have been reported of ginseng affecting platelet aggregation when coadministered with warfarin, which are considered to have a pharmacological basis [74].

19.6.3 Bitter Orange (*Citrus aurantium*, Family Rutaceae)

Bitter orange is included in several herbal weight loss products and is believed to possess antioxidant properties, although a literature review reported by Bent *et al.* [75] determined that the weight loss produced by treatment with bitter orange was not statistically significant. The effect of bitter orange on the CYP isozymes was evaluated by Gurley *et al.* [76] after daily dosing for 28 days, and it was observed that there was no change in the CYP activity.

19.6.4 Black Cohosh (*Actaea racemosa*, Family Ranunculaceae)

The dried roots of Black cohosh (formerly known as *Cimicifuga racemosa*) contain glycosides, isoferulic acids with anti-inflammatory effects, and phytoestrogens, among several other active substances [77]. Hence, it is often used as an antirheumatic and antispasmodic as well as to treat dysmenorrhea and to ease menopausal symptoms [78].

At a dose of 2180 mg/day taken for 28 days, which is significantly higher than the recommended daily dose of 40–200 mg, a clinical study reported by Gurley *et al.* [79] using CYP-selective probe substrates for CYP1A2, CYP2D6, CYP2E1, and CYP3A4 showed that there was no effect of black cohosh on CYP1A2, CYP2E1, and CYP3A4. However, a weak inhibition of CYP2D6 was clinically observed based on altered PK of

debrisoquin as the probe substrate in the same study. A later study, also conducted by Gurley *et al.* [80], using the same probe, showed that black cohosh after a daily dose of 80 mg for 14 days did not inhibit CYP2D6. Another clinical study, also reported by Gurley *et al.* [81], confirmed the absence of any CYP3A4-mediated interaction based on midazolam PK, although the dose of black cohosh was relatively lower (80 mg/day for 14 days). Clinical evaluation of digoxin PK after 40 mg/day dose for 14 days with black cohosh showed no potential for black cohosh to elicit drug interaction due to P-gp inhibition [82].

19.6.5 Cranberry (*Vaccinium macrocarpon*, Family Ericaceae)

The ripe fruits of Cranberry are known for anticoagulant, anti-inflammatory, and antimicrobial properties including antiparasitic activity. It is often used as a prophylaxis and/or treatment of urinary tract infections [83].

Six clinical studies have been reported for herb–drug interactions with cranberry administered either in the form of juice (5 of 6 studies) or in the form of a capsule (one of six studies) containing concentrated extract of cranberry. Cranberry juice did not alter the PK of flurbiprofen [84], cyclosporine [85], midazolam [86], tizanidine [86], and warfarin [86–88]. However, the area under the international normalized ratio (INR)–time curve (AUC_{INR}) for warfarin was slightly increased (30% increase) when capsules of concentrated cranberry extract (daily dose of 3 g for 14 days) were used as reported by Abdul *et al.* [89]. In this study, there was no impact on baseline INR, warfarin PK, or platelet aggregation suggesting that cranberry pretreatment did not have a significant effect on the clotting of blood.

19.6.6 Curcumin (*Curcuma longa*, Family Zingiberaceae)

Curcumin, a hydrophobic polyphenolic compound belonging to the curcuminoid class [90], is one of the principal active ingredients of turmeric which is a commonly used spice in Indian cuisine as well as a coloring pigment. Turmeric is obtained from the dried rhizomes of *Curcuma longa* and has been used for its medicinal properties in ayurvedic medicine for centuries. More recently, systematic study of the pharmacology of curcumin has gained interest due to evidence that it has anti-inflammatory, antioxidant, antiviral, antifungal, and anticancer properties [91,92]. Curcumin has potential against various malignant diseases such as diabetes mellitus, arthritis, and Alzheimer's and has been described as the "ideal spice for life" by Aggarwal *et al.* [93]. Baum *et al.* [94] showed that oral doses of up to 4 g/day for six months were found to be safe. Cheng *et al.* [4] demonstrated that curcumin is not toxic to humans up to 8000 mg/day when taken by mouth for three months. This study as well as another clinical study conducted by Sharma *et al.* [5] provided preliminary evidence that curcumin has the potential to serve as a chemopreventive agent for cancer.

Appiah-Opong *et al.* [95] studied the interactions of curcumin, a mixture of its decomposition products, and four of its individually identified decomposition products (vanillin, vanillic acid, ferulic aldehyde, and ferulic acid) on five major human drug-metabolizing CYPs using human recombinant CYPs *in vitro*. It was observed that curcumin strongly inhibited CYP2C9 (IC_{50} 4.3 μM) and showed moderate inhibition of CYP3A4 and CYP2B6 with IC_{50} values of 16.3 and 24.5 μM , respectively. Curcumin was observed to be a noncompetitive inhibitor of CYP2C9 (K_i 11.5 $\pm$ 0.8 μM),

whereas inhibition of CYP3A4 (K_i $7.4 \pm 3.5 \mu\text{M}$) and CYP2B6 (K_i $33.2 \pm 14.0 \mu\text{M}$) was of a competitive type. In a separate study reported by Volak *et al.* [96], curcumin as well as curcuminoid extract and curcuminoid mixture were evaluated *in vitro* for its potential effects on drug-metabolizing enzymes *in vitro*. In their study, they determined that curcumin was a potent inhibitor of sulfotransferase (SULT) > CYP2C19 > CYP2B6 > UDP-glucuronosyltransferase (UGT) > CYP2C9 > CYP3A with IC_{50} values ranging from 0.99 ± 0.04 to $25.3 \pm 1.3 \mu\text{M}$. SULT inhibition study with curcumin was conducted in LS 180 cells, CYP studies in human liver microsomes and UGT separately in both LS 180 cells and human liver microsomes. The inhibition of CYP3A activity by curcuminoid extract was determined to be competitive inhibition (K_i of $11.0 \pm 1.3 \mu\text{M}$) which was consistent with the results reported by Appiah-Opong *et al.* [95], whereas mixed competitive–noncompetitive inhibition was attributed to the inhibition of CYP2C9 and CYP2C19 activities with corresponding K_i values of 10.6 ± 1.1 and $7.8 \pm 0.9 \mu\text{M}$, respectively.

Chen *et al.* [97] conducted a clinical study in healthy male volunteers by administering 1 gm of curcumin orally (once daily) for 14 consecutive days and on the 15th day coadministering with 100-mg caffeine. Curcumin was shown to inhibit CYP1A2 activity by 28.6% as measured by decreased formation of caffeine metabolite, 1,7-dimethylxanthine. An increase (48.9%) in CYP2A6 activity was also observed in this study. Additional clinical studies are needed to fully validate curcumin's ability to interact with pharmaceutical medications by inhibition of CYP1A2 and other drug-metabolizing enzymes.

19.6.7 Danshen (*Salvia miltiorrhiza*, Family Lamiaceae)

Danshen is the dried root or rhizome of *Salvia miltiorrhiza*. It is primarily used for the treatment of cardiovascular diseases, including angina pectoris, stroke, and myocardial infarction [98]. The major active components of danshen are tanshinones.

Tanshinones are substrates for P-gp and are potent competitive inhibitors of CYP1A2 with K_i of less than $1 \mu\text{M}$ [39]. Izzat *et al.* [99] reported that patients on chronic warfarin therapy with coadministration of danshen had enhanced anticoagulation and bleeding. Additional clinical studies are needed to demonstrate that inhibition of warfarin metabolism (i.e., through inhibition of CYP2C9) may result in the enhanced anticoagulation observed in combination therapy with danshen. Qui *et al.* [100] reported that danshen induces CYP3A4 in healthy volunteers. Midazolam oral clearance in this study increased by 35% and C_{max} and AUC decreased by 31 and 27%, respectively.

19.6.8 Dong Quai (*Radix angelica sinensis*, Family Apiaceae)

Dong quai, a root preparation, is used to treat menstrual cramps, regulate menstrual periods, and lessen menopausal symptoms. Dong quai is thought to promote natural progesterone synthesis, a hormone that declines during menopause. It has also been reported to have medicinal use in certain cardiovascular conditions. The root of this herb is used to treat fatigue, anemia, and high blood pressure [6].

Sevior *et al.* [101] reported that dong quai inhibited CYP2B6 and CYP2C19 *in vitro* in human liver microsomes with IC_{50} values ranging between 3 and $5 \mu\text{M}$. Clinical herb–drug interaction studies involving dong quai and substrates of CYP2C19 and CYP2B6 need to be conducted to determine if the interactions are clinically relevant.

In a clinical study conducted by Page and Lawrence [102], coadministration of dong quai and warfarin increased the anticoagulant effect of warfarin resulting in higher INR ratios. It has been hypothesized that this effect is PD rather than PK. Additional studies are needed to understand the risks associated with coadministration of dong quai and other blood thinning agents.

19.6.9 Echinacea (*Echinacea* sp., Family Asteraceae)

Roots and sometimes other plant parts from multiple species of the *Echinacea* genus are used, namely, *E. purpurea*, *E. angustifolia*, and *E. pallida* in the treatment of upper respiratory tract infections [103]. Echinacea products contain alkylamides that are known to modulate CYP activity, in particular, CYP1A2 and CYP3A4 [104]. However, the type and concentration of the alkylamide present can vary depending on the *Echinacea* species [105]. This is perhaps the cause for the conflicting reports in the literature regarding the effect of Echinacea products on CYP activity *in vivo* [76,80].

Coadministration of Echinacea has been reported to either decrease the clearance of caffeine as reported by Gorski *et al.* [104] or have no effect on the PK of caffeine as reported by Gurley *et al.* [76]. A potential mechanism suggested for the lower clearance of caffeine is the inhibition of CYP1A2 by Echinacea. In this study, slight inhibition of CYP2C19 was also observed; however, the extent of inhibition was considered to be clinically insignificant [104]. Similarly, these two clinical studies also reported different outcome on the effect of Echinacea on CYP3A4. Gorski *et al.* reported increased systemic clearance of midazolam associated with increased oral bioavailability of midazolam suggesting inhibition of intestinal CYP3A4 and induction of hepatic CYP3A4 [104], whereas Gurley *et al.* reported no effect on CYP3A4 activity [76]. Also, the PK of digoxin, a P-gp substrate, was unaffected after administration of Echinacea [106]. Thus, currently, there are no clinical case reports that point to serious safety-impacting interactions of this herb with other drugs.

19.6.10 Evening Primrose Oil (*Oenothera biennis*, Family Onagraceae)

Evening primrose oil (EPO) is an oil that is extracted from the flowers of *Oenothera biennis* and is rich in linoleic acid and γ -linolenic acid (GLA), a polyunsaturated fatty acid [107]. EPO is generally used for the treatment of allergy-induced eczema, premenstrual syndrome, breast pain and tenderness, diabetic neuropathy, rheumatoid arthritis, and osteoporosis [108,109]. There are no published literature reports on interactions of EPO with other drugs.

19.6.11 Garlic (*Allium sativum*, Family Liliaceae)

Garlic, which is a common herb used in various cuisines, is also used in the reduction of hypertension and hyperlipidemia including prevention of arteriosclerosis [110]. It is also thought to possess some immune system strengthening and antimicrobial effect [111,112], which makes it the herb of choice in HIV-infected patients [113]. There are many known active ingredients in garlic cloves; however, the organosulfur compounds, such as allicin, alliin, *S*-allylcysteine, *S*-allylmercaptocysteine, diallyl sulfide, dipropyl sulfide, and dipropyl disulfide, are believed to be the therapeutic ingredients [114–117].

Case studies have been reported in the literature implicating interactions with garlic and other drugs such as anticoagulants, antiretrovirals, hypoglycemic, and analgesics.

Administration of garlic capsules twice daily for 14 days to normal healthy volunteers did not alter the PK of probe substrates that are metabolized by CYP3A4 such as midazolam [118] and alprazolam [119] or of dextromethorphan [119] which is metabolized by CYP2D6. However, garlic inhibited CYP2E1, as determined by the assessment of PK of the CYP2E1-selective probe substrate, chlorzoxazone [67].

After administration of garlic orally for three weeks to healthy volunteers, a twofold decrease in the plasma exposure of the coadministered HIV protease inhibitor, saquinavir, was observed [120]. This was hypothesized to be due to decreased bioavailability of saquinavir, which could be a result of P-gp inhibition, as saquinavir is a known substrate of CYP3A4 [121] and P-gp [122], and it has been shown that garlic does not modulate the *in vivo* activity of CYP3A4 using midazolam [118] and alprazolam [119] as probes, as discussed earlier. In fact, it was later shown that raw garlic and garlic products cause slight to moderate inhibition of P-gp *in vitro* [123], although the inhibition was not as strong compared to verapamil, a potent P-gp inhibitor. However, in a separate study reported by Gallicano *et al.* [124], single-dose PK of ritonavir, another HIV protease inhibitor, was unaltered after consecutive daily dosing of garlic for four days. It is postulated that a longer duration of dosing for garlic might contribute to alteration of ritonavir PK. In contrast, Laroche *et al.* [125] reported severe gastrointestinal toxicity for two HIV-infected patients who were taking garlic capsules for a little over two weeks before beginning ritonavir (400 or 600 mg, bid) treatment. This toxicity was attributed to the coadministration of garlic and ritonavir as it was found to be reversible when either drug was discontinued and recurred when a lower dose of ritonavir (100 mg) was coadministered with garlic. Although the underlying mechanism for the interaction between garlic and ritonavir needs to be further investigated, it has been postulated that either ritonavir modulates the metabolism of garlic leading to production of toxic metabolites or the garlic constituents might inhibit the CYP3A4-based metabolism [126] or P-gp-mediated transport of ritonavir [125], thereby leading to increased ritonavir concentrations. It is also possible, given that ritonavir autoinduces its metabolism during the first two weeks of therapy [127], that the effect of concomitantly administered garlic may be more pronounced after a single dose of ritonavir as compared to after multiple doses. Studies conducted with garlic products or the known active ingredients have shown some potential for interaction with P-gp as well as inhibition of cDNA-expressed CYP enzymes *in vitro* [123].

Interaction with docetaxel, a CYP3A4 substrate, was assessed clinically in women with metastatic breast cancer receiving docetaxel weekly for three weeks [128]. Three days after the initial dose of docetaxel, patients received 600 mg of garlic twice daily for 12 consecutive days. Docetaxel PK was assessed after each dose, and it was found that garlic did not significantly affect the disposition of docetaxel. However, there was some potential that garlic decreased the clearance of docetaxel in patients carrying a CYP3A5*1A allele.

Following a three-month dosing of garlic extract, assessment of acetaminophen PK showed a slight increase in sulfation of acetaminophen, however, did not alter the extent of oxidative and glucuronidation metabolites [129]. Overall, the authors concluded that garlic did not affect the PK of acetaminophen. In addition, diallyl sulfone has

been shown to inhibit the oxidation of acetaminophen to its hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinone imine, in human liver microsomes [130].

PD-based herb–drug interactions have also been reported for garlic with anticoagulants and antihyperglycemic agents. *In vitro* and *in vivo* studies conducted with certain organosulfur compounds present in garlic have demonstrated that garlic inhibits platelet aggregation in humans [131–134]. There are case reports of postoperative bleeding [135,136] and spontaneous spinal epidural hematoma that are considered to be linked to ingestion of garlic products [137,138]. Thus, the anticoagulant effect of garlic is responsible for increased clotting time and INR after concomitant dosing of garlic and warfarin [139–142]. Similarly, garlic extracts have been shown to produce antihyperglycemic effects in animals [143–145] and humans [146,147]. A literature report [148] described that the concomitant administration of chlorpropamide (which is used to treat diabetes), garlic, and bitter melon led to enhanced hypoglycemic response. This is another example of a PD herb–drug interaction, as each by itself exhibits antihyperglycemic properties.

19.6.12 Ginger (*Zingiber officinale*, Family Zingiberaceae)

Ginger is a commonly used botanical in many culinary preparations and is most commonly used as a household remedy for nausea, especially during the first trimester of pregnancy [149]. It is also reported to have antiemetic property including treatment of gastrointestinal infectious diseases, anti-inflammatory, and antiplatelet activity [150].

There are no reported clinical studies that have assessed the potential for its interaction with other drugs via CYP- or P-gp-related mechanism. On the basis of its pharmacological properties, Jiang *et al.* [151] assessed its potential to cause bleeding when coadministered with warfarin and reported no alteration in warfarin PK or in the coagulation parameters after administration of ginger.

19.6.13 Ginkgo (*Ginkgo biloba*, Family Ginkgoaceae)

The leaves of *Ginkgo biloba* contain powerful antioxidants and are mainly used for improving memory and for cerebral insufficiency and peripheral vascular disease [103,152]. The known active constituents, ginkgolide and bilobalide, have been shown to function as antagonists of the platelet-activating factor (PAF) receptor and therefore have antiplatelet aggregation activity [153].

In vitro study with cDNA-expressed CYPs has shown that the ginkgolic acids present in ginkgo biloba were potent inhibitors of CYP1A2, CYP2C9, and CYP2C19 with IC₅₀ values ranging from 2.41 to 4.88 μ M [154]. Ginkgo also moderately inhibited CYP2C8 [155], CYP2C9 [156], and CYP3A4 [157] *in vitro* in human liver microsomes; however, the studies reported in the literature to determine the potential of *Ginkgo* to inhibit CYP2C19 and CYP2D6 present conflicting results [154,158–160].

In contrast to the moderate (apparent K_i of 14.8 μ M) inhibition of CYP2C9 observed *in vitro* in human liver microsomes [156], ginkgo did not show any potential for interaction with CYP2C9 probe substrates, as evidenced by unaltered steady-state PK of diclofenac or on the urinary metabolic ratio of tolbutamide [156]. Consistent with this *in vivo* finding, the PK of another CYP2C9 substrate, flurbiprofen, was also unaffected by ginkgo [161].

There was a case report of elevated levels of antiepileptic drugs, valproic acid and phenytoin, in a 55-year-old man taking ginkgo in addition to herbs, and since the common CYP that metabolized both is CYP2C19, inhibition of CYP2C19 by ginkgo is suggested as the underlying reason [162]. Yin *et al.* [163] conducted a clinical study to look at effects of ginkgo treatment on the PK of omeprazole and hydroxylation of omeprazole. They reported a significant decrease in the ratio of AUC of the parent to AUC of its hydroxylated metabolite, with the decrease being higher in a poor metabolizer than an extensive metabolizer with regard to CYP2C19 genotype, thus indicating that ginkgo induced CYP2C19.

Smith *et al.* [66] reported a clinical study in healthy volunteers to assess the effect of ginkgo on the PK of nifedipine, a CYP3A4 substrate. A 53% increase in the peak plasma concentration of nifedipine at 30 min postdose was reported in this study, suggesting inhibition of CYP3A4. In contrast, another clinical study reported later by Yoshioka *et al.* [164] showed that ginkgo did not alter nifedipine PK. In addition, Markowitz *et al.* [165] reported a clinical study to assess the effect of ginkgo on CYP3A4 using alprazolam as a CYP3A4 substrate and CYP2D6 using dextromethorphan as a substrate. The data indicated that administration of ginkgo had no effect on both these CYP isozymes. A similar outcome for CYP3A4 was observed in a separate study reported by Yasui-Furukori *et al.* [166]. In this study, ginkgo extract was administered once daily for 30 days to 14 patients undergoing treatment with donepezil, a cholinesterase inhibitor, for Alzheimer's disease. Neither the plasma concentration of donepezil, which is metabolized by CYP3A4 and CYP2D6 [167], nor the cholinesterase activity in the red blood cell or the cognitive function of the patients over the duration of the study was significantly altered by ginkgo. In addition, a few other studies to clinically evaluate the effect of ginkgo on CYP3A4 are reported below. Robertson *et al.* [168] reported that ginkgo did not significantly alter the PK of antiretrovirals (lopinavir and ritonavir); however, it was observed that the AUC of midazolam decreased, suggesting induction of CYP3A metabolism. Uchida *et al.* [169] reported that ginkgo inhibited CYP3A metabolism resulting in increased AUC of midazolam and induction of CYP2C9 as evidenced by the decrease in AUC of tolbutamide. Additional studies are needed to elucidate the induction or inhibition effects of ginkgo on CYP3A metabolism since the existing reports in the literature are conflicting.

Similarly, assessment of the potential of ginkgo to cause P-gp-related interaction with drugs that are P-gp substrates gave mixed results when studied using digoxin and talinolol as P-gp probes. Ginkgo did not cause any significant alteration of digoxin PK [170], while it significantly increased the bioavailability of talinolol [171]. Therefore, similar to CYP3A4, the clinical potential for ginkgo to cause herb-drug interactions due to P-gp-related mechanism needs to be investigated further.

Several clinical reports of hemorrhage have implicated ginkgo either postoperatively [172–175] or when taken concomitantly with drugs that have anticoagulant activity such as warfarin [176,177] or clopidogrel [178] and nonsteroidal anti-inflammatory agents such as ibuprofen, aspirin, and rofecoxib [179]. Since, ginkgo does not seem to modulate any of the CYP activities to produce metabolism-based interactions with other drugs based on PK of known probe drugs for various CYP enzymes [156,180–182], the underlying mechanism for these case reports appears to be PD-based rather than metabolism related. However, the outcome of a prospectively designed repeat-dose (14 days) clinical study that was randomized, double-blind, and placebo-controlled in

young healthy male volunteers was that the administration of an extract of ginkgo does not cause significant changes in blood coagulation parameters [183]. Similarly, another study evaluated the effect of coadministration of ginkgo and acetylsalicylic acid on blood coagulation parameters and demonstrated that ginkgo did not produce any additional effect on bleeding time or other coagulation parameters [184].

Other sporadic reports of clinical interactions of ginkgo with other drugs, such as antihyperglycemic metformin [185] or antipsychotic risperidone [186] and antidepressant trazodone [187,188], are available and these are thought to be PDs based and hence are not discussed here.

19.6.14 Green Tea (*Camellia sinensis*, Family Theaceae)

Green tea is a popular beverage and is also available in the form of capsules containing concentrated extract. It is commonly recognized as an antioxidant and has been shown to have anti-inflammatory [189] and antineoplastic properties [190] and is often used as a prophylactic for cancer [191] as well as to aid weight loss [192,193]. There are several catechins in green tea extract which are active ingredients, of which the most abundant is epigallocatechin gallate and its recommended daily dose for therapeutic effect is 100–750 mg [194,195].

Green tea was shown to strongly inhibit CYP2C9, CYP2D6, and CYP3A4 activities in human liver microsomes using tolbutamide, bufuralol, and testosterone as probe substrates [196]. Also, Mirkov *et al.* [195] demonstrated that the catechins from green tea inhibited the CYP3A4-catalyzed oxidation of irinotecan and UGT1A1-catalyzed conjugation of its metabolite, SN-38, in human liver microsomes; however, they did not induce CYP3A4 in human hepatocytes.

In contrast to the *in vitro* studies, a clinical study conducted by Donovan *et al.* [197] to assess the effect of decaffeinated green tea on CYP2D6 and CYP3A4 showed no potential for herb–drug interaction via these two isozymes. In a later study conducted by Chow *et al.* [198] using a similar dose but with a longer duration (4 weeks vs 14 days in the prior study), it was observed that there was no effect on the activities of CYP1A2, CYP2C9, CYP2D6, or CYP3A4, although there was a slight inhibition of CYP3A4 activity as evident from an increase in buspirone AUC, however, it was considered to be clinically insignificant.

19.6.15 Kava (*Piper methysticum*, Family Piperaceae)

Kava (roots and rhizome) is used as an anxiolytic [103]. There have been numerous clinical cases of hepatotoxicity with coadministration of kava with other drugs [199].

Clinical studies with specific probe substrates have shown that kava inhibits CYP2E1 [79]; however, no effect was found on other CYPs such as CYP1A2, CYP2D6, and CYP3A4 [79,200]. A separate clinical study conducted by Gurley *et al.* [201] showed no significant alteration in digoxin PK due to coadministration of kava, due to which there does not appear to be any potential for clinical interaction of kava with other drugs that are P-gp substrates.

Clinical lack of efficacy has been reported for levodopa used to treat Parkinson's disease [202]. Serious adverse effect of being in a semi-comatose state has been reported for concurrent use of benzodiazepine alprazolam [203], while concomitant use of paroxetine to treat depression led to a lethargic state with diffuse muscular weakness and

some cognitive difficulty [204]. However, these interactions are thought to be PD in nature due to the direct effect of kava at dopamine and GABA receptors [205] rather than metabolism dependent.

19.6.16 Licorice (*Glycyrrhiza glabra*, Family Leguminosae)

The root of licorice has traditionally been used to treat sore throat and has recently been clinically shown to be effective [206]. It is a commonly used herb used in Chinese and Japanese traditional medicine. Licorice is also recognized as a natural sweetener, since the active ingredient, glycyrrhizin or glycyrrhizic acid, is 50 times sweeter than sugar [207,208].

In vitro studies conducted with expressed human P4503A4, 2B6, and 2C9 and glabridin, an isoflavone present in licorice, demonstrated time- and concentration-dependent inactivation of CYP2B6 and CYP3A4, while CYP2C9 was competitively inhibited [209]. Another *in vitro* study conducted by Budzinski *et al.* [210] in human liver microsomes with an extract of licorice showed that CYP3A4-mediated metabolism of benzyloxyresorufin was inhibited. However, Shon *et al.* [211] observed no significant change in the PK of midazolam in healthy volunteers who were given licorice extract for seven days, indicating that licorice does not show the potential for interaction with drugs that are substrates for CYP3A4. Thus, the nonconcordance of *in vitro* data to clinical studies could presumably be due to the different sources of the herb in these studies that could presumably have led to varying chemical composition.

There have been clinical reports of life-threatening hypokalemic paralysis reported in association with long-term licorice consumption [212,213]. It is unclear whether the hypokalemic paralysis is related to the inhibition of 5 α -, 5 β -reductase and 11 β -dehydrogenase which *in vitro* studies have shown glycyrrhizin and glycyrrhetic acid to be potent inhibitors of [214,215]. These enzymes play a role in steroid metabolism; hence, the *in vitro* inhibition studies suggest that the decreased steroid metabolism could potentiate the effect of endogenous and administered steroids [216]. Homma *et al.* [217] evaluated the effect of three Chinese herbal products, containing equal amounts of glycyrrhizin, on prednisolone PK and also measured the ratio of AUC of prednisone to AUC of prednisolone which is an indicator of 11 β -hydroxysteroid dehydrogenase activity. In this study, a significant increase (15.2%) in plasma prednisolone AUC was observed with one of the herbal preparation with a corresponding decrease in the ratio, while a significant decrease (17.2%) in prednisolone AUC was observed on coadministration of another herbal product with a corresponding increase in the ratio. Coadministration with the third product did not significantly alter the prednisolone plasma AUC or the ratio. This difference could be due to the presence of other components in the herbal product that could have different effects on steroid metabolism. These studies, taken as a whole, emphasize the challenges faced in assessing herb–drug interactions.

19.6.17 Milk Thistle (*Silybum marianum*, Family Asteraceae)

The extract of the seeds of *Silybum marianum* has been reported to have a hepatoprotective effect and is often used to reverse drug-mediated hepatotoxicity [218]. It is often used by patients diagnosed with viral hepatitis [219]. A typical extract of milk

thistle contains ~4–6% silymarin, which is considered to be the primary active ingredient, about 65–80% silymarin (a flavanolignan complex), and 20–35% fatty acids, including linoleic acid [220].

Several studies have been reported with drugs such as aminopyrine [221], irinotecan [222], indinavir [223–225], metronidazole [226], nifedipine [227], phenylbutazone [221], ranitidine [228], and rosuvastatin [229] as well as with standard probe substrates [118] such as caffeine (CYP1A2), debrisoquin (CYP2D6) [230], chlorzoxazone (CYP2E1), and midazolam (CYP3A4) [231], which assessed the effect on the activity of various CYP isozymes after daily dosing with milk thistle for 7, 14, 17, 21, or 28 days and observed no significant impact. There was a minor reduction in the AUC of indinavir in the two clinical studies reported by Piscitelli *et al.* [223] and by DiCenzo *et al.* [224]; however, it was considered to be clinically insignificant for indinavir. Consistent with these results, another active ingredient, silibinin, was reported to have no effect on the isozyme activity *in vitro* in human liver microsomes when evaluated by Beckmann-Knopp *et al.* [232] using erythromycin (CYP3A4), chlorzoxazone (CYP2E1), S(+)-mephenytoin (CYP2C19), caffeine (CYP1A2), or coumarin (CYP2A6) as probe substrates. However, Venkataramanan *et al.* [233] reported that silymarin at a high concentration of 0.1–0.25 mmol/L, significantly decreased CYP3A4-mediated 6 β -hydroxylation of testosterone as well as glucuronyltransferase activity in human hepatocytes. Sridar *et al.* [234] have reported that silibinin is a mechanism-based inhibitor of CYP3A4 and CYP2C9. These studies indicate that a systematic assessment is important to be able to predict interaction of milk thistle with drugs metabolized by CYP2C9, CYP2D6, CYP2E1, or CYP3A4.

Milk thistle did not alter digoxin PK *in vivo* indicating that it did not inhibit P-gp [82]. However, in contrast, a decrease in serum metronidazole concentrations was observed and it was attributed to an upregulation of P-gp [226]. In addition, *in vitro* studies with silymarin demonstrated increased accumulation of daunorubicin in cells expressing P-gp phenotype compared to cells that did not express P-gp, and photoaffinity labeling showed a direct interaction with P-gp binding [235]. Similarly, the cytotoxic effects of doxorubicin were potentiated *in vitro* by silibinin [236] and high affinity labeling to P-gp was inferred to be the cause [237]. Thus, the literature reports of significant modulation of P-gp by the active constituents silymarin and silibinin show the potential for clinical interaction of milk thistle with other P-gp substrates. Therefore, additional studies are required to understand the impact of milk thistle treatment on P-gp.

19.6.18 Saw Palmetto (*Serenoa repens*, Family *Arecacea/Palmae*)

Dried ripe fruits of Saw Palmetto are widely used for the treatment of stages I and II of benign prostatic hyperplasia [238]. Fatty acids, flavanoids, and plant sterols are the active ingredients in saw palmetto [239]. No serious clinical side-effects have been reported in patients taking saw palmetto [240].

Yale and Glurich reported potent inhibition of cDNA-expressed human P450C9, 2D6, and 3A4 by saw palmetto [241]. In contrast to these data, two independent clinical studies conducted in healthy volunteers by Gurley *et al.* [118] and Markowitz *et al.* [242], with selective CYP substrates for CYP1A2, CYP2D6, CYP2E1, or CYP3A4, showed no significant potential for herb–drug interactions mediated through these CYPs.

19.6.19 Siberian Ginseng (*Eleuthrococcus senticosus*, Family Araliaceae)

The roots of Siberian ginseng have immunomodulatory properties and are used as an adaptogen to confer resistance to the effects of stress [243]. The major pharmacological constituents of Siberian ginseng are the eleutherosides.

The effect of Siberian ginseng on CYP2D6 and CYP3A4 activities was assessed after daily dosing for 14 days using dextromethorphan and alprazolam as probe substrates, respectively [244]. No significant change in the PK of both these probes was observed in this study. This was consistent with the results of the *in vitro* study using cDNA-expressed human CYP3A4 where the extract of Siberian ginseng did not inhibit CYP3A4 [210]. A case report of increased serum digoxin concentrations without any signs of toxicity in a patient taking Siberian ginseng was investigated further [245]. It appeared that Siberian ginseng contained some digoxinlike entities that posed interference in the serum digoxin assay and ruled out involvement of P-gp interaction [246].

19.6.20 St. John's Wort (*Hypericum perforatum*, Family Hypericaceae)

SJW is a very popular herb used in the treatment of mild, moderate, and major depression [247,248], which is typically used in a chronic setting. Two major classes of biologically active compounds include the hypericins which are anthraquinones with antiviral and anticancer activity and the hyperforins which are prenylated acylphloroglucinols which display potent antimicrobial activity and are thought to be the primary bioactive ingredient for antidepressive effects of the herb [249].

When used as a monotherapy, treatment with SJW is quite safe. However, there are several clinical reports of adverse effects on concomitant administration of SJW with different drugs as a result of PK-based interaction due to either or both induction of CYP and intestinal P-gp which have been reviewed by Zhou and Lai [250]. This review cites studies reporting interactions with anticancer agents, anti-inflammatory drugs, antimicrobials, antiretrovirals, cardiovascular drugs, CNS drugs, contraceptives, hypoglycemic, immunosuppressants, and drugs acting on the respiratory system. The interaction of SJW with other drugs was observed ~14 days after daily administration of SJW; studies conducted after acute administration of SJW showed no potential for interaction, thereby suggesting that induction of CYP enzymes and/or P-gp could be the underlying mechanism [251–253].

Several clinical reports and clinical studies with SJW have confirmed the ability of SJW to induce CYP3A4, CYP2C19, and CYP2E1, and/or P-gp with no effect on activities of CYP1A2, CYP2C9, or the noninducible CYP2D6 [254–260]. Typically, the observed enzyme induction is reversible one week after cessation of SJW treatment [261]. It has been demonstrated that SJW extract and hyperforin treatment of primary human hepatocytes causes induction of CYP3A4 via the activation of PXR [262,263].

The interaction of SJW with P-gp substrates was evident from the lowered plasma concentrations of well-known P-gp substrates including digoxin [47,106,264] and fexofenadine [265,266]. Decreased plasma concentrations have also been observed for talinolol, another clinical probe for P-gp, when administered after pretreatment with SJW [267]. These talinolol PK data combined with a corresponding increase in MDR1 mRNA as well as protein levels of P-gp in the intestinal mucosa in humans, reported in the same study, indicate that SJW induces P-gp [267]. It is also believed that the hypericin in SJW is responsible for the induction of P-gp [264].

The use of SJW is very common in patients who have undergone organ transplant procedure and are undergoing treatment with an immunosuppressant such as cyclosporine or tacrolimus, since these patients also tend to experience depression. The interaction of SJW and cyclosporine has been the most well documented with reports of patients transplanted with heart, liver, or kidney and stabilized on cyclosporine showing acute rejection of the transplanted tissue which was associated with decrease in plasma cyclosporine level, after taking SJW at therapeutic doses [268]. Improvement was observed in most patients after discontinuation of SJW. Mai *et al.* [269] reported that after dosing stable renal transplant patients with SJW, a decrease in tacrolimus concentration but not of mycophenolic acid was observed, thereby confirming that induction of CYP3A4 and P-gp is responsible for the increased drug clearance, since tacrolimus is a substrate for both P-gp and CYP3A4, while mycophenolic acid is primarily metabolized by glucuronidation.

Patients on antineoplastic therapy also tend to self-medicate with SJW. Two independent clinical trials in healthy volunteers have been reported with SJW and two anticancer agents, imatinib [270,271] and irinotecan [272]. Imatinib is a potent inhibitor of Bcr-abl and c-kit tyrosine kinase and is mainly metabolized by CYP3A4 and is a P-gp substrate, while SN-38 is the active metabolite of irinotecan which is a CYP3A4 substrate. In both these studies, decreased plasma concentration of the anticancer agent, imatinib, or the active metabolite of irinotecan, SN-38, was reported after pretreatment with SJW.

Failure of anti-HIV therapy has been observed with antiretrovirals such as indinavir [273] and nevirapine [274], which are both CYP3A4 substrates. Decrease in the plasma concentration of the protease inhibitor, indinavir, was observed in healthy volunteers dosed with SJW for 14 days in an open-label trial that is attributed to the induction of CYP3A4. The nonnucleoside analog, nevirapine, which is metabolized by CYP3A4 and CYP2D6, showed increased oral clearance in five HIV-infected patients, when SJW was coadministered suggesting induction of CYP3A4. HIV viral load, as determined from the measurement of HIV mRNA, was also significantly increased in a patient after he took SJW concomitantly with indinavir and lamivudine [275]. It is postulated that the reported interaction between SJW and the antiretrovirals was due to the induction of CYP3A4 by SJW.

Induction of CYP3A4 has also caused failure of therapy for several cardiovascular drugs which are metabolized by CYP3A4 to a significant extent such that the autoinduction leads to a decrease in the serum concentration of these drugs making them less effective. These include the calcium channel blockers nifedipine [276] and verapamil [277], and the antihyperlipidemic drugs, atorvastatin [278], and simvastatin [279] but not pravastatin [279] which is not a substrate for CYP3A4 or for P-gp. In addition, clinical cases of increase in coagulation parameters when taking anticoagulants such as warfarin (racemic mixture of *R*- and *S*-enantiomers) have been reported, and it is documented that while *S*-warfarin is metabolized by CYP2C9, the *R*-enantiomer is metabolized by CYP1A1 and CYP3A4 [280].

SJW has been shown to increase the clearance of contraceptives such as ethinylestradiol, norethindrone, and ketodesogestrel, which was attributed to the induction of CYP3A4 as these drugs are substrates for CYP3A4 [281–283].

Hypoglycemic drugs such as tolbutamide is metabolized predominantly by CYP2C9 and its coadministration with SJW did not alter its PK [284], suggesting that SJW does not modulate CYP2C9 activity. In contrast, the PK of gliclazide, another CYP2C9

substrate used in the treatment of diabetes, was significantly altered by SJW in the absence of any significant change in CYP2C9 genotype [285], thus suggesting that the mechanism was independent of CYP2C9.

SJW has been shown to reduce plasma concentrations of fexofenadine, a long-acting antihistaminic drug, which is used as a P-gp substrate [265,266].

Plasma levels of voriconazole, an antifungal agent and CYP2C19 substrate, decreased after long-term coadministration of SJW in a clinical study [252]. This observation supports the enzymatic induction of CYP2C19.

Clinical study that involved coadministration of omeprazole with SJW over a period of 2 weeks resulted in decreased plasma concentrations of omeprazole and increased concentration of metabolites, CYP3A4-mediated sulfoxidation, and 2C19-catalyzed hydroxylation, indicating induction of both these CYP enzymes by SJW [286].

19.6.21 Valerian (*Valeriana officinalis*, Family Valerianaceae)

The extract of dried roots of valerian has been historically used to treat insomnia as an alternative to benzodiazepine and sometimes as an anxiolytic, hypnotic, and anticonvulsant [287,288]. It is sometimes also used in the treatment of migraine due to claims of its muscle relaxant property as well as in the treatment of gastrointestinal cramps and irritable bowel syndrome. The primary chemical components of valerian are valerenic acid and its derivatives, valepotriates, alkaloids, furanofuran lignans, and free amino acids [289].

Organic extracts of the valerian root have shown inhibition of CYP3A4 *in vitro* up to 88% [290], whereas valerenic acid, an active component in valerian, has demonstrated a much lower inhibitory potential toward CYP3A4 and minor inhibition of CYP2C9 and CYP2C19 [291]. Donovan *et al.* [292] evaluated the effect of valerian extract dosed at 1000 mg/day for 16 days and observed minimal alteration of CYP3A4 activity that was not clinically significant and no effect on CYP2D6. Gurley *et al.* [79] reported that dosing valerian at a total daily dose of 375 mg for 28 days had no impact on CYP1A2, CYP2D6, CYP2E1, and CYP3A4. While there are no literature reports of the potential for valerian to interact with P-gp substrates, no clinical reports of adverse events linked to valerian are currently available.

19.7 CONCLUSIONS AND FUTURE PERSPECTIVES

It is estimated that ~30% of patients in the United States currently use herbal dietary supplements [293]. Similarly, in other developed countries such as Australia, New Zealand, and some of the European countries, an estimated one third of adults use herbal supplements for promoting health and managing diseases [294]. The proportion of adults using herbal remedies increases sharply in many developing countries such as China and India, where more than 80% of the population is known to use herbal products [294]. Reasons for the increased use of herbal products include the popular belief that remedies of natural origin are safe, affordable, and easily available (i.e., local health foods, neighborhood grocery stores, and discount supermarkets) [295]. Many patients while taking herbal dietary supplements do not inform their physicians and a reason cited for this lack of communication may be fear of disapproval by their physicians or a desire to control personal therapy [296]. An important and real

safety concern associated with the use of herbal dietary supplements is that the ADME properties are rarely known, leading to an increased risk of unexpected interactions when concomitantly administered with prescription medications. The PK interactions of prescription medications by herbs that have been reported are primarily associated with inhibition or induction of CYP and P-gp enzymes; however, in many cases, the *in vitro* data do not accurately predict the clinical outcome. Thus, further studies are necessary to be able to develop a predictive assessment for clinical herb–drug interactions.

Currently, a knowledge gap exists with the interaction of prescription medication on the PK and PD of herbs and herbal formulations. With the cost of prescription medications skyrocketing and an increased health-awareness in the general population, it is inevitable that the use of herbal dietary supplements will increase. It is the need of the hour, therefore, that studies are conducted with scientific rigor and acceptable norms to characterize herbal medicines with regulatory oversight, much like is done today with pharmaceutical drugs.

ABBREVIATIONS

AUC	Area Under the Concentration Curve
$C_{\max}$	Maximum Concentration
CYP	Cytochrome P450
EMA	European Medicines Agency
FDA	Food and Drug Administration
P-gp	P-glycoprotein
$T_{\max}$	Time Required to Reach $C_{\max}$
$t_{1/2}$	Half-Life
UGT	Uridine Glucuronosyltransferase

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20 ADME of Anticancer Drugs

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20.1	Summary	1
20.2	Tegafur and CYP2A6	2
20.3	Letrozole and CYP2A6	3
20.4	Tamoxifen and CYP2D6	4
20.5	Thalidomide and CYP3A4/5	6
20.6	Irinotecan and UDP-glucuronosyltransferase 1A1	7
20.7	6-Mercaptopurine and thiopurine-S-methyltransferase	8
20.8	Carboplatin and hyperbaric oxygenation	8
20.9	Conclusions	9
	References	10

20.1 SUMMARY

Cytochrome P450 (P450 or CYP) comprises a superfamily of heme-thiolate monooxygenases involved in the oxidation of a large number of endogenous and exogenous compounds [1]. In human liver, CYP3A4 is the major P450 enzyme, followed by CYP2C9 [2]. An important role for polymorphic CYP3A5 in drug oxidation in African and Asian populations has been suggested [3,4]. CYP2D6 and CYP2C19 also catalyze the oxidation of many marketed drugs [5], but their content in human liver is relatively low [6]. Recently, CYP3A enzymes have been identified as being involved in the biotransformation of many anticancer drugs, as shown in Table 20.1. Large interindividual variations in the contents and activities of several P450 forms in human livers lead to different roles of P450s in the oxidations of substrates associated with pharmacological or toxicological actions [1].

Pharmacogenetics is a rapidly developing field, especially in oncology. In an ideal future scenario, pharmacogenetics will allow oncologists to personalize therapy based on patients' individual germline genetic test results. This would help improve efficacy, reduce toxicity, and predict nonresponders in a way that would allow an alternative approach to be chosen or individual dose adjustments to be made. Multiple pathways of drug metabolism have been studied extensively, for example, variations in the *UGT1A* gene and the *ABCC2* gene influence irinotecan metabolism and disposition [7]. Other genetic changes result in reduced DNA repair capacity related to platinum efficacy

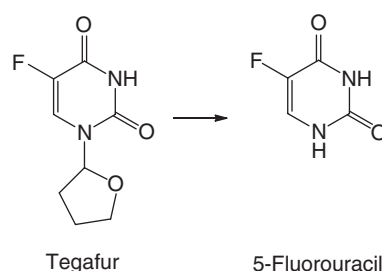
TABLE 20.1 Drug-Metabolizing Enzymes Involved in Biotransformation of Anticancer Drugs

Drug-Metabolizing Enzymes	Anticancer Drugs
P450 2A6	Anastrozole, fadrozole, letrozole, tegafur
P450 2B6	Cyclophosphamide, ifosfamide
P450 2C8	Paclitaxel
P450 2D6	Tamoxifen
P450 3A4	Cyclophosphamide, docetaxel, etoposide, gefitinib, ifosfamide, imatinib, irinotecan, retinoic acid, tamoxifen, teniposide, vinca alkaloids
UGT1A1	Irinotecan
DPYD	5-Fluorouracil
TPMT	Azathioprine, mercaptopurine

or reduced cytochrome CYP2D6 activity related to tamoxifen efficacy. Despite the extensive number of pharmacogenetic studies and their promising results, it is still unclear when and how pretreatment genetic screening will be implemented in oncology. Future prospective studies should focus on the effects of pharmacogenetics on patient outcome and then combine this with evaluations of cost effectiveness. In this section, recent findings on anticancer drug metabolism are summarized.

20.2 TEGAFUR AND CYP2A6

Tegafur [5-fluoro-1-(2-tetrahydrofuryl)-2,4(1*H*, 3*H*)-pyrimidinedione], an anticancer prodrug of 5-fluorouracil (5-FU), has been used clinically for over 30 years for chemotherapy [8]. It has been reported that tegafur is bioactivated to 5-FU (Fig. 20.1) mainly in the liver and that 5-FU may inhibit the growth of cancer cells by the inhibition of thymidylate synthase or by its incorporation into RNA [9]. 5-FU is further biotransformed to an inactive molecule by dihydropyrimidine dehydrogenase (DPYD) in the liver cytosol [10]. Two pathways have been proposed for the conversion of tegafur to 5-FU, the major pathway occurring in the microsomal fraction and the minor pathway in the cytosolic fraction [11]. Liver microsomal 5-FU formation from tegafur is catalyzed by the phase I drug-metabolizing enzymes CYP2A6, CYP1A2, and CYP2C8 [12,13]. On the other hand, cytosolic 5-FU formation from

**Figure 20.1** Tegafur and its metabolite, 5-fluorouracil.

tegafur is thought to be catalyzed by thymidine phosphorylase [14]. Thymidine phosphorylase, purified from human gastric tumor tissues, has been shown to catalyze 5-FU formation from tegafur as well as thymine formation from thymidine [15]. Thymidine phosphorylase levels were reported to be higher in various tumor tissues than in normal tissues [16]; therefore, 5-FU formation from tegafur in tumor tissues is considered to be mainly catalyzed by thymidine phosphorylase. The involvement of human liver microsomes and cytosol in 5-FU formation from tegafur in the presence of 5-chloro-2,4-dihydropyridine, a DPYD inhibitor, has been reported [17]. Moreover, it was confirmed that cytosolic 5-FU formation is mainly catalyzed by thymidine phosphorylase, not by uridine phosphorylase.

Tegafur has been clinically used for the treatment of various cancers. Recently, it has been used in combination with modulators such as 5-chloro-2,4-dihydropyrimidine (CDHP) and potassium otastat [18]. Many enzymes are known to be involved in the metabolism of tegafur, although P450 and dihydropyrimidine dehydrogenase are rate-limiting enzymes activating tegafur to 5-FU and inactivating 5-FU, respectively [10,19].

If a cancer patient lacks CYP2A6 activity, the patient is expected to have a poor metabolic phenotype in terms of the efficacy of tegafur. In a clinical study, a newly developed anticancer drug, TS-1 capsules, containing tegafur and CDHP, an inhibitor of DPYD, were orally administered to several gastric cancer patients [20]. The total area under the plasma tegafur concentration–time curve (AUC) in one patient was fourfold higher than that for the other patients. From this subject, a novel CYP2A6 mutation was found that causes an amino acid change from serine to proline at residue 224 [20]. It was concluded that the poor metabolic phenotype of this patient was caused by the existence of two mutant alleles: *CYP2A6*4* (whole deletion of *CYP2A6*) and a new variant, *CYP2A6*11* [20].

20.3 LETROZOLE AND CYP2A6

Aromatase inhibitors directed toward CYP19A1 are a good alternative to anti-estrogens in the treatment of hormone-dependent breast cancer. Aromatase (or CYP19A1) is the enzyme responsible for the rate-limiting step in estrogen synthesis and it is by inhibiting CYP19A1, and as a consequence inhibiting estrogen formation, that aromatase inhibitors present pharmacological activity [21]. The aromatase inhibitors used today, that is, anastrozole, letrozole (4,4'-[1*H*-1,2,4-triazol-1-ylmethylene]bis-benzonitrile, Fig. 20.2), and exemestane, are third-generation drugs, all having high specificity for the aromatase enzyme [22].

Letrozole is an orally active, nonsteroidal competitive inhibitor of aromatase [23] or CYP19A1. CYP19A1 catalyzes the three-step oxidation of androgens to estrogens [24], and its expression is known to be relevant to estrogen-dependent tumors [25]. Although letrozole is currently used worldwide for treatment of advanced breast cancer in postmenopausal women [26], knowledge is still limited regarding the roles of different P450 isoforms, including the pharmacological target site (CYP19A1), in drug disposition in the therapeutic setting.

After a single oral dose of 2.5 mg to human subjects, the mean maximum plasma concentration of letrozole was 13 μ M [27]. Owing to the slow elimination half-life, plasma concentrations significantly accumulate with repeated doses, achieving mean



Carbinol metabolite

trough and maximum concentrations of 0.3 and 0.5 μM , respectively, at the steady state [23]. In clinical studies, nonlinear pharmacokinetics have been observed for letrozole at high single doses (up to 30 mg) or after repeated doses (up to 10 mg per day) [23], resulting in an ~ 10 -fold higher plasma exposure than the therapeutic plasma levels in subjects given clinical doses. Large intersubject variation in plasma concentration has generally been observed, regardless of the dose range or regimen used.

Letrozole is cleared from the body by oxidative metabolism to a pharmacologically inactive carbinol metabolite (Fig. 20.2). More than 85% of administered letrozole has been recovered as the glucuronide of the carbinol metabolite [27,28]. Two P450 forms, CYP2A6 and CYP3A4, are reported to mainly contribute to the metabolism, based on *in vitro* biotransformation studies [29,30], but the quantitative contributions of each P450 isoform to the overall clearance, or the contributions of other enzymes such as CYP19A1 (the pharmacological target), remain unclear.

The metabolic activities of individual human liver microsomes showed a significant correlation with coumarin 7-hydroxylase activities (CYP2A6 marker) at a letrozole concentration of $0.5 \mu\text{M}$, and a good correlation was also seen with testosterone 6 β -hydroxylase activities (CYP3A4 marker) at a substrate concentration of $5 \mu\text{M}$. Significantly low carbinol-forming activities were observed in human liver microsomes from individuals possessing *CYP2A6*4/*4* (whole *CYP2A6* gene deletion) at a letrozole concentration of $0.5 \mu\text{M}$. The $V_{\text{max}}/K_{\text{M}}$ value measured for CYP2A6.7 (amino acid substitution type) in human liver microsomes in the presence of anti-CYP3A4 antibodies was approximately sevenfold smaller than that for CYP2A6.1 (wild type). CYP2A6 and CYP3A4 are recognized as catalyzing the conversion of letrozole to its carbinol metabolite *in vitro*, at low and high concentrations of letrozole, respectively [30]. In addition, CYP2A6 has been recently identified as a polymorphic P450 resulting in poor metabolizers associated with the whole gene deletion (*CYP2A6*4*) and/or activity-depressed genes (*CYP2A6*7* and *CYP2A6*9*), which are known to be more abundant in the Asian population [31] than in Caucasian or African populations [32]. Polymorphic variation of CYP2A6 is considered to be relevant to intersubject variation in the therapeutic exposure of letrozole.

20.4 TAMOXIFEN AND CYP2D6

Tamoxifen (Fig. 20.3) is an estrogen receptor (ER) modulator used worldwide in the prevention and treatment of ER-positive breast cancers. The clinical benefit of this agent for the treatment of ER-positive early breast cancer is evident by the eminent

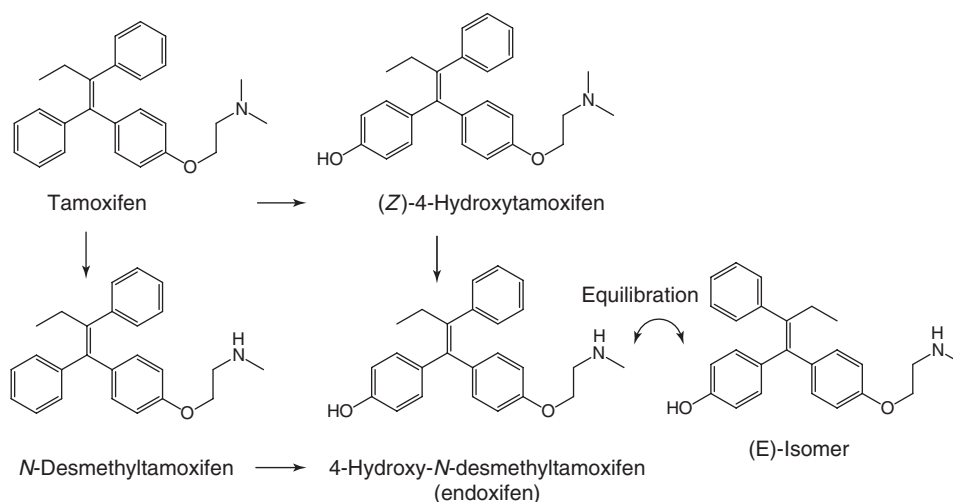


Figure 20.3 Tamoxifen and its metabolites.

reduction in recurrence and mortality rates [33,34]. Despite this success, the mechanisms underlying the ineffectiveness of tamoxifen in a subset of patients are not fully understood; interindividual differences in the formation of active metabolites could be an important factor affecting variability in the response to tamoxifen.

Tamoxifen is a prodrug that requires metabolic activation, carried out mainly by CYP2D6, to elicit its pharmacological activity with clinical outcome [7]. The metabolites 4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen) are believed to be the active therapeutic moieties (Fig. 20.3). Compared with the parent drug, these two metabolites have 100-fold greater affinity to ER and 30- to 100-fold greater potency in suppressing estrogen-dependent cell proliferation [35–37].

The *CYP2D6* gene locus is extremely polymorphic, having over 75 allelic variants (<http://www.cypalleles.ki.se/cyp2d6.htm>), and this contributes to the wide interindividual and ethnic differences in CYP2D6 activity *in vivo*. Women with a poor metabolizer phenotype with respect to CYP2D6, and consequently with low production of the active metabolite, have a greater risk of breast cancer relapse while on tamoxifen, both as a result of genetic variation (e.g., *CYP2D6**4) and also as a result of enzyme inhibition (e.g., by intake of CYP2D6 inhibitor drugs). In Asians, the major defective allele, *CYP2D6**10, causes decreased CYP2D6 activity, and a significantly reduced disease-free survival has been shown for *CYP2D6**10 homozygous patients treated with tamoxifen [38–40] in addition to the minor frequency of the whole gene deletion (*CYP2D6**5). In contrast, an opposite relationship between outcome of tamoxifen treatment and CYP2D6 polymorphism has been published [41]. The basis for this difference is not yet evident. Endocrine therapy tailored to CYP2D6 genotype could be considered for women diagnosed with breast cancer.

Tamoxifen metabolism is mediated by several P450 enzymes, of which CYP2D6 is the most prominent. However, the *in vitro* participation of CYP2C19 in the conversion of tamoxifen to (Z)-4-hydroxytamoxifen, *N*-desmethyltamoxifen, and (E)-4-hydroxytamoxifen and in the trans to cis isomerization of 4-hydroxytamoxifen is well documented (Fig. 20.3), especially at high concentrations [42,43]. In addition, patients

carrying the *CYP2C19**17 rapid activity allele [44] have been shown to have a more favorable clinical outcome during tamoxifen therapy, as measured by the relapse-free time [45]. It is noteworthy that two groups have investigated the effects of the defective *CYP2C19**2 allele on tamoxifen treatment [45,46] but found no significant difference as compared to the wild-type allele, indicating that the *CYP2C19**17 allele might be linked to another polymorphism influencing the phenotype/outcome of the treatment. It is interesting to note that an increased number of *CYP2C19**17 alleles correlated with a decreased risk of suffering from breast cancer [47]. These findings collectively suggest that more studies are needed before application of *CYP2D6* and *CYP2C19* pharmacogenetics to tamoxifen prescription can become a reality. Large prospective studies in this area are necessary to determine the benefit of *CYP2D6* genotyping in the selection of ER modulators for breast cancer.

20.5 THALIDOMIDE AND CYP3A4/5

Thalidomide [α -(*N*-phthalimido)glutarimide] (Fig. 20.4) was withdrawn from clinical use in the early 1960s because of its teratogenic effects in humans but has been approved for the treatment of refractory multiple myeloma by the United States and Japan since 2000 [48,49]. The *CYP2C19* genotype is reported to be associated with cancer treatment outcome using thalidomide [50], and the clinical response rate in 62 patients undergoing thalidomide (with dexamethasone) treatment was twice as high in extensive metabolizers than in poor metabolizers [50]. Decreased formation of active thalidomide metabolites (if those contribute to therapy) would be expected with defective *CYP2C19* alleles compared to the wild type [50]. No evidence has been obtained for drug interactions between thalidomide and hormonal contraceptives [51,52].

Thalidomide-inhibited *CYP2C19*-dependent (*S*)-mephenytoin 4'-hydroxylation at high concentrations but enhanced *CYP3A5*-dependent midazolam hydroxylation and

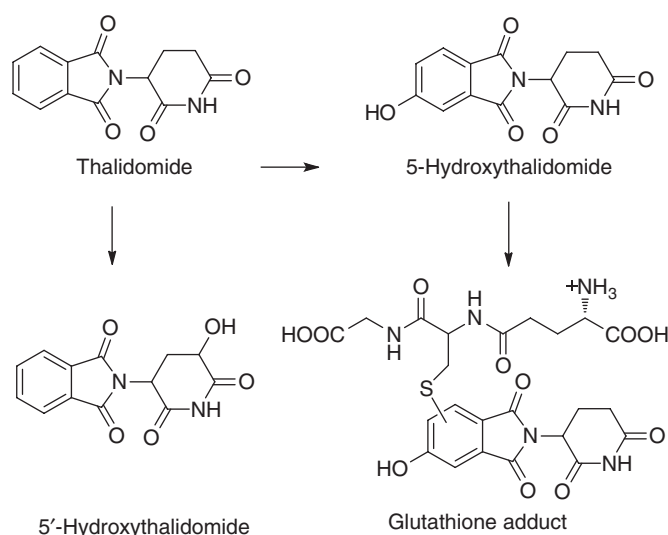


Figure 20.4 Thalidomide and its metabolites.

cyclosporine A clearance at clinically relevant concentrations [53]. Two hydroxylated metabolites of thalidomide (5-hydroxythalidomide and 5'-hydroxythalidomide) are reportedly formed at very low concentrations after incubation with recombinant human CYP2C19 [54]. Recently, although ligand cooperativity with CYP3A5 similar to that for CYP3A4 has been observed [55] and homotropic cooperativity of CYP3A5 with thalidomide was shown [56]. Liquid chromatography–mass spectrometry analysis revealed formation of a glutathione conjugate from (*R*)- and (*S*)-5-hydroxythalidomide, a process catalyzed by liver microsomal CYP3A4 and CYP3A5 in the presence of glutathione (assigned as a conjugate of 5-hydroxythalidomide formed on the phenyl ring) (Fig. 20.4) [56]. These findings established that human CYP3A4 and CYP3A5 mediate thalidomide 5-hydroxylation and further oxidation leads to a glutathione conjugate, which may be of relevance in the pharmacological and toxicological actions of thalidomide.

20.6 IRINOTECAN AND UDP-GLUCURONOSYLTRANSFERASE 1A1

Irinotecan is mainly used in the treatment of colon cancer, particularly in combination with other chemotherapy agents. Irinotecan (known as *CPT-11* during development), a semi-synthetic analogue of the natural alkaloid camptothecin, is activated by enzymatic hydrolysis to SN-38 (Fig. 20.5), an inhibitor of topoisomerase I. The inhibition of topoisomerase I by the active metabolite SN-38 leads to inhibition of both DNA replication and transcription. SN-38 is inactivated by glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1).

Subjects harboring the *UGT1A1**28 gene (called *TA₇* in the promoter region) express fewer UGT1A1 enzymes in their livers and often suffer from Gilbert's syndrome [57]. During chemotherapy, these patients effectively receive a larger than expected dose because their bodies are unable to clear irinotecan as fast as other subjects; this corresponds to higher incidences of severe diarrhea and neutropenia [58]. A clinical study was performed in 2004 that validated prospectively both the association of *UGT1A1**28 with greater toxicity in patients undergoing treatment with irinotecan and the ability of genetic testing to predict toxicity before chemotherapy administration [59]. The US FDA made changes to the labeling of irinotecan to add pharmacogenomics recommendations regarding which patients carrying genetic polymorphism of the *UGT1A1* gene

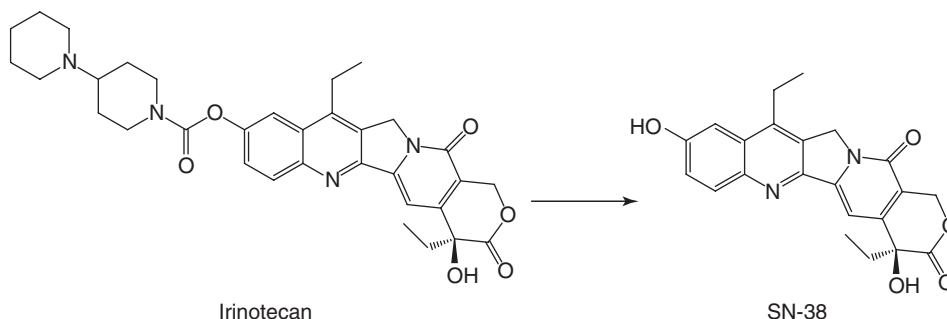


Figure 20.5 Irinotecan and its metabolite.

(specifically *UGT1A1**28) might benefit from reduced drug doses. Irinotecan is one of the first widely used chemotherapy agents for which personalized medicine according to genotype has been applied [60].

20.7 6-MERCAPTOPURINE AND THIOPURINE-S-METHYLTRANSFERASE

6-Mercaptopurine (Fig. 20.6) is used to treat leukemia and pediatric non-Hodgkin's lymphoma and is an immunosuppressive agent [61]. 6-Mercaptopurine inhibits purine nucleotide synthesis and metabolism and alters the synthesis and function of RNA and DNA. Some of the adverse reactions of taking 6-mercaptopurine include black or tarry stools (melena), bloody stools, and bloody urine [62].

6-Mercaptopurine causes myelosuppression, suppressing the production of white and red blood cells and may be toxic to bone marrow. Weekly blood counts are recommended for patients on 6-mercaptopurine, and treatment is interrupted if there is an unexplained abnormally large drop in white blood cell or any other blood count. Subjects who exhibit myelosuppression or bone marrow toxicity should be tested for thiopurine-S-methyltransferase (TPMT) enzyme deficiency [63,64]. Although patients with TPMT deficiency are much more likely to develop dangerous myelosuppression [65], it may be possible to continue using 6-mercaptopurine at a lower dose [66].

20.8 CARBOPLATIN AND HYPERBARIC OXYGENATION

Carboplatin (Fig. 20.7), *cis*-diammine-1,1-cyclobutane dicarboxylate platinum(II), is a second-generation platinum antineoplastic agent with modest activity against brain tumors [67]. The efficacy of intravenous administration of 400-mg carboplatin/m²

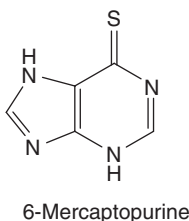


Figure 20.6 Chemical structure of 6-mercaptopurine.

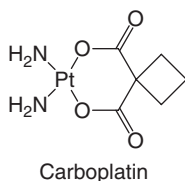


Figure 20.7 Chemical structure of carboplatin.

body surface area over 60 min combined with hyperbaric oxygenation (HBO) therapy (0.2 MPa for 60 min) was investigated in several patients with malignant or brainstem gliomas [68]. Plasma ultrafiltrate samples were analyzed by high performance liquid chromatography [69] to evaluate the relationship between efficacy and pharmacokinetics. Brain tumor response was evaluated by magnetic resonance imaging as a function of maximum plasma concentration, area under the curve, or mean residence time (MRT) for carboplatin. The MRT for carboplatin in the complete or partial response group was significantly longer than that in the progressive disease group [68], but maximum plasma concentration and area under the curve had no effect on outcome. These results suggest that HBO therapy prolongs the biological residence time of carboplatin with modification of chemotherapy and/or clinical antitumor effects in patients with malignant gliomas.

Carboplatin had low brain penetration potential in a cultured blood–brain barrier system. Therefore, HBO therapy at 0.2 MPa for 60 min resulted in apparently higher concentrations of carboplatin in the brain than expected. The present findings of good clinical response may suggest that HBO therapy modifies the pharmacokinetics of carboplatin. With a rat model, changes in the pharmacokinetics of carboplatin with HBO therapy were also suggested by the detection of carboplatin in the brain at 60 min (from the start of administration) after HBO treatment at 0.2 MPa for 20 min [70,71]. Oxygen-induced cerebral vasoconstriction could result in some modification of drug pharmacokinetics, presumably because of various biochemical changes in the brain such as inactivation of intracellular enzyme systems or changes in blood flow rates. Any resultant prolonged biological half-life of carboplatin might explain the effect of combined cisplatin/HBO therapy for malignant gliomas.

Carboplatin had similar endothelial permeability to that of doxorubicin or verapamil, typical P-glycoprotein substrates, in an *in vitro* blood–brain barrier system [71]. Increased permeability of P-glycoprotein-dependent carboplatin as a result of HBO (similar to that resulting from verapamil) may rapidly reach pharmacologically significant concentrations in the central nervous system. Moreover, increased distribution of carboplatin to rat brains with the aid of HBO *in vivo* has been reported [70]. Prolonged biological residence time of carboplatin may be achieved by combination with HBO therapy, resulting in improved efficacy against malignant gliomas. The MRT may be important for predicting continuation or modification of chemotherapy and/or clinical antitumor effects on malignant gliomas.

20.9 CONCLUSIONS

P450 enzymes responsible for the activation or inactivation of anticancer drugs are polymorphic, and much research has been directed at elucidating the ways in which genetic polymorphism in the corresponding genes can affect the efficacy of cancer therapy. With respect to anticancer therapy, the influence of CYP2D6 polymorphism on tamoxifen treatment merits additional study to establish the relationship. Further interesting results can be anticipated leading to more detailed labeling of irinotecan with pharmacogenomic recommendations on which patients carrying genetic polymorphism of the *UGT1A1* gene (specifically *UGT1A1**28) should perhaps receive reduced drug doses. In the future, increased understanding regarding the role of P450 expression will also provide additional information on the ADME of anticancer drugs.

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21 ADME of Cardiovascular Drugs

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21.1	Summary of cardiovascular drug metabolism	1
21.2	Drug-metabolizing enzymes	2
21.3	Cytochrome P450	2
21.4	Drug-metabolizing enzymes expressed in the cardiovascular system	7
21.5	Role of CYP metabolites in cardiovascular health	8
21.6	Drug–drug interactions	9
21.7	Transporters in CV drug metabolism	10
21.8	Biomarkers and metabolomics	11
21.9	LIPID-lowering agents	12
21.10	Diuretics	17
21.11	Antithrombotics	17
21.12	Antiarrhythmics	20
21.13	ACE inhibitors	25
21.14	Angiotensin receptor blockers	26
21.15	The future of cardiovascular drug metabolism	28
	References	28

21.1 SUMMARY OF CARDIOVASCULAR DRUG METABOLISM

Effective drug therapy for cardiovascular diseases (CVDs) is determined by systemic exposure of the therapeutic agent and specificity for the target. Drug concentration at the target site is dependent on the distributional characteristics of the drug (e.g., membrane permeability and unbound fraction) and by its concentration in blood or plasma. Since the systemic exposure of a drug is usually directly related to maximize desired effect and minimize undesired effects, the factors that influence the concentration of the agent over time determine the success of the therapy. Accordingly, those factors that control clearance and elimination of the drug ultimately control safety and effectiveness.

Clearance of most cardiovascular drugs is mediated by one or more enzymatic or transport processes. Isoforms of cytochrome P450 (CYP) make up the most prevalent oxidative or the so-called phase I clearance pathway. Some cardiovascular (CV) drugs are metabolized by primary or secondary conjugation, forming glucuronides, sulfates,

or other conjugates, the so-called phase II pathway. A few drugs, notably angiotensin-converting enzyme (ACE) inhibitors are metabolized by hydrolysis pathways. Although the human CYP superfamily is composed of more than 50 members, CV drugs interact primarily with CYP3A4, 2D6, or 2C9. Given that CYP3A4 is the most highly expressed CYP in the liver and has the largest active site, the association with CV drug metabolism is not surprising. However, liver CYP2D6 abundance is relatively low (about 2% of total) and the active site more restricted, yet it is the primary clearance mechanism for a number of CV drugs.

The most notable CV adverse events have stemmed from drug interactions due to changes in the enzymatic clearance process. Enzymatic processes are subject to saturation, inhibition, induction, and genetic polymorphism, each of which can change systemic exposure and therapeutic efficacy. In the case of the thienopyridine platelet inhibitors, CYP2C19 directly controls effectiveness by virtue of the activation pathway for generation of the active thiols. Other oxidative or hydrolytic enzymes also control drug clearance, particularly in the case of ACE inhibitors.

The enzymatic and transporter processes that control drug clearance can be capacity-limited processes, subject to variation in activity or expression due to competing substrates or regulation by intrinsic and external factors. Each of these attributes has the potential to influence effectiveness of cardiovascular drugs as well as cardiotoxic effects of noncardiovascular therapeutics.

21.2 DRUG-METABOLIZING ENZYMES

Drug-metabolizing enzymes (DMEs) play an important role in cardiovascular health and disease. These enzymes, especially CYP, control the clearance of many small-molecule cardiovascular drugs and are involved in the metabolism of endogenous substances (such as leukotrienes, steroids, and bile acid) that are in turn implicated in several cardiovascular-related pathways, including inflammation, lipid metabolism, and blood pressure regulation. In general, drugs can be metabolized by oxidation, reduction, hydrolysis, or conjugation, generally accepted pathways for excretion of drugs from the body. The enzymes involved in metabolism are present in many tissues but are most concentrated in the liver. These enzymes are divided into two groups. Phase I metabolizing enzymes, including CYP450s and flavin monooxygenases (FMOs), catalyze the introduction of an oxygen atom into substrate molecules often resulting in hydroxylation, dealkylation, and hydrolysis. Phase II reactions involve conjugation with an endogenous or exogenous substance. Phase II conjugating enzymes include the UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), *N*-acetyltransferases (NATs), and glutathione transferases (GSTs). Metabolites formed from either or both processes are more polar and more readily excreted by renal and/or hepatobiliary processes. Among these enzymes, CYPs often play a critical role in the disposition of cardiovascular drugs.

21.3 CYTOCHROME P450

CYP is a superfamily of hemoprotein found in human liver and other tissues such as small intestine that play an important role in the metabolism of exogenous as well

TABLE 21.1 Cardiovascular Drugs as CYP Substrates

Substrates	CYP3A4/5	CYP2C19	CYP2C8	CYP2C9	CYP2D6	CYP1A2
<i>Lipid lowering agents</i>						
Simvastatin	×	—	×	—	—	—
Lovastatin	×	—	—	—	—	—
Atorvastatin	×	—	×	—	—	—
Fluvastatin	×	—	×	×	—	—
Pravastatin	×	—	—	—	—	—
Rosuvastatin	×	×	—	×	—	—
Pitavastatin	—	—	—	×	—	—
Gemfibrozil	—	—	×	—	—	—
<i>Diuretics</i>						
Torsemide	—	—	×	×	—	—
<i>Antithrombotics</i>						
Warfarin	—	—	—	×	—	×
<i>Antiarrhythmics</i>						
Quinidine	×	—	—	×	—	—
Procainamide	—	—	—	—	×	—
Lidocaine	×	—	—	—	—	×
Propafenone	×	—	—	—	×	×
Carvedilol	—	—	—	—	×	—
Propranolol	—	—	—	—	×	×
Metoprolol	—	—	—	—	×	—
Amiodarone	×	—	×	—	—	—
Debrisoquine	—	—	—	—	×	—
Flecainide	—	—	—	—	×	—
Mexiletine	—	—	—	—	×	—
Verapamil	×	—	—	—	—	×
Diltiazem	×	—	—	—	—	—
<i>Antiplatelet</i>						
Clopidogrel	×	×	—	×	—	×

Data were collected from drug interaction database from the University of Washington.

as endogenous molecules. CYP enzymes are so named because they absorb light at a wavelength of 450 nm when carbon monoxide binds to the enzymes at their reduced state. There are more than 50 CYP enzymes in human, although many of these enzymes have the capacity to metabolize drugs, the majority of CYP-mediated drug metabolism in human is catalyzed by subfamily CYP3A, 2C, 2D6, and 1A enzymes (Table 21.1).

21.3.1 Cytochrome P450 3A Subfamily

CYP3A is the most highly expressed isoform in human, representing ~30% of the spectroscopically detectable CYP in the liver [1] and have an important role in the oxidative, peroxidative, and reductive metabolism of endogenous steroids, many procarcinogens, and at least 50% of all drugs. The most abundant CYP3A isoform expressed in liver and gut is CYP3A4 [2]. Interindividual expression of CYP3A4 can vary by 50-fold among individuals and functionality (i.e., drug clearance) by about 20-fold. High interindividual variation can be associated with genetic and environmental factors [3].

Despite extensive efforts to identify genetic variation within the CYP3A4 gene associated with altered enzyme activity, multiple groups have failed to identify common CYP3A4 genotypes that can explain variable CYP3A4 expression [4,5]. Although the substrate specificity of CYP3A5 is similar to CYP3A4, it is thought to be less important for drug metabolism because it is expressed at only 10–30% of CYP3A4 levels. A unique characteristic of CYP3A is the autoactivation or heteroactivation, which is associated with the increased catalytic activity of a particular substrate by the addition of another xenobiotic or same substrate. This positive cooperativity or activation of CYP3A occurs with midazolam by α -naphthoflavone, alprazolam, flunitrazepam, and triazolam by testosterone and so on [6]. The clinical significance of activation is not clear, complicating the interpretation of *in vitro* studies and extrapolation to human pharmacokinetics (PK).

CYP3A4 is often implicated in drug–drug interactions (DDIs) as a result of inhibition or induction by drugs or dietary components, sometimes leading to adverse drug reactions. Azole antifungal agents, ketoconazole, itraconazole, and fluconazole are potent competitive CYP3A4 inhibitors with K_i values below $1\ \mu\text{M}$. Macrolides such as erythromycin, and troleandomycin and antidepressant such as fluoxetine and nortriptyline are CYP3A inhibitors acting via the formation of metabolite–intermediate complexes. HIV protease inhibitors such as ritonavir and 17- α -ethinyl-substituted steroids have been found to be mechanism-based CYP3A4 inhibitors. Rifampin, carbamazepine, phenytoin, and phenobarbital are reported as potent CYP3A inducers. Terfenadine, a well-known early example of drug interactions, was approved in the United States in 1985 for the treatment of seasonal allergic rhinitis. The drug is primarily eliminated by CYP3A4-mediated metabolism. Coadministration with potent CYP3A4 inhibitors such as ketoconazole and erythromycin markedly elevated terfenadine plasma level to produce serious cardiac arrhythmias leading to death in some instances [7]. Terfenadine was removed from the market when fexofenadine, the active metabolite of terfenadine, was approved and marketed. Fexofenadine does not block cardiac potassium channels. Prolonged QT interval and syncope were also reported for nonsedating antihistamine, astemizole, when coadministered with ketoconazole, itraconazole, or erythromycin [8]. Nevertheless, most clinically significant drug interactions result agents with a narrow therapeutic index coadministered potent inhibitors or inducers. More modest interactions are generally of lesser concern since the consequences are within the window of normal variability.

21.3.2 Cytochrome P450 2D6 Subfamily

CYP2D6 accounts for about 2% of total hepatic CYP but is responsible for about 25% of the metabolism of known drugs, including many of those used in the treatment of CVD. Genetic variants of CYP2D6, which result in varying levels of metabolic activity, are of clinical importance in some settings. Currently, more than 75 allelic variants have been identified; all variant alleles are presented at the home page of the human CYP allele nomenclature committee (<http://www.imm.ki.se/cypalleles/cyp2d6.htm>). The variant CYP2D6 alleles result from point mutations, deletion, or duplication of the entire gene and result in an abolished, decreased, normal, increased, or qualitatively altered catalytic activity. Establishing which phenotype an individual has often depends on the administration of a probe drug that is a substrate of CYP2D6 (e.g., dextromethorphan, debrisoquine, or sparteine) and measuring the metabolite–parent

ratio. However, there are some limitations for estimating *in vivo* enzyme activity using urinary metabolite to parent ratio [9]. For dextromethorphan, there is not a linear correlation between enzyme activity and urinary parent/metabolite ratio, probably a result of pH dependence and renal or hepatic transport. Dextromethorphan is also a substrate of CYP3A. In addition, cost and time effectiveness of urine analysis have precluded widespread, routine phenotyping determinations. As a practical alternative, genotyping of the CYP2D6 gene is increasingly used in clinical practice to assess the risk of undesired drug effects or therapeutic failure for individual patient and eventually to apply a dose adjustment or change in therapeutic strategy. Considering the growing number of CYP2D6 alleles and corresponding ranges of activity, prediction of a subject's phenotype based on their genotype is a significant challenge. Currently, standard process of translating genotype data into a phenotype prediction is not established. Several different genotype–phenotype systems for CYP2D6 have been proposed, including the CYP2D6 “activity score” (AS) system [9,10]. In this system, over 25 CYP2D6 allelic variants were genotyped in 672 subjects of Caucasian and African-American heritage. For each variant CYP2D6 allele, a value relative to the fully functional CYP2D6*1 reference allele is assigned based on published literature on *in vivo* and *in vitro* activity toward commonly used probe drug, such as dextromethorphan (DM), debrisoquine, or sparteine. The AS of a genotype is the sum of values assigned to each allele with the assumption of both alleles contributing equally to overall CYP2D6 activity. A total 95 genotypes are categorized into six AS groups. A final model (AS-model A) is generated to relate AS groups to CYP2D6 activity based on urinary metabolite data (DM/dextromethorphan ratios). The authors also emphasize that the probability of belonging to a particular phenotype group using AS-model A may be more accurately predicted if ethnicity is considered. Although there are some limitations of using dextromethorphan urinary ratios to estimate *in vivo* enzyme activity, AS system is a simplified tool for translating vast genotype data into CYP2D6 activity prediction.

It is important to establish a more quantitative system to precisely predict CYP2D6 activity *in vivo* and transform this information to dose adjustment for individual patient for drugs that are primarily metabolized by CYP2D6, such as the antiarrhythmics flecainide and propafenone or β -blocker metoprolol.

21.3.3 The Cytochrome P450 2C Subfamily

The CYP2C subfamily of P450 enzymes metabolize ~20% of clinically used drugs; CYP2C8, 2C9, and 2C19 are of clinical importance. Next to CYP3A4, CYP2C9 is the most predominantly expressed CYP enzyme in the human liver and the most highly expressed of the 2C subfamily. It metabolizes many clinically important drugs, including the diabetic agent tolbutamide, the anticonvulsant phenytoin, the *S*-enantiomer of the anticoagulant warfarin, Δ^1 -tetrahydrocannabinol, and numerous anti-inflammatory drugs such as ibuprofen, diclofenac, piroxicam, tenoxicam, and mefenamic acid [11], the antihypertensive losartan [12], the antidiabetic drug glipizide, and the diuretic torsemide [13]. Defective alleles of CYP2C9 [14,15] have the potential to affect the safety of those CYP2C9 drugs with smaller therapeutic indices such as warfarin, phenytoin, and certain antidiabetic drugs. CYP2C9*2 and 2C9*3 are known to affect catalytic function. CYP2C9*2 has a frequency of ~8–10% in Caucasians but has a lower frequency in African-Americans and appears to be virtually absent in Asians

[16,17]. CYP2C9*3 has a frequency of ~6% in Caucasians (the frequency of homozygous CYP2C9*3 is 0.3%); the frequency in Asians is probably 2%. Results from the human studies published to date have concluded that CYP2C9 genotype contributes significantly to the variability in disposition and dose–response of substrates such as warfarin, phenytoin, tolbutamide, glipizide, losartan, candesartan, irbesartan, ibuprofen, flurbiprofen, and celecoxib. Individuals carrying the CYP2C9*2 and *3 alleles also have lower warfarin and phenytoin daily dose requirements and appear more susceptible to adverse events during the initiation of therapy. CYP2C9 genotype-guided dosing may be clinically useful and warrants prospective investigation [18].

CYP2C19 is the principle catalyst for metabolism of several well-known drugs such as omeprazole, diazepam, and mephenytoin; it is also responsible for activation of proguanil to cycloguanil. Genetic variation attributable to CYP2C19 was first described in the 1980s [19], about the same time, the thienopyridine antiplatelet drug ticlopidine was brought to market. Although clopidogrel is the current standard of care for coronary artery disease patients undergoing a percutaneous coronary intervention (PCI), ~25% of the patients experience a subtherapeutic antiplatelet response [20]. Clopidogrel is a prodrug, about 85% of the clopidogrel dose is hydrolyzed by esterase to an inactive metabolite, and the remaining clopidogrel is available for the conversion to the active metabolite via two sequential metabolism-mediated steps [21]. The role of CYP2C19 in its metabolism was unknown until several years after its approval that a report suggested possible polymorphism in CYP2C19 might be a cause of variable pharmacodynamic response [22]. This was confirmed in a number of subsequent reports, thoroughly reviewed by Farid *et al.*, pointing to the conclusion that CYP2C19 is the major contributor to 2-oxo-clopidogrel (active metabolite) formation from clopidogrel. CYP2C19 variant allele carriers exhibit a significantly lower capacity to metabolize clopidogrel and inhibit platelet activation and are therefore at higher risk of adverse cardiovascular events. Finally resulting in a US Food and Drug Administration (FDA) recommendation that patients be genotyped for CYP2C19 PM to avoid inadequate platelet inhibition [20].

21.3.4 Cytochrome P450 1A2 Subfamily

CYP1A2 accounts for ~13% of total hepatic CYP expression [1], CYP1A2 shows up to 40-fold variability between individuals and corresponding variability of enzyme activity and drug metabolism. Although there is evidence that genetic factors have major influence on observed enzyme activity [23], the role of polymorphisms identified to date is unclear. Apart from smoking, coadministration of drugs that are inhibitors, inducers, or competing substrates for CYP1A2 can influence CYP1A2 activity [24]. Phenotyping represent a suitable method to shed light on the actual enzyme activity. Caffeine is the most commonly used substance for CYP1A2 phenotyping. The first step in its metabolism is almost exclusively mediated by CYP1A2, and concentrations in biological matrices can be determined using a simple HPLC method. An epidemiological study examining the role of diet and caffeine in cardiovascular health found an association of CYP1A1*1F allele with nonfatal myocardial infarction (MI) [25]. Carriers of *1F allele are “slow metabolizers” of caffeine compared to wild type, probably due to reduced expression of the enzyme. Overall, the involvement of CYP1A2 in cardiovascular drug metabolism is relatively low.

21.3.5 Other Cytochrome P450s and Phase II Enzymes

Other CYP enzymes such as CYP2B6, CYP1A1, UGT, or SULT are generally not critically involved in the metabolism of CV drugs, although it should be noted that 1-*O*-glucuronide of gemfibrozil was found to be a potent mechanism-based CYP2C8 inhibitor. Several reported drug interactions between gemfibrozil and CYP2C8 substrates such as antidiabetic agents rosiglitazone and pioglitazone are probably due to CYP2C8 inhibition by 1-*O*-glucuronide of gemfibrozil [26,27].

21.4 DRUG-METABOLIZING ENZYMES EXPRESSED IN THE CARDIOVASCULAR SYSTEM

Although significant efforts have been focused on hepatic CYP-mediated cardiovascular drug interactions and genetic polymorphism of hepatic CYPs leading to adverse drug events, limited information is available regarding cardiac metabolism [28,29]. Differences in cardiac drug metabolism may also influence pharmacological efficacy or adverse drug effects. For example, lack of efficacy for ACE inhibitors in treating right-ventricular hypertrophy was attributed to metabolic inactivation of drug in the right ventricle but not in the left ventricle [30]. Further, endogenous CYP metabolites, epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acid (HETEs), prostacyclin (PGI₂), aldosterone, and sex hormones have pivotal roles in the maintenance of cardiovascular health.

Evidence for expression or at least transcription has been observed for a number of CYPs in cardiac tissue. CYPs identified in mammalian species include CYP1A1, 1B1, 2A1/2, 2A6/7, 2B6/7, 2C8/9, 2D6, 2E1, 2J2, 3A4, 4A1/2, 4B1, and 4F1 [29].

CYP1A1 mRNA was detected in the right ventricle, pulmonary aorta, ascending aorta, and the left atrium of patients with dilated cardiomyopathy and in the left ventricle of healthy subjects [31]. CYP1A2 mRNA is absent in healthy human heart [32]. CYP1B1 mRNA expression was observed in the normal human heart in relatively high abundance [32]. CYP1A1 mRNA and CYP1B1 are inducible in vascular smooth muscle cells (SMCs) [33].

In humans, CYP2B, 2C, 2D6, 2E1, and 2J2 were detected in heart tissues [30,34]. CYP2B6/7, 2C8-19, and 2D6 were predominantly expressed in the right ventricle; the unilateral expression of the 2D6 gene in right-ventricular tissue [30], whereas CYP2E1 mRNA was expressed in various parts of the heart, including the right and left atria, right and left ventricle, aorta, and the ventricular septum. CYP2E1 protein was found in the endocardium and coronary vessels of human autopsy samples [35]. CYP2C8, 2C9, and 2J2 mRNA and protein are present in various amounts in the human heart, coronary artery, and aorta tissues [34]. CYP2J2 mRNA levels in the heart were ~10 times higher than those of CYP2C9 or 2C8. CYP2J2 and 2C8 protein were shown to be predominant CYP expressed in human heart, CYP2J2 is also expressed in blood vessels. Beside higher expression in heart tissue, CYP2J2 is most abundant in small intestine [36,37]. In fact, CYP2J2 was shown to be responsible for the first-pass metabolism of ebastine and astemizole [38]. In a recent screening of CYP2J2 substrates using 139 marketed therapeutic agents, several novel substrates including terfenadine, astemizole, amiodarone, albendazole, danazol, thioridazine, tamoxifen, cyclosporine, nabumetone, and mesoridazine were identified [39]. Interestingly, the substrates identified for CYP2J2

are also metabolized by CYP3A4. Although CYP2J2 active site can accommodate large molecules such as CYP3A4, CYP2J2 metabolism was more restricted to a single site of molecule, whereas CYP3A4 commonly metabolizes compounds at multiple sites of molecules. Little information is available on the expression of CYP3A family in cardiovascular tissues. CYP3A4/5/7 mRNA was not found in human heart tissues [30]. CYP4F3/12 mRNA was detected in the human and dog heart [40,41]. The role and regulation of CYP expression in the heart is not clear but it is unlikely that it plays any meaningful role in drug clearance.

21.5 ROLE OF CYP METABOLITES IN CARDIOVASCULAR HEALTH

Metabolism of eicosanoids in the cardiovascular system is well described and partly dependent on CYPs. There are three major metabolic pathways involved in arachidonic acid metabolism [42]. First, hydroperoxyeicosatetraenoic acids (HPETEs) and dihydroxyeicosatetraenoic acid (DiHETE) are formed by lipoxygenase and subsequently converted to HETEs by peroxidases; in the second pathway, cyclooxygenase catalyzes arachidonic acid to prostaglandin G₂, in a subsequent peroxidase reaction, PGG₂ undergoes a two-electron reduction to PGH₂. PGH₂ can be further metabolized to other eicosanoids such as PGI₂ and thromboxane A₂ (TXA₂). Third, CYP metabolizes arachidonic acid to regio- and stereospecific EET and HETEs.

Several CYPs can metabolize arachidonic acid to EETs, including CYP1A, 2B, 2C, 2E, and 2J subfamilies [43,44]. CYP2J isoforms have been proposed as the predominant enzymes responsible for epoxidation of endogenous arachidonic acid pools in human and rat heart [36,45]. The induction of CYP2C in native porcine coronary artery endothelial cells by β -naphthoflavone enhances the formation of EET, as well as endothelium-derived hyperpolarizing factor (EDHF)-mediated hyperpolarization and relaxation. Transfection of coronary arteries with CYP2C antisense oligonucleotides results in decreased levels of CYP2C and attenuates EDHF-mediated vascular responses, indicating that the EDHF synthase in the porcine coronary vascular bed is a CYP2C isoform. EETs are important components of many intracellular signaling pathways involved in vasodilatory, anti-inflammatory, and cardioprotective responses in cardiovascular system [29].

In addition to generation of the vasorelaxant 11,12-EET, CYP2C9 was shown to be potential major source of reactive oxygen species (ROS) [46]. ROS generated in arteries reacts with NO to produce peroxynitrite (ONOO⁻) resulting in a reduction of NO bioavailability that impairs endothelium-dependent vascular function in atherosclerosis [47]. Hunter *et al.* recently demonstrated that CYP2C inhibition during the peritransplant period prevented development of cardiac allograft vasculopathy (CAV) by inhibiting early SMC proliferation and intimal hyperplasia. This effect could also be a result of reduced postischemic oxidative damage, contributing to increased NO bioavailability and/or prevention of interferon γ (IFN- γ) production [48]. Interestingly, it was reported that concomitant pretreatment with 11,12- or 14,15-EET in combination with the free radical scavenger, 2-mercaptopropionyl glycine (2-MPG), completely abolished the cardioprotective effect of 11,12- and 14,15-EETs, suggesting part of the cardioprotective effects of EETs in rat hearts against infarction is the result of an initial burst of ROS and subsequent activation of both the sarcK_{ATP} and mitoK_{ATP} channel [49].

CYP ω -hydroxylases, such as CYP4A and 4F, biosynthesize 20-HETEs [50]. CYP1A1/2, 1B1, and 2E1 were reported to produce different regioisomers of HETE [28]. HET0016 is a potent and fairly selective inhibitor of 20-HETE formation with little effect on the activities of cyclooxygenase and other CYP enzymes [51]. The IC_{50} values of HET0016 for the formation of 20-HETE by human recombinant CYP4F2, 4F3, and 4A11 enzymes are 125, 100, and 42 nM, respectively [52]. In addition to CYP inhibitors, antisense of cDNA oligonucleotides (ODNs) has been developed to block the formation of 20-HETE. CYP4A1/4A2/4A3-specific antisense ODNs can inhibit protein expression and the corresponding catalytic activity [53]. The role of HETEs in the control of cardiovascular functions has been reviewed [54]. Altered expression and function of arachidonic acid ω -hydroxylases in models of hypertension, diabetes, inflammation, and pregnancy suggest that 20-HETE may be involved in the pathogenesis of these diseases [50].

21.6 DRUG-DRUG INTERACTIONS

Pharmacokinetic drug interactions can cause excess or insufficient drug exposure leading to, respectively, toxicity or ineffectiveness and in some cases to termination of drug development, severe prescribing restriction, and withdrawal of drugs from the market. The majority of DDIs stem from changes in enzymatic capacity. The existing guidance issued by the FDA covers mainly CYP450-mediated drug interactions, the importance of other mechanisms, such as transporter-based drug interaction, non-CYP-based interaction, and interactions involving therapeutic proteins, have been recognized more recently. Many cardiovascular drugs are metabolized in the liver; CYP3A4, 2D6, and 2C9 are primary enzymes mediating cardiovascular drug interactions. CYP inducers such as phenytoin, barbiturates, rifampin, and St John's wort can induce CYP3A4, 2B6, and 2C8/9 and increase metabolism of susceptible cardiovascular drugs (e.g., some statins, nifedipine, or warfarin), resulting in decreased plasma concentrations and reduced efficacy. On the other hand, systemic exposure of cardiovascular drugs can be increased by potent CYP inhibitors (e.g., erythromycin, grapefruit juice, or omeprazole).

Some cardiovascular drugs are transporter substrates or inhibitors. For example, most statins are hepatic organic anion transporting polypeptide (OATP)1B1/1B3 substrates; cyclosporine causes up to 20-fold increase in area-under-the-curve (AUC) values of statins in organ transplant patient via OATP1B1/1B3 inhibition leading to greater risk of myopathy [55]. Quinidine and verapamil can cause significant increase in digoxin absorption via gut P-glycoprotein (PGP) inhibition [56].

The interaction between clopidogrel, an antiplatelet drug, and omeprazole, a proton pump inhibitor, is illustrated in words from a regulatory agency. "New data show that when clopidogrel and omeprazole are taken together, the effectiveness of clopidogrel is reduced. Patients at risk for heart attacks or strokes who use clopidogrel to prevent blood clots will not get the full effect of this medicine if they are also taking omeprazole." The updated label acknowledges that "concomitant use of drugs that inhibit CYP2C19 (e.g., omeprazole) should be discouraged." The underlying mechanism for this drug interaction is that omeprazole inhibits the CYP2C19, which is the key enzyme for the conversion of clopidogrel into its active form [57]. Other examples of adverse pharmacokinetic drug interaction include increased risk of myopathy with

statins during the coadministration of gemfibrozil via CYP2C8 inhibition [58] and market withdrawals of mibefradil, a calcium channel blocker, because of severe interactions with multiple drugs via inhibition of CYP3A4 and PGP [59]. Detailed drug interactions of major classes of cardiovascular drugs are discussed in later sections.

Of course, noncardiovascular drug interactions may produce significant cardiovascular side effects. Coadministration of CYP3A4 inhibitors (such as ketoconazole, itraconazole, and erythromycin) with terfenadine, methadone, or cisapride have been reported to increase plasma concentration of these drugs and the risk of Torsade de Pointes [56].

21.7 TRANSPORTERS IN CV DRUG METABOLISM

Although more than 400 membrane transporters have been identified in humans, 7 transporters account for much of the apparent drug transport activity in humans: two efflux transporters [PGP and breast cancer resistance protein (BCRP)] and five uptake transporters (OCT2, OAT1, OAT3, OATP1B1, and OATP1B3) [60]. The efflux transporters PGP and BCRP are both members of the ATP-binding cassette (ABC) family and function on the apical or luminal side of the intestinal epithelia, kidney proximal tubules, hepatocytes (canalicular membrane), and blood–brain barrier (capillary endothelial cells). The five uptake transporters are members of the solute carrier (SLC) family. OATP1B1 and OATP1B3 function on the sinusoidal side (blood side) of hepatocytes. OCT2, OAT1, and OAT3 function as uptake transporters on the basolateral side (blood side) of kidney proximal tubule cells and function by selectively pumping substrates from the blood. In this way, transporters serve a protective role by pumping substrates out of the cells. Examination of recent publications on drug transporters shows considerable involvement of cardiovascular drugs [60]. Indeed, the field of membrane transporters has grown in part because of the need to understand the role of uptake and efflux transporters in the disposition of the statin drugs. Of the five major drug transporter families, DDIs with four are characterized primarily by cardiovascular drugs [60]. Although the discovery and understanding of transporters in drug disposition and interactions has lagged that of CYPs, progress in recent years has provided a much more complete understanding of the situation to the point where guidelines for evaluation of DDI has been proposed and the role of transporters in disposition of drugs is expected.

The efflux transporter PGP (MDR1 and ABCB1) appears to serve as a protective mechanism by pumping its substrate back into the lumen of the small intestine, out of the brain, liver, and kidney. It is a member of the ABC family of transporters, characterized by ATP-dependent directional flow. It has broad substrate specificity and plays an important role in limiting the absorption of many drugs through the gut wall and blood–brain barrier. It also facilitates excretion of many drug substrates from the liver and kidney. The cardiac glycoside digoxin is a prototypic inhibitor of PGP.

BCRP is a related ABC transporter expressed in tissues similar to PGP and functioning in similar manner but with different overlapping substrate specificity. Although it might play a role in the disposition of some statins, it appears to have few interactions with cardiac drugs.

The OATP family represents a family of SLC transporters with substrate specificity for amphiphilic compounds. Notable are OATP1B1 and OATP1B3, both of which

appear to be involved in hepatic uptake of statins and might be important in controlling PK and toxicity of some statins.

Two additional transporters that have limited drug substrates but nevertheless important for disposition of ACE inhibitors (e.g., zofenopril and fosinopril) are PEPT1 and PEPT2. Both are members of the SLC family, functioning as uptake transporters on the apical membrane (i.e., luminal side) of intestinal epithelia and kidney proximal tubule cells.

Although some drugs are cleared solely by metabolism involving one or more enzymes and a few are cleared only by transporters, many are cleared by a combination of transporters and DMEs. This can result in vectorial transport of some drugs or complex disposition interactions for others [61].

Bosentan, a dual antagonist of endothelins A and B, is used in treatment of pulmonary artery hypertension. Although its clearance is dependent on the CYP-mediated metabolism, the hepatic uptake via OATP1B1 and 1B3 becomes rate determining in the overall elimination [62].

21.8 BIOMARKERS AND METABOLOMICS

CVD is one of the major causes of human mortality. Methods to minimize CVD risk is a major challenge in the prevention and treatment of CVD. Clinical assessment is the keystone of patient management; however, such evaluation has its limitations [63]. Biomarkers could provide an important approach to understand the spectrum of CVD with applications in at least five areas: screening, diagnosis, prognostication, prediction of disease recurrence, and therapeutic monitoring of CVD [64]. However, close scrutiny of a range of biomarker studies shows shortcomings of some biomarker hopes [65].

The so-called metabolomics is the study of complex metabolite profiles in biological samples, such as urine, plasma, cerebral spinal fluid, or tissue biopsies. Disease changes the concentration and fluxes of small biochemicals that may be captured by metabolite fingerprint in the cells, tissues, and biofluids. In contrast to classical biochemical approaches that often focus on single metabolites or reactions, metabolomics involves the collections of quantitative data on broad series of metabolites in an attempt to gain an overall understanding of metabolism and/or metabolic dynamics associated with disease status and drug action [66]. The metabolome consists of a variety of chemicals with different physicochemical properties such as amino acids, peptide, glucose, lipids, organic acid, nucleotides, ATP, and biogenic amine neurotransmitters. The large numbers of metabolites present in human (over 20,000) and their wide concentration range spreading over nine orders of magnitude make it impossible to achieve with a single analytical method. The two most important tools employed presently in metabolomics are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) [67–69].

Sabatine *et al.* [70] reported the application of metabolomics to acute myocardial ischemia. Metabolite profiles in plasma were obtained before and after exercise stress from 18 patients who demonstrated inducible ischemia (cases) and 18 of whom did not (control). Lactic acid and metabolites involved in skeletal muscle adenosine monophosphate (AMP) catabolism increased after exercise in both cases and controls. Statistically

significant change in six members of the citric acid pathway were found in myocardial ischemic group but remained unchanged in controls.

Metabolomic studies of adipose tissues have been demonstrated to be useful in discovery of new biomarkers related to CVD. Several hormones and factors produced in this tissue have been associated with obesity, insulin resistance, and CVD, particularly, the accumulation of visceral adipose tissue increases the risk of developing metabolic disease and CVD [69]. In addition, alteration in lipid metabolism has been linked to several diseases such as atherosclerosis, diabetes, obesity, and stroke [69,71]. Altered lipoprotein particle composition has been detected by NMR in patients with hypertension [68].

21.9 LIPID-LOWERING AGENTS

21.9.1 Statins

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are widely used and well-tolerated drugs in the treatment of hypercholesterolemia, lowering the risk of CVD. However, myopathy is a potential adverse effect of these agents. Cerivastatin was withdrawn from world markets as a result of the relatively high rate of fatal rhabdomyolysis that raised concerns on the safety of statins [72]. The mechanism of statin-caused myopathy is unclear; however, it appears to be dose dependent, the risk increases significantly when high dose is used and in particular when statins are prescribed in combination with agents that are also myotoxic or that increase the plasma concentration of statins [73,74]. Statins are highly selective inhibitors of HMG-CoA reductase inhibitors, showing almost no relevant affinity toward other enzymes or receptors [75], making pharmacodynamic interaction less likely. Clinically significant DDIs with statins are thought to result from altered clearance and distribution of statins. It was reported that the rate of myopathy is twofold greater (0.22%) in statin combination therapy with potential inhibitors than statins alone (0.12%) [76].

21.9.1.1 Drug Interaction by CYP Enzymes. Drug-specific interactions with each statin are dependent on metabolic pathway of individual statins (Tables 21.2 and 21.3). Simvastatin and lovastatin are administered as lipophilic lactone prodrugs, whereas other statins are given as active acid forms. Simvastatin, lovastatin, and atorvastatin are predominantly metabolized by CYP3A4 [77–79] and fluvastatin is metabolized primarily by CYP2C9 and to a lesser extent by CYP2C8 and 3A4 [80]. Rosuvastatin shows minimal metabolism, being excreted largely unchanged via the biliary route, and almost no meaningful pharmacokinetic interactions [81,82]. Pravastatin is partly degraded to 3/ α -isopravastatin and 6/ ϵ -epipravastatin in the stomach and partially metabolized by CYP enzymes or excreted as the parent compound into urine and bile [81].

Potent CYP3A inhibitors used with simvastatin or lovastatin significantly increased the exposure of these statins, probably a result of inhibited first-pass metabolism. Strong CYP3A inhibitors, such as itraconazole and ketoconazole, can increase the AUCs of simvastatin and lovastatin up to 20-fold [83–85]; however, only a slight AUC increase was observed with pravastatin, atorvastatin [86,87], rosuvastatin [88], and fluvastatin. Combination of saquinavir with ritonavir as a boosting agent increased AUC of simvastatin acid 30-fold; similarly, the AUC of atorvastatin was increased 3.5-fold [89].

TABLE 21.2 Pharmacokinetic Properties, Metabolizing Enzymes, and Transporters of Statins

Parameter	Simvastatin	Lovastatin	Atorvastatin	Fluvastatin	Pravastatin	Rosuvastatin	Pitavastatin
Lactone prodrug	Yes	Yes	No	No	No	No	No
Lipophilicity	+++	+++	+++	+++	+	++	+++
Absorption (%)	60–85	30	30	98	35	50	80
Bioavailability (%)	<5	5	12	30	18	20	60
Hepatic extraction (%)	≥ 80	≥ 70	70	≥ 70	45	63	—
Protein binding (%)	>95	>98	>98	>98	50	90	96
Half-life (h)	2–5	2–5	7–20	1–3	1–3	20	10–13
Metabolism	Extensive	Extensive	Extensive	Extensive	Mainly unchanged	Mainly unchanged	Limited
Metabolizing enzymes	CYP3A4, CYP2C8*	CYP3A4	CYP3A4, CYP2C8*, UGT*	CYP2C9, CYP3A4*, CYP2C8*	CYP3A4*	CYP2C9, CYP2C19*, CYP3A4*	CYP2C9*
Uptake transporters	OATP1B1	OATP1B	OATP1B1, OATP2B1	OATP1B1*, OATP1B3, OATP2B1	OATP1B1, OATP1B3, OATP2B1	OATP1B1, OATP1B3, OATP2B1	OATP1B1, OATP1B3, OATP2B1
Efflux transporters	PGP, BCRP (lactone)	PGP	PGP, BCRP	BCRP	BCRP, PGP, MRP2	BCRP, MRP2	BCRP*, PGP, MRP2

Source: Adapted from Ref. 55.

TABLE 21.3 Percent AUC Increase in Statins Following Coadministration of CYP and/or Transporter Inhibitors

Inhibitor/inducer	Simvastatin	Lovastatin	Atorvastatin	Fluvastatin	Pravastatin	Rosuvastatin	Pitavastatin
Itraconazole	900	1380, 3540	47, 150	—	48, 71	26, 37	—
Clarithromycin, erythromycin	521, 895	—	32–345	—	111	—	179
Verapamil, diltiazem	99–381	26	—	—	31	—	—
Cyclosporine	155, 696	406	645	89–254	1077, 2183	608	351
Grapefruit	37–1513	91, 1426	—	—	—	—	—
Gemfibrozil, fenofibrate	43	—	24	—	22–102	88	25, 36
PI or PI combination	507	—	74–836	—	90	36–212	31

Data collected from drug interaction database (University of Washington).

Grapefruit juice, an intestinal CYP3A4 inhibitor, can increase lovastatin and simvastatin AUC up to 15-fold and atorvastatin by 3.3-fold but no change for pravastatin. The extent of interaction depends on the amount of grapefruit juice; <1 quart daily consumption appears to have minimal effect [90]. Weak or moderate CYP3A4 inhibitors, such as verapamil, diltiazem, erythromycin, and clarithromycin, increase the AUC of simvastatin acid by about three- to ninefold [87,91]. There are few reports of clinically relevant DDI between fluvastatin and CYP2C9 inhibitors, even potent CYP2C9 inhibitor fluconazole appears to only slightly increase the plasma concentration of fluvastatin [92]. CYP2C8 contributes to the metabolism of simvastatin and lovastatin acid. Gemfibrozil and its glucuronide metabolites are CYP2C8 inhibitors. Gemfibrozil markedly increase the AUCs of the active simvastatin acid and lovastatin acid [93,94]. Generally, induction-mediated statin interaction is less clinically significant than that caused by potent inhibitors. CYP and transporter inducers, such as rifampin and carbamazepine, reduced the AUCs of simvastatin by 80–90% [95,96], rifampicin decreased fluvastatin by 50% and that of pravastatin by 30% [80,97].

21.9.1.2 Drug Interaction by Transporters. Many statins are substrates of efflux or uptake transporters expressed in the intestine and liver, for example, PGP, MRP2, bile salt export pump (BSEP), BCRP, OATP1B1, OATP1B3, and OATP2B1 (Table 21.2). Since most statins are eliminated by metabolism or biliary excretion, their active hepatic uptake, metabolism, and biliary excretion can regulate their total clearance. The interplay between intracellular CYP3A4 and the efflux transporter PGP in the intestinal wall may contribute to the high presystemic extraction of simvastatin and lovastatin, as well as mediate DDI [55].

The significance of BCRP in the pharmacokinetics of different statins has been shown in several studies [98,99]. Individuals with ABCG2c.421AA variant allele showed AUCs 70–144% higher for atorvastatin, rosuvastatin, fluvastatin, and simvastatin than mean AUC values for individuals with the c.421cc genotype.

OATP1B1, 1B2, and 1B3 are expressed on the sinusoidal membrane of hepatocytes and can facilitate liver uptake of their substrate drugs. All statins are OATP1B1 substrates; some are also substrates of other uptake transporters. OATP1B1 appears to be the most important liver-specific uptake transporter of statins [100]. It is believed that OATP1B1 inhibitors decrease the liver uptake of statins and increase plasma to liver concentration ratio, potentially leading to increased risk of skeletal muscle toxicity and attenuated cholesterol-lowering effect. In addition, genetic variability in the gene encoding OATP transporter can result in marked interindividual differences in pharmacokinetics. In one study, SLCO1B1c.521CC participants had a 91% and 74% larger pravastatin AUC than those with the c.521TT or c.521TC genotype, respectively [101]. The plasma concentrations of active simvastatin acid, pitavastatin, atorvastatin, and rosuvastatin have been 221%, 162%, 144%, and 65% higher in c.521CC homozygotes than c.521TT homozygotes; however, no significant effect was observed for fluvastatin [100].

Several drugs including cyclosporine, rifampicin, clarithromycin, and ritonavir act as OATP1B1 inhibitor *in vitro*. Cyclosporine is a potent OATP1B1 inhibitor ($K_i = 0.242 \mu M$), it also inhibits several influx and efflux transporters, such as OATP1B3, OATP2B1, MDR1, MRP2, and CYP3A4. Cyclosporine causes a 2- to 20-fold increase in AUC values of statins in organ transplant patient [102–109]. Since fluvastatin, pitavastatin, pravastatin, and rosuvastatin are not significantly

metabolized by CYP3A4, plasma concentration increases in these statins may be primarily due to OATP1B1 inhibition. A study by Bergman *et al.* [110] in pigs explored the mechanism of cyclosporine–rosuvastatin interaction. Intravenous infusion of cyclosporine markedly decreased the hepatic extraction of a single intrajejunal rosuvastatin dose, causing a 9.1-fold increase in AUC in hepatic vein with a 2.1-fold decrease in bile exposure. It was concluded that strong effect of cyclosporine resulted from the inhibition of OATP1B1-mediated sinusoidal transport, rather than canalicular transporters.

21.9.2 Gemfibrozil

Gemfibrozil [5-(2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic] is an oral lipid-lowering agent, which is classified as a fibric acid derivative. Gemfibrozil decreases serum triglycerides, very low density lipoprotein, and increases high density lipoprotein cholesterol.

Gemfibrozil is completely absorbed after oral administration in human [111]; ~70% of an orally administered dose is excreted in urine, mostly as glucuronide conjugates, with <2% excreted as unchanged gemfibrozil. Six percent of the dose is accounted for the feces. Three major metabolic pathways of gemfibrozil were characterized. The first metabolic pathway is oxidation of *meta*-methyl group, yielding a benzyl alcohol that is further oxidized to benzoic acid metabolite. The second pathway is the hydroxylation of aromatic ring to phenol. The third pathway is glucuronide conjugation of gemfibrozil and its oxidative metabolites. Mano *et al.* [112] demonstrated that UGT2B7 is the primary isozyme responsible for gemfibrozil glucuronidation, although other UGT isoforms such as UGT1A1, 1A3, 1A9, and 2B15 also possess catalytic activity [113]. Also, Ogilvie *et al.* [58] showed human liver microsomes and recombinant CYP2C8 both convert gemfibrozil glucuronide to a hydroxylated metabolite. Gemfibrozil is shown to be a CYP2C9 inhibitor (IC_{50} of $30\ \mu M$), whereas its glucuronide was found to be a more potent mechanism-based CYP2C8 inhibitor (IC_{50} of $1.8\ \mu M$). Coadministration of gemfibrozil with the CYP2C9 substrate warfarin does not increase the plasma concentrations of warfarin [114]. However, gemfibrozil can increase the plasma concentration of CYP2C8 substrates. The AUCs of antidiabetic agents rosiglitazone and pioglitazone and μ -opioid receptor agonist loperamide were increased to over twofold [115–118] and the AUC of repaglinide can be increased up to eightfold [26,27].

In addition, gemfibrozil caused a sixfold increase in the mean AUC value of cerivastatin [119] and moderate AUC increase in active simvastatin acid [93], lovastatin acid [94], atorvastatin [120], pravastatin [121], and rosuvastatin [122]. Gemfibrozil–statins interaction may be based on the dual inhibition of both OATP1B1 and CYP2C8; the extent of interaction may depend on the relative importance of OATP1B1 and CYP2C8 in the pharmacokinetics of the statin. Gemfibrozil and its 1-*O*-glucuronide appear to be relatively weak inhibitors of OATP1B1 ($K_i \sim 22\text{--}25\ \mu M$) [123]. *In vitro* experiments showed that gemfibrozil ($200\ \mu M$) reduced OATP1B1-, 2B1-, and 1B3-mediated fluvastatin transport from 62% to 97% [124].

Combination treatment of statins and fibrates is a potentially useful strategy to improve lipid and lipoprotein profiles and reduce cardiovascular risk in patients with diabetes mellitus and metabolic syndrome. However, statin–fibrate combination regimens have potential adverse effects on skeletal muscles including myopathy. The

mechanism of statin-related or statin-fibrate-related muscle toxicity are not clear, may result from both pharmacodynamic and pharmacokinetic mechanism [125]. Since other fibrates, such as fenofibrate and bezafibrate, do not have significant effects on OATP1B1, and CYP2C8 showed only minor pharmacokinetic interaction [126], it is expected that treatment with fenofibrate in combination with statin might be less likely to cause adverse muscle effects than gemfibrozil–statin regimes on the base of pharmacokinetic interaction.

21.10 DIURETICS

The loop diuretic torsemide is well absorbed and yields a bioavailability of 80% in healthy individuals [127]. Torsemide undergoes extensive hepatic metabolism, only 20% of the parent drug is recovered unchanged in the urine. The major portion of renal excretion of torsemide occurs via tubular secretion. Renal failure seems to have minimal effect on total plasma clearance, whereas ~50% decrease in plasma clearance was observed in patients with liver disease and an increase in elimination half-life. Hydroxylation of the methyl group of the phenyl ring, M1, and further oxidized metabolite, M5 (carboxylic acid), are major metabolite detected in urine. The metabolite M5 is pharmacologically inactive, metabolite M1 may be ~10% as active as the parent drug. M1 formation is primarily mediated by CYP2C9 with an apparent K_m value of 11–23 μM [128]. CYP2C8 contributed the M1 formation to a minor extent [129]. A clinically significant drug interaction of torsemide involving the CYP450 system has not been reported.

Thiazide diuretics, indapamide and xipamide, undergo extensive hepatic metabolism; however, the underlying enzymes are poorly characterized [130]. Other loop diuretics, such as furosemide, bumetanide, ethacrynic acid, and thiazide diuretics, including chlorothiazide, cyclothiazide, hydrochlorothiazide, and trichlormethiazide, are primarily eliminated in urine via tubular secretion. These diuretics, which carry a common chemical characteristic of a sulfamoyl group, are weak organic acids. It has been reported that organic anion transporters are involved in the tubular secretion of diuretics [131]. OAT1 is suggested to play an important role in the basolateral uptake of thiazides, whereas OAT3 is mainly responsible in the uptake of loop diuretics, it is also suggested that bumetanide taken up by OAT3 and/or OAT1 is excreted into the urine by OAT4. In rats, OAT1 contributed to the renal tubular secretion of thiazides and loop diuretics [132].

Concomitant administration of probenecid and furosemide results in a ~2.5-fold increase in furosemide AUC [133] and 60% decrease in furosemide oral clearance, suggesting probenecid inhibits furosemide secretion, probably by the organic anion transporter (OAT) system.

21.11 ANTITHROMBOTICS

21.11.1 Warfarin

A widely prescribed oral anticoagulant for the treatment and prevention of thrombotic diseases, warfarin has a narrow therapeutic range and a >10-fold interindividual variability in the dose required to attain a therapeutic response [134]. The most common

adverse event associated with warfarin overdose is bleeding. Warfarin initiation doses are often prescribed empirically and typically range from 2.5 to 10 mg/day with peak effects usually observed after ~5 days [135]. It usually takes several weeks to establish a patient's optimal maintenance dose by intense International Normalized Ratio (INR) monitoring and dose adjustments based on multiple influencing factors, such as diet, disease state, concomitant use of other medications, and genetic factors. At least 30 genes have been associated with the metabolism and action of warfarin [136]; single nucleotide polymorphisms in CYP2C9 and VKORC1 are strongly associated with warfarin dose requirements [134,135,137]. In August 2007, the FDA revised the prescribing information for warfarin to encourage, but not require, pharmacogenomics testing when initiating warfarin therapy [138].

Warfarin tablets contain a racemic mixture of R- and S-isomers. The S-isomer exhibits three to five times more anticoagulant activity than the R-isomer in humans. Under steady-state conditions, S-warfarin accounts for 60–70% of warfarin anticoagulant effect and R-warfarin for 30–40% [139]. Warfarin is completely absorbed after oral administration with peak concentration generally attained within the first 4 h. Elimination of warfarin is almost entirely by metabolism. The warfarin isomers undergo stereoselective metabolism; S-warfarin is metabolized primarily by CYP2C9 to 7-hydroxywarfarin, R-isomer is metabolized primarily by CYP1A2 to 6- and 8-hydroxywarfarin, by CYP3A4 to 10-hydroxywarfarin, and by carbonyl reductase to diastereoisomeric alcohol [14]. CYP2C9 tends to have more clinical significance than CYP3A4 or carbonyl reductase. Two common variants CYP2C9*2 and *3 have 12% and 5% reduced catalytic activity compared to wild type (*1) [136]. A systematic review and meta-analysis of nine studies has showed that patients carrying at least one variant allele of CYP2C9*2 have a 17% reduction of daily warfarin dose and a 37% reduction of daily warfarin dose for patients carrying CYP2C9*3 [140]. Patients carrying at least one copy of variant alleles have a increased relative risks of bleeding as compared to wild type (relative risk of 1.9 for CYP2C9*2 and 1.77 for CYP2C9*3).

Vitamin K epoxide reductase (VKOR) is involved in making vitamin K-dependent clotting factors and is the target enzyme for warfarin. Multiple variants in the genes encoding VKOR have been identified and also correlate with warfarin dose requirements. Several studies suggest that variations in CYP2C9 and VKOR can potentially account for 5–22% and 6–37% of the interindividual variability of warfarin dose [134]. A pharmacogenetic algorithm was developed by the International Warfarin Pharmacogenetics Consortium using genotypes from VKORC1 and CYP2C9 and clinical variables to predict the stable therapeutic dose [141]. The data are from a large and diverse cohort of patients (4043 patients) and validation cohort (1009 patients). The algorithm appears to predict the stable therapeutic dose of warfarin better than a fixed dose approach and better than a clinical algorithm built from the same large data set. The pharmacogenetic was more accurate at identifying patients who required low doses (21 mg or less per week) and those requiring high doses (49 mg or more per week). In January 2010, the FDA updated the warfarin label specifying that “a patient's CYP2C9 and VKORC1 genotype information when available, can assist in selection of the starting dose.” The agency also provides initial dosage recommendations for patients with different variant combinations. However, the FDA does not require that genetic testing be done before prescribing warfarin.

21.11.2 Antiplatelet Drugs

Coronary artery disease, one of the leading causes of mortality in the United States, can be effectively managed by administration of various antithrombotic strategies. The most widely used is treatment with one of several antiplatelet regimens. There are three marketed thienopyridine antiplatelet prodrugs that function by inhibition of ADP P2Y₁₂, resulting in diminished platelet aggregation and lessened risk of thrombosis.

The thienopyridine ticlopidine, clopidogrel, and prasugrel are prodrugs that require biotransformation to produce their respective active metabolites. Ticlopidine is the first compound of this class, introduced to the market in 1979 for prevention of thrombotic stroke. After a single oral dose of [¹⁴C]ticlopidine to human, about 60% of the administered radioactivity was recovered in the urine and 23% in the feces [142,143]. 2-Chlorohippuric acid was the main metabolite detected in urine, whereas ticlopidine was present in trace amount in urine. Other lower level metabolites such as 2-oxo-ticlopidine, ticlopidine-*N*-oxide, and *o*-chlorohippuric acid were also detected. The pharmacologically active metabolite was not detected or identified. The structure of the active metabolite was first deduced based on the structures of prasugrel and clopidogrel active metabolites and later on characterized after *in vitro* incubation of 2-oxo-ticlopidine with homogenates prepared from phenobarbital-induced rat livers [144]. CYP2C19 and 2B6 were shown to contribute to the formation of 2-oxo-ticlopidine [145,146]; however, the CYPs involved in the metabolic transformation of 2-oxo-ticlopidine to active metabolite are unknown.

Ticlopidine is dosed at 250 mg BID and although generally safe is characterized by a number of side effects, including gastrointestinal disturbances, skin rash, and in rare cases hepatotoxicity, agranulocytosis, neutropenia, and aplastic anemia [143,147]. The later could be due to formation of reactive metabolites and high dose [147,148]. Clopidogrel and prasugrel were introduced at much lower dose of 75 and 10 mg QD and are characterized by generally better safety.

Metabolism of clopidogrel proceeded with two competitive pathways with the primary pathway leading to the formation of inactive acid metabolite by carboxylesterase I (hCE1) [149,150]. The minor pathway proceeded with the formation of 2-oxo-clopidogrel, an intermediate to further ring opening active metabolite. Several studies suggested CYP1A2, 2B6, and 2C19 contribute to 2-oxo-clopidogrel formation; the active metabolite was formed from the thiolactone by CYP2B6, 2C9, 2C19, and 3A4. Several clinical trials have shown that efficacy of clopidogrel is associated with CYP2C19 functionality such that subjects who were carriers of the reduced-function CYP2C19 allele showed significantly poorer clinical response and lower AUC of active drug [143]. The FDA has added a black-box warning to the clopidogrel label, indicating that mutations in the CYP2C19 gene render certain patients unable to respond to the drug, which places them at increased risk for heart attack and stroke.

Prasugrel is the newest thienopyridine antiplatelet drug approved for use in Europe and the United States for the reduction of thrombotic cardiovascular events in patients with acute coronary syndrome, who are to be managed with PCI. Prasugrel is rapidly absorbed and extensively metabolized such that it is not detected in plasma. It is rapidly hydrolyzed by hCE2, primarily an intestinal enzyme, to form the thiolactone, which is further metabolized to the active metabolite primarily by CYP3A4 and 2B6 and to a lesser extent by CYP2C9 and 2C19 [143]. The active metabolite is the major metabolite circulating in plasma, representing 26% of the AUC_{0–12h} of the radioactivity [143].

In vitro, ticlopidine was a potent mechanism-based inhibitor of CYP2C19 slowing the clearance of omeprazole and phenytoin, resulting in toxicity of the latter [151]. Clopidogrel showed similar though less pronounced inhibition of CYP2C19 and both drugs showed inhibition of CYP2B6 [146]. Prasugrel did not meaningfully inhibit either isoform due to its activation via noncup processes. This series of drugs presents an illustrative example of progressive drug discovery.

21.12 ANTIARRHYTHMICS

Antiarrhythmic agents are a group of pharmaceuticals that are used to suppress fast rhythms of the heart such as atrial fibrillation, atrial flutter, ventricular tachycardia, and ventricular fibrillation. Antiarrhythmic drugs comprise many different drug classes with different mechanisms of action. The Vaughan–Williams classification is one of the most widely used classification schemes for antiarrhythmic agents. This classification scheme is based on the primary mechanism of its antiarrhythmic effect. There are five main classes in the Vaughan–Williams classification of antiarrhythmic agents. Class I agents interfere with the sodium channel, class II agents are β -blockers, class III agents affect potassium efflux, class IV agents affect calcium channel and the atrioventricular (AV) node, and class V agents work by other or unknown mechanisms.

21.12.1 Class I Agents

Quinidine is the oldest clinically used antiarrhythmic agent. In human liver microsomes, two major metabolites of quinidine are identified: (3*S*)-3-hydroxy-quinidine (3-OH-Q) and quinidine-*N*-oxide (Q-N-OX) [152]. Formation of 3-OH-Q is mediated exclusively by CYP3A4 with the K_m value of $74\mu M$. CYP3A4 is also a primary enzyme catalyzing Q-N-OX formation with a K_m of $76\mu M$; CYP2C9 and 2E1 catalyze minor proportions of the N-oxidation. *in vivo* studies to assess the effect of diclofenac, disulfiram, itraconazole, grapefruit juice, erythromycin, and fluvoxamine on the pharmacokinetics of quinidine confirm the important role of CYP3A4 in the oxidation of quinidine *in vivo*, particularly in the formation of 3-OH-Q [153,154]. It has been suggested that the formation of 3-OH-Q may serve as an indicator of CYP3A4 activity *in vivo* [155]. Quinidine is also a potent competitive CYP2D6 inhibitor with K_i value in the range of $0.027\text{--}0.4\mu M$ and a PGP substrate with an efflux ratio of 3 in caco2 assay [156].

Quinidine has been involved in numerous DDIs. Coadministration of quinidine with cimetidine, itraconazole, verapamil, and diltiazem led to a moderate increase in quinidine plasma concentration and a 30–60% decrease in total plasma clearance. The effect appears to be mediated through inhibition of CYP3A4 and/or inhibition of renal tubular secretion of the parent drug [157–160]. Plasma concentration of quinidine can be significantly decreased by CYP inducers such as rifampicin, phenytoin, and phenobarbital [161]. Coadministration of metoprolol, a CYP2D6 substrate, with quinidine resulted in a fivefold increase in the metoprolol AUC [162]. Similar quinidine cotherapy with dextromethorphan led to a 40-fold increase in AUC values of dextromethorphan, a CYP2D6 substrate [163]. Quinidine was associated with >threefold increases in plasma digoxin concentrations when coadministration with quinidine. Several studies suggested that inhibition of PGP-mediated digoxin efflux by quinidine leading to

increased absorption and decreased renal and biliary clearance may be the underlying mechanism [98,164].

Procainamide is an antiarrhythmic medication that is primarily used to treat ventricular tachydysrhythmias. Oral doses of procainamide are rapidly and nearly completely absorbed [165]. At therapeutic doses, 48% of dose is excreted unchanged in urine. *N*-acetyl procainamide (NAPA) was the major metabolite in the urine (15% of the dose). The $C_{\max}$ of NAPA was 50% of that of procainamide in the 10 human subjects; however, plasma concentration of NAPA was higher than that of procainamide in 2 patients. The formation of NAPA was mainly mediated by the cytosolic polymorphic NAT. The NAPA to procainamide ratios in urine and plasma were found to be higher in rapid than in slow acetylators [166–168]. Genetic variant NAT2*4, NAT2*6A, and NAT2*7B were shown to metabolize procainamide with K_m values in the range of 2–3 mM [169]. NAPA is an active metabolite with ~70% of the antiarrhythmic activity of the parent drug [170]. It is important to consider both procainamide and NAPA levels when evaluating the status of a patient. In addition to *N*-acetylation, Uetrecht and coworkers [171] demonstrated that procainamide was metabolized to the reactive *N*-hydroxylamine-procainamide (NOH-PA), further formed highly reactive nitroso procainamide (NO-PA), NO-PA may covalently bind to macromolecules, leading to the drug-induced lupus erythematosus syndrome observed during the procainamide chronic therapy. *In vitro* chemical inhibition and correlation analysis suggested that CYP2D6 was the major human CYP isozyme involved in the formation of the reactive metabolite of procainamide, namely, *N*-hydroxyprocainamide [172]. It was reported that coadministration with amiodarone, antibacterials trimethoprim and ofloxacin, propranolol, and cimetidine increased the plasma concentration of procainamide and NAPA [161].

Lidocaine is a widely used local anesthetic and antiarrhythmic drug that undergoes extensive metabolism in the liver [173,174]. Following a single oral dose of 250 mg to two healthy volunteers, the major urinary metabolite was 4-hydroxy-2,6-dimethylaniline and its conjugating metabolites (72% of the dose). Monoethylglycinexylidide (MEGX) and other aromatic hydroxylated metabolites represented <5% of the dose in urine. In human liver microsomes, lidocaine was mainly oxidized to *N*-deethylated metabolite, MEGX, whereas 3-hydroxylation on the aromatic ring was a minor metabolic pathway [175]. Both CYP1A2 and 3A4 were capable of catalyzing the formation of MEGX and 3-hydroxyl lidocaine [93], CYP1A2 was the major isoform catalyzing lidocaine *N*-deethylation at low substrate concentration (5 μM), CYP3A4 contributed more at higher lidocaine concentration (800 μM), and 3-hydroxylation was primarily catalyzed by CYP1A2. MEGX appeared to have similar antiarrhythmic and convulsant activities to lidocaine and may be synergistic to the effectiveness and toxicity of lidocaine [176]. CYP3A4 inhibitors, erythromycin and itraconazole, increased lidocaine peak plasma concentration by 40–70% [177]. Isohanni and coworker [178] further studied the effect of fluvoxamine (CYP1A2 inhibitor) and erythromycin on the pharmacokinetics of lidocaine, fluvoxamine alone increased the mean AUC of lidocaine to 305% and the $C_{\max}$ to 220% compared to the placebo phase, but there was no significant effect on the $T_{\max}$ or half-life of lidocaine. The combination of fluvoxamine and erythromycin increased the mean AUC of lidocaine to ~360% and the $C_{\max}$ of lidocaine to 250%. It was concluded that inhibition of CYP1A2 by fluvoxamine considerably reduces the presystemic metabolism of oral lidocaine and may increase the risk of lidocaine toxicity if lidocaine is ingested. Lidocaine disposition is significantly affected by changes in hepatic blood flow [179]. β -Blocker propranolol has

been shown to decrease lidocaine clearance secondary to decreased hepatic blood flow [161], subsequently elevated lidocaine plasma concentration has been associated with central nervous system (CNS) toxicity. Similar interaction may be expected with other β -blockers since most appear to decrease hepatic blood flow. In addition, coadministration with amiodarone, cimetidine, and HIV protease may also alter plasma concentration of lidocaine.

Propafenone is a class 1C antiarrhythmic drug with local anesthetic effects and a direct stabilizing action on myocardial membranes. The metabolism of propafenone was studied in human after a single oral dose [180]. Propafenone is completely absorbed and metabolized, <1% of the dose was recovered as parent drug. The major metabolites in excreta are conjugates of 5-hydroxypropafenone (5-OH-PF) and hydroxy-methoxypropafenone and propafenone glucuronide. Conjugates of hydroxylated derivatives of propafenone are predominant components in the plasma. Propafenone is administered as a racemate of *S*(+)- and *R*(-)-enantiomers. (*R,S*)-propafenone was mainly metabolized to 5-OH-PF and *N*-dealkylated propafenone (NDPF) in human liver microsomes [181]. The formation of 5-OH-PF is primarily mediated by CYP2D6; CYP3A4 and 1A2 mediated the formation of NDPF. The 5-OHPF formation from racemic propafenone and from its individual enantiomers followed one-enzyme Michaelis–Menten kinetics with a mean K_m of 0.12 μM . Stereoselectivity in V_{max} and K_m values was observed, with (*S*)-propafenone displaying higher K_m and V_{max} values. *N*-Depropylpropafenone (*N*-DPP) was monophasic with a mean K_m of 116 μM . No stereoselectivity in propafenone *N*-dealkylation was observed [182]. Large interindividual variations in plasma concentration observed in human can be explained by genetically determining metabolism of propafenone via CYP2D6. A clinical study in 28 patients with chronic ventricular arrhythmias (22 extensive metabolizers (EMs) and 6 poor metabolizers (PMs)) concluded that propafenone metabolism was polymorphic and cosegregated with that of debrisoquine 4-hydroxylation [183]. The data also suggested that PMs are at higher risk of developing CNS side effects presumably due to the high plasma concentration achieved in these patients. Therefore, monitoring of the plasma concentration of propafenone may be clinically useful for the prediction of CNS side effects. It was proposed to use urinary excretion of intact glucuronides of propafenone to determine the metabolic phenotype of propafenone [184]. Propafenone is also a potent CYP2D6 competitive inhibitor with a K_i value of 34 nM [185] and a weak CYP1A2 inhibitor with the K_i value of 21 μM [186]. Moreover, propafenone was not a substrate of PGP, whereas propafenone and its metabolites 5-OH-PF and NDPF inhibited PGP-mediated digoxin transport with IC_{50} values of 6.8, 19.9, and 21.3 mM, respectively, therefore contributing to the digoxin–propafenone interaction observed in human [161,187–189]. Propafenone increased the plasma concentration of metoprolol, propranolol, mexiletine, and warfarin [141,190–192] and led to adverse effects. Thus, the dose of these drugs should be adjusted when coadministration with propafenone.

21.12.2 Class II Agents: β -Adrenergic Blockade

β -Adrenoreceptor blockers are widely prescribed for the treatment of CVD, including hypertension, coronary heart disease, and heart failure. Over 30 agents are available worldwide and are distinguished by the presence of β_1 selectivity, partial agonism, membrane stabilizing effect, the presence of β -receptor antagonism, direct vasodilating

properties, and so on. From a pharmacokinetic perspective, these drugs can be divided into two general categories [193]: those primarily metabolized by the liver and those that are predominantly excreted unchanged by the kidney. The former include propranolol, metoprolol, carvedilol, and so on and the latter include atenolol, nadolol, sotalol, and so on.

Carvedilol is metabolized primarily to glucuronide conjugates in human (22% of total plasma radioactivity and 32% of total urine radioactivity) [194,195]. Three UGT isoforms including UGT1A1, 2B4, and 2B7 can catalyze the reaction with similar K_m values in the range of 20–50 μM [196]. Stereoselective metabolism of racemic carvedilol by UGT1A1 and 2B7 was observed [197]. Oxidative metabolites 4 or 5-hydroxy carvedilol were also observed in urine with a small portion of dose (6.4%). CYP2D6 was mainly involved in the formation of these two oxidative metabolites [198]. A clinical study evaluating the effects of polymorphisms in UGTs and CYP2D6 on pharmacokinetics of carvedilol in Japanese suggested that genetic polymorphisms of UGT2B7 and CYP2D6 may be partially responsible for interindividual variation in carvedilol clearance [199].

Propranolol is cleared almost entirely by metabolism with <1% of the dose recovered as unchanged drug [200]. The drug is metabolized through three primary pathways: direct glucuronidation (17% of dose), aromatic hydroxylation (mainly 4-hydroxylation, 42% of dose), and side-chain oxidation. Four major metabolites are characterized as propranolol glucuronide, naphthyloxylactic acid, glucuronide, and sulfate conjugates of 4-hydroxyl propranolol. *In vitro* studies indicate that CYP2D6 is the predominant propranolol 4-hydroxylase; however, CYP1A2 may also significantly contribute propranolol 4-hydroxylation, especially in the 2–10% of the Caucasians who are poor metabolizers of CYP2D6 [201]. Both CYP1A2 and 2D6 catalyze the side-chain oxidation [202]. UGTs 1A9, 1A10, 2B4, and 2B7 catalyze propranolol glucuronidation [203]. On average, the contribution of CYP2D6 to the overall elimination of propranolol is insufficient to cause differences in its pharmacokinetics between phenotypes [204]. However, coadministration of several CYP2D6 inhibitors, such as quinidine, propafenone, or fluoxetine, resulted in significant increases in propranolol plasma level and altered the clinical response [190,205,206]. Rifampicin is a potent inducer of propranolol metabolism, resulting in two- to fourfold increase in propranolol clearance [207,208]. Propranolol is also an inhibitor of CYP2D6 and 1A2, the major isoforms catalyzing its metabolism. Coadministration with theophylline (CYP1A2 probe substrate) resulted in the increased plasma level of these drugs [209].

Metoprolol undergoes extensive hepatic metabolism with <5% of the dose recovered unchanged in urine [210]. Three major metabolic pathways were identified: O-desmethylation (65% of dose), deamination (10% of dose), and α -hydroxylation (10% of dose). CYP2D6 phenotype has a significant effect on metoprolol plasma concentration with PMs having sixfold higher steady-state concentration than EMs [204,211]. Coadministration of CYP2D6 inhibitors profoundly affects metoprolol plasma level and may result in phenotype change from EM to PM. Quinidine coadministration causes a 60% decrease in metoprolol plasma clearance [212], paroxetine increased mean metoprolol AUC about fourfold [213]. Although the expected effects of CYP2D6 genotype on pharmacokinetics of metoprolol were observed, the effect of CYP2D6 genotype on efficacy and toxicity of metoprolol is controversial, most studies suffer from small numbers of CYP2D6 PMs [204,214]. A recent clinical study with 1533 patients demonstrated metoprolol subject homozygous for the CYP2D6*4 allele had a significantly

lower heart rate and diastolic blood pressure than those with the wild-type genotype [214].

21.12.3 Class III Agents: Potassium Channel Blockade

Class III antiarrhythmic drugs, including amiodarone, bretylium, dofetilide, ibutilide, and sotalol, are effective for the management of various types of cardiac arrhythmias both atrial and ventricular in origin. Among the class III antiarrhythmic drugs, bretylium and sotalol are primarily excreted unchanged in urine. Ibutilide is extensively metabolized in the liver to primarily ω -oxidative metabolites followed by sequential β -oxidation of the heptyl side chain of ibutilide. These reactions are not mediated by CYP3A4 and 2D6, but possibly by CYP4 families. No significant pharmacokinetic drug interactions for these agents have been identified [161,215]. Dofetilide is mainly cleared in the kidney via the cationic transport system. Cimetidine, ketoconazole, trimethoprim, prochlorperazine, megestrol, and thiazide diuretics can inhibit tubular secretion. Thus, drugs that inhibit CYP3A4 and/or the renal transport system may interact with dofetilide [215].

Amiodarone has a large volume of distribution and is distributed extensively to extravascular tissues such as liver, lung, and adipose [216]. High dose amiodarone has been associated with pulmonary toxicity. A recent study suggests that amiodarone is a OATP2B1 substrate and that amiodarone uptake via OATP2B1 might lead to accumulation of amiodarone in the lung and amiodarone-induced pulmonary toxicity [186]. Amiodarone undergoes extensive metabolism in the liver, the primary and principle metabolite is desethylamiodarone [217], this metabolite is found to parallel amiodarone concentration in plasma but has variable concentration in tissues. Desethylamiodarone has higher concentration than that of amiodarone in most tissues but lower concentration in adipose. Desethylamiodarone may also contribute to amiodarone-induced pulmonary toxicity. Desethylamiodarone metabolite is pharmacologically active and may be synergistic to the pharmacological effect of amiodarone [176]. Multiple CYP enzymes including CYP1A1, 3A4, 1A2, 2D6, 2C8, and 2C19 catalyzed amiodarone N-deethylation. However, CYP2C8 and 3A4 were significantly involved in amiodarone N-deethylation in human liver microsomes. CYP2C8 has been suggested to be a predominant isoform at clinically relevant concentrations of amiodarone [218,219]. Amiodarone weakly inhibits CYP2C9, 2D6, and 3A4 with K_i values of 45–271 μM . Desethylamiodarone inhibits CYP2D6, 2A6, 2B6, 1A1, 1A2, 2C9, and 2C19 with lower K_i values of 2.3–15.7 μM [220]. Amiodarone and desethylamiodarone markedly inhibited the basal–apical transport (renal secretion) of [3H]digoxin and increased the apical to basal transport (reabsorption) with IC_{50} of 5.48 and 1.27 μM , respectively. The clinically significant DDI between digoxin and amiodarone could be due to the increased digoxin bioavailability via inhibition of PGP present in the gastrointestinal tract [215,221]. Numerous drug interactions have been reported between amiodarone and other drugs. Coadministration with phenytoin, warfarin, and cyclosporine increases the plasma concentrations of these drugs through inhibitions of CYP2C9, 2C9 and 1A2, and 3A4, respectively [161,215]. The magnitude of interaction appears to be dependent on the dose of amiodarone. Plasma concentration of these drugs should be monitored and dosage adjusted accordingly. Other antiarrhythmic agents, such as dofetilide, flecainide, lidocaine, ibutilide, lidocaine, procainamide, and sotalol, showed drug interaction with amiodarone [215].

21.12.4 Class IV Agents: Calcium Channel Blockade

Verapamil is a class IV L-type calcium channel blocker of the phenylalkylamine chemical class. It has been used in the treatment of hypertension, angina pectoris, cardiac arrhythmia, and most recently, cluster headaches. Verapamil is administered as a racemic mixture of S- and R-enantiomers. The two enantiomers possess different pharmacokinetic and pharmacodynamic properties. The pharmacological properties (i.e., the negative dromotropic effect) are up to 20 times more potent for S-verapamil than R-verapamil [222]. In human, the metabolism of verapamil is stereoselective, the clearance of S-enantiomer is fourfold more rapid than that of R-enantiomer [223] after oral administration of racemic mixture. AUC ratio of R-verapamil to S-verapamil is ~ 5 . However, when administered intravenously, the AUC of S-verapamil is $\sim 50\%$ lower than that of R-verapamil [224]. Verapamil is extensively metabolized; only 3–4% of dose is excreted in urine as the unchanged drug [225]. N- and O-dealkylation are prominent metabolic pathways, producing a secondary amine (D-617) and norverapamil [226]. The N-dealkylation of verapamil in human liver microsomes is not stereoselective. CYP3A and 1A2 are the primary enzymes responsible for N-dealkylation and N-demethylation [227], whereas O-demethylation is mainly mediated by CYP2C family [228]. Grapefruit juice, a moderate CYP3A inhibitor, increases verapamil AUC by 40%; smokers had significantly lower AUC and $C_{\max,ss}$ values than nonsmokers by (means) 0.61- to 0.85-fold for verapamil and norverapamil enantiomers, respectively [229]. While the CYP3A inducer rifampin causes a 90% reduction in verapamil AUC, *in vitro* studies suggest that verapamil is a PGP substrate with an efflux ratio in the range of 2–5 [230–232]. Lovastatin and atorvastatin increase verapamil AUC by 40–60% in healthy Korean subjects, possibly via inhibiting both PGP and CYP3A [233,234]. Verapamil is a moderate CYP3A4, 2D6, 1A2, and 2C19 inhibitor *in vitro* [235–238]. Verapamil increases the AUCs of CYP3A4 probe substrates, midazolam, buspirone, and simvastatin by three- to fivefold [87,239,240]. Coadministration with verapamil only slightly increases theophylline AUC [241,242].

21.13 ACE INHIBITORS

ACE inhibitors are important therapies in the treatment of hypertension, congestive heart failure, postmyocardial infarction, and diabetic nephropathy. By inhibition of ACE, the drugs reduce angiotensin II synthesis in the circulation and tissues. The ACE inhibitors are currently classified into three classes based on their molecular structures. The first class represents sulfhydryl-containing agents, such as captopril (the first ACE inhibitor) and zofenopril. The second class, the dicarboxylate-containing agents, are the largest group including enalapril, lisinopril, ramipril, perindopril,trandolapril, quinapril, benazepril, imidapril, temocapril, spirapril, and moexipril. The third class represents phosphorus-containing inhibitors; fosinopril is the only member of this group.

Captopril is the first ACE inhibitor introduced to the market for the treatment of hypertension and congestive heart failure. After oral administration of captopril, $\sim 70\%$ of dose is absorbed with a bioavailability of 60% [203]. The compound is primarily cleared by urine. For patients with renal impairment, the dose may be reduced based on the degree of renal failure. The most common adverse effects associated with captopril use is skin rash, taste disturbance, neutropenia, and more severe forms of cutaneous

disorders, such as toxic epidermal necrolysis, lichenoid eruption, or pemphigus. The latter have been attributed both to a sulfhydryl substituent and overdosage [203,243]. Second-generation ACE inhibitors lacking the sulfhydryl group do not show the cutaneous adverse events. All dicarboxylate-containing agents except lisinopril are prodrugs designed to improve oral absorption from gastrointestinal tract and are metabolized by esterase in the liver and/or small intestine to the active components. CYP is not involved in the metabolism of ACE inhibitors. The predominant elimination pathway of most ACE inhibitors including enalapril, benazepril, quinapril, trandolapril, moexipril, and imidapril is via the kidney [203,244]. For other ACE inhibitors, such as fosinopril, ramipril, lisinopril, spirapril, and temocapril, the proportion of a dose recovered in feces increased. For most ACE inhibitors, AUC and elimination half-life increase by three- to sixfold in patient with $CL_{cr} < 30$ mL/min. A dose reduction is often recommended in patients with moderate to severe impairment of renal function. Dosage justification is not needed in patients with hepatic disease for most ACE inhibitors except for moexipril and spirapril. A 30–100% lower C_{max} and AUC were observed in patients with hepatic disease compared with healthy young volunteers.

Most ACE inhibitors resemble Ala-Pro dipeptide or Xaa-Ala-Pro tripeptide structures. It is known that di- and tripeptides are taken up into intestinal cells by the low affinity H^+ /peptide cotransporter PEPT1. In the kidney tubule, di- and tripeptides are reabsorbed by PEPT1 and by the high affinity H^+ /peptide cotransporter PEPT2 [245]. The involvement of PEPT1 and PEPT2 in the transport of ACE inhibitors is controversial. A recent investigation on the interaction of ACE inhibitors with PEPT1 and PEPT2 by Knutter and coworker suggests that most ACE inhibitors have low affinity with peptide transporters with the K_i values in the range of 0.3–3 mM; zofenopril and fosinopril show stronger affinity with K_i values of 13–30 μM . Further studies measuring transport current show low or no measurable electrogenic transport activity for all tested ACE inhibitors. Knutter *et al.* conclude that peptide transporters do not control intestinal absorption and renal reabsorption of ACE inhibitors. *In vitro* data suggests that enalapril is a substrate of OATP1B1/1B3 and MRP2 [246] and quinapril is a substrate of renal hOAT3 [247].

21.14 ANGIOTENSIN RECEPTOR BLOCKERS

The angiotensin receptor blockers (ARBs) are a group of antihypertensive drugs that act by blocking the effects of the hormone angiotensin II (Ang II) in the body, thereby lowering blood pressure. Their main indications are mild to moderate hypertension, chronic heart failure, and secondary stroke prevention and diabetic nephropathy. Saralasin, the first Ang II blocker, is an octapeptide analog of Ang II. This compound is not orally bioavailable, has short biological half-life, and shows partial agonist activity; therefore, it is not suitable as a drug [248]. However, early studies with saralasin provided the foundation for the therapeutic potential of Ang II receptor blockade. Great efforts by a group at DuPont and research investigators at Takeda focused on developing a smaller nonpeptide substance with similar binding affinity and acceptable oral bioavailability. The first successful Ang II blocker, losartan, was approved in 1995. Since then, five other ARBs including candesartan cilexetil, eprosantan, irbesartan, telmisartan, and valsartan and three combinations with hydrochlorothiazide (irbesartan, losartan, and valsartan) have been approved as antihypertensive agents. All ARBs

have a carboxylic acid group except for irbesartan. Candesartan cilexetil, a racemic prodrug, is rapidly and completely metabolized by esterase in the gastrointestinal tract to the active candesartan [249]. Candesartan is not metabolized by CYP system; after oral administration, candesartan is excreted mainly unchanged in feces via bile and in urine.

Losartan is rapidly and completely absorbed in healthy adult volunteers [250]. Losartan undergoes extensive first-pass metabolism with oral bioavailability of ~33%. It is converted to aldehyde intermediate E-3179 and further oxidized to a carboxylic acid metabolite (E-3174) that is responsible for most of the angiotensin II receptor antagonism. About 14% of an oral dose is converted to this active metabolite. *In vitro* and *in vivo* studies employing chemical inhibition, inhibitory antibody, and recombinant human hepatic CYP isoforms indicate that CYP2C9 is the predominant enzyme in losartan metabolism to its active metabolite D-3174 [251]. Relatively modest changes in the concentration of losartan and E-3174 were observed when the CYP2C9 inhibitor fluconazole [83,252] or inducer phenobarbital [12] was coadministered with losartan. Fluconazole decreased the E-3174 concentration by 70% and increased losartan AUC by 27%. Coadministration of phenytoin significantly reduced the oral clearance of losartan and AUC_(0–24) only in wild-type CYP2C9 individuals [245]. This result suggests that DDI studies with losartan should be evaluated in the context of CYP2C9 genotype of the study subjects. Losartan is a PGP substrate [253]; however, it does not alter pharmacokinetics of digoxin [254]. To date, DDIs have not been reported to influence the blood-pressure-reducing ability of losartan.

Valsartan is primarily eliminated by biliary excretion in feces (83% of the dose) with a small portion in the urine (13%) [255]. Unchanged drug in the excreta accounted for 81% of the dose with about 20% of dose recovered as metabolites including 4-hydroxyvaleryl metabolite (9%). *In vitro* studies show that CYP2C9 is the only form responsible for 4-hydroxylation of valsartan [256]. Valsartan is significantly taken up into OATP1B1 and OATP1B3 expressing HEK293 cells. Saturable ATP-dependent transport into membrane vesicles expressing human MRP2 has been observed. Taken together, these results suggest that OATP1B1 and OATP1B3 as the uptake transporters and MRP2 as the efflux transporter are responsible for the efficient hepatobiliary transport of valsartan [257]. No clinically meaningful pharmacokinetic drug interactions are observed when valsartan is coadministered with simvastatin, amlodipine, atenolol, digoxin, furosemide, glyburide, hydrochlorothiazide, indomethacin, and warfarin [249,258].

Similar to valsartan, telmisartan is minimally metabolized by CYP; a small amount of acyl glucuronide is found in circulation. Most of the administered dose is eliminated unchanged in feces via biliary excretion (>97%) [259]. Telmisartan has been shown to be an OATP1B3 substrate *in vitro* [61], and MDR1, MRP2, and BCRP are involved in the hepatic export of telmisartan acyl glucuronide [61]. A recent study shows that telmisartan is a potent inhibitor of PGP with an IC₅₀ of 0.38 μ M and a less potent BCRP and MRP2 inhibitor (IC₅₀: 16.9 and 25.4 μ M, respectively). MRP2 genetic polymorphisms appear to strongly influence interindividual variation in telmisartan pharmacokinetics in Japanese renal transplant recipients [260]. Telmisartan causes variable increases in serum digoxin levels [261], it is therefore recommended that digoxin levels need to be monitored in patients taking this drug combination. There are no clinically significant DDIs of telmisartan with warfarin, acetaminophen, amlodipine, glibenclamide, ibuprofen, and hydrochlorothiazide [249].

21.15 THE FUTURE OF CARDIOVASCULAR DRUG METABOLISM

The enzymology of CYP and transporters is reasonably well understood and methodology for prediction of DDI is sufficiently mature so that there should be fewer, if any, postmarketing drug withdrawals due to single-point DDI-mediated adverse events, such as mibefradil and cerivastatin. The understanding of genetic variation on drug disposition has made considerable contributions to cardiovascular drug safety and knowledge will continue to grow. Although the influence of biomarkers on drug safety and efficacy has not been as dramatic as promised, it is likely that improvements in sensitivity, selectivity, quantitation, and lower cost will bring modest success. Given the successes of physiologically based pharmacokinetic (PBPK) and pharmacokinetic-pharmacodynamic (PKPD) modeling, it is likely the next big thing will be integration of multiple parameters into models resulting in better predictivity of efficacy and DDI. In particular, the ability to quantitatively combine several pathways for drug disposition, such as vectorial uptake, efflux, and metabolism by multiple active systems, and incorporating genetic population variation should be possible in the coming years. Similarly, recent years have shown steady improvement in development of drugs with larger safety margins and more directed actions, as seen with antiplatelet drugs and antithrombotics.

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22 Disposition of Biological Therapeutics: Monoclonal Antibodies, Proteins, and Peptides

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22.1	Introduction	1
22.2	Renal clearance of peptides and proteins	3
22.3	Enzymatic clearance mechanisms of peptides and proteins	3
22.4	Reducing peptide and protein catabolism	7
22.5	Half-life extension of peptides and proteins fused to serum proteins	10
22.6	Half-life extension of peptides and proteins through posttranslational modification	11
22.7	Antibodies as therapeutic proteins	13
22.8	Summary	18
	References	18

22.1 INTRODUCTION

The past decade has witnessed a major shift in the relative distribution of therapeutic agents being considered to treat human illness. This change in therapeutic landscape has been the result of an overall decrease in the number of traditional or small-molecule drugs being approved, with a concomitant increase in the approval of novel therapeutic biologics or large-molecule drugs. This marked growth of biopharmaceuticals is a reflection of the maturation of biotechnology, both in terms of discovery and development of large-molecule drugs as well as the ability to consistently manufacture these complex molecules. However, despite an impressive evolution of the science, a sound understanding of the factors that control the systemic disposition and pharmacokinetics (PK) of these molecules remains to be defined.

In general terms, a biopharmaceutical is a protein- or nucleic-acid-based pharmaceutical substance used for therapeutic purposes, which is produced by means other than direct extraction from a native (nonengineered) biological source. An example of the latter is seen with insulin, which was extracted from pancreatic tissue of slaughterhouse animals [1]. A high level definition of biopharmaceuticals may be regarded as

molecules that are produced using recombinant DNA technology in expression cells that are amplified in bacterial or mammalian cell culture.

In addition to the dramatic differences in manufacturing processes between biopharmaceutical drugs and traditional drugs, molecular size is another obvious distinction: the molecules of a biological medicine are much larger, have far more complex spatial structures and are much more diverse, or heterogeneous, than the small molecules that make up traditional drugs. A molecule of a biological medicine is typically a protein made of a chain of 20 to several hundred amino acids within a complex three-dimensional structure. Examples of biopharmaceuticals include monofunctional antibodies that bind one antigen or target, bifunctional antibodies that bind two targets, antibody fragments, peptides, fusion proteins, plasma-derived proteins, and various soluble receptors or receptor ligands.

A second marked difference between biopharmaceuticals and small molecules is observed in the routes of administration. The route of administration of a biopharmaceutical is very different from a traditional drug taken as a pill or capsule, and each drug is developed with a unique route of administration. Typically, biopharmaceuticals are given intravenously, subcutaneously, or intramuscularly, as they generally cannot be orally administered due to degradation in the gastrointestinal (GI) tract. However, the oral delivery of biopharmaceuticals is an active area of research [2]. In addition, many biopharmaceuticals are administered in a clinical setting, although recently for some chronic indications (e.g., rheumatoid arthritis), molecules exist that are self-administered subcutaneously with autoinjector devices.

Another aspect in which biopharmaceuticals are very different from small-molecule drugs is observed with the routes of elimination and observed PK. The elimination pathways for small-molecule drugs are governed by hepatic metabolism (e.g., cytochromes P450) and the subsequent half-lives are in the range of minutes or hours [3]. In contrast, the half-lives of biopharmaceuticals, antibody drugs, for example, are in the order of weeks [4]. For small-molecule drugs, many safety problems occur around the time of $C_{\max}$; however, for biopharmaceuticals, the focus is typically around total drug exposure or area under the curve (AUC) instead of $C_{\max}$.

Biopharmaceuticals present several advantages over small organic molecule therapeutics in treating many diseases. Foremost, the selectivity of biologics is unsurpassed, and because of this inherent specificity in biological function, protein therapeutics present minimal disturbances to normal biological functions, which limits the potential for untoward side effects. Biopharmaceuticals can be grossly separated into different categories: peptides, proteins (enzymes), and antibodies. For the purpose of this chapter, peptides and proteins have been combined based on similar clearance mechanisms and strategies used to enhance their PK properties as therapeutic agents. Antibodies are described in a separate category. The overall focus of this chapter is to highlight the factors that govern the disposition and PK of the aforementioned biopharmaceuticals.

Peptide or protein biopharmaceutical agents are often designed to replace a deficient or abnormal protein pathway in patients or introduce a novel biological function in order to circumvent a particular physiological deficiency. Peptides of various size and derivation are used as drugs across a wide range of therapeutic regimens and represent one of the fastest growing classes of new drugs in the arena of drug discovery. Within the literature, the distinction between peptide and protein therapeutics can be somewhat inconsistent; thus, by definition, any polymer consisting of two or more amino acids linked by amide bonds is termed a *peptide*.

The increasing importance of peptide therapeutics in drug discovery today can be attributed to two primary factors. First, the evolution of drug discovery techniques, such as phage display and combinatorial chemistry, has led to the identification of numerous peptide drug candidates that may provide hope as biopharmaceutical agents [5,6]. Second, advances in genetic engineering and chemical synthesis have enabled the large-scale production of peptides that previously were only available in small quantities [7]. Despite the technological advances detailed above, however, when compared with antibodies and proteins, the disposition of peptide drug candidates is plagued with a critical drawback; namely, they are rapidly eliminated from the systemic circulation.

Oral absorption of peptide and protein therapeutics is prohibited by numerous properties associated with the GI tract, including the acidic environment of the stomach (pH 1–3), which is destabilizing to most peptides and proteins, and the production of large amounts of proteolytic enzymes that are secreted into the digestive tract (discussed in detail below). This barrier to the oral delivery of peptides and proteins has required the pursuit of alternate, more invasive routes of administration, including intravenous and subcutaneous dosing. The frequency of using these alternate routes can be minimized with biologic therapeutics that have sufficiently prolonged half-lives (>25 days), which should promote less frequent dosing. However, peptides and proteins are typically cleared from the bloodstream within minutes to hours after administration, and as a result in many cases, there is insufficient drug exposure in the target tissue to elicit a pharmacological effect. The short plasma half-lives observed for peptides typically result from rapid renal clearance due to the small size of the peptides (<60 kDa for the kidney filtration cutoff threshold) and/or from enzymatic digestion in the blood, kidneys, or liver [8,9]. To remedy this issue, a number of strategies have been developed to reduce renal excretion. Consequently, strategies employed to increase the half-life typically require mechanisms to reduce amide hydrolysis or degradation in order to maintain biological activity of the chimeric peptide or protein once serum residence times have been extended.

22.2 RENAL CLEARANCE OF PEPTIDES AND PROTEINS

As described above, one of the major problems for therapeutic peptides is that these drug candidates are rapidly cleared from the circulation via the kidneys [10]. To address the issue of rapid renal elimination, numerous technologies have been developed to increase the *in vivo* plasma residence time of peptide drugs and these will be discussed in more detail in the subsequent sections of this chapter. Overall, these approaches are all designed to make the peptide larger (generally >60 kDa) to retard excretion through the kidneys.

22.3 ENZYMATIC CLEARANCE MECHANISMS OF PEPTIDES AND PROTEINS

Despite the fact that the molecular weight for most peptides is typically below 10 kDa and therefore they should be readily filtered by the glomerulus in the kidney, rapid elimination of peptides due to renal clearance cannot completely account for the exposure

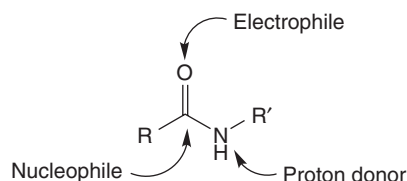


Figure 22.1 Chemical interactions and reactions of the peptide backbone.

issues observed for some peptides, as for example, some half-lives exceed the glomerular filtration rate (GFR) of about 100 mL/min for a normal 70 kg man [11]. To this end, the short half-lives of many small peptides (<10 min) cannot be completely explained by renal clearance and is most likely due to rapid enzymatic degradation.

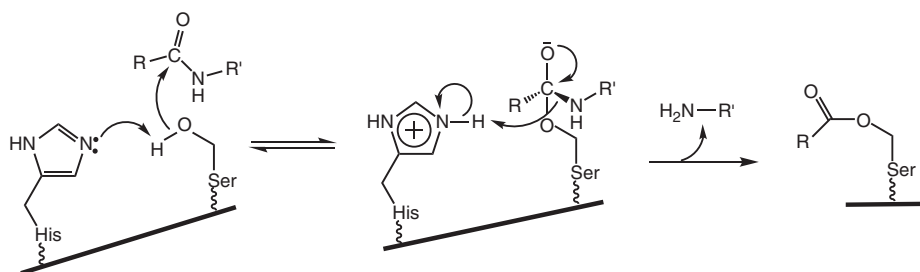
The process of this metabolic degradation is also called *proteolysis* and is best described in terms of chemical hydrolysis (Fig. 22.1). For peptides, blood, liver, and kidney are probably the most important sites of metabolism, as they contain a wide variety of peptidases [9]. However, because of the physical–chemical properties (e.g., hydrophilicity) of many peptide drugs, proteolytic enzymes in the blood may play a more important role in their metabolism [12].

The mechanism for peptide hydrolysis, like all enzyme-mediated catalytic reactions, requires interactions with appropriate active site groups (Fig. 22.2). For the hydrolysis reaction to occur, the combined action of the active site catalytic groups is essential [13,14], as the peptide bond represents a strong linkage that has a high degree of double-bond character (a consequence of the degree of delocalization of the nitrogen lone pair into the carbonyl group). The strength of the peptide bond weakens in the first step, when a nucleophile, such as water or a serine OH group, attacks the carbonyl carbon atom, which results in a tetrahedral intermediate bearing an oxyanion. In this sequence, the nucleophilic attack is aided by a general base that accepts the proton from the nucleophilic OH group (Fig. 22.2a). The intermediate is stabilized by electrophilic catalysis, which is provided by hydrogen bonds from the oxyanion-binding site to the carbonyl oxygen. This is followed by the expulsion of the leaving group, in this case the amine, from the tetrahedral intermediate. Because the amine is a poor leaving group, it must be protonated by a general acid to be able to depart (Fig. 22.2b). In short, similar to chemical hydrolysis reactions, proteolysis of the peptide bond cleavage proceeds via the formation and decomposition of the tetrahedral intermediate, which is facilitated by general acid–general base catalysis, mediated by a single proton carrier, often an active site histidine side chain in many cases [15].

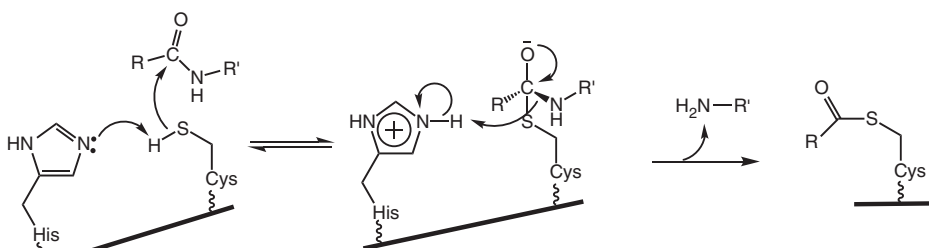
22.3.1 Mechanism of Peptide Hydrolysis

In general terms, there are four distinct peptidase mechanisms, which are loosely linked to the primary means of the hydrolysis reaction (e.g., serine, cysteine, aspartic, and metallopeptidases). The most obvious difference between the enzyme mechanisms is the presence or absence of a covalent acyl-enzyme intermediate in the reaction pathway [16–18]. The catalyses of serine and cysteine peptidases involve the covalent intermediate (ester and thiolester, respectively) (Fig. 22.2a), while the aspartic and the metallopeptidase catalyses do not (Fig. 22.2b). For hydrolysis reactions carried out by

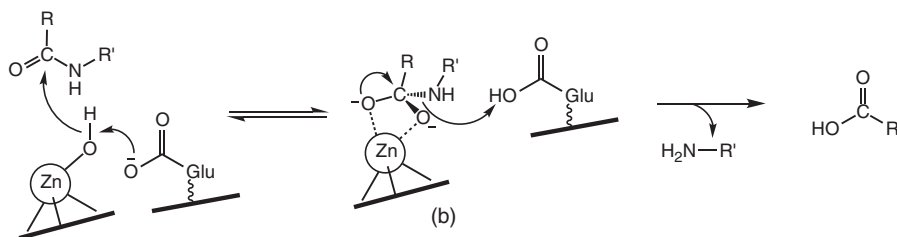
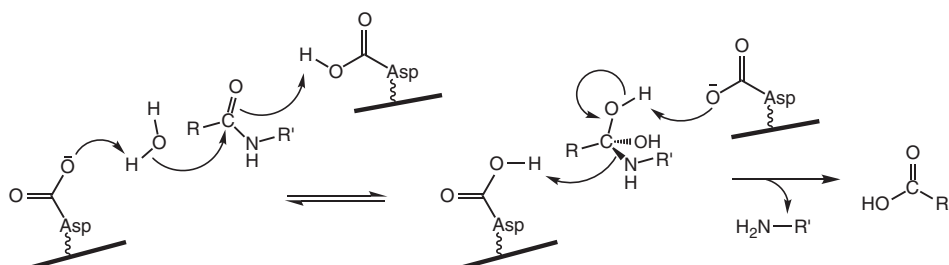
Serine protease



Cysteine protease



(a)



(b)

Figure 22.2 (a) Active-site nucleophiles present in serine and cysteine proteases that form an acyl tetrahedral intermediate. (b) Active-site waters acting as nucleophile in metalloproteases.

the latter two groups, the substrate is attacked directly by a water molecule rather than by a serine or cysteine residue.

Interestingly, despite the mechanistic differences, the basic catalytic features of the enzymes described above is quite similar. Hydrolysis of the peptide bond is an addition–elimination reaction involving a tetrahedral intermediate. For these reactions, metal ions and functional organic groups promote hydrolytic cleavage peptides through (i) electrostatic activation of the substrate in the ground state or stabilization of the transition state by metal ion coordination, hydrogen bonding, or proton transfer; (ii) stabilization of leaving group by metal coordination, hydrogen bonding, or proton transfer; (iii) nucleophilic attack on the substrate by functional group or by metal-coordinated hydroxide, which is generated at neutral pH by Lewis-acid activation of metal-coordinated water; and (iv) generation of the nucleophile via proton abstraction by a basic group.

22.3.2 Peptidase Selectivity

Peptide-metabolizing enzymes can be separated into two major classes (Fig. 22.3): the endopeptidases, which break peptide bonds within the molecule of peptides, and the exopeptidases, which catalyze the removal of one or two amino acids from the N-terminus or the C-terminus of a peptide [9]. Exopeptidases that remove dipeptides from the N-terminus of peptides are called *dipeptidyl peptidases*, while those that remove dipeptides from the C-terminus are termed *peptidyl dipeptidases* [19]. In terms of substrate recognition, each peptidase appears to have its own cleavage specificity of peptides. In some cases, multiple enzymes may hydrolyze a particular peptide to

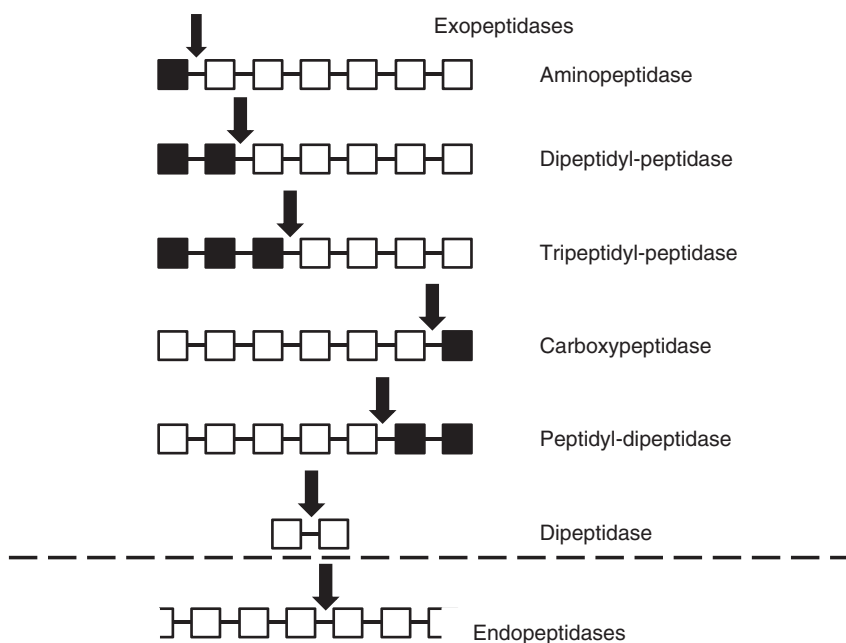


Figure 22.3 An illustration of the two types of peptidases and their general activity.

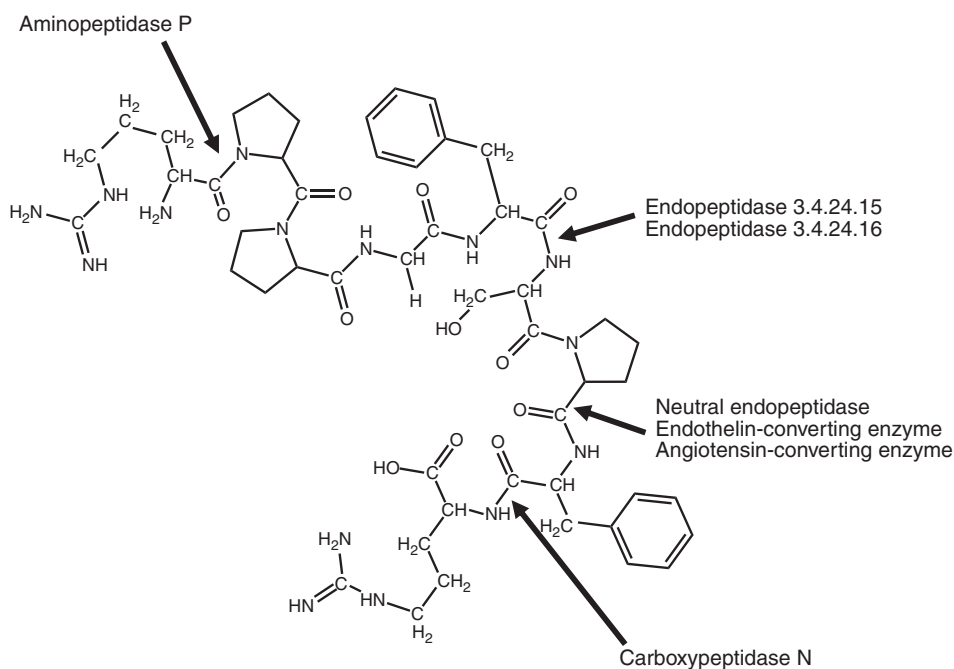


Figure 22.4 Peptidase activity on bradykinin.

yield several products. For example, during a single passage through the pulmonary vascular bed of the lung, no fewer than five of the eight peptide bonds of bradykinin are hydrolyzed (Fig. 22.4) by a variety of peptidases [20]. In contrast, glucagon-like peptide 1 (GLP-1) is specifically hydrolyzed by removal of His-Ala dipeptide of the N-terminus by dipeptidyl peptidase IV (DPPIV) [21]. In this fashion, the information of cleavage specificity of a given peptide drug is important, because knowing the cleavage site allows a rational modification of the peptide drug in order to improve its metabolic stability.

22.4 REDUCING PEPTIDE AND PROTEIN CATABOLISM

22.4.1 Blocking Proteolysis of Peptides and Proteins to Increase Plasma Half-Life

The attachment of polyethyleneglycol (PEG) to a drug molecule, termed *PEGylation*, was first pioneered by Davis and colleagues in the 1970s [7]. The primary objective of this chemical modification was to modify the PK profile of a peptide drug by extending the half-life of the molecule on systemic administration. PEGs are amphiphilic and relatively chemically inert polymers consisting of repetitive units of ethylene oxide [8]. PEGs are named based on the number of ethylene oxide units in the polymer chain; a large variety of PEG molecules with different molecular weights are commercially available. PEGs can be presented with various configurations categorized in two main

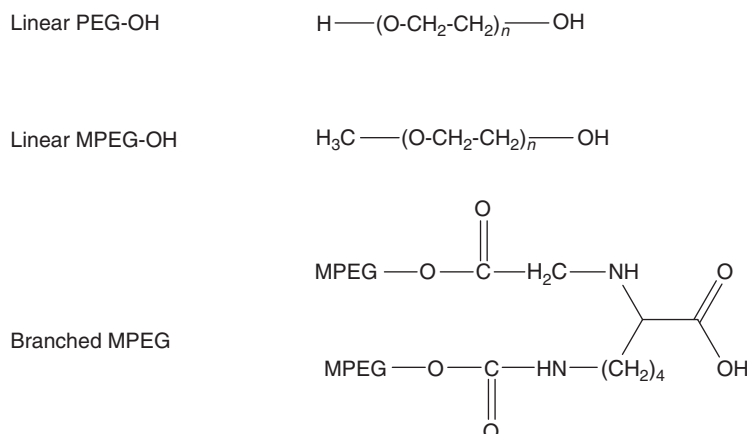


Figure 22.5 Types of pegylation for protein modification.

classes of linear or branched polymers (Fig. 22.5). Moreover, PEGs can be further divided into two groups: PEGs with free hydroxyl groups at both ends and PEGs with one or two methoxylated end group(s) [9]. Multiple low molecular weight PEG molecules (5 kDa), or single high molecular weight PEG molecules (40 kDa) can often be attached at the N-terminus or C-terminus of a peptide without affecting biological activity [10].

The attachment of PEG to peptides is a widely accepted method to increase serum residence times [11]. For example, PHA-794428 is a PEGylated version of somatropin (human growth hormone). The PK of PHA-794428 somatropin is mediated by two mechanisms. The primary mechanism is via clearance by glomerular filtration [12]. When the PK of both molecules (PHA-794428 and somatropin) were evaluated in humans following single subcutaneous administration it was observed that the PEGylation of somatropin with a 40 kDa PEG resulted in a 10- to 20-fold increase in AUC with a similar increase in half-life when compared with somatropin [13].

Site-specific PEGylation has also been achieved in combination with the application of protein chemistry. For example, GLP-1-(7-36) is an endogenous hormone with potential as an antidiabetic agent but due to its extremely short half-life has limited therapeutic potential as the native peptide. Through PEGylation of lysine 34, the residue directly adjacent to the DPPIV peptidase cleavage site, the stability of GLP-1 in the presence of DPPIV is increased >50-fold [22]. The *in vivo* half-life is extended as well but remains short (<1 h) likely due to the renal clearance component of the small PEGylated peptide. Site-specific PEGylation also demonstrated the ability to significantly increase the half-life of IL-15 from minutes to days [23]. In addition, the authors suggest that the strategic placement of the PEG can be useful in the modulation of protein-protein interactions. Several strategies have been developed to introduce PEG molecules specifically into proteins.

The introduction of PEG can also reduce the potential for immunogenicity of some proteins or specific protein sequences. Although site-specific conjugation strategies have been developed, which improve the yield and specificity of the conjugation site, issues remain with the cost and intrinsic heterogeneity of the PEG itself. PEGylated

proteins can also give rise to toxicity. Specifically, renal tubular vacuolation has been observed in animal models. Renally cleared PEGylated proteins or their metabolites may accumulate in the kidney, causing formation of PEG hydrates that interfere with normal glomerular filtration.

The replacement of an L-amino acid by a D-amino acid of a peptide to reduce its susceptibility to enzymatic cleavage is a common strategy to prolong intrinsic plasma half-life of the peptide [24]. For example, natural gonadotropin-releasing hormone (GnRH) has a very short half-life in plasma, which limits its clinical usefulness [25]. However, when a glycine was substituted with a D-tryptophan at position 6 of the natural GnRH, the resulting molecule, triptorelin, has a half-life about 10-fold longer (2 h) than that of natural GnRH, which affords a role for the treatment of central precocious puberty in pediatrics [26]. However, the strategy of substitution of L-amino acids with D-amino acids does not always work and care must be taken to introduce the amino acid substitution in a position where proteolysis occurs [27].

A second example where unnatural amino acid linkages have been employed is observed with β -amino acid peptides [28]. In peptides that comprise α -amino acids, the carboxylic acid group and the amino group are bonded to the same carbon center (also known as the α -carbon because it is one atom away from the carboxylate group). In contrast, β -amino acids have the amino group bonded to the β -carbon (Fig. 22.6). The strategy for this modification is that substitution of an α -amino acid located at the hydrolytically liable peptide cleavage site with a β -amino acid would introduce an extra carbon in the peptide backbone and thus confer resistance to proteolytic cleavage (Fig. 22.7) without necessarily abolishing enzyme binding [29].

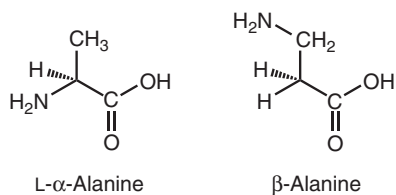


Figure 22.6 Illustration of the natural (L) amino acid compared to non natural (D) amino acid.

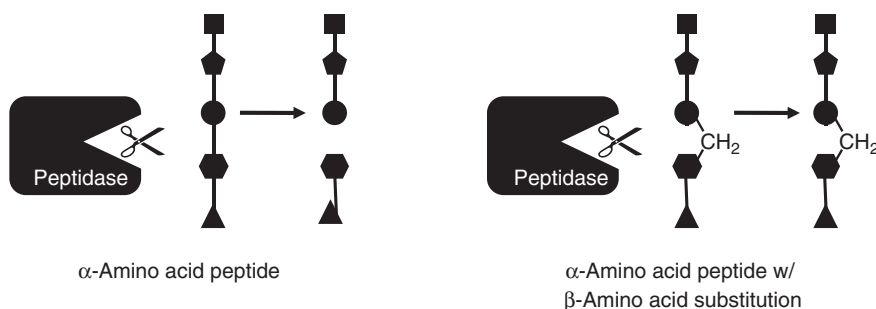


Figure 22.7 Illustration of the natural (L) amino acid compared to non natural (D) amino acid.

As mentioned above, a separate approach to enhance peptide stability is to modify either or both the C-terminus and N-terminus of peptides to minimize their susceptibility to exopeptidases. For instance, N-pyroglutamylation of GLP-1 led to an improved enzymatic stability of and a subsequent increase in *in vivo* glucose-lowering effect in adult obese diabetic (ob/ob) mice [30].

The conjugation of high polymeric mass to peptide drugs has previously been described to decrease the fraction of renal clearance of a peptide and also represents another way to improve their metabolic stability [22,30]. In this fashion, the conjugation of a peptide with PEG increases its hydrodynamic radius and improves its stability toward peptidases. For example a dual-acting peptide's (DAPD) pharmacological effects are attenuated because of its poor metabolic stability. In an effort to overcome a short plasma half-life of the hybrid peptide, DAPD was PEGylated at the C-terminus to enhance its metabolic stability and prolong the duration of pharmacological action [31].

Alternate methods to develop proteolytic resistant peptides have been demonstrated through the cyclization of peptides with olefin metathesis. Specifically, ring-closing metathesis (RCM) links *N*-amino acid to *N* + 4-amino acid by forming a carbon-carbon bond [32]. The end result is the generation of peptides with increased helical structure and proteolysis resistant peptides that are more suitable as therapeutic candidates due to their increased stability. For example, Fuzeon or enfuvirtide is a 36-amino-acid peptide that confers resistance to HIV-1 infection but remains a last option due to short lived efficacy because of rapid elimination [33]. Through engineering in two ring-closing or stapled sites, the PK of the ring-closed peptide (RCP) showed an ~200-fold increase in half-life from 11 min for the parental peptide to 2040 min [34]. Numerous additional RCP have demonstrated similar increases in stability and proteolysis resistant behavior [35–37]. Alternatively, strategic replacement of amino acids that trigger proteolytic degradation can be selectively replaced with more stable amino acids [38]. Yet another strategy is to replace labile amino acids with the introduction of nonnatural amino acids, which has also demonstrated the ability to increase *in vivo* half-life of peptide and protein fragments.

The primary disadvantage of techniques that focus solely on controlling proteolysis, where a reduction in catabolism can be demonstrated in most of the examples, the principal elimination pathway for the peptide/protein still results in significant renal clearance of the modified peptide/proteins. Future efforts will have to combine proteolytic stabilizing techniques with specific strategies to reduce renal clearance.

22.5 HALF-LIFE EXTENSION OF PEPTIDES AND PROTEINS FUSED TO SERUM PROTEINS

In addition to PEGylation, other approaches toward improving the PK of peptides that are based on binding to or fusion with long-circulating serum proteins such as albumin have been developed. Albumin is the most abundant protein in the blood plasma and is produced in the liver as a monomeric protein of 67 kDa. In addition to its role in regulating the osmotic pressure of plasma, albumin also functions as a transporter of endogenous small molecules such as long-chain fatty acids, bilirubin, and steroid hormones [14]. Albumin also binds with high affinity to a broad range of drugs influencing their PK properties [15].

22.5.1 Attachment to Carrier Proteins

Albumin has a simple molecular structure and is highly stable. It is abundantly present in vascular and extravascular compartments with a circulation half-life of 19 days in humans [16]. Recent studies have shown that this long serum half-life is due to a recycling process mediated by the neonatal Fc receptor (FcRn), similar to that observed for IgG molecules [17]. Some companies have taken advantage of these properties and have fused human serum albumin (HSA) with hormones to serve as a macromolecular carrier for drug delivery [18].

22.5.2 Attachment to Antibody Fragments

A second approach pioneered by Amgen is the attachment of Fc regions to peptides to improve PK. One example is romiplostim, an Fc-fusion protein analog of thrombopoietin, a hormone that regulates platelet production [19]. In this instance, the company made use of the natural antibody constant region half-life extension mechanism, provided by FcRn recycling of antibodies, and any other peptide or protein fused to an antibody Fc domain [20].

22.6 HALF-LIFE EXTENSION OF PEPTIDES AND PROTEINS THROUGH POSTTRANSLATIONAL MODIFICATION

22.6.1 N-Terminal Acetylation

The N-terminus of proteins contains a methionine residue capable of undergoing multiple modifications. With the exposed N-terminus, methionine can be acetylated, triggering methionine-aminopeptidase activity, which cleaves off the acetylated methionine residue. The fate of the newly exposed residue is determined partially by size; specifically, if the amino acid residue is small enough, it too will be acetylated. The acetylations can trigger the ubiquitin degradation system.

22.6.2 Phosphorylation

Phosphorylation of serine or threonine residues has demonstrated the ability to increase clearance of endogenous proteins. For example, the phosphorylation of huntingtin at Ser13 and/or Ser16 leads to accelerated clearance of huntingtin. The phosphorylation also triggers additional modifications, including ubiquitination and acetylation, which has been discussed in further detail later in this chapter. Alternatively, a reduction in autophagy has been observed for Ataxin-7 on posttranslational modification of lysine 257. Blocking anticipated phosphorylation sites may lead to unique biodisposition of protein therapeutics.

22.6.3 Engineered Glycosylation

Protein glycosylation sites also contribute to the diverse biological properties of proteins [39]. Specific changes in glycosylation patterns influence all facets of protein behavior, including expression, proper folding, tertiary stability, biodistribution, activity, and

ultimately the half-life of a protein [40–43]. The ability to affect half-life through changes in the glycosylation state of proteins has been recognized and a significant effort has been made to harness the power of glycosylation in the development of therapeutic proteins [44–46]. The two most common forms of glycosylation are N-linked and O-linked. The general motif for N-linked glycosylation of proteins is Asn-X-Thr/Ser, where X can be any amino acid residue except proline [47]. *N*-Glycans share a common pentasaccharide core and are designated by subcategory as high mannose type, complex type, or hybrid type. In contrast, O-glycosylation does not appear to have a formal consensus sequence and O-linked sugars are commonly attached to serine or threonine residues through an *N*-acetylgalactosamine (GalNAc). Two additional types of glycosylation, *C*-mannosylation and glycosylphosphatidylinositol are not discussed further due to limited scope in therapeutic protein design at this time. In addition to the primary sequence motif that indicates potential for glycosylation, it has been proposed that glycosylation sites commonly occur at regions of alternating secondary structure [48]. The increased ability to predict glycosylation sites from primary structure combined with other rule-based computational approaches should aid in the design of novel sites for glycan introduction into proteins in order to enhance the PK properties of protein therapeutics [47].

A number of methods have been employed to alter glycosylation of recombinant proteins. Foremost, the choice of cell line can influence the type and extent of glycosylation, which can further be manipulated by altering expression conditions and media content. Selective pressure of CHO cell lines has been used to isolate mutants in the glycosyltransferase genes [49,50]. Other cell lines are also capable of manufacturing glycoproteins, including human embryonic kidney (HEK) cells, baby hamster kidney (BHK) cells, mouse myeloma cells such as NS0, and the human retinal cell line PERC.6.

In addition to the modification of glycan patterns through strategic choice of expression system, new glycan sites can also be introduced in order to try to improve protein stability, formulation conditions, and PK properties. Site-directed mutagenesis to engineer the glycosylating motif into the therapeutic protein can be attempted [44]. However, this strategy is not trivial and often numerous attempts have to be made in order to introduce novel sites for glycan structures; however, the population of these novel sites to a high degree of fidelity remains quite variable [51]. The variable success of this approach may be indicative of additional factors that contribute to the potential for different protein glycoforms, including microconformations adopted by the protein during biosynthesis and protein folding process. To circumvent these uncertainties, a site-directed mutational approach in combination with specific labeling chemistry has been employed to introduce stoichiometric amounts of glycosylated protein to yield highly homogenous protein preparations [52].

Introducing novel glycan sites may also lead to changes in receptor binding and therefore *in vivo* activity. For example, the specific activity of wild-type DPPIV is significantly higher than that of Gln3191-DPPIV. Alternatively, Gln83 and Gln686 only displayed modest changes in activity compare to wild-type (fully glycosylated enzyme) indicating specific regions of protein glycan structure may have greater impact on function than others [53]. To minimize reduction in activity as a result of introducing novel glycosylation sites internal to the protein, use of the termini of the protein sequence for the introduction of a glycan motif is another strategy that appears more predictive and reproducible [44].

The impact of N-linked glycosylation in native and engineered proteins is readily apparent on review of physical and PK properties of different proteins [54,55]. Another example is follicle-stimulating hormone (FSH), which is a highly glycosylated protein that offers medical advances as a treatment for infertility. The hormone contains two subunits: α and β . Each subunit has two potential sites for glycosylation and structural characterization demonstrated glycol forms with increased sialic acid content tended to possess longer circulation half-lives. Through N-terminal extension of the protein sequence to introduce two novel glycan sites, a modified FSH coined FSH1208 displayed prolonged half-life and efficacy *in vivo* [44]. Lastly, L-asparaginase derived from *Escherichia coli* was chemically modified with *N*-acetylneuraminyl lactose [56]. The thermal stability as well as proteolytic stability was improved for this modified protein. In addition, mouse PK studies were performed with the two materials, which illustrated that the modified protein has a twofold increase in half-life.

22.6.4 Amino Acid Extension Techniques

A polypeptide tethering technique was explored largely based on the premise of the advantages of PEGylation. This strategy focuses on the design of a soluble, chemically stable, and predominantly unstructured polypeptide that provides a maximal hydrodynamic radius to enhance protein PK. The intrinsic physiochemical properties of individual amino acids were carefully explored in designing the proper polypeptide chain; hydrophobic amino acids (e.g., F, I, L, M, V, W, and Y) that typically contribute to compact structures and/or protein aggregation were avoided. Hydrophobic amino acids also play a critical role during binding of peptides to major histocompatibility complex (MHC) class II-driven immune responses. Other amino acids have properties that are undesirable in a molecule intended for clinical usage. For instance, amide-containing side chains such as N, Q, and W are subject to chemical instability during long-term storage of protein drugs, and positively charged side chains such as H, K, and R can facilitate binding to cell membranes. Cysteine residues can cause heterogeneous disulfide cross-linking.

22.7 ANTIBODIES AS THERAPEUTIC PROTEINS

Antibodies, or immunoglobulins, are generated during a humoral immune response critical for the protection of higher organisms. These macromolecules are grouped into five subclasses or isotypes based on the structure of their heavy chains and consist of IgA, IgD, IgE, IgG, and IgM. IgG represents the most important class of antibodies as it is the most abundant in serum at ~ 12 mg/mL. The IgG isotype is further divided into four subclasses, IgG1, 2, 3, and 4, with each subclass playing particular roles during an immune response [57]. In general, IgG antibodies have half-lives in human of ~ 21 days with the exception of IgG3, which has a 7-day half-life [58], which has been shown to be due to a single amino acid in the Fc region (H435R) which results in less efficient FcRn-dependent recycling (see below [59]).

22.7.1 General Antibody Structure

Each antibody contains two identical heavy and light chains made up of variable and constant regions. Each chain contains an N-terminal antigen-binding variable domain,

or Fab, and a C-terminal constant domain, or Fc. The antigen-binding site is contained within the Fab fragment and is formed by the combination of the heavy and light chain variable region domains, in particular, through three complementary determining regions (CDRs) in each of the heavy and light chain, which define the specificity and affinity of a given antibody for its antigen or target. The light chain constant region has one domain, CL, whereas the heavy chain constant region has three domains, CH1, CH2, and CH3, and a hinge region. Interchain disulfide bonds between CL and CH1 stabilize the association of the heavy and light chain, and multiple interchain disulfide bonds reside in the hinge region between CH1 and CH2 and function to stabilize the heavy chains, while the hinge allows for flexibility of the Fab region relative to the Fc [57,60]. The heavy chain constant domains or Fc region of an immunoglobulin molecule, in particular CH2 and CH3, are important for imparting function onto the molecule; they interact with Fc receptors (FcRs) on numerous effector cells, including macrophages, monocytes, and natural killer cells to potentiate effector functions either through antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), or with complement proteins to initiate complement-dependent cytotoxicity (CDC) [61,62]. These heavy chain domains also interact with the FcRn (described below) to regulate the functional lifespan of IgG molecules. The general structure of an IgG is shown in Fig. 22.8.

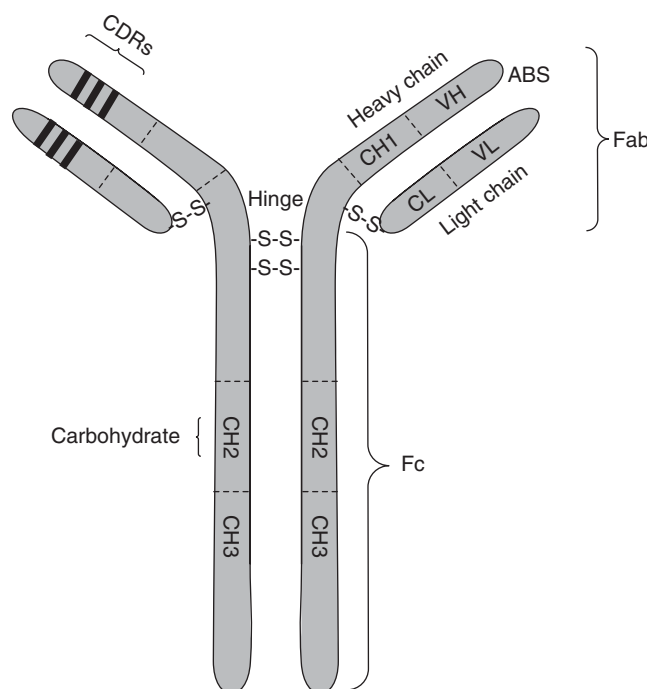


Figure 22.8 General antibody structure. Fc, crystallizable fragment; Fab, antigen-binding fragment; ABS, antigen-binding site; CDR, complementarity-determining regions; Light chain, VL and CL; Heavy Chain, VH, CH1, CH2, and CH3. The carbohydrate-binding region is located in CH₂ of Fc.

Monoclonal antibodies (mAbs) have become an important therapeutic option for many diseases, including cancer, inflammatory, and infectious diseases (reviewed in Refs 63 and 64). Antibody development platforms include but are not limited to murine hybridomas, chimeric mAbs with human Fc domains, humanized antibodies to remove the mouse/rodent components through molecular biology techniques to reduce immunogenicity, and fully human mAbs derived from phage libraries or transgenic mice that express human IgG loci to generate mAbs [65]. In general, IgG antibody half-life increases with increasing amount of humanization, ranging from mouse to chimeric to humanized to fully human [58,66,67].

22.7.2 Antibody Recycling Through the Neonatal Fc Receptor (FcRn)

FcRn is a heterodimer of the MHC class I such as H-chain and the β -2 microglobulin L-chain [68,69]. The presence of FcRn was first proposed by Brambell *et al.* [70,71], who predicted the presence of a saturable receptor that played a role in pre- and postnatal epithelial transcytosis of IgG across the placenta in humans or intestinal epithelium in rodents, respectively, and in the protection of IgG from catabolism leading to long serum half-life. An additional role for FcRn in immunoglobulin homeostasis was further confirmed in β -2 microglobulin KO [72–74] and FcRn KO mice [75], where IgG isotype Abs were rapidly cleared, as they were present at 10- to 15-fold less concentration than normal and thus lack protection, whereas other isotypes such as IgM and IgA had no change in serum concentration.

The vascular endothelium is the main site where FcRn is expressed [75], but it is also expressed on intestinal, kidney, and bronchial epithelial cells [76] as well as hepatocytes and phagocytic cells of the reticuloendothelial system (RES) and plays a role in clearance and recycling [66].

More recent studies have confirmed a role in immunoglobulin homeostasis by regulating the turnover of IgG [75,77]. Interaction of IgG isotypes with FcRn, particularly IgG1, 2, and 4, occurs after pinocytosis of immunoglobulin molecules from the bloodstream into intracellular endosomes, where, in a pH-dependent manner, antibodies now bind to FcRn and are protected from degradation; those that fail to bind FcRn are catabolized in the lysosomes. On recycling to the cell surface, the IgG molecule is released in the pH 7.4 environment of the bloodstream, thus extending the half-life of the molecule. The proportion of IgG processed through the recycling versus destructive pathway is believed to be important in determining the persistence of IgG in the circulation [78].

There is growing evidence that increasing affinity for FcRn can positively influence the half-life of IgG molecules. By altering Fc-region amino acids that interact with FcRn to increase the endosomal binding at pH 6 and promote recycling, while maintaining release into the bloodstream at pH 7.4, it is this differential pH-dependent binding that has been targeted through Fc engineering approaches to generate mAbs with extended half-lives [62,79]. Extending therapeutic antibody half-life would be advantageous to allow for reduced dosing frequency, decreased dosing amount, increased bioavailability, and improved patient compliance. Much effort has been expended to map the FcRn-binding sites within the IgG constant regions, and the majority of the amino acid residues described as affecting binding have been found to lie within the CH2–CH3 interface of the constant region [59,80] as this region interacts directly with FcRn. The pH dependency of binding is due minimally to conserved histidine residues at positions H310 and H435, which are protonated at acidic and neutral pH or deprotonated at physiological ($\sim$ 7.2–7.4) pH [81,82].

To date several groups have generated Fc region mutants that demonstrate increased half-life in preclinical animal species, including mice and nonhuman primates. Dall'Acqua and colleagues have described the "YTE" mutant after phage display mutagenesis of Fc yielded several sequence variants that improved *in vitro* binding to mouse FcRn at acidic pH and also at pH 7.4 [83]. The residues of interest for the YTE site-directed mutational variant mapped to the CH2 domain, specifically to residues M252(Y), S254(T), and T256(E). PK studies in mice demonstrated those variants that bound more tightly to mouse FcRn at both pH 6 and 7.4, including the YTE variant, and PK profiles with lower exposures and reduced half-lives compared to the parental antibody. The YTE mutations were engineered into the anti-respiratory syncytial virus (RSV) mAb MEDI-524 and yielded improved human FcRn *in vitro* binding at pH 6 but not at pH 7.4. The resulting PK profile of this variant in cynomolgus monkeys demonstrated a fourfold improvement in half-life compared to the parental molecule [84]. This variant, MEDI-557, is currently in phase I clinical trials where validation of improved half-life could be demonstrated.

Using molecular modeling to identify Fc residues that might interact with FcRn, Hinton *et al.* [85,86] demonstrated extended mAb PK profiles by incorporating T250Q/M428L mutations into an IgG2 and IgG1 anti-hepatitis B virus mAb and showed a 2- to 2.5-fold improved half-life in rhesus monkeys, respectively. These variants demonstrated an $\sim 28\times$ and $37\times$ higher affinity for rhesus FcRn binding by surface plasmon resonance at pH 6 versus the parental mAb and showed no binding at pH 7.4 and above for the IgG2 and IgG1 variants. Assuringly, there was little to no negative effect on ADCC or CDC activity, or loss of protein A binding (i.e., a matrix used for purification of monoclonal antibodies) by the variant antibodies, which suggests that the CH2–CH3 interface important for all these interactions is not affected by the incorporation of the mutations.

In the first of several reports to look at the importance of altering Fc region residue N434, Petkova *et al.* [87] generated variants N434A and T307A/E380A/N434A that demonstrated enhanced pH-dependent binding to human FcRn *in vitro* and a correlative half-life improvement *in vivo* versus the parental antibody in mice transgenic for the expression of human FcRn. However, these variants showed no improved half-life in wild-type mice, a result that demonstrates the importance of screening modified Fc variants in the correct molecular context [88].

A second analysis of position N434A by Yeung *et al.* [89] revealed an increased binding to human and primate FcRn at pH 6.0 but negligible binding at pH 7.4 by this variant, which led to an increased exposure and half-life with decreased clearance compared to the wild-type parental mAb. Interestingly, variant N434W, which had 80-fold improved binding to human and primate FcRn at pH 6 as well as increased binding at pH 7.4 revealed a PK profile similar to the wild-type mAb, which demonstrates the importance of preserving the efficient release of IgG at pH 7.4 from FcRn into the bloodstream [89]. This is the first group to demonstrate a potential ceiling for the amount of improvement in differential binding to FcRn in a nonhuman primate system.

Finally, Deng *et al.* demonstrated differential PK with an anti-tumor necrosis factor (TNF) variant mAb with changes in the Fc region of N434A and N434H [90]. The N434A variant mAb had similar *in vitro* binding properties to FcRn as the parental mAb and thus in mice had a similar PK profile, whereas N434H had increased pH 7.4 FcRn binding *in vitro* and demonstrated a more rapid clearance and lower bioavailability versus the parental mAb *in vivo*. Furthermore, both variants demonstrated higher affinity

in vitro binding at pH 6 compared to the parental mAb and demonstrated improved PK characteristics in cynomolgus monkeys, including higher exposures *in vivo*, again confirming the importance of tuning the differential binding effect to promote improved pH 6 binding, while maintaining low binding affinity to promote efficient release at pH 7.4.

In addition to improved PK profiles through FcRn modifications, recent examples of improved pharmacodynamics due to improved FcRn binding have also been reported. Zalevsky *et al.* [91] demonstrated three- to fivefold improved half-lives with the anti-vascular endothelial growth factor (VEGF) mAb bevacizumab and the anti-epidermal growth factor receptor (EGFR) mAb cetuximab with the LS series of mutations (M428L/N434S) in cynomolgus monkeys and huFcRn Tg mice, respectively, and both demonstrated an improved *in vivo* efficacy in xenograft tumor models, the first demonstration of improved pharmacology manifested as increased tumor-suppressing pharmacodynamics due to antibodies with longer half-lives.

Yeung *et al.* from Genentech also demonstrated an improvement of PK in cynomolgus monkeys of bevacizumab variants that contained modified Fc residues. Interestingly, the variant T307Q/N434A also demonstrated improved pharmacology in mouse colorectal tumor xenograft models despite having no improved pH-dependent murine FcRn binding or improved PK parameters in mouse [92]. With a finding similar to Deng *et al.* [90], Yeung *et al.* also demonstrated that a 10-fold improvement in pH 6 binding to FcRn may be the upper limit as a greater than 10× increase in binding affinity did not lead to improved PK or half-life but instead led to some degree of a reduced or deleterious effect on PK.

While it appears that there is a direct correlation between improved *in vitro* binding to FcRn at pH 6 and improved *in vivo* PK profiles from the examples presented so far, other Fc-region modification examples that improve FcRn binding *in vitro* have not shown such enhancement *in vivo*. For example, incorporation of the half-life improving T250Q/M428L mutations described by Hinton into an anti-TNF antibody demonstrated ~40-fold improved *in vitro* binding to cynomolgus FcRn but showed no improved PK in cynomolgus monkeys [93,94]. Likewise, Gurbaxani *et al.* have shown no correlation between mAb Fc:FcRn affinity and *in vivo* half-life improvement [95]. Thus, other factors in addition to Fc:FcRn affinity are involved in improvement of PK parameters, which could include absolute (vs relative) FcRn:IgG Fc affinity at both pH 6 and 7.4, the kinetics of the interactions, and the contribution of other clearance mechanisms, including target-mediated disposition, in addition to the FcRn-mediated clearance pathway [79,90]. Interestingly, in a recent report, Suzuki *et al.* [96] analyzed the ~25 FDA, EU, and Japanese approved therapeutic antibody and five Fc-fusion protein drugs and demonstrated differential FcRn binding due to affinity differences between Fc interactions with FcRn, which suggests that the overall CH2–CH3 Fc structure may contribute to the relatively rapid clearance of Fc-fusion proteins when compared to full antibodies. Ultimately, clinical validation of several of these modifications or of one modification in several applications will be needed to understand the global utility of this Fc-modified half-life extension approach.

22.7.3 Antibody Targeting and Influence on Pharmacokinetics

The characteristics of an antigen targeted by a monoclonal antibody may play a role in the disposition and elimination of the dosed antibody. In general, anticytokine or antisoluble protein antibodies do not seem to be markedly altered in clearance due to their interaction with their target as evidenced by dose proportional linear clearance.

Cell surface receptors on the other hand may have a significant impact on clearance particularly for mAbs that are cleared through internalization of an antibody–antigen complex [66,97,98]. For example, cetuximab, and trastuzumab, an anti-human epidermal growth factor receptor 2 (HEGFR2) mAb, both demonstrate nonlinear clearance [97,98]. The extent that a membrane-associated antigen affects mAb clearance depends on the density and distribution of the target antigen on the cell surface and the relative internalization efficiency and turnover rate of the receptor [66].

Other influences on mAb PK include the generation of antidrug antibodies that may act to neutralize the drug or increase its clearance, thus reducing its efficacy. The majority of the currently marketed therapeutic proteins and mAbs demonstrate some level of immunogenicity, although use of humanization technologies and fully human antibodies [63] or engineering efforts to remove immunogenic epitopes [99] has reduced the incidence compared to that observed for first-generation mouse mAbs or next-generation chimeric mAbs. Fully human mAbs theoretically are less immunogenic but still contain CDRs that are unique to each therapeutic protein. In general, the fully human proteins or mAbs have demonstrated lower or no degrees of immunogenicity.

22.8 SUMMARY

As the number of approved biologics increases over the coming years, numerous opportunities for the clinical development of novel protein and antibody drugs will occur. Increasing and expanding the understanding of the critical PK and pharmacodynamic parameters will be necessary in order to speed development and to continue to deliver new biological therapeutic agents.

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23 ADME of Natural Toxins

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23.1	Introduction	1
23.2	Aflatoxins	2
23.3	Aristolochic acid	4
23.4	Arsenic	6
23.5	Selenium	7
23.6	Ibotenic acid and muscimol	9
23.7	α -amanitin	9
23.8	Pyrrolizidine alkaloids	10
23.9	Germander (teucrin A)	11
23.10	Macrocyclic trichothecene (MT) mycotoxins	11
	Acknowledgments	12
	References	12

23.1 INTRODUCTION

23.1.1 What are Natural Toxins?

A common misperception among the public is that any product derived from nature is inherently safe. Nothing could be farther from the truth. Some of the compounds that are most toxic to humans and other mammals occur in nature. History is rife with stories of murder and poisonings from natural toxins, from the death of Socrates via ingestion of poison hemlock (coniine alkaloids from *Conium maculatum*) in 399 BC to the 1978 murder of Bulgarian writer Georgi Markov with an umbrella outfitted with a ricin-filled pellet.

For the sake of this chapter, natural toxins are defined as potentially harmful chemicals produced by living organisms, such as plants, animals, fungi, and algae, or those found naturally occurring in the environment, such as toxic metals. Of course, the

Paracelsan tenet notes that all substances are potentially poisonous depending on the dose. Here, we will consider representative, small-molecule compounds most commonly associated with severe adverse reactions and death.

The production of natural toxins by living organisms is most often the result of secondary metabolism of natural products. Natural toxins may be ingested, inhaled, or absorbed through the skin. Of greatest concern is that natural toxins may enter the food supply at many different points and may be ingested by animals or humans, therefore representing a threat to human health. Increasing attention is also being given to toxic mold contamination of buildings and the risks of inhaled natural toxins.

Over decades, there have been reports on the acute, chronic, and late effects of exposure to natural toxins in both isolated communities and across large populations. Moreover, there have been correlations of their consumption and disposition with late effects of exposure, such as increased risk of cancer from aflatoxins [1]. As a result, there has been much interest in understanding the mechanisms of absorption, distribution, metabolism, and excretion of these compounds and minimizing exposure.

Given the relevance of natural toxins in human health, much attention has been given to identify their sources and understand their effects on the human body. Organizations such as the World Health Organization (WHO), International Program on Chemical Safety (IPCS), US Food and Drug Administration (FDA), and the American Association of Poison Control Centers will commonly issue warnings on toxicology and poisoning trends as well as emergency management of adverse effects of natural toxins.

In the following chapter, we discuss some examples of natural toxins, their sources, and notable mechanisms of metabolism and excretion that influence the toxicity of these chemicals.

23.2 AFLATOXINS

Aflatoxins are the most studied of all fungal toxins or mycotoxins. Aflatoxins are produced primarily by two fungal species, *Aspergillus flavus* and *Aspergillus parasiticus*. These organisms are common saprophytes and opportunistic pathogens with high occurrence in tropics or semitropic regions [2]. Aflatoxins are by-products of fungal metabolism with the major types of metabolites aflatoxin B₁ (AFB₁), aflatoxin B₂, aflatoxin G₁, aflatoxin G₂, and aflatoxin M₁ [2]. Aflatoxins are detected occasionally in milk, cheese, corn, peanuts, cottonseed, Brazil nuts, walnuts, pistachios, pecans, almonds, figs, spices, and a variety of other foods and feeds. Milk, eggs, and meat products are sometimes contaminated because of animal consumption of aflatoxin-contaminated feed. However, the materials with the highest risk of aflatoxin contamination are corn; cereals such as rice, groundnuts, and peanuts; and cottonseed [2].

Factors contributing to the presence or production of mycotoxins in foods or feeds include storage, environmental, and ecological conditions, particularly high humidity environments that encourage growth of *Aspergillus* species normally found in soil [3]. Mycotoxicoses in humans or animals are characterized as food- or feed- related, non-contagious, nontransferable, noninfectious, and nontraceable to microorganism other than fungi [3].

Aflatoxins were originally identified in 1960 as the cause of the death of thousands of turkeys due to hepatic necrosis [4]; their feed was identified as the source of the toxin. To date, the largest case of aflatoxicoses reported in humans occurred in western India in 1974, resulting in 397 recognized cases and 106 deaths [5]. However, additional outbreaks have been reported throughout the world, some examples include Eastern and Central provinces of Kenya in 1981 and most recently, in 2004, where the outbreak resulted from widespread aflatoxin contamination of locally grown maize [6]. The incidence of aflatoxin-driven outbreaks often generates concern about the safety of those exposed and the rate at which neighboring developments show the symptoms. For every symptomatic case of aflatoxicosis identified, several other persons likely were exposed and might face future adverse health consequences. Thus, there is a need to execute routine food inspections to ensure food safety and local education and assistance to ensure that foods such as maize are harvested correctly, dried completely, and stored properly.

Aflatoxin exposure can result in acute aflatoxicosis (poisoning caused by ingestion of high levels of aflatoxin) often manifested as severe, acute hepatotoxicity. Among the early symptoms of hepatotoxicity include anorexia, malaise, and low grade fever. After the acute exposure, the symptoms progress to lethal hepatitis with vomiting, abdominal pain, jaundice, fulminant hepatic failure, and death [7]. In contrast, chronic aflatoxicosis (poisoning caused by chronic ingestion of low to moderate levels of aflatoxin) typically presents with impaired food conversion and slow growth. In addition to aflatoxicosis, aflatoxin exposure can lead to carcinogenesis, particularly hepatocellular carcinoma [1,8,9] as recently reviewed in Ref. 10).

A somewhat controversial approach to prevent aflatoxin exposure via corn and peanuts has been undertaken by the international agribusiness firm Syngenta with the introduction of their Alfa-Guard product. Alfa-Guard is a hulled barley delivery system for spores of a non-aflatoxin-producing strain of *A. flavus* that actively competes for growth by toxic *A. flavus*. The product originated in the US Department of Agriculture's Agricultural Research Service with the work of microbiologist Joe Dorner at the National Peanut Research Laboratory in Dawson, GA.

Despite all of our knowledge on the symptoms and detection of aflatoxin poisoning, it is likely aflatoxins will continue to be a public health problem until appropriate storage methods for food are implemented worldwide. But even in areas of the world with ideal crop storage conditions, *A. flavus* may be unavoidable. The most critical public health approach is continued monitoring of food products for aflatoxins and vigilant surveillance and detection of aflatoxin exposure.

23.2.1 ADME of Aflatoxins

The primary route of absorption of aflatoxins is via ingestion of contaminated food. However, aflatoxins can be found in the dust of contaminated grains and inhalation has been associated with increased risk of lung cancers [11].

Aflatoxins are a classic example of natural toxins that must undergo metabolism to exert their untoward effects. In fact, this theme is common among microbial and plant-based xenobiotics that are toxic to mammals [12].

Aflatoxin activation is mediated via cytochrome P450s (CYPs). Initial debate focused on whether CYP3A4 is the dominant enzyme for AFB₁ metabolism [13,14] relative to the contribution of CYP1A2 [15]. A later study from Gallagher and

colleagues suggested that CYP3A enzyme(s) are capable of AFB₁ oxidation at relatively high substrate concentrations; however, CYP1A2 appears to be the high affinity isoform that is active at lower substrate concentrations when examined in human liver microsomes [16,17]. However, a more recent study on AFB₁ metabolism indicates a predominant role of CYP3A4 for AFB₁ disposition in human liver [18]. In lung cells transfected with cDNA expression constructs for CYP1A2 or CYP3A4, both were capable of AFB₁ activation at low substrate concentrations [11].

Among AFB₁ biotransformation products, activation to the reactive aflatoxin B₁-8,9-epoxide (AFBO) is necessary to exert its hepatocarcinogenic effects, since AFBO binding to cellular DNA, is highly correlated to the carcinogenic potency of AFB₁ [16]. Studies of aflatoxin metabolites have demonstrated covalent binding of AFB₁ to DNA as the AFB₁-N⁷-guanine adduct, 2,3-dihydro-2-(N⁷-guanyl)-3-hydroxy AFB₁ [19]. These adducts can cause G > T transversions and other mutations that lead to tumor formation [20].

In addition to DNA adduct formation, AFBO can also bind other proteins and critical cellular nucleophiles [10]. An example of AFB₁ binding proteins is albumin, the major transporter of AFB₁ in blood [21]. Serum AFB-albumin adducts, are associated positively with hepatocellular carcinoma in humans [22], and analysis of serum adducts has also indicated a positive correlation between dietary AFB₁ exposure and serum AFB₁-albumin adducts [23].

Another example of a toxic adducts is the interaction of AFB₁ with the signal recognition particle (SRP), which acts as an escort for polyribosomes with signal peptides to be transported and bound to the cytoplasmic face of the endoplasmic reticulum (ER) during protein targeting [24]. The interaction of AFB₁ with SRP is now known to generate structural alterations to prevent biosynthesis and translocation of secretory proteins that are important components of the plasma membrane, gap junctions, and intercellular matrix. Singh *et al.* [25] hypothesize that these interactions could disturb cell-to-cell communication and contribute to tumorigenesis.

Aflatoxins are metabolized in liver to oxidized derivatives, including AFBO and AFM₁, that are secreted in the bile and excreted in urine, with a small proportion (about 1% of the ingested dose) being activated through the epoxide to form covalent adducts with DNA [8]. In humans and most animals, the principal route of AFB₁ detoxification is through conjugation with endogenous glutathione (GSH), a reaction catalyzed by glutathione-S-transferases (GST) [10]. Circumstantial evidence is suggestive that GST disposition of aflatoxin metabolites plays a major role in determining the sensitivity of cells to AFB₁ [26]. Essigmann *et al.* [27] first evaluated the possibility of detecting DNA adducts caused by AFB₁ ingestion in human urine, with great success. Since then, detection of DNA adducts or AFBO-albumin complexes in the urine have been a reliable marker for the presence of aflatoxin metabolites.

23.3 ARISTOLOCHIC ACID

Aristolochic acid (AA) is a chemical found in the extract of the plant *Aristolochia fangchi* [28]. Usually, AA is a mixture of structurally related nitrophenanthrene carboxylic acids, with 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid I known as *aristolochic acid I* (AAI) and the demethoxy analog also known as *aristolochic acid II* (AAII) [29]. For many years, AA was utilized for a variety of

treatments summarized elsewhere [29,30] until it was found that the use of herbal drugs containing AA can cause permanent kidney damage, including end-stage kidney failure, requiring dialysis or kidney transplantation, and cancer associated with the urinary tract.

As a result, in 2001, the FDA released a list of compounds containing AA (<http://www.fda.gov/Food/DietarySupplements/Alerts/ucm095297.htm>) [31]. Moreover, despite the long use of AA in herbal remedies for the treatment of numerous symptoms [29], it was not until the early 1990s when cases of advanced renal failure were reported in Belgium, where it was reported in 1991 that consumption of AA in herbal preparations inadvertently included in slimming pills had a correlation with Chinese herb nephropathy (CHN), a type of progressive renal fibrosis [32,33]. In addition to this incident, AA-driven DNA adducts have also been correlated with Balkan endemic nephropathy patients [34]. Once the role of AA was identified as the cause of CHN, the disorder was then proposed to be referred to as AA nephropathy [35].

In addition to the nephrotoxic effects of AA, correlation with the effects of this toxin in carcinogenesis and mutagenesis [36,37], and with the formation of AA DNA adducts [38], has also been demonstrated. More specifically, the activation of AA to AAI has led to various reports indicating that the AAI-driven DNA adducts are the main cause of malignant transformation [29,30] by inserting an A > T mutagenic transversions affecting adenines of codon 61 of the H-Ras mouse gene [37] or purines in the human tumor suppressor p53 gene [39]. Grollman *et al.* [40] further confirmed the presence of aristolactam-dA and aristolactam-dG lesions in the p53 genes in patients with Balkan endemic nephropathy (EN), defined in this chapter as a chronic tubulointerstitial disease frequently associated with urothelial carcinomas of the upper urinary tract. The authors cite the source of the exposure as *Aristolochia clematitis*, a plant endemic to wheat fields of the region. Consumption of baked breads made from grains contaminated with low levels of *A. clematitis* is believed to be responsible for this chronic disease.

23.3.1 ADME of Aristolochic Acid

AA is primarily absorbed in the body via oral ingestion, either via contaminated grain or in weight-loss diet pills containing extracts of *A. fangchi* [41]. Given that AA is predominantly metabolized by hepatic CYP1A2, the kidney and urinary tract are the predominant sites of toxicity [30]. More specifically, there is also evidence that epithelial cells of the proximal tubule are the primary cellular target of AA [42]. Therefore, the liver, kidney, and transitional uroepithelial cells are the main tissues in which AA metabolites are distributed.

In vitro studies, under anaerobic conditions, have suggested that the most important human enzymes activating AA to AAI are hepatic microsomal CYP1A2, renal microsomal NADPH:CYP reductase [43], hepatic and renal cytosolic NAD(P)H:quinone oxidoreductase (NQO1), as well as cyclooxygenase (COX), which is highly expressed in the urothelial tissue [28]. Of all the activating enzymes mentioned above, one of the most efficient AA activating enzymes is NQO1, ubiquitously present in all tissue types [30].

On the other hand, there is also evidence that under aerobic conditions, hepatic microsomes from rats and humans demethylate AAI to form AAIIa [44]. AAIIa (8-hydroxy-6-nitro-phenanthro-(3,4-*d*)-1,3-di-oxolo-5-carboxylic acid) is the nontoxic

derivative of AA. *In vivo* studies by Xiao and colleagues [45] suggested that hepatic CYP1A enzymes, specifically CYP1A2, also play a critical role in detoxification and prevention of AAI-induced kidney injury. Therefore, depending on the systematic conditions, CYP enzymes can play the role of AA activator or AAI excretion.

AA metabolites are excreted in urine. As described above, the main effects of AAI in the human body is the formation of DNA adducts in animals and humans. As a result, AA–DNA adducts have been validated and used as biomarkers of exposure to AA and to investigate the mutagenic and carcinogenic potential of AA. The major AA–DNA adducts found in rodents exposed to AA and in patients suffering from AAN were identified as 7-(deoxyadenosin- N^6 -yl) aristolactam I (dA-AAI), 7-(deoxyguanosin- N^2 -yl) aristolactam I (dG-AAI), and 7-(deoxyadenosin- N^6 -yl) aristolactam II (dA-AAII) [30,46].

23.4 ARSENIC

Arsenic (As) is a highly poisonous metallic element. Inorganic As occurs naturally in the environment and exposure can occur via leaching from geological formations and occupational exposure such as in mining activities, metal processing, and application of pesticides [47].

As exposure can occur from food, air, and water, but the major chronic As exposure comes from the most precious and needed element for survival, water. Among the arsenic species identified in natural water are inorganic arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}), and dimethylarsinous acid (DMA^{III}); As^{III} and As^{V} are by far the most toxic to humans [48]. Therefore, it is vital to regulate the levels of As in water sources all around the world as a method to control As poisoning (arsenicosis) throughout the world. As exposure is correlated with several types of cancer such as the skin, bladder, and lung, reviewed in detail by Kapaj and colleagues [47] and others [49]. In addition to cancer, As also has effects in memory and intellectual functions, cardiovascular disease, respiratory system diseases, and diabetes among others [47].

The mechanisms by which As causes carcinogenicity is by exerting either genotoxic effects (e.g., chromosomal abnormalities, oxidative stress, and gene amplification) or nongenotoxic effects (e.g., altered growth factors, enhanced cell proliferation, and altered DNA repair) as reviewed in detail elsewhere [50].

23.4.1 ADME of Arsenic

As can be absorbed through either the respiratory or the digestive system. Human inhalation exposure to inorganic arsenic can occur through occupational exposure (e.g., coal-fired power plants) or during cigarette smoking [51]. The process starts when As particles are first deposited in the lungs, where it is later absorbed into the bloodstream, depending on the solubility of the chemical form of As. Evidence of As absorption can be detected in the urine, making possible to determine the As exposure in humans as reported by Offergelt *et al.* [52], Yager *et al.* [53], and others.

After the absorption of As through either the respiratory or digestive system, As metabolites enter the bloodstream, to be distributed to the different parts of the body. Exposure to As leads to an accumulation of As in tissues such as skin, hair, and nails,

resulting in various clinical symptoms such as hyperpigmentation and keratosis [47]. The analysis of Scottish and Japanese individuals after long-term chronic exposure showed that As can also accumulate in bone, teeth, muscle, brain, and heart among other organs as summarized in Ref. 48.

In humans, As is excreted as both methyl arsenic (MAs) and dimethyl As (DMA) being the second the main metabolite excreted by the body and detected in the urine of exposed patients [54,55]. The metabolism of arsenic is characterized in many species by two types of reactions: (1) reduction of pentavalent to trivalent arsenic and (2) oxidative methylation reactions in which trivalent forms of arsenic are sequentially methylated to form mono-, di-, and trimethylated products using *S*-adenosyl methionine (SAM) as the methyl donor and GSH as an essential cofactor [51].

Each oxidative methylation reaction is preceded by reduction of arsenic from the pentavalent to the trivalent oxidation state. These reactions are catalyzed by a cytosolic enzyme, arsenic (+3 oxidation state) methyltransferase (As3mt), which uses *S*-adenosylmethionine (AdoMet) as a methyl group donor and requires a dithiol-containing reductant (e.g., thioredoxin) for catalysis [56,57].

Urinary excretion of arsenic begins when arsenic metabolites in the blood are removed by the kidneys about 5 h after ingestion [54]. Early reviews in the field have reported that the arsenic metabolites entering the bloodstream are excreted in several forms, including arsenite (As+3), arsenate (As+5), methyl dimethylarsinic acid (DMAA), and other organically bound arsenic compounds [54]. As can also be excreted through the skin, nails, hair, and sweat but to a far lesser quantitative extent.

While arsenic is considered toxic during acute or chronic oral or inhalation exposure, intravenous arsenic trioxide (As₂O₃) was investigated in China during the early 1990s as a treatment for acute promyelocytic leukemia (APL) [58]. The same research team that had popularized the use of all-*trans*-retinoic acid for APL had been investigating traditional Chinese remedies and identified an antileukemia component as arsenic trioxide [59]. The anticancer effects of arsenic trioxide appear to be due in part to inducing BH-3 family proapoptotic mediators [60]. Second-generation organic arsenicals may continue to have efficacy in cancers that have become resistant to arsenic trioxide [61].

23.5 SELENIUM

Of all the natural products to which humans are exposed, not all can be classified as either beneficial or toxic. This is the case for the trace element Selenium (Se), which can be both beneficial or toxic depending on the exposure and/or doses administered. Se is an essential trace element for humans, animals, and some bacteria [62].

Se is considered essential due to its association with proteins, known as *selenoproteins* [63]. Selenoproteins have biological functions in oxidoreductions, redox signaling, antioxidant defense, thyroid hormone metabolism, and immune responses [64]. Given the important role of selenoproteins in physiological functions, selenoproteins possess a strong correlation with human diseases such as cancer, Keshan disease, virus infections, male infertility, and abnormalities in immune responses and thyroid hormone function [64].

While an essential nutrient, Se can also be toxic depending on the exposure levels. For example, Se compounds at low concentration may have protective anticarcinogenic

properties, whereas at higher concentration, they can be genotoxic and possibly carcinogenic [65]. Chronic toxicity of selenium in humans results in a condition termed *selenosis*, characterized by hair and nail loss and brittleness, gastrointestinal problems, skin rash, garlic breath odor, and nervous system abnormalities [66]. Other related toxic effects caused by Se are disruption of endocrine function, including synthesis of thyroid hormones and growth hormones, and insulin-like growth factor metabolism [67]. The mechanism of Se toxicity has not been clarified but is mostly attributed to its ability to induce oxidative stress both *in vitro* and *in vivo* [62,68].

Se exists mostly in organic forms in normal diets. Organic Se is present in foods mainly in the form of selenomethionine, selenocysteine, and Se methylselenocysteine, whereas inorganic Se either as selenite or as selenate occurs much less frequently and in very low amounts [69]. Of the organic forms, selenomethionine is the predominant form in most Se-rich diets. Both organic and inorganic forms of Se appear to be utilized with similar efficacy in the body to produce selenoproteins [70]. Given that Se is a nutrient whose deficiency and toxic concentrations are very close to each other, it is important to know about its abundance or deficiency in food and diet to establish the right balance of Se in humans and animals [67] and to establish clear parameters of the maximum tolerated doses to benefit from it and also prevent its toxic effects.

23.5.1 ADME of Selenium

The main source of Se is food, and the content of Se can vary much depending on the geographical location, seasonal changes, protein content, and food processing [67]. Se *exposure* occurs mostly through ingestion of Se-containing foods, with much of dietary Se coming from nuts, cereals, meat, fish, and eggs. The levels of Se in different foods and beverages vary and have been reviewed in detail in Ref. 67. Se bioavailability strongly depends on the chemical form of Se found in food, and generally, organic forms of Se are more bioavailable than the inorganic forms [67,71]. Approximately 80% of dietary Se is absorbed, with wheat and meats the most important Se dietary sources [67]. The absorption of Se occurs in the small intestine as evaluated in many animal models by numerous researchers in the field.

Following Se absorption, it is then distributed throughout the body where Se can be located in the skeletal muscles, although organs such as the kidneys, testes, and liver have the highest relative concentration of Se. In contrast, cells that reveal a higher consumption of Se are those of the immune system, erythrocytes, and platelets [67].

Inorganic forms of selenium (selenite and selenate) are reduced by GSH to generate hydrogen selenide (H_2Se) intermediates that later become incorporated into selenoproteins. Organic forms of Se can also become the H_2Se intermediate, where H_2Se is the intermediate compound between the reductive metabolism of Se and its methylation pathway [69]. Selenium compounds such as selenite, selenodiglutathione, and selenocystine are substrates for *antioxidant enzymes* thioredoxin reductase, thioredoxin, and glutathione peroxidase among others [72]. A by-product from Se metabolism is the formation and accumulation of reactive oxygen species (ROS), which are considered as a source of Se-driven toxicity in the form of DNA damage as reviewed by [69,73,74].

Se is eliminated from the body primarily via the urine, although significant losses via feces also occurs and low amounts of Se are also lost through the skin and respiration [67]. More specifically, there are studies confirming that Se excretion can be rapidly

detected in the urine, where 50–78% of the ingested element is excreted [75], and the levels of secreted Se change depending on the intake of Se in an individuals diet.

23.6 IBOTENIC ACID AND MUSCIMOL

Amanita muscaria is a mushroom with a rich history of use by shamanic cultures of Siberia and northeast Asia. Known also as fly agaric for its use as an insecticide, *A. muscaria* contains the alkaloids ibotenic acid and muscimol and is easily recognizable owing to its red cap with white spots. Saar [76] has described the religious use of *A. muscaria* and English language accounts extending back to the 1700s describe an unusual practice of metabolic significance. A Swedish colonel, Philip Johan von Strahlenberg, held as a prisoner by the Koryak tribe of Siberia wrote in 1730 of his experiences:

“Those who are rich among them, lay up large provisions of these mushrooms, for the winter. When they make a feast, they pour water upon some of the mushrooms, and boil them. Then they drink the liquor, which intoxicates them. The poorer sort . . . post themselves, on these occasions, round the huts of the rich, and watch the opportunity of the guests coming down to make water; and then hold a wooden bowl to receive the urine, which they drink off greedily. . . , and by this way they also get drunk [77]”

The practice, substantiated by George Steller in 1774, owes to the decarboxylation of ibotenic acid to muscimol and a much higher urinary concentration of muscimol than in the mushrooms. Muscimol is an agonist for GABA(A) receptors and produces a lucid dreamlike state that can be perceived as either desirable or disturbing depending on the context. Often mischaracterized as a hallucinogen, muscimol is better described as a deliriant. The receptor specificity of muscimol has led to its use as a radioligand for defining GABA(A) receptors. The parent compound, ibotenic acid, is also a N-methyl-D-aspartate (NMDA) glutamate receptor agonist and is responsible for jerky muscle movements often accompanying accidental ingestion of the mushrooms. In the laboratory setting, ibotenic acid can be used to specifically lesion parts of the brain owing to its excitotoxic effect at NMDA receptors.

Ingestion of *A. muscaria* is rarely fatal. According to the American Association of Poison Control Centers, 61 cases of *A. muscaria* poisonings were reported in 2009.

23.7 α -AMANITIN

While this chapter focuses on small-molecule toxins, the previous discussion of *Amanita* species necessitates some treatment of this classic peptide hepatotoxin. Unlike mushrooms that produce ibotenic acid and muscimol, other species of *Amanita*, most notably not only *Amanita phalloides* but also *Amanita virosa* and *Amanita bisporigera*, are recognized for their production of the cyclic octapeptide, α -amanitin.

Work on the chemistry of toxic “death cap” mushrooms can be traced by to Heinrich Wieland, 1927 Nobel laureate in chemistry for work on the bile salts. Wieland’s student at Munich (and later son-in-law), Feodor Lynen, conducted his 1937 PhD dissertation work entitled “On the Toxic Substances in *Amanita*,” and Wieland’s laboratory later

crystallized the substance in 1941. Lynen went on to investigate the biochemistry and synthesis of cholesterol and fatty acids, work for which he shared the 1964 Nobel Prize in Physiology or Medicine with Konrad Bloch. Work on the amatoxins and related phalloidins was instead undertaken by Heinrich's son Theodor and nephew Ulrich, respectively. While phalloidin exerts toxicity by triggering depolymerization of F-actin, it requires hepatic bioactivation for maximum toxicity. Fluorescent conjugates of phalloidin are used widely today for microscopic imaging of cellular actin networks.

Theodor Wieland worked tirelessly on the chemistry and biochemistry of amatoxins and was the first to separate the α , β , and later γ isoforms by solid-phase electrophoresis [78], a method later used to demonstrate isozymes among enzyme families. The Italian group of Stirpe and Fiume were first to demonstrate in 1967 that α -amanitin rapidly and potently inhibited RNA synthesis [79,80]. Lindell *et al.* [81] later showed in 1970 that the proximal target of α -amanitin was nuclear RNA polymerase II, the RNA polymerase responsible for messenger RNA synthesis. In fact, the selectivity of α -amanitin for the type II polymerase is used to distinguish between the biosynthetic activities of RNA polymerase I (primarily ribosomal RNAs) and III (primarily transfer RNAs).

Symptoms of amatoxin poisoning do not usually present until 6–8 h after ingestion, with severe abdominal cramps, vomiting, and gastrointestinal bleeding that gives rise to hepatic and renal failure [82]. Reports vary based on amount ingested and time of treatment initiation, but most sources cite a 15% fatality rate within 10 days of *A. phalloides* ingestion. Liver transplantation can be curative but is usually not practical. One challenge in antidote management is that α -amanitin undergoes enterohepatic recirculation.

European literature had suggested the potential utility of an intravenous preparation of partially purified extract of milk thistle (*Silybum marianum*), silibinin hemisuccinate (Legalon SIL). This intravenous preparation is produced by Madaus AG and is currently only available in Europe. Two hepatoprotective mechanisms of action are possible: the silibinin compounds may inhibit reuptake of the toxin by intact hepatocytes and/or competitively interfere with glucuronide hydrolysis. Several highly publicized and successful treatment cases in the United States led to an initiation of an open-label clinical trial [83]. The outcome of the ongoing trial is likely to influence the regulatory status of Legalon in the United States.

The reader is referred to an outstanding review in *Science* by Theodor Wieland in 1968 [84] that exhaustively addresses the toxins of all *Amanita* species, from ibotenic acid and muscimol to amatoxins and phalloidotoxins.

23.8 PYRROLIZIDINE ALKALOIDS

Over 350 pyrrolizidine alkaloids can be found in numerous plant species, typically considered noxious weeds, but may also be contaminants of honey, milk, and grains. The most common historical exposure to pyrrolizidine alkaloids has been with herbs purported to have medicinal value. Comfrey, for example, is still sold for making medicinal teas yet is a well-known source of the alkaloids, symphytine, and echimidine [85].

Pyrrolizidine alkaloids are bioactivated by CYP to cause veno-occlusive disease of the liver and cirrhosis after long-term use [11]. This life-threatening disease was first described in 1954 from the ingestion of Jamaican bush tea (*Senecio discolor* and

Crotalaria fulva) but literature reports consistent with this pathology can be found as early as 1920 [86].

CYP3A4 and flavin-containing monooxygenases are responsible for the C- and N-oxidation of pyrrolizidine alkaloids to form the reactive pyrrolic ester and *N*-oxides, respectively.

23.9 GERMANDER (TEUCRIN A)

While pyrrolizidine alkaloids and AA are among the best known plant toxins historically, some localized cases of hepatotoxic herb exposure appear sporadically. In 1991, use of a French weight loss supplement containing wall germander (*Teucrium chamaedrys*) was associated with seven cases of hepatitis that required hospitalization. Pathology ranged from hyperbilirubinemia and jaundice to hepatic necrosis [87]. The hepatotoxicity of two furan-containing diterpenoid components, teucrin A and teuchamaedryn A, is mediated via CYP3A4 oxidation of the 3-substituted furan to an epoxide [88]. The epoxide is proposed to rearrange to form an electrophilic reactive enedial. Druckova *et al.* [89] used teucrin enedial–albumin adducts to create an antibody probe that revealed 46 protein adduct targets in rats administered with teucrin A. The majority of adducted target proteins were found in the mitochondria and ER, such as those involved in lipid metabolism, energy production, cellular respiration, drug metabolism and clearance, and protein chaperones.

While these incidents are infrequent, herbal folkloric use of germander species continues today. A February 2012 case report from Istanbul detailed a case of vomiting and moderate hepatic injury in two-month-old twin sisters who had been given 10 mL of polygermander tea (*Teucrium polium*) added daily to their infant formula for colic [90]. The infants recovered with supportive intravenous fluids and vitamin K administration and were discharged after five days. The continued occurrence of these and other cases of “natural” hepatotoxicity should serve as warning to clinicians to query patients about alternative medicine use on presentation with liver disease of unknown cause [11].

23.10 MACROCYCLIC TRICHOTHECENE (MT) MYCOTOXINS

This class of compounds has become of increasing toxicological importance due to the appreciation of the so-called toxic black mold in human diseases. *Stachybotrys chartarum* and *Stachybotrys chlorohalonata* can colonize water-damaged building materials and cause indoor air exposure to macrocyclic trichothecenes (MTs). These and other species can produce one or more sesquiterpene trichothecenes whose presence can be quantified in indoor air of contaminated buildings. The most common trichothecenes are verrucarins B and J; roridin E; satratoxins F, G, and H; and isosatratoxins F, G, and H.

These compounds are broadly cytotoxic in the nanomolar range and had originally been evaluated by the US National Cancer Institute for potential anticancer activity but their lack of selectivity caused their study to be discontinued. The most noteworthy structural features in their toxicity are the presence of an epoxide moiety (present in all but three MTs) and a diene in the sesquiterpene skeleton. Little work has been done on

the metabolism and excretion of MTs, but it is likely that phase I and phase II enzymes are involved in activation and detoxification of these increasingly relevant toxins.

Work in the yeast *Saccharomyces cerevisiae* indicates that mitochondrial protein synthesis is the target of the trichothecene, trichothecin [91,92]. Several of the compounds are known to cause respiratory symptoms by activating cytokine secretion in human macrophages [93].

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24 Influence of Changes in Physiology, Transporters, and Enzyme Expression on Disposition and Metabolism of Drugs during Pregnancy and Clinical Implications

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24.1	Introduction	1
24.2	Physiological changes occurring in pregnant mothers and their influence on drug disposition	2
24.3	Changes in transporters during pregnancy	6
24.4	Drug-metabolizing enzymes (DMEs)	13
24.5	Clinical implications of drug therapy during pregnancy	19
24.6	Conclusions	19
	Acknowledgment	19
	References	21

24.1 INTRODUCTION

During pregnancy, progesterone, a steroidal sex hormone, plays an important role in the discontinuation of female menstrual cycle and in the embryogenesis. The increase in progesterone levels starts during ovulation, continues to increase during pregnancy, and

helps in the development of the uterine lining for implantation of the fertilized egg. In reverse, decreased progesterone levels are associated with the risk of miscarriage. The levels of progesterone during menstrual cycle and in the first trimester of pregnancy are controlled by ovaries, but as the placenta grows (ninth–tenth week), it takes over this role of the ovary and maintains progesterone during pregnancy.

The changes in levels of progesterone and other associated hormones are linked to changes in physiological conditions of the pregnant mother, such as increase in blood plasma volume, extracellular fluids, cardiac output, renal flow, white blood cells, platelet and decreases in albumin, hemoglobin, urea, creatinine, and so on. These physiological adaptations are to favor the development of the fetus, but this also results in alterations to drug-metabolizing enzymes (DMEs) and xenobiotic transporters, thereby affecting pharmacokinetics of drugs in the body and leading to increased potential for side effects.

During pregnancy, human placenta functions as both a barrier and a transport organ, helping in exchange of nutrients and oxygen as well as waste products between the mother and the fetus. These functions are assisted by transporter proteins expressed on the placental syncytiotrophoblasts, which have differential localization in the maternal-facing brush border membrane and fetal-facing basal membrane. The transporters expressed on maternal-facing brush border membrane facilitate the entry of xenobiotics from mother to fetus. The passage of nutrients and xenobiotics across the placental barrier is chemically regulated by enzymes such as hydrolases, transferases, oxidoreductases, lyases, and isomerases. These enzymes have been implicated in xenobiotic metabolism, the generation of reactive metabolites, and in rare cases, birth defects and teratogenicity.

The purpose of this chapter is to review the aspects of transport, metabolism, and clinical implications of administered drugs during pregnancy. It covers the nature of physiological changes occurring during pregnancy, types of transporters expressed, differential enzymes present, and clinical implications of these factors on drug therapy for the pregnant mother and fetus.

24.2 PHYSIOLOGICAL CHANGES OCCURRING IN PREGNANT MOTHERS AND THEIR INFLUENCE ON DRUG DISPOSITION

The physiology of a pregnant woman is significantly different from that of a non-pregnant woman. Practically, physiological changes begin in early gestation and are most pronounced in the third trimester of pregnancy. Some of these are normalized within 24 h of delivery, while others return to normal in 12 weeks after delivery [1]. Table 24.1 lists the nature of physiological changes in the body through the pregnancy period and postpartum. Major changes occur within the gastrointestinal tract (GIT), cardiovascular, renal, and hepatic systems. These changes have the potential to influence absorption, distribution, metabolism, and excretion (ADME) of drugs. A few examples are included in Table 24.2.

24.2.1 Physiological Changes in the Cardiovascular System

Cardiovascular changes occurring during pregnancy include alterations in cardiac output, blood volume, blood pressure, extracellular fluid space, plasma albumin, and

TABLE 24.1 Physiological Changes during Pregnancy

Physiological Parameter	Nonpregnant Women	Pregnant Women	References
BP	SBP = 120 ± 9.8 mm Hg DBP = 80.0 ± 7.2 mm Hg	SBP = 119.6 ± 9.8 mm Hg DBP = 65.0 ± 7.2 mm Hg	2
Cardiac output	2.90 ± 0.17 L/min	6.7 L/min at 8–11 wk and 8.7 L/min at 36–39 wk	3
Blood plasma volume	4–5 L	Increase by 40–50%	3
RBC count	4.2–5.4 million cells/mL	Increased by 20–30%	3
Hb	10.5–13.5 g/dL	Decrease in Hb due to relatively less increase in RBC count in comparison to blood plasma volume	4,5
Alkaline phosphatase, plasma	26–110 IU/L	Increased during pregnancy	6
Platelets count	$200\text{--}600 \times 10^9$ cells/L	Unchanged/slightly increased	3
Urine creatinine	37–250 mg/dL	Decreased in pregnancy	3
Albumin	24–31 g/L	Decreased in pregnancy	6
Fasting glucose	3.0–5.0 mmol/L	Unchanged in pregnancy	3
Alanine	407 ± 66 μ mol/L	10–20 wk = 321 ± 58 μ mol/L, 30–40 wk = 321 ± 58 μ mol/L	7
Citrulline	43 ± 11 μ mol/L	10–20 wk = 24 ± 6 μ mol/L, 30–40 wk = 29 ± 9 μ mol/L	7
Arginine	79 ± 10 μ mol/L	10–20 wk = 51 ± 10 μ mol/L, 30–40 wk = 39 ± 8 μ mol/L	7
Ornithine	50 ± 4 μ mol/L	10–20 wk = 23 ± 5 μ mol/L, 30–40 wk = 21 ± 4 μ mol/L	7
Total bilirubin	0.0–1.0 mg/dL	Decreased during pregnancy	6

Abbreviations: BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; Hb, hemoglobin; RBC, red blood cells; SBP, systolic blood pressure; wk, weeks.

RBC count. Cardiac output rises by 30–50% by the end of the second trimester and remains the same until delivery. Likewise, blood volume increases progressively from six to eight weeks to reach a maximum at 32–34 weeks with little change thereafter, with overall increase being 40–50% during normal pregnancy [23–26]. The increase in blood and total body water volumes alter the volume of distribution of various drugs. These changes affect drug disposition and elimination, as well as the terminal elimination half-life [27]. Albumin content has been shown to decrease, while there is increase in α 1-acid glycoprotein during pregnancy. These changes lead to alterations in protein binding of drugs and result in increase in their plasma concentration beyond the therapeutic window. For example, diazepam, phenytoin, and sodium valproate have significantly elevated free fractions in the last trimester because of the decrease in the

TABLE 24.2 Select List of Drugs Showing Alterations in Pharmacokinetics and Metabolism during Pregnancy

Drug	Gestational Age	ADME	Enzyme/Transporter	Significance	References
Methadone	27–30 wk	Increased metabolism and decreased absorption	NS	Higher elimination rate constant and lower half-life compared to nonpregnant controls	8
Caffeine	Second and third trimesters	Decreased metabolism	CYP1A2	Decrease in clearance	9
Nelfinavir	31–36 wk	Increased metabolism	Induction of hepatic CYP3A4 or inhibition of CYP2C19	Safety and efficacy (therapeutic drug monitoring)	10
Indinavir	Correlation between during pregnancy and postpartum	Increased metabolism	NS	Warrant dosage adjustment during pregnancy	11
Cotinine	NS	Increased metabolism	CYP2A6	Clearance increased by 140% and half-life by 50%	12
Lamotrigine	Second and third trimesters	Drug distributed in fetus	NS	Leads folate deficiency that finally results in teratogenic effects	13
Labetalol	NS	Increased metabolism	UGT1A1, UGT2B7	Clearance increased	14
Metoprolol	Third trimester	Increased metabolism	CYP3A4	Clearance increased	15,16
Nifedipine	Third trimester	Increased metabolism	CYP3A4	Clearance increased	17
Phenytoin	NS	Altered absorption or metabolism	NS	Seizures appear	18,19
Citalopram	20, 30, and 36 wk	Increased metabolism	NS	Dose adjustment required	20
Escitalopram	20, 30, and 36 wk	Increased metabolism	NS	Dose adjustment required	20
Sertraline	20, 30, and 36 wk	Increased metabolism	NS	Dose adjustment required	20
Proguanil	Third trimester	Decreased metabolism	CYP2C19	Active metabolite formation decreased, so dose required to be increased by 50%	21,22

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; CYP, cytochrome; NS, not specified; UGT, UDP glucuronosyltransferase.

albumin content. The increase in body weight during pregnancy further results in a decrease in dose/kg of administered drugs and leads to a significant lowering of a drug's steady-state concentration with resultant suboptimal treatment.

24.2.2 Physiological Changes in the Gastrointestinal System

Motility disturbances of the GIT are common during pregnancy. These disturbances are largely due to alterations in sex hormone levels, including heartburn, nausea and vomiting, abdominal bloating, and constipation [28]. Among these symptoms, nausea and vomiting have greater influence on absorption and bioavailability of drugs, especially during the first trimester [29]. Also, increase in plasma progesterone concentration during pregnancy corresponds to decrease in gastrointestinal motility, with associated prolonged gastric emptying and intestinal transit times. This may lead to delayed drug absorption and reduced peak concentration [30]. In addition, gastric pH is slightly increased during pregnancy due to the reduced gastric acid secretion, which may affect ionization and absorption of weak acidic and basic drugs [31].

24.2.3 Physiological Changes in the Renal System

Renal physiological changes are characterized by marked vasodilatation that leads to an increase in the glomerular filtration rate (GFR) and renal plasma flow (RPF). These changes are initiated during the first trimester, by the end of which GFR and RPF increase by 50% and 60–80%, respectively. Apparently, there is higher increase in RPF in comparison to GFR, resulting in a significant decrease in filtration fraction (GFR/RPF). In addition, creatinine clearance is increased during pregnancy, while its production remained unchanged. This results in lowering of serum creatinine. As a fallout of the above conditions, there is increased clearance of polar drugs via kidney during pregnancy, with the likelihood of failure of the treatment [32–34].

24.2.4 Physiological Changes in the Hepatic System

During pregnancy, the mass of the liver is increased up to 50%, which results in a decrease in the per milligram expression of DMEs. In addition, alterations in sex hormone levels also affect expression of different phase I and phase II DMEs. Davis *et al.* [35] found that hepatic microsomal enzymes started rising progressively from the twelfth week of pregnancy and continued till delivery, with a gradual fall to normal levels by the sixth week postpartum. Sulfate conjugation was decreased during pregnancy, and this was attributed to the inhibitory effect of the high levels of estrogen during pregnancy. In a similar manner, inhibitory effects of progestogens on the glucuronide conjugation of drugs were also established [7]. Additional pregnancy-induced changes included decrease in bilirubin and γ -glutamyltransferase and increase in aminotransferases, alkaline phosphate (used in the diagnosis of hepatotoxicity), and 5'-nucleotidase (used in the diagnosis of cholestasis) [6]. Some changes during pregnancy were similar to those observed in hepatotoxicity, for example, increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity during the third trimester, although this typically remained below the upper margin of normal limit. However, other reports indicate that there were no changes in ALT and AST activity during pregnancy [36–40]. However, their activity increased during labor due to the contractions of the uterine muscles [41,42].

24.3 CHANGES IN TRANSPORTERS DURING PREGNANCY

The efflux [ATP-binding cassette (ABC)] and uptake [solute carrier (SLC)] transporters are altered during pregnancy and ontogeny. The changes occurring at placental and fetal levels are primarily meant to provide nutrition to the fetus and provide protection from undesired xenobiotics and endogenous waste products. This barrier is not absolute, and some of the xenobiotics and drugs do cross and influence the fetus.

24.3.1 Transporters in Human Placenta

Drugs used during pregnancy are for the treatment of either the mother or, to a lesser extent, the fetus. During pregnancy, drug selection is mainly dependent on the ability of the drug to cross the physical barrier between the maternal and fetal compartments. If therapy is needed only for the mother then transfer of drugs through the placenta can lead to toxic effects in the fetus. On the other hand, if the fetus requires therapy, transfer of the drugs from the mother to the fetus becomes an important determinant.

Major functions of the placenta are to transfer nutrients from the mother to the fetus and to eliminate metabolic waste products from the fetus through the mother, which is performed by transporters that are expressed in the maternal-facing brush border membrane and the fetal-facing basal membrane of the syncytiotrophoblasts. These transporters are involved not only in maintenance of the flow of nutrients and macromolecules that are needed for placental function and fetal development but also in the transportation of xenobiotics, including therapeutic agents, environmental pollutants and toxins, and even drugs of abuse. The ABC efflux transporters expressed in placenta are P-glycoprotein (PgP/MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance proteins 1–3 and 5 (MRP1–3 and 5/ABCC1–3 and 5). The SLC influx transporters are organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), monocarboxylate transporters and nucleoside transporters [43–46]. Table 24.3 lists placental transporters along with their drug substrates and their clinical impact on the fetus. The human placental barrier at term, the microstructure of placental barrier, and the main transporter proteins expressed in human placental barrier and their localization are illustrated in Fig. 24.1 [46].

24.3.1.1 ABC Transporters. ABC efflux transporters are located on the maternal-facing and the fetal-facing sides of syncytiotrophoblasts and/or the endothelium of fetal capillaries. These are involved in transport of exogenous and endogenous compounds and metabolites across the placenta, so as to limit the fetal exposure to potential toxins from maternal circulation, thereby protecting it. Drug substrates for this category of transporters include cardiovascular, anticancer, and anticonvulsant drugs; anti-HIV protease inhibitors; and so on. The ABC efflux transporters expressed in placenta are PgP/MDR1/ABCB1, BCRP/ABCG2, MRP1–3 and 5/ABCC1–3 and 5.

24.3.1.1.1 PgP/MDR1/ABCB1. This transporter is a product of the multiple drug resistance (MDR) ABCB1 gene and is expressed in the placental trophoblast layer located in the brush border membrane. PgP is involved in active efflux of lipophilic

TABLE 24.3 Transporters Involved in Drug Transfer Across the Placenta

Transporter	Substrate	Clinical Impact	References
OATP	Streptomycin	Hearing loss in infants	47
Na ⁺ -dependent organic cation transporter	Tetracycline	Deformations in bone and formation of teeth, enamel in infants	48,49
Active transport	Warfarin	Bleeding in fetal brain	50,51
Mrp2	ACE inhibitor	Damage to fetal kidney	52,53
PgP	Glyburide	Active efflux reduces toxicity in fetus	54
<i>mdr1a</i> -/-/ <i>1b</i> -/-	Saquinavir, paclitaxel	16 times more drug required	55
PgP	Avermectin	Protects from cleft palate in fetus	56
PgP	Indinavir	Lower fetomaternal clearance, bidirectional transfer	57
PgP	Vinblastine	Increased maternofetal clearance	56
PgP	Methadone	Active efflux reduces toxicity in fetus	58
PgP	Anthracyclines, taxanes	Decreased exposure to fetus and reduced toxicity	43
PgP	HIV protease inhibitor	Reduced mother to child HIV transmission	59
Passive transport	Ganciclovir	Teratogenic and embryotoxic effects	60
ABC transporters	Antiepileptic drugs	Congenital malformations, minor anomalies, congenital syndrome, and developmental disorders	61
Passive transport	Lidocaine, bupivacaine	Epileptiform discharges, immaturity of the autonomic nervous supply to the cardiovascular system, fetal methamoglobinemia	62,63
Carrier-mediated transport	Metformin	Polycystic ovary syndrome	64,65

Abbreviations: ABC, ATP-binding cassette transporter; ACE, angiotensin converting enzyme; HIV, human immunodeficiency virus; OATP, organic anion transporter polypeptides; PgP, P-glycoprotein; MRP, multiple drugs resistance protein.

substances from the cell. Transport by PgP is unidirectional, and the substrate is effluxed out from the cell due to its asymmetrical membrane topology. Thus, by moving out toxic substances/xenobiotics, PgP protects the fetus from potential toxins that can cross the placenta [66].

The placental expression of PgP is significantly high in preterm as compared to term placenta and gradually decreases with gestational age [67]. Cyclosporine was used to directly evaluate the influence of PgP on transplacental

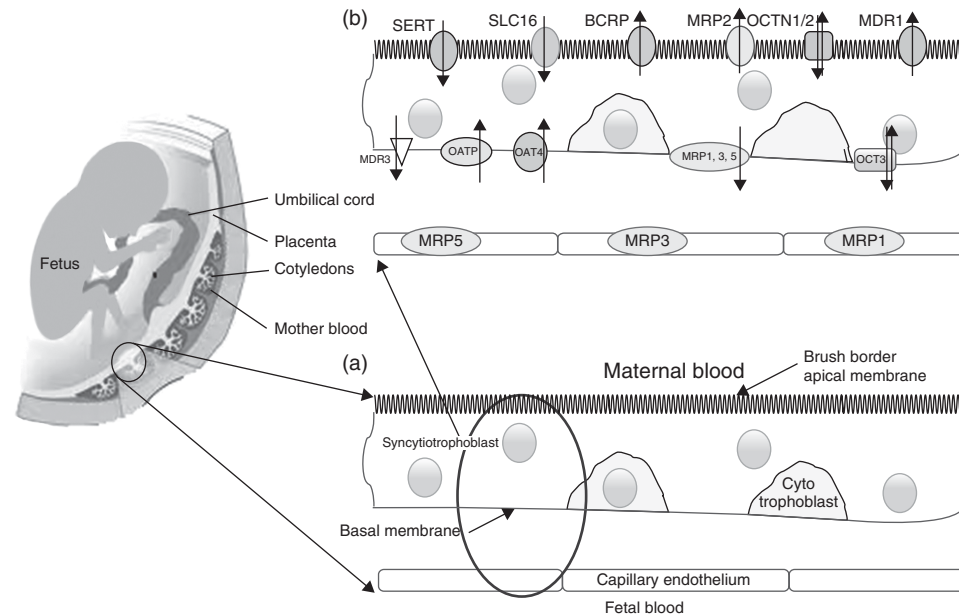


Figure 24.1 Pictorial representation of term placenta along with the developed fetus, indicating the expression of transporter proteins (BCRP, breast cancer resistance protein; MDR, multidrug resistance protein; MRP, multidrug-resistance-associated protein; NET, noradrenalin transporter; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCTN, novel organic cation transporter; SER, serotonin transporter). (a) Zoomed section of placenta. (b) Enlarged view of placental cellular structure. (See color insert.)

transfer with a dually perfused human placenta model. The clearance of cyclosporine was increased significantly when it was coadministered with quinidine (a potent PgP inhibitor) [68]. PgP decreased the uptake of rhodamine123 from the maternal to the fetal side, while accelerating elimination of PgP substrates from the fetal side [69]. This perfused placenta model has also been used to demonstrate efflux of the PgP substrate drugs, saquinavir [70], methadone [38], indinavir [58], and prazosin [71]. Interestingly, a reverse pattern was found in the rat, where an increase in Mdr1a and Mdr1b expression was observed with advancing gestation [72].

24.3.1.1.2 BCRP. BCRP expression is very high in the human placenta, suggesting a role similar to PgP in limiting fetal exposure to xenobiotics and facilitating elimination of xenobiotics from the fetal compartment. In membrane vesicles prepared from human term placenta, BCRP transported mitoxantrone [73] and glyburide [74], and the perfused human placenta model revealed BCRP-mediated efflux of glyburide [75] and cimetidine [76]. A role has been suggested for BCRP (and OATP2B1) in regulating placental estrogen synthesis by mediating the concentrations of dehydroepiandrosterone sulfate and estrone 3-sulfate [77]. BCRP and its mRNA expression was twice as high in the preterm (28 ± 1 weeks) relative to the term (39 ± 2 weeks) human placenta [78]. Similar decrease with gestation was also observed in the rat [79].

24.3.1.1.3 MRPs. MRP1 is present in the apical membrane of the placental syncytiotrophoblasts [80] and the human choriocarcinoma cell line BeWo, where it transports unconjugated bilirubin [81] and mediates 2,4-dinitrophenyl-*S*-glutathione efflux [82]. MRP1 expression in BeWo cells is induced by zearalenone, a nonsteroidal estrogenic mycotoxin [83]. Similar to MRP1, MRP2 is also expressed in the apical membrane of the placental syncytiotrophoblasts [84]. The maternal-to-fetal transfer of talinolol is increased by probenecid, an MRP2 inhibitor, demonstrating functional significance in the human placenta [85]. There is increase in placental MRP2 mRNA and the protein with gestation, indicating developmental control of MRP2 in human placenta [84]. MRP3 is localized in the fetal vessel endothelial cells and at lower concentrations on the apical side of the placental syncytiotrophoblasts [80]. MRP5 is expressed at the basal membrane of syncytiotrophoblasts and endothelial cells of the fetal vessels [86]. MRP5 protein expression decreases with pregnancy, with the highest expression in early preterm placenta [86]. The excretion of potentially toxic anions such as bile acids and biliary pigments is performed by the fetus in conjunction with the placenta and the maternal liver. Bile acids and bilirubin are transferred to the maternal blood by MRP1–3 and BCRP [87]. Under physiological circumstances, fetal bile acids and bilirubin are transported across the placenta and taken up by the maternal liver, which eliminates them via MRP2 and bile salt export pump (BSEP).

24.3.1.2 SLC Transporters. These are uptake proteins present in the placenta and are involved in the transportation of nutrients and proteins toward the developing fetus and of potential toxins and endogenous waste toward the maternal side. The SLC

uptake transporters include OATPs, OATs, OCTs, monocarboxylate and nucleoside transporters.

24.3.1.2.1 OATP. These are bidirectional transporters, which work along the substrate concentration gradient. To transport organic anions, including steroid conjugates, thyroid hormones, prostanoids, digoxin, statins, methotrexate, and antibiotics. OATP1B1, OATP1B2, OATP2B1, OATP3A1, and OATP4A1 are expressed in the human placenta [88–91]. OATP2B1 is abundantly expressed in the placenta and localized in the basolateral membrane of the syncytium [88] and OATP1B3 is located on the apical surface of syncytiotrophoblasts [90].

Repaglinide, a known OATP substrate was used in the *ex vivo* placental perfusion model to evaluate the placental transfer of this drug. Using antipyrine as a reference for passive diffusion, it was observed that the rate of repaglinide transport from the mother to the fetus was low, while this was higher in the fetal-to-maternal direction. This highlighted the importance of active transport by the OATP transporters in fetal exposure to repaglinide [92]. OATPs play a role in bile acid and bilirubin efflux from fetal hepatocytes toward the fetal blood and from the trophoblast to the maternal blood, facilitating the transfer of these potential toxins from the fetus to the maternal circulation [87]. The bile acid transporter, OATP1A2, is localized to the vasculosyncytial membrane and apical surface of syncytiotrophoblasts and OATP1B3, to the vasculosyncytial membrane of the syncytiotrophoblasts. In intrahepatic cholestasis occurring during pregnancy, the expression of both transporters is significantly reduced, relative to the normal placenta [93].

24.3.1.2.2 OAT. OATs are involved in the elimination of a variety of endogenous substances, xenobiotics, and their metabolites from the body and have weak structural similarity to the OCTs. The endogenous substrates of OATs are cyclic nucleotides, prostaglandins, urate, dicarboxylates, and so on, while exogenous substrates are anionic drugs and environmental substances. Most OATs are expressed in the kidney and some are expressed in the liver, brain, and placenta. These transporters are involved in renal secretory pathways for organic anions and in the distribution of organic anions throughout the body. OATs expressed in the human placenta were OAT1/SLC22A6, OAT3/SLC22A8, and OAT4/SLC22A11. OAT1 and 3 are present in syncytial basement membrane and involved in the elimination of toxins and xenobiotics from the fetal circulation, whereas OAT4 is expressed in the cytotrophoblast layer that underlies the syncytium [52].

24.3.1.2.3 OCT. OCTs expressed in human placenta are OCT1, OCT2, OCT3 (SLC22A3), OCTN1 (SLC22A4), and OCTN2 (SLC22A5). OCT1–3 are high capacity nonneuronal monoamine transporters. Their substrates include amiloride and cimetidine. The OCT inhibitor quinidine is reported to inhibit acetylcholine release in human placental tissue [94]. OCTN1 is localized to the brush border membrane of the syncytiotrophoblasts of term placenta, and involved in transport of fluoxetine, citalopram, and verapamil [52]. OCTN2 is a high affinity L-carnitine uptake protein localized on the apical surface of syncytiotrophoblasts of the placenta. It has been reported to be involved in the transport of L-carnitine across the placenta, which is

inhibited by levamisole and progesterone in human trophoblast cells. The transporter is additionally involved in the movement of methamphetamine, quinidine, verapamil, cephaloridine, glibenclamide, and imipramine. Thus, it plays an important role, as some of these drugs have been shown to be toxic for the fetus [52,94].

24.3.1.2.4 Other SLC Transporters. Other SLC transporters expressed in placenta are monoamine transporter (SLC6A), nucleoside transporter (SLC29A), and carboxylate transporter (SLC16). SLC6A transporters that are involved in the transport of serotonin and dopamine are named *serotonin transporters* (SERT/SLC6A4) and *dopamine transporters* (NET/SLC6A2), respectively. They are involved in the transport of amphetamines and cocaine, respectively. SLC29A is located on the brush border membrane of the syncytium and transports purine and pyrimidine across the placenta. It was also found to be involved in the transport of chemotherapeutics, nucleoside analogs, and vasodilator drugs such as dipyridamole and dilazep. SLC16 is an H⁺ cotransporter that is involved in the movement of carboxylates across the syncytium [52].

24.3.2 Transporters in the Fetus

There are relatively limited drug-transporter-based studies on the human fetus. PgP is the most studied transporter, and its distribution in the fetus is represented in Fig. 24.2. Highest expression is in the cardiac muscles and the kidney tubules during the gestation period. It is not expressed in the pneumocyte and bronchiole of the lungs, but the expression is higher in the bronchi during 18 to 20 weeks' gestation. In the adrenal gland, PgP is expressed only in the definitive cortex zone and no expression is reported in the fetal zone and medulla of the adrenal gland [95]. The early fetal brain has low expression of microvessel PgP, rendering the fetus more susceptible to the toxins in maternal circulation. With advancement of pregnancy, there is a significant increase in PgP expression in the fetus and is likely the reason for the observed higher responsiveness of PgP function in primary brain endothelial cells to glucocorticoids [95]. Synthetic glucocorticoids administered to pregnant women at risk of preterm delivery were found to increase the protective capacity of the developing fetal blood–brain barrier, depending on the time of exposure [96]. Immunohistochemical staining was used to visualize the expression and localization of PgP in the developing human fetus during cerebral cortex formation [97]. At 12 weeks of gestation, PgP was diffusely located in the endothelial cells lining the primary cortex microvessels. At 18 weeks, PgP was present in the cortex and subcortical vessels, and at 22 weeks, it was concentrated in the endothelial cell membranes. Radial glial cells and astrocytes did not stain for PgP in any of the samples. There was low expression of PgP in the radial glia at 18 weeks' gestation and at midgestation, where it was observed in the abluminal endothelial cell membrane. These studies demonstrate that PgP is expressed early during human cerebrocortical microvessel development and at midgestation, where its efflux activity is regulated by interactions with the caveolar endothelial cell compartment [97].

MRP1 and BCRP are also expressed in the developing human central nervous system (CNS). While MRP1 is expressed in choroid plexus and ependymal cells, BCRP is most prominently expressed in microvessel endothelial cells of the CNS. Both PgP and MRP1 are also found in large pyramidal neurons. Immunostaining during

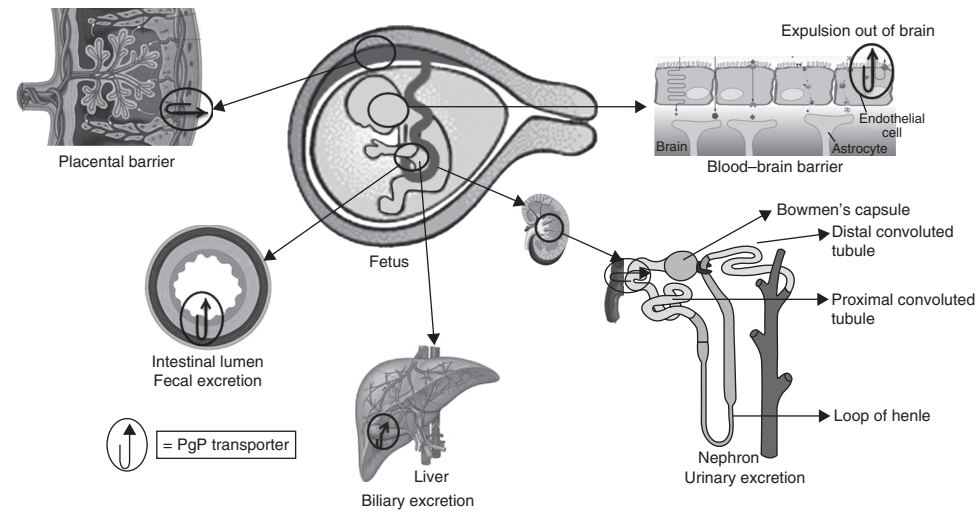


Figure 24.2 Pictorial representation showing distribution of PgP transporter in placenta and organs of the fetus. (See color insert.)

different stages of gestation showed increase in PgP expression during gestational maturation, whereas MRP1 and BCRP expression remained constant [98]. In embryonic and fetal membranes, BCRP expression helped to ensure proper function of the fetoplacental unit [99].

BSEP, MRP2, and MDR3 are the major hepatic canalicular transporters, mediating bile secretion in the fetus [100]. Human liver samples from the fetus at 14–20 weeks of gestational age and adults were tested for mRNA expression of BSEP, MDR3, MRP2, Na⁺-taurocholate cotransporting polypeptide (NTCP), and farnesoid X receptor (FXR) genes. Immunohistochemical staining of BSEP, MDR3, and MRP2 was performed on the fetal and adult livers. All genes tested were expressed at midgestational age. MDR3 and NTCP showed significantly lower levels in fetal liver as compared to the adult. Immunohistochemical staining of MRP2 in the fetal liver was canalicular, BSEP was both intracellular and canalicular, and there was low expression of MDR3, which was found only occasionally along the canaliculi.

In a mouse model, Ntcp and Bsep were the key transporters for hepatic bile acid uptake and excretion. Ntcp and Bsep mRNA levels in the liver were low in the fetus and reached the highest levels at parturition. After birth, mouse Ntcp and Bsep mRNA levels decreased to less than half, before gradually increasing to adult levels by day 30 [101].

24.4 DRUG-METABOLIZING ENZYMES (DMEs)

Owing to changes in the sex hormones progesterone and estrogen in different trimesters of pregnancy, alterations occur in the expression and activity of DMEs in the mother, particularly in the placenta [14,102–106]. Some of the DMEs are typically absent in the fetus and develop either immediately or subsequently after birth.

24.4.1 DME Changes in the Pregnant Mother

The sex hormones are ligands of pregnane X receptor (PXR) and thus significantly influence the expression of CYPs. For example, levels of CYP2A6, CYP2C9, CYP2D6, and CYP3A4 were increased, while expression of CYP1A2 and CYP2C19 was lowered during pregnancy [107]. Also, expression of UGT1A4 and UGT2B7 was enhanced. Such changes in DME expression have the potential to alter the pharmacokinetics of drugs and even carry the propensity to form toxic metabolites, with the possibility to even cause birth defects. For example, a genetic defect in arene oxide detoxification increased the risk of birth defects in case of an epileptic mother who has been treated with phenytoin [108]. A study to assess potential contribution of enzymatic bioactivation of phenytoin to a teratogenic metabolite, using a murine embryo culture model, suggested that the embryo had expression of responsible DMEs [109]. Similarly, a direct relationship between pharmacogenetics and drug-induced birth defects has been proposed for folate metabolism [110]. It has been suggested that enhanced metabolic conversion of valproate to its toxic metabolites (e.g., 2-propyl-4-pentenoic acid) during pregnancy is associated with its teratogenicity at higher doses [111].

24.4.1.1 CYP450. Endogenous sex hormones, namely, progesterone and estrogen, and exogenous substances, such as rifampicin, phenobarbital, and polyaromatic hydrocarbons (PAHs), influence expression and activity of CYPs via influence on nuclear

receptors and/or immunomodulators [112]. Hepatic transcription factors such as aryl hydrocarbon receptor (AhR), PXR, and constitutive androstane receptor (CAR) play a critical role in the upregulation of the expression of hepatic CYPs [113].

Among the CYPs, CYP1A2 activity reduces gradually from early to late pregnancy [107]. The respective reduction was as much as 33%, 48%, and 65% during 14–18, 24–28, and 36–40 weeks of pregnancy relative to the postpartum period [114]. A typical example of the influence of lowering in activity of this enzyme is the decrease in clearance of caffeine that becomes half in the second and third trimesters, with no change during first trimester or after delivery [9]. Similarly, CYP2C9-mediated metabolism increased during pregnancy in humans, while CYP2C19-dependent metabolism was decreased. The clearance of phenytoin, a CYP2C9 substrate, was increased during pregnancy [115], and the dosage of the drug was required to be increased by 85% to maintain therapeutic plasma concentration [116]. In contrast, CYP2C19-mediated metabolism of proguanil to its active metabolite (cycloguanil) decreased during the pregnancy. The proguanil to cycloguanil plasma concentration ratio increased by 63% during second and third trimesters of pregnancy, as compared to postpartum [117]. In a rat study, *cyp2c6* and *cyp2c12* transcription and protein expression were not significantly changed during pregnancy, although intrinsic clearance of *cyp2c6*-mediated diclofenac 4'-hydroxylation was increased twofold on day 19 of gestation, when compared to nonpregnant controls. This was explained through the decrease in K_m for 4'-hydroxydiclofenac formation [118].

The expression of CYP2D6 is predominantly under the genetic control, and thus far, no chemical inducers of this enzyme have been reported. The enzyme's activity fluctuated from preovulatory to luteal phases and subsequently during different terms of pregnancy. CYP2D6 activity was found to be 25% lower during luteal phase, as compared to the preovulatory phase. This was attributed to fluctuations in the progesterone levels [119,120]. This steroid has also been implicated in increased CYP2D6 activity, which goes up by 26%, 35%, and 48% at 14–18, 24–28, and 36–40 weeks of pregnancy, respectively [114]. CYP2D6 mRNA is reported in the fetal liver and term placenta, while protein expression was not found [114,121–125]. In other reports, two classical CYP2D6 probe substrate drugs, dextromethorphan and metoprolol, were used to assess the enzyme's activity. Data showed significant increase in CYP2D6 activity [121,126]. At 26–30 weeks of gestation, metoprolol oral clearance was increased sixfold and its bioavailability decreased to half as compared to postpartum [126]. A significant decrease in the urinary dextromethorphan/dextrorphan metabolic ratio, indicative of an increase in CYP2D6 activity, was also observed during all trimesters of pregnancy [114]. In another study, 53% decrease and 63% increase in the dextromethorphan to dextrorphan plasma concentration ratio was found in extensive metabolizers and poor metabolizers of pregnant mothers, respectively [121].

The expression of CYP2E1 has been shown to be suppressed, particularly during late pregnancy [127]. Koh *et al.* [128] correlated CYP2E1 expression in mouse livers with that of peroxisome proliferator-activated receptor α (PPAR- α). The authors observed potential involvement of PPAR- α in the downregulation of CYP2E1 during pregnancy. CYP2E1 induction/suppression profile in pregnant females who consume alcohol has been correlated with congenital abnormalities including low birth weight, mandibular hyperplasia, and developmental delay [129]. In the rodents, *cyp2e1* expression has been demonstrated to be controlled at three different levels: posttranslationally by

ligand-dependent enzyme stabilization, translationally by stabilization of its RNA, and transcriptionally during ontogenesis [130].

CYP3A4 and CYP3A7 are the major isoforms expressed in adult and fetal livers, respectively. Unadkat *et al.* [113,131–136] studied changes in pharmacokinetics of CYP3A4 substrates (anti-HIV drugs) during pregnancy in different species, including humans, and suggested that decrease in $C_{\max}$ of anti-HIV drugs during pregnancy was due to increase in CYP3A4 expression. On the other hand, the expression of CYP3A7 was downregulated during intrauterine development in the first trimester of pregnancy [137].

24.4.1.2 Uridine 5'-Diphosphoglucuronosyltransferase (UGT). UGT is a phase II enzyme that consists of two major families, UGT1 and UGT2. UGT expression is significantly influenced by changes in female sex hormone levels and by activation of nuclear receptors [14,102]. UGT1A is expressed in the first trimester but not in the term human placenta [138,139]. UGT1A1 is involved in the glucuronidation of bilirubin. During pregnancy, its activity is enhanced by progesterone in a concentration-dependent manner, whereas estrogens have no influence [14]. UGT2B isozyme expression is not influenced by changes in progesterone and estrogen during pregnancy [14].

24.4.1.3 Glutathione S-Transferase (GST). GSTs play a central role in xenobiotic detoxification and have been classified as GST-alpha (GSTA), GST-mu (GSTM), GST-pi (GSTP), and GST-theta (GSTT) subtypes. These enzymes neutralize electrophilic metabolites by conjugating them with glutathione. Overall hepatic GST activity was induced during pregnancy [140].

24.4.1.4 Sulfotransferase (SULT). Sulfation is an important mechanism for regulating biological activity of numerous hormones and neurotransmitters. Tissue-specific regulation of SULT expression was observed during placental development. SULT activity assays and Western blot analysis in human placental cotyledon and membranes (amnion, chorion, and decidua basalis) from 13 to 42 weeks of gestation revealed expression of phenol and catecholamine sulfotransferases at the highest levels. These were generally higher in the villous than membranous tissues [141]. Estrogen sulfotransferase existed at extremely low levels during early pregnancy and gradually increased through midgestation and late gestation in the decidual component of the placenta [141].

24.4.2 Changes in DMEs in the Placenta

The placenta undergoes changes during different stages of gestation and this contributes to a high degree of variability in enzymatic activities and biochemical components. In the human placenta, many changes occur during the course of gestation periods, with variability contributed by factors, such as diet and exposure to environmental chemicals. However, biochemical processes related to the drug metabolism are not extensively studied in placental tissues, as compared to the liver. This is likely due to the strong association between the hemoglobin/methemoglobin and the homogenized placental membranes, which contributes to difficulty in the preparation of placental DMEs [142–144].

The enzymes CYP1A1, CYP1A2, CYP2C, CYP2D6, CYP2E1, CYP2F1, CYP3A4, CYP3A5, and CYP3A7 are expressed at mRNA levels in the first-trimester placenta. CYPs reported in full-term placenta are CYP1A1, CYP2E1, CYP2F1, CYP3A3/4, CYP3A5, and CYP3A7. The expression of these enzymes in placenta was much lower than that in the liver and lungs. The methodologies used for the detection of CYPs in human placenta in the first-trimester and full-term placenta are listed in Table 24.4.

The subtype CYP1A1 was expressed during both first trimester and full term, which was reflected by ethoxyresorufin-O-deethylase (EROD) activity in the human placenta. The enzyme was involved in the metabolism of xenobiotics that are widely used in pharmacotherapy or even present in the diet. It also played a key role in the metabolic activation of procarcinogens, such as arylamines and PAHs, that form adducts with placental and fetal DNA. CYP1A1 expression was transcriptionally upregulated through ligand-activated AhR, which plays an important role in adaptive response to xenobiotics. AhR was activated in the placenta by maternal cigarette smoking, polychlorinated biphenyls and dichlorodiphenyltrichloroethane (DDT) [150–152]. The toxicological consequences of CYP1A1 induction in the placenta of women smokers, have been summarized, which include premature birth, intrauterine growth retardation (IUGR), structural abnormalities, fetal death or placental abruption, risk of low birth weight, low birth length, and low head circumference [153]. The expression of the subtype CYP1A2 is reported in the first-trimester placenta, while it was absent in full-term placenta [123]. Similarly, CYP2C and CYP2D6 showed mRNA expression in first-trimester placenta, while protein expression was not detected at any stage of pregnancy [154].

CYP2E1 was detected at mRNA level in the placenta but was not detected immunohistochemically and by enzyme activity assays. This could be due to lower sensitivity of the assays or there is the possibility that mRNA might not have translated into active protein as both posttranscriptional and posttranslational mechanisms regulated its expression [155]. Placental CYP2E1 was induced on heavy alcohol consumption and is held responsible for susceptibility to alcohol-related birth defects. The proposed reason is the increase in the amount of circulating placental acetaldehyde [156,157]. The expression of CYP2F1 mRNA was established in both first-trimester and full-term placenta using RT-PCR, but protein expression and function values are yet not proven [154].

CYP3A isoforms were expressed in the placental trophoblast cell lines [158]. Although the activity of CYP3A *per se* was not found in the human placenta, the expression of CYP3A4/5 was found in the full-term placenta [123]. In comparison, CYP3A7 was detected in the first- and second-trimester placentas, while it was absent in full-term placenta [159].

24.4.3 Changes in DMEs in the Fetus

A study to assess the effect of smoking and genetic polymorphisms on the fetus in 293 women in Japan revealed that birth weight and length were significantly lower in infants born to heavy smokers with the wild-type *AhR* and the *CYP1A1* variant genotypes (m1/m2 + m2/m2) or the *GSTM1* null genotype [160]. CYP1A2 has been reported to be absent in the human fetal liver, appears gradually after birth, and takes several months to years to reach the adult levels [161]. Another important subtype is CYP2D6. Its mRNA is reported in the fetal liver but no protein expression was found [114,121–125]. The concentration of CYP2D6 is shown to increase a few days after birth [125], but remains low during the first month of life (about 20% of an adult's levels) [162]. Its level in

TABLE 24.4 The Presence of CYPs in Human Placenta during Different Gestational Stages and Methodologies Used for their Detection

CYP	First-Trimester Placenta	Methodology Used for Detection	Full-Term Placenta	Methodology Used for Detection	Metabolic Role
CYP1A1	+	Enzymatic activity determinations [139], immunoblot methods [124], RT-PCR [145], Western blot, slot-blot analyses [146]	+	Enzymatic activity determinations [123], mRNA in northern blot, RT-PCR [123], immunohistochemistry, immunoinhibition assays [123]	Detoxification of aromatic hydrocarbons 146,147
CYP1A2	+	RT-PCR	—	RT-PCR, diagnostic inhibitors [123]	Detoxification of aromatic amines [148]
CYP1B1	+	RT-PCR	+	RT-PCR [123]	Low molecular weight procarcinogens (nitrosamines), organic solvents, and drugs such as paracetamol and chlorzoxazone [149]
CYP2C	+	RT-PCR [124]	—	RT-PCR [123]	
CYP2D6	+	RT-PCR [124]	—	RT-PCR [123]	
CYP2E1	+	RT-PCR [124]	+	RT-PCR [123], western blot	
CYP2F1	+	RT-PCR [124]	+	RT-PCR [123]	
CYP3A4/7	+	RT-PCR, immunoblot methods [123], Western blot,	+	Northern blot, RT-PCR [123], Western blot	

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction.

newborn is low even during lactation, and the same is independent of the functional genotype. Thus, fetuses and neonates have been categorized as poor metabolizers of CYP2D6 substrates (such as dextromethorphan), as the clearance of the latter is low. Therefore, individualization of dosing has been suggested in order to prevent drug accumulation or toxicity of CYP2D6 substrates [163]. The variability of CYP2D6 activities in infants older than 1–2 weeks is found to be similar to the adults, and is also influenced by the genotype [164].

On the other hand, CYP2E1 is not detected in the fetal liver but was expressed in the first few hours following birth, and the same was independent of the gestational age (between 25 and 40 weeks). The steady increase in expression continued during the first year [130]. Similarly, CYP3A4 expression level was initially very low (10 pmol/mg protein), which increased with age [137]. Against it, the expression of CYP3A7 in fetal liver microsomes falls between 311 and 158 pmol/mg protein and showed significant expression till six months of postnatal age [165].

In a report on UGTs [166], UGT1A1 was found to be absent in the prenat and its expression was triggered during birth. It reached adult levels by three to six months of postnatal age. UGT1A3 expression was observed in the fetus and neonate liver, which was one-third of the adult expression. The same conclusion is drawn in other studies where UGT1A subfamily members were found to be absent in the fetus, their expression appeared in neonates and gradually reached adult levels at 10 years of age [167,168]. UGT2B7 is the only UGT2B isoform observed in the liver of fetus aged 15–27 weeks, where its expression was 10–20% of the adults. These levels remained consistent till birth, after which there was a rapid increase in expression that reached adult levels by two to six months of age [169,170].

Expression of GSTs has been found to vary to a large extent during fetal growth. At gestational age of 10 weeks, expression of hepatic GSTA1 and GSTA2 was to the extent of 182.4–247.2 and 14.2–31.2 pmol/mg cytosolic protein, respectively. During the initial two years, the levels of GSTA1 and GSTA2 increased by 1.5- and 4-folds, respectively. Thereafter, they were detected in the liver throughout life. Like GSTA, the GSTM subtype also exhibited very low expression during early stages of life and the expression increased fivefold by adulthood. GSTP1 was detected at the level of 18.0–25.2 pmol/mg cytosol proteins during 10–22 weeks of gestational age and decreased continuously in the subsequent trimester. The expression is not reported in the adult liver. The pattern of fetal kidney expression of GSTP1 before 35 weeks' gestational age was similar to that observed for GSTA1/A2. After 35 weeks of gestation till term, the same was restricted to the collecting tubules and the distal loop of Henle [171–173]. The presence of GST isoforms in the urinary epithelia, digestive tract, and respiratory tract highlighted the importance of GST in detoxification reactions at a very early age, suggesting that an embryo has the capability to defend against toxic compounds to some extent [163].

No activity of SULT1A3 has been reported in adult human liver, although it is expressed at high levels early in the fetal development [174]. The same decreased significantly in the late fetal and early neonatal development. The fetal lung had significant SULT1A3 activity, while neonatal levels were considerably lower. The expression of SULTs in the developing fetus is more widespread than that in the adult. Studies on enzyme activity and protein and mRNA expression of SULT1A, SULT1B, SULT1C, SULT1E, and SULT2A families in a variety of human fetal and adult tissues revealed that SULTs were expressed in the human fetus at levels equivalent to or higher than

the adults [175]. SULT1B1 was expressed at high levels in the fetal small intestine, while SULT1C2 was expressed in fetal liver, kidneys, and small intestine but not in the adult liver or colon [175].

24.5 CLINICAL IMPLICATIONS OF DRUG THERAPY DURING PREGNANCY

As discussed above, pregnancy results in physiological changes, development of the placenta and changes related to the development and protection of the fetus. The placenta plays a significant role in drug metabolism and transport of xenobiotics between maternal and fetal compartments. Apart from genetic factors and maternal infections, it is the unwanted transfer of drugs from the mother to the fetus that can affect the latter through different stages of its growth and lead to drug-related birth defects and teratogenicity. Drugs influence the development of the fetus at three stages of pregnancy: (i) fertilization and implantation (from conception to 17 days), leading to the failure of pregnancy, which is generally unnoticed; (ii) organogenesis (18–55 days), wherein deformities in organs can occur; and (iii) growth and development (56 days onwards), where the end result is developmental and functional abnormalities. Toxicity of some drugs depends on the stage at which these are administered.

Numerous drugs prescribed to pregnant women, such as antihypertensives, antiepileptics, anticancer drugs, and anti-inflammatory agents, have the potential to cause birth defects in the fetus after crossing the placenta. Drug-related birth defects are heart malformations, including holes in the heart chambers; premature birth; persistent pulmonary hypertension of the newborn; brain defects; facial and spinal defects and fetal alcohol syndrome. Table 24.5 summarizes the examples of drugs that can cause teratogenic abnormalities.

24.6 CONCLUSIONS

Pregnancy presents additional challenges for safe and efficacious drug administration to the prospective mother. The physiological changes in the body as a whole, modifications in the drug transporters, alterations in expression of DMEs, and the presence of polymorphic forms make the prediction of pharmacokinetics and pharmacodynamics of any given medication difficult. Added are the ethical concerns with respect to the safety of the fetus, so hurdles exist in testing and following the paths taken by drugs in pregnant women. This is the reason for the relatively limited research in the field. There is a need for improvement in preclinical models for evaluating the contribution of DMEs and transporters early in the drug development. This may help to design and develop safer drugs, with reduced fetal exposure.

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TABLE 24.5 Drugs Administered to Pregnant Mothers (under Various Disease Conditions) during Different Trimesters and Their Teratogenic Effect on Fetuses

Drugs Crossing Placenta	Trimester	Fetal Birth Defect and Toxicity	References
Selective serotonin uptake inhibitors	First trimester	Omphalocele, craniosynostosis, congenital heart defects, laterality defects, conotruncal defects, atrioventricular defects, right ventricular outflow tract obstruction defects, left ventricular outflow tract obstruction defects, septal defects, anomalous pulmonary venous return.	176,177
Female sex hormones	First trimester (taken unknowingly)	Cardiovascular birth defects	178
Lamotrigine	First trimester	Orofacial cleft, ventricular septal defect, bilateral hip dislocation, club feet	179
Folic acid antagonists	Second and third months	Cardiovascular defect	180
Bendectin	First trimester	Isolated cleft palate, cleft lip with or without cleft palate, heart defects	181
Tumor necrosis factor antagonists	NS	Vertebral abnormalities, anal atresia, cardiac defect, tracheoesophageal, renal, and limb abnormalities association	182
Tranquilizers (meprobamate or chlorthalidone)	First 42 d	Teratogenic	183
Cocaine	NS	Embryonic or fetal vascular disruption	184
Aspirin	First trimester	Teratogenic	185
Methamphetamine	First and second trimesters	Teratogenic	186
Acetaminophen	First trimester	Gastroschisis	187
Trimethadione	NS	Malformed ears, cleft palate, cardiac defects, urogenital malformations, and skeletal abnormalities	188
Carbamazepine	NS	Spina bifida	189

Abbreviation: NS, not specified.

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1 When and How to Apply ADMET Principles to Drug Discovery and Development

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1.1	Summary	1
1.2	Introduction: The stages of drug discovery	2
1.3	Target space defines the broad properties of the drug	4
1.4	ADMET space limits the possibilities of success for an oral drug	4
1.5	Properties needed in an oral drug	6
1.6	Hierarchical and horizontal <i>in vitro</i> screening sequences	9
1.7	Too early or too late	12
1.8	Integration of <i>in vivo</i> and <i>in vitro</i> data	15
1.9	When all else fail—use and abuse of prodrugs	16
1.10	Development: Human metabolism the pivotal study	18
1.11	Summary and future perspective	19
	References	19

1.1 SUMMARY

The oral delivery of small drug molecules is still the favored target of most drug discovery programs. Medicinal chemistry practices are skilled in the introduction of potency and selectivity against a desired target; however, an oral drug has to combine solubility and permeability, and rates of elimination low enough to sustain therapeutic concentrations. To achieve this screening cascades need to be introduced to select molecules with the right pharmacokinetic properties and to guide structure–activity relationships (SAR). Although there appears considerable freedom to target the right properties, many of the pharmacokinetic properties are dictated by the characteristics of the target and its active/binding site. Open solvent exposed binding sites often lead to larger drug molecules with high polar surface areas (PSAs), whereas smaller hydrophilic pockets lead to smaller molecules with lower PSA. This dictates the intrinsic permeability of a molecule that determines the fate of its overall disposition. Highly permeable molecules are usually cleared exclusively by metabolism, whereas complex

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processes involving metabolism and transporters clear less permeable molecules. With these molecules it is difficult to unravel the rate-limiting step.

Designing the screening cascade correctly is vital since information may not be helpful or may be misleading at the wrong stage. Moreover, even the basic absorption, distributions, metabolism and toxicity (ADMET) screens for solubility, permeability, and metabolic vulnerability can give results compromised by factors such as cosolvents (DMSO), the presence of transporters or membrane binding. Other systems, such as induction screening, may not provide information that can be put into context until the potency and likely dose of the candidate molecules can be estimated. As such these screens probably should be confined to the final phases of the discovery program. For instance, plasma protein binding information is only valuable to determine free fraction in *in vivo* experiments and thereby calculate unbound drug concentrations. Unbound drug concentrations allow understanding of the relationship between *in vitro* pharmacology measurements and *in vivo* activity. They also allow calculation of intrinsic clearance, which relates directly to *in vitro* clearance experiments such as microsomal metabolism rate. Protein binding does not determine *in vivo* unbound drug concentration, which is determined by intrinsic clearance. Attempts to modify drug series to reduce protein binding to achieve better efficacy are scientifically incorrect and may lead discovery programs down false alleys.

1.2 INTRODUCTION: THE STAGES OF DRUG DISCOVERY

Around 90% of newly approved drugs are small molecules. Within small molecules the oral route is the most favored with nearly 90% of drugs administered this way. Other routes include intravenous (5%) inhaled and transdermal. The oral route is considered the optimum for small molecule delivery as it allows a wide range of doses to be administered, allows convenient patient self-administration, is adaptable to varying dosage regimens, allows convenient pack size and needs no special equipment. To provide an oral drug the molecule must undergo dissolution in the gastrointestinal tract (GIT), absorption across the GIT, and penetration to the target, if it is intracellular or in the central nervous system (CNS). Moreover, the drug has to be present in the circulation and body for sufficient time and at concentration which activate or inhibit the pharmacological target, to provide a convenient dosage interval.

Whether we will see a change to new modalities in the next decade, in 2010 drug discovery and development programs were still mainly directed toward oral drugs. The methods used to discover drugs have gradually changed to what can be termed *strictly rationale led*. In these programs, a drug is developed against a known target, and usually the drug is optimized to only have affinity and selectivity toward this target. Many technologies including genomics, molecular biology methods of protein expression, microplate high throughput screening technology (HTS), X-ray crystallography have helped facilitate this direction.

A simple scheme for the stages of drug discovery and development is illustrated below:

Stage I: Target Identification. The protein of interest becomes apparent in the disease process. The identification may be triggered by genomics and proteomics. For instance, a group may be particularly resistant or sensitive to a disease and share a common genetic mutation, or a protein may be consistently overexpressed

or lacking in a disease. Both clearly are connected but the technology used for identification varies. Validation of the target is also important not only in terms of efficacy, but also safety. This will be discussed in more detail in Section 1.6.1.

Stage II: Series/lead identification comprising HTS leading to groups of compounds often termed *HITS*. These are compounds active in the HTS screen but need to be validated in terms of more rigorous pharmacology, structural inspection, purity, and so on. Some of the *HITS* may be deemed leads and trigger a chemical series. Although used in different ways, the term lead and lead series usually describes the compounds validated from *HITS* and seen as having sufficient chemical attractiveness to be predicted as capable of being modified into a potential drug. Lead series may also be assembled from fragment-based approaches (which assemble the lead from fragments shown to interact with the target) followed by chemistry follow up in literally joining these molecules together. This approach uses early examination of the modes and forms of interaction of the molecule with the target such as X-ray crystallography.

Stage III: Compound identification comprising series/lead development generating SAR, leading to compounds with most of the desirable properties. This is the time-consuming process of turning a potential lead, which may have low affinity for the target, low selectivity against some other proteins, and pharmacokinetic issues such as high metabolic instability. The process culminates in the production or nomination of a development candidate (and back up selection). Often, all the problems identified in the lead series are not completely resolved, and most drug candidates and even drugs are a compromise rather than a “perfect candidate.”

Stage IV: Candidate Development. The drug candidate is profiled in human volunteers (phase 1) for tolerance and pharmacokinetics (adequate oral drug properties to activate/inhibit the pharmacological target with an acceptable dosage regimen). The drug is then administered in increasing numbers (phases 2 and 3) to patients to demonstrate safety and efficacy. Each stage is supported by preclinical toxicology, pharmacy (formulations), and so on.

Following Stage III, the properties of a molecule are fixed, and while ADMET work is an essential part of drug development, it cannot improve the intrinsic properties of a molecule. It does of course optimize the use of the drug in patients in terms of efficacy and side effects. Dosage regimens can be optimized for patients particularly where comeds are likely to be administered and ADMET preclinical work in combination with clinical pharmacokinetic studies is pivotal.

The statement concerning most drugs being a compromise is largely due to two factors.

1. Humans (and animals) have evolved to have multiple defense mechanisms against foreign molecules. Like many biological systems, there is considerable overlap and compensation. Moreover, these systems are usually promiscuous and able to accept a wide range of chemical forms and structures.
2. The drug targets chosen dictate to a considerable degree the nature of the chemical matter that interacts with them. Thus the medicinal chemist is constrained in his choices as to what molecules he can synthesize and what properties he wants in those molecules.

Even before a single molecule is synthesized or screened certain ADMET properties are therefore defined. The nature of the target largely determines the type of chemical matter and its complexity.

1.3 TARGET SPACE DEFINES THE BROAD PROPERTIES OF THE DRUG

Targets for drug discovery programs such as aminergic GPCRs, some ion channels, and certain enzymes such as cyclooxygenase and acetylcholinesterase have small cavity volumes (less than 1000 Å³ buried within the protein target) and a large hydrophobic surface (representing 30% or greater of the total area). Polar ionic interactions are restricted and confined to this buried active site. Lead series, development candidates and drugs against these targets are small with moderate or low PSA and lipophilicities. These properties are conducive to discovering oral drugs. Some targets (usually lipid or lipid acid enzymes and carriers) require high lipophilicities to interact with the target. For instance, cholesterol ester transfer protein was an attractive target based on initial genomic data. Cholesterol esters have extremely high lipophilicities and it is not surprising, therefore, that inhibitors of the protein are all of very high lipophilicity. The development of these compounds has been hampered by poor solubility, inadequate dissolution, and the need for complex formulations. For some the natural substrate is a protein a considerable portion of which interacts with the active site. Aspartyl proteases, the family of which includes HIV protease, rennin and β-secretase targets for AIDS, hypertension and Alzheimer's respectively, have open binding cavities of greater dimensions, a smaller proportional hydrophobic surface and the need for more polar ionic interactions. Lead series, development candidates and drugs against these targets are large (MW >500) with high PSAs and lipophilicities. The consequences of these properties are explained below.

1.4 ADMET SPACE LIMITS THE POSSIBILITIES OF SUCCESS FOR AN ORAL DRUG

Membrane permeability is pivotal to ADMET properties defining absorption, rapid equilibrium between tissues, cell interiors and the circulating unbound drug in plasma, and the route of drug clearance and elimination. Drugs of high membrane permeability are not cleared renally because of tubular absorption. Permeable drugs rapidly establish equilibrium across membranes. This means an absence of influence by active transport processes since the passive flux is faster than any active transport flux. Such drugs are normally cleared by metabolism, whether this is by default, or that the groupings and structure giving good permeability are also amenable to metabolism by enzymes such as CYP450s is an introduction into how metabolism may be attenuated but is unlikely to be abolished. The influence of permeability on clearance is shown in Table 1.1. Drugs with lower membrane permeability may have their disposition influenced by transporters, both efflux and influx. The rate of transport flux now is (much) greater than the intrinsic passive flux so these molecules move in a largely unidirectional way across membranes with the requisite transporters present. Efflux transporters (e.g., Pgp) are particularly important in the GIT and the blood brain barrier. These transporters also play a role in active renal excretion and biliary excretion. The effects in the GIT

TABLE 1.1 Classification of the Disposition Fate of a Compound Based on Its Permeability Across a Biological Membrane

Permeability	Low	Medium	High
PSA/Log <i>P</i> Absorption	High Low (e.g., aliskeran) unless MW is less than 250 Da and absorbed by paracellular route (e.g., atenolol)	Medium Variable Influenced by permeability and transporters (e.g., nelfinavir)	Low High via transcellular route (e.g., propranolol)
Bioavailability	As for absorption	As for absorption and metabolism	Variable. Influenced by metabolism
Clearance	Renal or biliary (possible transporter involvement)	Transporters and metabolism	Metabolism

Permeability of a biomembrane is favored by lipophilicity and attenuated by polar functionality (PSA). Cells have tight junctions, which act as pores. The pore size is large in most capillaries allowing polar drugs access to extracellular receptors such as GPCRs. Pores are small in the capillaries of the blood brain barrier and moderate in the gastrointestinal tract.

are somewhat attenuated by the effects of saturation of the transporter by the high concentrations of drug present after the dissolution process. Most calculations suggest that the volume of gastrointestinal fluid in the small intestine is around 250 mL in which the entire administered dose can dissolve. In contrast, saturation of those present in the blood brain barrier is unlikely to occur at normal efficacious concentrations. Thus, drugs of lower membrane permeability may have reasonable oral absorption and bioavailability but not penetrate the CNS to a marked degree.

Membrane permeability depends on the drug being able to cross the lipoidal core of the membrane and is dependant therefore on the drug having affinity for lipid (lipophilicity). Lipophilicity (log *P* or log *D*) can be measured or calculated and can be conceived as being composed of two components that of molecular weight and polar surface area (PSA). Transfer through a membrane involves the complete removal of the molecule from water. Lipophilic groups have a positive solvation free energy and are readily soluble in the hydrocarbon core of a membrane. In contrast, the desolvation of polar groups is unfavorable and the groupings do not readily dissolve or penetrate the hydrocarbon core of the membrane. PSA is recognized as one of the most important measurements incorporating both structure and the ability of a molecule to form hydrogen bonds with solvent. A cutoff value for poorly permeable and (therefore, poorly orally absorbed) compounds has been set at >140–150 Å². Expressing the above concept as a virtual formula log *P* equates to molecular weight—PSA. This virtual formula defines ADMET space as a conceptual boundary that encloses the properties most likely to be associated with successful drugs. The dimensions of such a space are illustrated in Fig. 1.1. The formula explains the interconnectivity of the physicochemical properties. If we consider absorption, lipophilicity will always give high permeability across the membranes but, at its upper limits, solubility is so low that adequate dissolution is not achieved at clinical doses. The interconnection with molecular weight has spawned a belief that this characteristic is important per se.

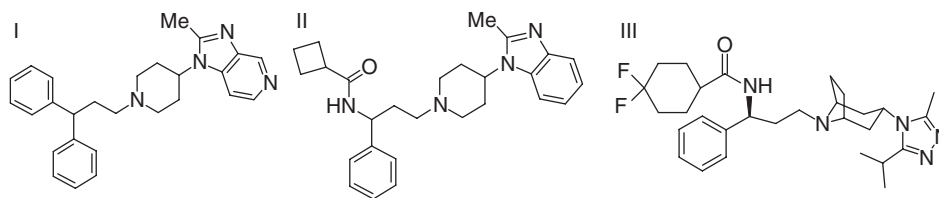


Figure 1.1 Structures of I the initial HIT/lead compound in the CCR5 project leading to the discovery of maraviroc and illustrating the unhindered pyridine nitrogen leading to potent CYP450 inhibition and its replacement with benzimidazole II. The other structural changes produced a compound antivirally *active in vitro*. Changes to decrease lipophilicity used the triazole group and resulted eventually in maraviroc III.

Most likely, it is the fact that drug molecules are made from carbon (lipophilicity), oxygen, and nitrogen (PSA) and that as molecular weight approaches 500, the chances of too high a lipophilicity or too great a PSA increase so that a molecule does not dissolve in the aqueous media of the GIT or the molecule has low permeability to membranes.

When a lead is being advanced it is usual for the lipophilicity of the newly synthesized analogs to show an increase in lipophilicity particularly as the medicinal chemist tries to increase the potency of a molecule to low nanomolar from often low micromolar. The loss of solvent is a major contributor to the binding free energy. Lipophilic groups have a positive solvation free energy; this, together with favorable direct van der Waals interactions, make lipophilic interactions a key force in drug binding. In contrast, the desolvation of polar groups is unfavorable even though hydrogen bonds between the substrate and enzyme or receptor, are favorable. Overall, the contribution of polar contacts to the binding free energy, is likely to be variable owing to the balance of desolvation and actual binding. Lipophilic groups often, therefore, guarantee potency increases usually to the extent of 0.7 kcal/mol and compounds become increasingly lipophilic to achieve drug like (low nanomolar) potencies.

1.5 PROPERTIES NEEDED IN AN ORAL DRUG

1.5.1 Solubility

Material in the early phases of drug discovery is amorphous. Moreover, most screening systems are run with the compound handling systems using DMSO stock solutions. Thus, the usual measurement is kinetic solubility in which a (DMSO) stock solution of compound is added to aqueous solvent and the solubility rapidly determined. This method has inaccuracies due to the presence of DMSO and the rapid nonequilibrium conditions [1]. Crystallizing a compound (by definition) produces a less soluble form rendering the kinetic solubility measurements redundant and possibly misleading. More accurate measures are provided by thermodynamic solubility, which refers to the measurement of solubility by addition of the solid material, to aqueous media under conditions of equilibrium and requiring a long incubation time [1]. Such solubility methods are not adaptable to high speed screening and material handling systems. The dilemma then is how useful early measurements of kinetic solubility are on the

amorphous material and how timely are tedious thermodynamic measures of crystalline material. A final problem is the appearance of new polymorphs of the crystal, which can emerge anytime in the discovery, development, and even product cycle.

1.5.2 Permeability

During the discovery phase most screening systems rely on Caco-2 cells, MDCK cells, or artificial membranes. The cell lines provide a more realistic model of membrane systems, but usually have the assessment complicated by the presence of transporters systems. Thus the output is often not true passive flux (for poorly permeable compounds) but a composite of permeability and transport. The transporters are saturable rendering the choice of concentrations critical in making intercompound comparisons. It is noticeable also that interlaboratory variation is very high [2] making the data very difficult to apply across compounds sets from different laboratories.

1.5.3 Metabolic Stability

The rate of metabolism of compounds can be measured using liver microsomes, human hepatocytes, or recombinant enzymes. Microsomes offer a robust oxidative metabolism screen when fortified with NADPH regenerating systems. Consideration needs to be given to microsomal binding, particularly with lipophilic basic molecules and for comparative data a correction is needed for this [3]. Owing to the large variation in CYP450 enzyme content screening usually uses microsomes prepared from a pool (5–10 individuals) of livers.

Hepatocytes provide the full complement of oxidative and conjugation metabolism. Cryopreserved hepatocytes are normally utilized in compound screening, but caution should be exercised in comparing pathways as the freeze thawing process can lead to loss of cofactors. While microsomal binding is relatively easy to predict or measure, hepatocyte binding is much more difficult to estimate, and to scale, into an intrinsic clearance prediction.

Apart from obvious differences, in the metabolic pathways supported, microsomes and hepatocytes may give conflicting results for compounds of low cell permeability, where the rate of metabolism in microsomes is attenuated by the slow rate of flux across the hepatocyte cell membrane [4]. Much of the work supporting the use of *in vitro* systems such as microsomes to predict *in vivo* clearance used series largely populated with permeable compounds.

1.5.4 Drug–Drug Interactions

The effect of the emerging candidate drugs on existing concomitant therapy (drug–drug interactions, DDIs) can be examined by the use of fluorogenic substrates with recombinant enzymes, particularly CYP450s or human liver microsomes and selective substrates [5]. Often the screens are run in two phases with most compounds synthesized submitted to a high throughput screen using recombinant enzymes with relatively nonselective fluorescent probe substrates (the selectivity being gained by use of the individual isoforms). These screens tend to use a single substrate concentration (around 20–50 μM) and look for percentage of inhibition of the reaction against control. Incubations are conducted with and without preincubation

to look for competitive and time-dependent “mechanism-based” irreversible or quasi irreversible inhibitors. A second phase assay will often be used to examine compounds of high interest. This assay uses human liver microsomes and selective drug substrates. CYPs screened are generally 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4. The selective substrates for these are universally recognized and are often phenacetin (1A2), diclofenac (2C9), *S*-mephenytoin (2C19), bufuralol (2D6), chlorzoxazone (2E1), and testosterone (3A4). These screens are now firmly established as selectivity screens.

1.5.5 Induction

Induction screening can use reporter gene assays, cultured human hepatocytes, tissue slices, or immortalized cells such as HepaRG [6,7]. Reporter gene assays offer the closest to a general screening system but may give false negatives due to the absence of interplay with other receptors involved in the induction process. This criticism also applies to cell lines. Hepatocytes, while a comprehensive system, are variable from donor to donor. The concern around induction varies greatly from company to company and is often related to intracompany experiences and choice of therapeutic area. Clinical induction is actually quite rare and is seen almost exclusively (not absolutely) with high dose drugs. The high doses of these drugs are necessary because of weak affinity for their primary pharmacology target (antiepileptics such as phenytoin and carbamazepine, 30–50 μM against their Na^+ channel target and around 10 μM against the PXR receptor mostly involved in CYP3A4 induction) or the need to maintain plasma concentrations above the IC^{90} (anti-infectives such as nonnucleoside reverse transferase inhibitors for HIV). The only compound identified with high potency is hepatorin (low nanomolar) which cannot really be termed *drug like*.

1.5.6 Plasma Protein Binding

Methods to determine protein binding and free drug largely rely on the separation of the unbound drug from the bound drug by dialysis, filtration, or centrifugation or a combination of these methods. During development, traditionally the drug was radiolabeled to make the assay simpler. The methods work well for moderately bound drugs but loose accuracy when drugs are more than 95% bound. Much of the accuracy concerns the variable nonspecific binding to the apparatus used. The accuracy was also confounded, traditionally, by the purity of radiolabeled compound available. Thus, many drugs are categorized as greater than 99% bound. This applies typically to acidic drugs such as nonsteroidal anti-inflammatory agents.

BIACORE SPR is a biosensor-based technique using surface plasmon resonance (SPR) technology and can be used to determine the kinetics of protein–ligand interactions including albumin and other plasma proteins [8]. This technique gives actual affinity data rather than just percentage unbound. From affinity the fraction unbound can of course be calculated. For instance, warfarin binds to albumin (c99% bound). Using BIACORE technology, it was shown that at high concentrations warfarin bound at more than one site on human serum albumin. The kinetics of the high affinity site were $K_d = 3.7 \pm 1.2 \mu M$ and a dissociation rate constant of $1.2 s^{-1}$. From the K_d value, binding of 99.4% was calculated.

1.6 HIERARCHICAL AND HORIZONTAL *IN VITRO* SCREENING SEQUENCES

Two process models of screening cover most approaches to drug discovery screening. The hierarchical model places the various screens in a sequence such that only compounds possessing certain criteria move to the next phase. For instance, a newly synthesized compound or series of compounds could be screened for target binding, close neighbors to target binding (selectivity), functional activity (particularly if an agonist), and then for solubility, permeability, metabolic stability, and DDIs. Appropriate compounds may be further progressed into *in vivo* screens for pharmacology and pharmacokinetics. In the hierarchical model, therefore, ADMET data is collected only on compounds with adequate potency and selectivity, and even the collection of this can be staged.

In the horizontal model, ADMET data is collected on all compounds synthesized in a single screening phase incorporating *in vitro* potency and selectivity and the suite of ADMET screens. The horizontal model may be considered optimum for looking for exceptions to druglike property rules and can build SAR streams rapidly so potentially allowing real time SAR of the type normally reserved for retrospective analysis.

These two models indicate a divergence of how the data is handled. The hierarchical model means that full data is available on a few compounds, can be manipulated on a spreadsheet and can be interpreted easily by a medicinal chemist. Certain screens can be moved up in priority as the project SAR reveal key flaws and problems. The horizontal model could result in more than 5000 data points to collate as SAR. This immediately requires computational systems and complex analysis to process and optimize. Moreover, the cost of providing this number of data points makes screening a very expensive process.

1.6.1 When are ADMET Properties Critical in Influencing Compound Progression

At any stage, ADMET arguments could be advanced for the progression or cancellation of a project. At target identification the structure of the protein, close family members, nature of the possible drug binding site can provide information on whether the target is druggable. Targets such as aspartic acid proteases may be considered undruggable based on the information, but while being difficult have yielded drugs for hypertension (aliskerin, renin inhibitor [9]), and HIV (various [10]). β -Secretase as a target for alzheimers [11] is a possible higher hurdle since the target of interest is now located in the CNS and drugs will have to cross the blood brain barrier. Perhaps, rather than discard targets on ADMET space grounds, appropriate pathways to a drug should be identified at this stage such as a biological or small chemical approach after the key points of

1. Pivotal point in pathway of disease effects or symptoms.
2. Effects of inhibition or activation of target not likely to be attenuated by competing pathways.
3. Likely overall beneficial effect influenced by interaction with drug molecule in most (all) patients.

4. Sufficient diversity for selection against other receptors including subtypes.

Target identification and selection (1–4) are exemplified in the postgenomic era by CCR5 and its role in AIDS [12,13]. A deletion in the CCR5 gene called *CCR5* Δ 32, results in a nonfunctional CCR5 protein that is not expressed on cells. People with two copies of the *CCR5* Δ 32 gene are resistant to HIV infection. The CCR5 protein allows a pathway for virus to enter the cell and a rational is therefore to produce an antagonist to mimic deletion of the gene. Encouragement for possible success with a small molecule is provided by CCR5 being a cell surface receptor of the family of nonaminergic GPCRs (G-protein-coupled receptors) which have been successful targets in a number of drug discovery programs. The genomic information also allows an indication of likely safety outcomes in this approach since those carrying the *CCR5* Δ 32 gene are otherwise healthy. This target has produced a recent drug maraviroc (III in Fig. 1.1) in the fight against AIDS. Points 3 and 4 are listed above, unfortunately are often only realized late in the discovery/development/product cycle. For instance, alosetron [14] was a selective 5-HT₃ antagonist. The rationale for the drug was the role 5-HT₃ antagonists have in suppressing the reflex colonic motor function in response to food ingestion. This reflex in diarrhea-predominant irritable bowel syndrome (IBS) is exaggerated resulting in cramping and diarrhea. The drug's side effects were noted as constipation. Wider use in patients after marketing produced several cases of ischemic colitis. Constipation can facilitate the development of ischemic colitis by increased colonic intraluminal pressure compressing the mucosal vessels and impeding circulation. Cilansetron, another 5-HT₃ antagonist, shows identical rates of ischemic colitis indicating that the effects are target related and not drug molecule specific. Although withdrawn, alosetron has been reinstated using very strict labeling and prescriber/patient practices. The antiobesity agents [14] were withdrawn for heart defects. Fenfluramine and dexfenfluramine bind weekly to 5-HT receptors and the major *N*-deethylated metabolite norfenfluramine is responsible for the pharmacological activity. The drugs reduce appetite by activating 5-HT_{2C} receptors via the metabolite. The metabolite is not selective and also activates the 5-HT_{2B} receptors on heart valves and pulmonary artery interstitial cells causing the formation of proliferative fibromyxoid plaques. While antagonists of subtypes of receptors are “routinely” possible there are considerably more demands to obtain selective agonists and the lack of a selective 5-HT_{2C} receptor agonist for obesity treatment may testify to this.

When a number of targets are being prioritized then it seems appropriate to consider the possibility of the binding site yielding molecules fitting within the confines of ADMET space.

Series/lead identification comprising HTS or fragment-based approaches followed by chemistry follow up is the first opportunity to gain actual experimental data, rather than theoretical. Considerable debate occurs as to how useful ADMET data is at this stage rather than physicochemical properties. The principal concern is that the molecular template, while giving potency and selectivity is flawed from an ADMET viewpoint and despite manipulation of the structure will not give favorable oral drug properties. Should lead molecules, therefore, be screened for ADMET properties to exclude them as leads or merely to show areas of improvement. Should the areas of improvement be prioritized early on

TABLE 1.2 Comparison of the Hydrophilic Drug Atenolol (log *D* 7.4–1.7) and the Lipophilic Drug Propranolol (log *D* 7.4–1.2) Illustrating the Need to Compare All Aspects of Screening Data to Choose Compounds

	Affinity pA ₂	Renal CL _i (u) (ml/min/kg)	Metabolic CL _i (u) (ml/min/kg)	Vd(u) (L/kg)	Half-life (h)	Daily dose (mg)
Atenolol	6.5	2	—	1	3–5	50–100
Propranolol	8.3	—	470	50	3–5	30–90

or should selectivity and potency be paramount until reasonable improvements in this area are seen.

- Compound identification comprising series/lead development followed by development candidate selection. At this stage, the use of ADMET data is unarguable. Most compounds will be close to the required potency and selectivity and usually one or two ADMET properties are being optimized. Potency and selectivity values should not be divorced from ADMET values and probably each compound should be evaluated in a horizontal manner obtaining full data. This is exemplified in Table 1.2 where two very successful β -adrenoceptor antagonists, with very different physicochemical, biological, and clearance properties, are compared. Owing to the compensatory nature of some of the processes the dose and dosing frequency of both drugs are very similar.

1.6.2 Is Drug Discovery a Linear or Nonlinear Process?

It is impossible to change one aspect of a compound's properties and not affect other properties. This interdependence of processes for the delivery and performance of an oral drug is the reason that drug discovery takes a long and somewhat circuitous and meandering route to the goal of a drug candidate. While many of the changes turn out to be deleterious, there is a counterside. Occasionally, this process works for the drug discoverer and a single change can turn an unproductive series to one that produces a drug candidate. The discoveries of amlodipine and fluconazole [15] contained a single chemical change or concept that led to very successful drugs. Unlike most other dihydropyridines calcium channel antagonists amlodipine has a basic primary amine side chain. Differentiating fluconazole was the introduction of a second triazole. Much of the thinking was to mainly to address solubility issues in the lead compounds nifedipine and ketoconazole. These were intuitive steps, based on the knowledge of the physicochemical nature of the new group and some freedom to retain activity by substitution in the selected region. These groupings dramatically enhanced the volume of distribution for amlodipine against other dihydropyridines, leading to a much longer half-life suitable for once a day administration. The bis-triazole series, of which fluconazole is member, had greatly improved solubility and at the lipophilicity of fluconazole (Log *P* 0.6) very little metabolism. Clearance was by glomerular filtration, with about 60–70% tubular reabsorption by passive reabsorption, again leading to a long half-life due to the low clearance value. However, there is an important corollary to this in that the lead compounds and lead series always were reasonably placed in ADMET versus target space.

Marviroc's discovery followed a more linear approach as the drug's discoverers worked through balancing permeability for oral absorption, rate of metabolism, and IKR

channel activity. The lead series and eventual drug are close to the edges of ADMET space versus target space. Screening in terms of permeability and metabolism was provided on almost all compound synthesized once sufficient potency had been attained in the lead series to see actual antiviral activity against the HIV virus (preclinical proof of concept).

The dilemma of all discovery projects is that the future is not predictable and the question is how far should progress be made before termination of a compound series or even a project. *N*-(1-(3,5-Dichlorobenzenesulfonyl)-2S-methyl-azetidine-2-carbonyl)-L-4-(2',6'-dimethoxyphenyl)phenylalanine was optimized to be a potent inhibitor of the (VLA) antigen-4 [16]. In terms of ADMET, space versus target space the properties dictated were not promising and the compound has physicochemical properties consistent with poor ADMET characteristics: molecular weight 607, PSA 131, log *P* 5.6. The compound was rapidly cleared in rat with values consistent with very high extraction by the liver (clearance equal to hepatic blood). In this particular case, further experimentation continued to show that the clearance of the compound was a result of the multiple processes of metabolism by oxidation and glucuronidation and biliary excretion of the parent molecule. The major contributor was the active hepatic uptake of parent compound (the compound was shown to be a substrate of the rat organic anion transporter, Oatp1b2) and metabolism did not influence clearance but determined excreted fate. The lead series was abandoned after these experiments.

1.7 TOO EARLY OR TOO LATE

The dilemma is how easy is it to remove a particular ADMET liability of a compound late on in the discovery process. In general, most discovery programs of major pharmaceutical companies have a screening cascade incorporating ADMET data into the earliest stage of compound synthesis and these screens tend to be comprehensive and the data arrives in a hierarchical manner. Even in this situation, a question of weighting needs to be applied.

1.7.1 Induction Screening

Induction screening is of low value until some understanding of the likely potency and concentration response of the project compounds is established. Many compounds including the reference standards such as rifampicin, show effects at concentrations approaching or exceeding $1\text{ }\mu\text{M}$ in *in vitro* systems. A detailed comparison of *in vitro* screening data and clinical results confirms that EC_{50} or maximum responses to an agent are of little value in determining which compounds will cause induction in the clinic. Only when *in vivo* exposure in humans can be determined (C_{max} or AUC plasma concentration data) can the compounds be correctly ranked. For instance, verapamil, which is not a clinical inducer, has an EC_{50} of $3.2\text{ }\mu\text{M}$ while phenytoin and carbamazepine, which are clinical inducers, have EC_{50} values of 8 and $15.6\text{ }\mu\text{M}$, respectively. The difference between the compounds is due to the clinical dose and resultant plasma concentrations which for the anticonvulsants are some two orders of magnitude greater than the calcium channel blocker. The structures of clinical P450 inducers of CYP3A4 show no obvious SAR and even detailed analysis suggests that the only common feature is some degree of lipophilicity. This is perhaps to be expected

from what is essentially a weak interaction between the compounds and the receptor [6,7]. In the absence of any other guidance, it is suggested that any induction screening is postponed if target potencies are below 50 nM. Because of the promiscuity of the receptors and the higher doses used in preclinical safety assessment, induction is a common finding and should not be viewed as a failure in screening. Moreover, owing to differences in receptors animal findings are not extrapolatable to the clinic.

1.7.2 Inhibition of CYP450 Enzymes

Similar comments can be made about the value of the data until an *understanding* of the likely potency and concentration response of the project compounds is established. However, unlike CYP3A4 induction, some structural features are associated with highly potent CYP inhibitors, both reversible and to a lesser extent irreversible. The presence of unhindered nitrogen in a saturated ring system (pyridine, imidazole, triazole) may result in the lone pair of the nitrogen being able to form a ligand interaction with the heme of the CYP450. Many of the potent CYP450 inhibitors bind in this manner and the interaction adds 6 kcal to the binding energy. This interaction is the basis for the action of azole antifungals and a number of aromatase inhibitors. As this interaction is commonplace and invariably leads to highly potent inhibitors such functionality is best avoided from the outset. In the case of the discovery of maraviroc, the original HTS hit and lead compound possessed an imidazopyridine [12] leading to very potent CYP450 inhibition. Replacement of the functionality occurred early on with benzimidazole (Fig. 1.1) and eventually a substituted triazole.

Mechanism-based time-dependent inhibitors form complexes with the heme moiety of CYP450 or bind irreversibly to the protein. Tertiary amines and their metabolism products classically are heme interactants, while irreversible protein binding inhibitors (suicide substrates) are classically formed by conversion to highly reactive short-lived metabolites.

Imidazole ring systems are also prevalent in mechanism-based time-dependent CYP450 inhibitors, although the relationships are more complex and are not accompanied by a mechanistic understanding [15]. Sufficient information is available to allow *in silico* filtering of compound structures to determine possible avenues which may lead to this problem [17].

1.7.3 Solubility, Permeability, and Metabolic Stability

Although a molecule has different properties, the three key factors that characterize an oral drug are intertwined by the physicochemical properties that define them. Increasing lipophilicity [which will often provide potency increases (see above)] will usually increase permeability and also increase the rate of metabolism while lowering solubility. This balancing act is a pivotal part of SAR development of a project and starts at the time of the lead series identification. Maraviroc [12] exemplifies the route from a lipophilic metabolically unstable lead to molecules with groupings such as triazole incorporated to counter the high rate of metabolism. The lower lipophilicities gave better metabolic stability but demonstrated low rates of flux in Caco-2 cells and resultant poor oral absorption. The eventual and successful development candidate maraviroc was a compromise of these properties but had sufficiently good pharmacokinetics to promise an IC_{90} on a reasonable oral drug regimen (300 mg od or bd).

As a general rule, lead compounds show rapid metabolism and hence high clearance is a widespread problem. The above example used the lowering of overall lipophilicity as a method to get stability; the other common tactic is the removal of vulnerable functionality or the introduction of blocking groups, particularly halogens, after identifying the sites of metabolism. This tactic forms an almost classical screening synthesis cycle, with potency, selectivity, flux, and overall metabolic rate being provided alongside and site of metabolism. This cycle is possible now because of the development of mass spectrometry technology. The cycle can be extended to use computational prediction of likely metabolites and then monitoring of these in high throughput manner [18]. The software, in this case MetaSite, is only 55% predictive of the compounds primary site of metabolism. However, by analyzing in addition 2nd and 3rd order rank predictions, the combination of *in silico* selection of likely metabolites and then monitoring these *in vitro* the method provides a favorable screening performance. The data on site of metabolism can usually not be provided on the same number of compounds. Typically, labile sites are explored alongside other sites in the generation of SAR. Intrinsic clearance data are calculated by screening compounds in human liver microsomes. This process is shown in the work on corticotropin-releasing factor-1 (CRF1) receptor antagonists [19]. The major sites of metabolism in the initial lead compound (potency 0.26 nM) were O-demethylation of both the methoxy groups (Fig. 1.2). These two positions were found to be the major sites of metabolism in all compounds generated and intrinsic clearance was influenced mainly by substitution at these sites. The 1-(1-cyclopropyl)ethyl group was selected as a stable (but less potent) alternative offering scope for potency improvements. The other labile *O*-methoxy position showed lower intrinsic clearance when replaced with groups such as trifluoromethoxy, trifluoromethyl, difluoromethoxy, chloro, cyano, and ethoxy. One of the difluoromethoxy substituted compounds combined potency ($IC_{50} = 1$ nM), functional antagonism, selectivity, and metabolic stability (Fig. 1.2).

1.7.4 Plasma Protein Binding

There is no common consensus and there are many misconceptions around protein binding and unbound drug. This arises because in a static *in vitro* test, the addition of

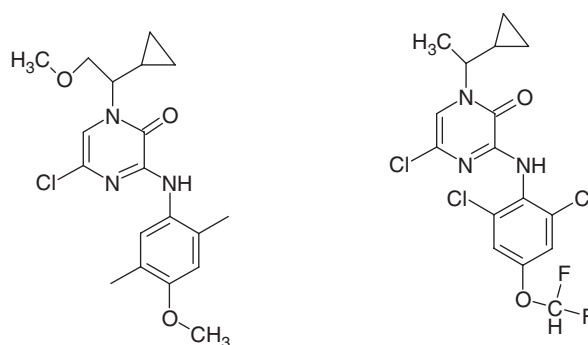


Figure 1.2 Corticotropin-releasing factor-1 (CRF1) receptor antagonists. The major sites of metabolism in the initial lead compound were O-demethylation of both the methoxy groups. These positions were stabilized by incorporations of the groupings shown.

albumin of plasma will often decrease the apparent potency of a drug. In this case, the change in free fraction directly relates to the concentration of free (unbound) drug. Because the addition of serum or plasma proteins is common in many assays, a biased view of compound affinity and potency may arise. Moreover, the static *in vitro* situation is then directly extrapolated to the dynamic *in vivo* situation and a belief formed that protein binding will similarly attenuate the effects of the drug. These beliefs drive a discovery project to attempt to lower plasma protein binding and this in turn requires plasma protein binding data to be available alongside data on potency, metabolic stability, permeability, and so on. If we concentrate on oral drugs, dosed to steady state unlike the static *in vitro* system, free concentration *in vivo* is not dependent on free fraction but on intrinsic clearance. The simple analogy is that if plasma protein was added to a clearance assay, and the drug is plasma protein bound, then there will be a lower rate of drug disappearance directly proportional to the reduction in potency. When the drug binds to proteins in a reversible manner the aqueous concentration of drug will be lowered by the amount bound. This will of course reflect the concentration of drug interacting at the receptor, but it will also lower the amount of drug interacting with the clearance enzymes: fewer drugs will be transformed per unit time. Thus, reversible interactions with proteins (or the phospholipids of membranes) will proportionately give lower drug concentrations than no interactions, but those concentrations will be sustained for proportionately longer. In pharmacokinetic terms over a defined period (the dosing interval) reversible interactions with proteins will lower maximum concentrations ($C_{\max}$) or aqueous drug but raise minimum concentrations ($C_{\min}$) to the exact extent that the average concentration (C_{av}) will be unchanged. Because the enzyme kinetics are essentially unchanged, providing first order is observed, daily dose is unchanged. Changing protein binding will not usually be reflected in changes in daily dose size to achieve the same average free drug plasma concentrations (although frequency of administration could be affected) [20]. Essentially, when all the processes of drug metabolism are modeled then $\text{AUC}_u = F_{\text{abs}} \cdot F_{\text{gut}} \cdot \text{Dose}/\text{CL}_{\text{int}}$, where AUC_u is the unbound drug area under the plasma concentration curve, F_{abs} is the fraction absorbed, F_{gut} is the fraction metabolized by passage through the gut wall (gut first pass) and CL_{int} is the intrinsic clearance. In the discovery setting, the latter is routinely assessed during metabolic stability screening (see above). Note that the fraction unbound is not present in the equation. It needs to be determined, however, at the stage when *in vivo* experiments are performed to complete the right-hand side. AUC_u is easily converted to C_{av} (average plasma concentration over specified time) by dividing AUC_u by time. C_{av} is a more useful parameter as it allows direct comparison with *in vitro* potency measurements [21].

1.8 INTEGRATION OF *IN VIVO* AND *IN VITRO* DATA

While *in vitro* data provides most of the ADMET data to a discovery project *in vivo* data is vital to ensure that what is being generated *in vitro* is consistent with what is observed *in vivo*. A major concern is around clearance processes. Table 1.1 indicates that highly permeable compounds are cleared metabolically and microsome data can be reliably applied. Exceptions to this include compounds with functionality likely to be metabolized by systems not present in microsomes such as aldehyde oxidase. This is one of several enzymes besides cytochrome P450 involved in the

oxidative metabolism of drugs [22]. Aldehyde oxidase [23] catalyzes the oxidation of aldehydes to carboxylic acids. Most importantly for drug discovery it catalyzes the oxidation of carbon–nitrogen double bonds present in aromatic azaheterocyclic compounds. Reliance on microsomal systems may give false indications of stability since the enzyme is soluble, and not bound to the endoplasmic reticulum, and thus appears in the supernatant. Aldehyde oxidase activities in dog are unusually low and strain dependent in rats so *in vivo* experiments may also be misleading.

Similarly, microsomes are normally fortified for oxidative metabolism only and direct conjugation of a substrate will not be observed. This suggests that for any compounds containing functionality likely to be labile to conjugation enzymes (hydroxyl, phenols, etc.) hepatocyte screening should also take place.

For less permeable compounds, the possibility of transporter involvement in clearance becomes likely. This interplay unless understood may lead to false assumptions about project priorities. For instance, the compound *N*-(1-(3,5-dichlorobenzenesulfonyl)-2*S*-methyl-azetidine-2-carbonyl)-L-4-(2',6'-dimethoxyphenyl)phenylalanine referred to above [16] was metabolized by oxidation and glucuronidation but when examined *in vivo* the major contributor to clearance was the active hepatic uptake of parent compound by a liver transport system. Reliance on microsomal or hepatocyte data may have led the project to attempt to stabilize the metabolic lability of the compound. The fate of the series, however, was largely determined by the physicochemical properties and their effects on permeability and transporter affinity.

1.9 WHEN ALL ELSE FAIL—USE AND ABUSE OF PRODRUGS

Often in medicinal chemistry programs, a stage is reached where many criteria for a molecule have been met, but certain elusive properties are unobtainable in the current series. Rather than search for an alternative series, attempts are made to produce a labile chemical derivative that yields the active drug at efficacious concentrations. These labile forms are called *prodrugs* [24] and have been attempted in a variety of forms to address solubility, permeability, clearance, and selectivity issues. Prodrug moieties have been added to molecules to

1. increase water solubility to aid dissolution, or to allow parental formulation;
2. increase permeability to aid absorption;
3. improve systemic pharmacokinetics by decreasing maximum concentrations and increasing drug half-life;
4. improve therapeutic index by targeting a drug to a tissue or cell type.

Actual examples of prodrugs successfully designed, developed, and marketed are largely limited to the first two categories.

The actual form of the prodrug varies. Testa [25] defined the chemical criteria that may be used to classify prodrugs:

- Bioprecursors, which do not contain a promoiety, yet are activated by oxidation, reduction, or hydrolysis.
- Carrier-linked prodrugs, where the drug is linked to a carrier and activation occurs by hydrolysis, oxidation, or reduction.

Macromolecular prodrugs, where the carrier is a macromolecule such as poly-ethyleneglycol.

Drug–antibody conjugates, where the carrier is an antibody, for instance, one with affinity for a particular tumor cell.

A number of drugs are prodrugs and termed *bioprecursors* but are not actually created by design including omeprazole, simvastatin, lovastatin, and clopidogrel. Many of these types of drug were discovered and developed without knowledge of the bioprecursor role. Clopidogrel's mechanism of action, as an example was not discovered until 2000 although US approval by the FDA was granted in 1997. The bioprecursor role of clopidogrel is the generation of its 2-oxo metabolite a metabolite, which hydrolyzes to a reactive thiol that can bind covalently to platelet ADP receptors and inhibit platelet aggregation [24].

To increase water solubility addition of water soluble functionality by pegylation and phosphate esters has been used. A recently marketed example of phosphate ester prodrugging is Fosfluconazole, a prodrug of fluconazole, an azole antifungal agent. The hydrolysis of fosfluconazole to fluconazole is catalyzed by alkaline phosphatases, which reside in most tissues especially the kidney and liver. Fosfluconazole is over 40 times more soluble than fluconazole, allowing it to be administered by bolus injection. The water solubility increase is due to a combination of intrinsic hydrophilicity and ionization [24].

The vast majority of successful prodrugs increase membrane permeability to allow oral absorption. These drugs conform to the ADMET space requirements in terms of polar surface area but are not lipophilic enough.

Prodrugging and acid function does little to mask polar functionality (same for alcohol groups) since the esterification of a carboxylic acid reduces PSA by only 11 Å². Thus, potential prodruggable molecules need to conform to PSA requirements prior to prodrugging. While this may be critical in some cases, this is a relatively small reduction and not what is envisaged in the scope of “masking” of polar groups. Major effect of esterification is to render the ionizable carboxylic acid neutral. The overall effect on lipophilicity is a combination of the loss of ionization and the intrinsic lipophilicity of the promoiety. For an acid of pK_a 3.4, approximately 4 log units of lipophilicity will be added to the log *D*_{7.4}. As a guide, therefore, the potential candidate needs a PSA less than 150 Å² to render a possible successful campaign [24].

Targeting a drug to a particular protein usually involves synthesizing a peptide or antibody delivery conjugate. These can show success *in vitro*, but usually fail *in vivo* for a number of reasons. Critical to success is the interplay of rates including [24]:

1. clearance of the drug conjugate from the systemic circulation;
2. rate of uptake by target;
3. rotency of drug;
4. release of drug in or at the target;
5. disappearance from target of the drug;
6. systemic clearance of the drug

Each of these factors is critical and needs to be precisely defined. For instance, for 2 and 3 many attempted delivery systems rely on an antibody conjugate and internalization. The antibody will often have subnanomolar affinity, whereas the drug may only

have micromolar activity against the target. This type of approach places unrealistic demands on the other factors even allowing that the antibody may be able to deliver more than one molecule of drug [24].

1.10 DEVELOPMENT: HUMAN METABOLISM THE PIVOTAL STUDY

Once a candidate is submitted for development a well-documented series of studies are conducted linking preclinical and clinical studies together. A pivotal study that causes much debate is the human metabolism study. This normally uses a radiolabeled version of the candidate drug. The emphasis of this study has changed with time but can be defined as a part of understanding the PK/PD (pharmacokinetics/pharmacodynamics) of the drug. It can be broken down into two elements: circulating and excreted metabolites. Smith and Obach [26] have suggested the primary purpose of these:

Circulating metabolites are of interest primarily because they can directly and probably reversibly interact with macromolecules, particularly proteins and cause a change in conformation and function of the protein to elicit a biological effect (beneficial or hazardous). These effects can be similar and additive to the parent molecule or may in some cases be different. Identifying and analyzing these metabolites in the same matrix as the parent allows concentrations to be measured and thereby assessment of PK/PD.

Excreted metabolites are of interest primarily, in human, because they allow the proportion of the parent converted to a particular metabolite to be determined and thereby support the *in vitro* enzymological evaluations for population variations and drug–drug interactions. In addition, they allow the detection of the downstream products of reactive metabolites and, moreover, allow an estimation of the amount (mass) formed. From a technical viewpoint, excreted metabolites often allow an easier workup of sample (than circulating metabolites) for detailed structural analysis and in some cases even yield sufficient sample for biological testing.

The species used in preclinical safety studies are also studied in this manner to allow comparisons with the human data. A variety of standards have been suggested for the comparison but clearly a metabolite present in much higher concentrations than seen in preclinical testing raises concerns and needs careful and thoughtful consideration. To obviate a late stage problem, there is considerable benefit in conducting the human metabolism study early; however, sufficient information needs to be available to make this expensive study pivotal. The most important factor is the clinical dose range, until this is known metabolism studies may be of a limited value. This information begins to emerge at the end of phase 2A or “proof of concept” studies, and it may be that the most opportune time is following these studies in parallel with phase 2B clinical studies. Various designs are employed but the following considerations need to be incorporated:

1. Polymorphically expressed enzymes: does the design need to incorporate representative variants in the volunteer population.
2. Single or multiple doses: can single dose results be extrapolated or does the design need to incorporate a steady state regime. An example would be a clinical enzyme inducer which could modify its own metabolism.

3. Unlabeled fragments of the molecule: depending on the site of label novel chemical matter may be liberated as unlabeled material. Should cold assay technology be used?
4. Does the radiolabel become incorporated into intermediary metabolism, does this render the use of a label unethical.

1.11 SUMMARY AND FUTURE PERSPECTIVE

The component parts of ADMET screening and their relative importance will continue to be discussed and debated in the foreseeable future. Most of the screening systems are now relatively generally established, but as this chapter tries to emphasize may be applied with the wrong emphasis or timing. There are considerable gaps in the systems to understand fully drug transport, something becoming more important as target and ADMET space ensure less permeable compounds. A part of the problem is the promiscuous SAR of the proteins, the difficulty of establishing how much of the protein is present, and the complexity of transport kinetics [27].

A second factor is the outsourcing and packaging of drug screens. As cost of research becomes an issue it is possible to design compounds in one place, synthesize them in another and screen them in a third. These places may represent different countries and even continents. The concept of a drug discovery team with representatives of the various disciplines intimately supervising the practical aspects becomes lost. Screening becomes a checklist, rather than a bespoke science and the danger of inappropriate data is maximized. Project objectives need to be agreed and careful examination of the appropriate screens and screening sequence conducted. Data from screens becomes available at different times and it is possible that the most rapid screens may disproportionately influence a project rather than the most important screens.

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2 Molecular Challenges in Front-Loading Toxicity Testing of Anticancer Drugs in Drug Discovery

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2.1	Introduction	1
2.2	Cytotoxics	2
2.3	Targeted therapies	2
2.4	Nonclinical studies: the shifting paradigm for toxicity testing	3
2.5	Early toxicology for discovery support: how much applies to anticancer drugs	4
2.6	Newer molecular targets and cancer therapeutics	6
2.7	Toxicities of targeted therapeutics	7
2.8	Toxicities emerging in clinical trials or post marketing	8
2.9	Immunomodulatory therapeutics	9
2.10	Molecular challenges in screening targeted therapies	10
2.11	Signaling pathways in cancer related to toxicities of therapeutics	10
2.12	Other toxicities	13
2.13	Do current preclinical studies predict human adverse events from molecular targeted therapies?	15
2.14	Exploratory and phase 0 trials in cancer patients	16
2.15	Conclusion and future perspectives	17
	References	18

2.1 INTRODUCTION

The oncology market is the fastest growing in the pharmaceutical industry, yet oncology still has one of the poorest records for success in clinical development, partly due to the less than optimal correlation between nonclinical models and the disease in humans [1]. Growth of the market is being fueled by the magnitude of the disease globally; currently, 25 million people are afflicted with cancer worldwide. The World Health Organization (WHO) predicts that cancer rates may increase by 50% from 2000 to

2015. The Association for International Cancer Research (AICR) estimates that by 2020, there will be 10 million deaths from cancer per year. Even in developed nations, ~50% of patients diagnosed with cancer will eventually die of this disease. In the United States, cancer is the second leading cause of death by disease, one of every four deaths—and is exceeded only by heart disease. Approximately 570,000 Americans are expected to die of cancer each year, which equates to more than 1500 people a day. Nearly 1.4 million new cancer cases are diagnosed annually.

2.2 CYTOTOXICS

For several years, drug discovery efforts have focused on cytotoxics, compounds that block essential cellular functions and kill dividing cells [1,2]. These agents have included those designed to interfere with precisely defined physiological processes and those with pleiotrophic mechanisms. Examples of mechanisms in the first category include metabolite synthesis (e.g., methotrexate), microtubule polymerization (e.g., Taxol), and chromosome topology (e.g., irinotecan). Pleiotrophic molecules include those considered to be DNA-modifying (e.g., cisplatin). The therapeutic margin of these compounds is typically assessed as the differential between on-target effects in cancer cells and on-mechanism effects in normal cells. Dose response and distribution within tumors and normal tissues result in estimates of the therapeutic window (margin between efficacy and toxicity), and these estimates are studied in animal models and early clinical trials. Another class of anticancer drugs includes antihormonal agents such as estrogen receptor modulators (e.g., tamoxifen) and aromatase inhibitors (e.g., letrozole) [1].

2.3 TARGETED THERAPIES

There is a rapidly growing pipeline of targeted therapeutics designed to modulate single or multiple targets known to be important in cancer [2]. Accordingly, there has been a trend to move away from molecules with broad cytotoxic activity to more molecular targeted therapies [3,4], particularly in small-molecule research and development. Therapeutics of all types are now being developed in a targeted fashion, keying in specific molecular targets and specific signaling transduction pathways [2,3]. It is possible that broader therapeutic windows may be seen with these types of agents, particularly, if the specificity in targeting is limited to families of proteins prominent in tumors versus normal cells.

Biotherapeutics, another category of targeted anticancer drugs, represent a rapidly growing therapeutic modality. Biotherapeutics or biologicals are drug products where the active substance is produced or extracted from a biological source. These include recombinant hormones and proteins, monoclonal antibodies (mAbs), cytokines, growth factors, gene therapy products, vaccines, cell-based products, gene silencing therapies, tissue-engineered products, and stem cell therapies. A high proportion of biotherapeutic molecules under development and targeted for cancer therapy are mAbs, with most possessing IgG1 Fc designs with immunomodulatory function [5].

For targeted therapies including biotherapeutics, all effects attributed to the pharmacological mechanism, whether positive or negative, are considered to be related to

(i) binding or modulating a target (including unintended targets), (ii) the downstream effects from the modulation, (iii) targeting biological or signal transduction pathways, (iv) localized immune responses based on the design of the molecule, and (v) mechanistically based effects such as exaggerated pharmacology at the intended site of action or another tissue not intended as the primary site of action. Differences in molecules and effects also relate to the extent of target-related and nontarget elimination, receptor synthesis and turnover, molecule–receptor binding affinity, and molecule–receptor complex turnover [5].

Regardless of the type of therapeutic, the classic estimate of safety or therapeutic window in nonclinical mouse xenograft efficacy models, the ratio of tumor volumes in treated and control animals with the estimate of toxicity defined as *percent loss in body weight* (<10%) has carried on in the drug discovery field, even with newer targeted therapeutics [6]. Although this estimate has been used for decades, it fails to distinguish between on- and off-target effects or to differentiate between mechanism- and chemistry-based toxicities.

2.4 NONCLINICAL STUDIES: THE SHIFTING PARADIGM FOR TOXICITY TESTING

Nonclinical studies for anticancer drugs, which cover *in vitro* and *in vivo* studies, are designed to identify and quantify the pharmacological properties of the therapeutic, to understand the toxicological properties of the therapeutic including target organ(s) and dose (exposure)–response relationships, and to establish safe initial dose levels for the first exposures in humans [5]. First-in-human (FIH) guidance based on preclinical markers of safety and efficacy has been issued by the EMEA (European Medicines Agency) [7,8] and US Food and Drug Administration (US FDA) [9], covering both small molecules and biotherapeutics, and a new International Conference of Harmonization (ICH) guideline on nonclinical evaluation for anticancer therapies is pending [10]. These guidance documents can be interpreted as “limiting” the amount and extent of toxicology testing required to enter the first clinical trials, with the underlying thought that the best information for developing an anticancer therapeutic will come from experience in cancer patients. This is exemplified in the current concepts for FIH dose selection for biotherapeutics, which have evolved into a target-mechanism-based model (TMBM) utilizing exposure–response relationships from both *in vitro* and *in vivo* studies including (i) minimally effective exposure concentrations for biological activity (e.g., ED₁₀), (ii) pharmacologically active levels (e.g., ED₅₀) particularly important for FIH cancer trials, (iii) no observable adverse effect level (NOAEL) and/or toxicodynamic endpoint estimates from toxicology studies, and (iv) exposure- or concentration-related biomarker changes [5]. Ideally, these parameters are measured in the nonclinical studies, but in some cases, they are predicted via modeling or allometric extrapolation, such as in the case for estimated human doses and/or exposures. Dose escalation to a maximum tolerated dose (MTD) is a goal of phase 1 trials in order to select an optimal biological dose for phase 2, and most researchers are in agreement that nonclinical studies should be conducted, which allow such dose escalation to occur as rapidly as possible. In cancer patients, starting at a suboptimal or potentially subtherapeutic dose is not generally viewed as being acceptable

because there is an inherent desire to give each patient a potential therapeutic dose level [5].

2.5 EARLY TOXICOLOGY FOR DISCOVERY SUPPORT: HOW MUCH APPLIES TO ANTICANCER DRUGS

Several schemes for incorporating toxicity testing in the discovery process have been proposed with the objective of identifying potential side effects in humans and in animals used for pivotal toxicology assessments for regulatory purposes. Lead compounds or development candidates that pass through these “filters” increase the probability of success for a molecule. Screens, which include both *in vitro* and *in vivo* studies, are designed to be related to chemical reactivity or metabolic instability, unintended off-target effects possibly related to the unanticipated interaction with other targets or receptors, certain physicochemical properties of the parent compound and/or metabolites, and those properties that are related to the intended therapeutic mechanism of action, where unanticipated biological consequences or interaction with an unanticipated target takes place [11]. For the most part these are focused on understanding chemically related toxicity, where a correlation between chemical structure and potential toxicity is assessed [12]. Molecular mechanisms and the consequences of biological target modulation are typically reserved for efficacy and potency screening, particularly to attempt to understand the kinetics and dynamics of the compound at the target site [13]. Compounds or classes of compounds that are known to confer toxicity in a specific tissue will typically lead to an *in vitro* assay with results correlated with potential findings in either animal or human *in vivo* assessments. With a defined quantifiable endpoint and structural similarities in libraries, detailed quantitative structure–activity relationship (QSAR) modeling can be used as a predictive process for both efficacy and toxicity [11]. Therefore, early toxicity screening typically involves *in silico*, *in vitro*, and/or *in vivo* analyses and studies based on known toxicities that have either occurred in animals or resulted in slowing down or eliminating projects, where toxicities have occurred in clinical trials or where toxicities seen in patients after a drug reaches the market. The primary concern with screening assays is that they must be predictive, and the predictive endpoint must be human toxicity. If the goal of any program is to predict toxicity in rodents because rodents will be used in pivotal toxicology studies, then the toxicity induced in rodents must be relevant and predictive of the same effect in humans. Great effort is made in early screening on rank ordering compounds to filter out “bad actors” and increase the probability of selecting molecular structures that eliminate certain unwanted attributes. For the most part, these assays are done in human cell-based systems, which maintain genotype and phenotype [14], and even then there are several examples where the relevance of screening cannot be determined. This includes assays designed to understand the chemical interactions with the constitutive androstane receptor (CAR) and peroxisome-proliferator-activated receptor (PPAR) nuclear receptor families [15]. In addition, the difficulties of modeling and predicting toxicity from immunomodulatory drugs are well established [14,16]. One of the primary challenges in this field is that assays meant to be predictive are developed retrospectively based on experience, and as such, they tend to validate the previous finding and/or may be used to define a mechanism. Questions still remain as to the quality of some cell-based assays used prospectively for predicting potential toxicities with previously untested compounds.

2.5.1 Screening for Liver Toxicity

Hepatotoxicity is a major cause of drug failures both pre- and postapproval; however, the fact that several mechanisms can be involved and some liver toxicities are idiosyncratic (rare in occurrence) makes this a difficult task for accurate screening [11]. There are several systems in current use including primary cell cultures, immortalized cell lines, liver slices, and whole perfused livers [17,18]. The two most widely used methods use primary cells from freshly isolated hepatocytes from humans and animals used in preclinical assessments, and immortalized cells that over- or under-express certain phase I CYP450 and conjugating enzymes allowing the assessment of maximal production of reactive intermediates. Although not as widely used, differences in metabolism may be assessed when polymorphisms affect metabolic rates in certain individuals [18]. Cytotoxic screening with newer technologies to assess multiple parameters such as nuclear morphology, cell proliferation, plasma membrane integrity, and mitochondrial function in a high content screening format are also being used [19]. An advantage of this type of screen is the ability to couple with assays measuring mechanistic endpoints such as oxidative stress, DNA damage, apoptosis, and cell cycle inhibition, again in a high content mode via multiplexed platforms [19]. It should be pointed out that these types of assays work the best when used to rank order compounds rather than as a predictive assay for an individual compound. Computational models for liver toxicity have also been developed [20,21]; however, there remains difficulty when several different mechanisms of liver toxicity are represented by compounds used to train computational models. Using cell-based assays in a predictive mode, prospectively, still carries a level of uncertainty because of differences seen between species and individual differences noted in freshly collected hepatocytes [18,19]. Array “omics” technologies have been utilized in virtually all these assay formats but have had limited success when used prospectively [14]. These technologies may be of greater utility in defining specific mechanisms rather than as predictive assays. An important limitation is the number of cells needed for assays and the fact that results are based on average changes from several cells [22]. Important work by Cohen and colleagues [23] noted differences in protein response of human cancer cells to drugs using central dogma (CD) tagging in human H1299 lung carcinoma cells and time-lapse fluorescence microscopy. These changes involved rapid translocation of proteins specific to the mechanism of the drug and slower, wide-ranging temporal waves of protein degradation and accumulation. They provide an understanding of how cells that seem identical show different responses to drugs [22,23]. Technologies that measure responses in individual cells rather than averages of several cells (with cell to cell variations) may offer a promising way to understand both efficacy and toxicity in the same assay.

2.5.2 Screening for Cardiotoxicity

Cardiotoxicity in humans is another type of toxicity seen with several classes of compounds including targeted therapeutics. Screening for QTc prolongation has been established for several years, and multiple screening approaches are being required and employed in most major industrial companies globally. Cardiotoxicities from targeted therapies (other than QTc prolongation) are more difficult to predict in preclinical studies, as these types of effects lead to defects in left ventricular function and congestive heart failure. Several screening approaches have been used in an attempt to

prospectively predict cardiotoxicity using *in vitro* models, which include cardiomyocyte cell lines or primary cells [24]. Primary cells are typically ventricular myocytes from neonatal or adult rats. The merits and limitations of these two approaches have been discussed and it has been concluded that cell lines are inferior to primary cells because the mitochondrial electron transport system is the dominant source of energy in cardiomyocytes, whereas cell lines rely on glucose for energy generation [24]. These systems are sensitive and may produce a high degree of false-positive results and will not detect toxicity from direct effects on the vasculature [24]. Several *in vivo* models have also been used, including echocardiography in mice, pharmacologic stressor probes in rodents, catheterization for contractile dysfunction, and transmission electron microscopy in rodents. Prospective use of these techniques as an effective predictive assay has not been reported to any extent [24].

Early screening programs are usually company specific based on toxicities seen in internal pipelines but tend to include structure–activity relationship (SAR) (alerts) by computational tools [11], identification of reactive moieties in chemical structures using both computational and capture assays [20], and screening in model systems based on suspected target organs as noted above. Early *in vivo* studies are typically designed to explore linearity of dose versus exposure, which may include assessing different vehicles and their effect on bioavailability, rising-dose studies to explore dose versus overt toxicity, and preliminary studies (7–10 days) to establish high and low dose levels for definitive toxicology studies [12].

2.6 NEWER MOLECULAR TARGETS AND CANCER THERAPEUTICS

New targets are continually being discovered by molecular genetic assessments of cancer biology. Molecular targeted therapies are composed of novel therapeutic agents that target biologically important processes central to the development and progression of cancer and generally fall into the following categories [25]:

- *Single-Target Signal Transduction Inhibitors:* These include inhibitors of any single target (primarily) such as EGFR (epidermal growth factor receptor), mTOR (mammalian target of rapamycin), PKC (protein kinase C), Bcr/Abl [e.g., imatinib (Gleevec; Novartis), trastuzumab (Herceptin; Genentech/Roche), and panitumumab (Vectibix; Amgen)]. Imatinib is generally considered to be a single-target agent but it also inhibits PDGFR (platelet-derived growth factor receptor) α/β and KIT to some degree.
- *Multitargeted Inhibitors:* These include any agent with more than one therapeutic target [e.g., sorafenib (Nexavar; Onyx), sunitinib (Sutent; Pfizer), dasatinib (Sprycel; BMS), and lapatinib (Tykerb; GSK)].
- *Angiogenesis Inhibitors:* These include inhibitors of VEGF/VEGFR (vascular endothelial growth factor/VEGF receptor), uPA, and integrin families [e.g., bevacizumab (Avastin; Genentech/Roche)].
- *Epigenetic Modulators:* These include the histone deacetylase (HDAC) inhibitors [e.g., vorinostat (Zolinza; Merck)].
- *Cell Cycle and Apoptosis Targeted Agents:* These include modulators of CDK, Bcl-2, TRAIL, and Chk proteins [e.g., bortezomib (Velcade; Millennium)].

- *Immunomodulatory and Immunoconjugated Therapeutics*: These include the cancer immunotherapies and radioimmunotherapies [e.g., rituximab (Rituxan; BiogenIdec/Genentech/Roche) and gemtuzumab ozogamicin (Mylotarg; Wyeth/Pfizer)].

Agents that target (i) cell division by newer mechanisms, such as the aurora kinase and cyclin-dependent kinase inhibitors, (ii) protein turnover (e.g., bortezomib), or (iii) chromatin modification (e.g., HDAC inhibitors) have been referred to as *neo-cytotoxics* [1].

A market report on molecular targeted therapies by Datamonitor in October 2007 [25] identified 329 different targeted therapeutics in the developmental pipeline. Of these, 44 (13%) were classified as angiogenesis inhibitors, 45 (14%) were single-target signal transduction inhibitors, 65 (20%) were multitargeted inhibitors, 95 (29%) were cell cycle or apoptosis targeted agents, 64 (19%) were immunomodulatory or immunoconjugated therapeutics, and 16 (5%) were epigenetic modulators. Datamonitor forecast these targeted therapy candidates to realize an aggregate seven major markets' sales potential of \$6.03 billion by 2016. A report by Bioseeker in October 2009 [26] identified 409 protein kinase drugs (990 projects) either ongoing or recently stopped within the portfolio of 160 companies or investigators. The data included 133 identified targets, 200 drug target profiles, and 57 different cancer indications.

2.7 TOXICITIES OF TARGETED THERAPEUTICS

The increased interest in discovering and developing molecular targeted therapeutics has changed the focus of toxicities in patients away from traditional cytotoxic side effects seen with chemotherapeutics, which potentially changes the types of early toxicity screening programs that must be instituted in drug discovery and early development. It should be noted, however, that several targeted therapies do get approved as combinations with cytotoxic therapies. Early discovery-based screening in oncology drug discovery concentrates on chemical-based toxicity, where modifications of the structure of potential candidate drugs are accomplished during lead optimization, and mechanism-based toxicity, where a specific toxicity or tissue has been identified as being an issue to overcome during the lead discovery and development process. Various tiered approaches have been developed in industry, which include SAR structural alerts for toxicity (including genotoxicity), identification of reactive moieties in proposed chemical structures, and primary and secondary screening in model systems [11,12,17]. These systems have, among other endpoints, included novel approaches utilizing cytotoxicity as the defining type of toxic event [17,19,20].

Since many toxicities associated with molecular targeted therapies are associated with the actual mechanism for pharmacological targeting, these agents require a detailed understanding of the molecular aspects of the target, affected signaling pathways, and potential or unsuspected off-target toxicities that may be encountered. This raises the question of whether traditional toxicity screening programs and studies can or should be front loaded into discovery development programs of individual targeted therapies without a more complete understanding of how to monitor mechanistic toxicities where specific biomarkers of affected pathways may be needed. Realistically, the issue here is the lack of a clear understanding of the potential mechanisms and putative events

that trigger toxicities with molecular targeted therapies and the lack of defined and validated biomarkers. Biomarkers are defined as *objectively measured characteristics* that are useful as indicators of normal and/or pathological biological processes, or pharmacological responses to a therapeutic intervention. Biomarkers theoretically can help to determine whether the molecule reaches and modulates the desired target, whether it affects biological activity as intended, and whether this may lead to a desired or, in some cases, an undesired outcome [5]. Examples of the difficulties with mechanism-based biomarkers used for safety endpoints are seen with biomarkers of mTOR inhibition: phosphorylation of 4E-BP1 and S6K1. These biomarkers have been used effectively in animal studies and clinical trials showing dose-related modulation of downstream events from mTOR. However, adverse events in humans cannot be correlated *per se* with these mechanistic markers. Toxicities that occur in greater than 50% of patients receiving mTOR inhibitors (e.g., rapamycin) include mucositis, stomatitis, cold sores, pneumonitis, hypersensitivity reactions, and skin rashes. The mechanisms for these types of reactions have not been determined, although all are usually managed adequately. Hyperglycemia can be associated with a disruption of insulin signaling [27], which is seen more commonly with inhibitors of targets upstream of mTOR, such as PI3K.

2.8 TOXICITIES EMERGING IN CLINICAL TRIALS OR POST MARKETING

Human adverse events from therapeutics are typically discovered during clinical trials or after market approval where exposure to the drug is expanded dramatically. One important type of analysis focuses on toxicities that have resulted in market withdrawal of the drug. Several authors have surveyed drug withdrawals and offered detailed analyses of potential mechanisms of individual drugs and the factors leading to human toxicity [28,29]. In these reports the reasons for market withdrawal have been classified as (A) based on the primary pharmacology (A1) of the drug and its intended target or (A2) secondary pharmacology due to nonselective activity against nonintended targets; (B) based on an immunological mechanism, which includes several idiosyncratic toxicities; (C) based on a chemical reaction with tissue macromolecules with a rapid ensuing response, typically occurring via bioactivation to a reactive chemical moiety; and (D) delayed responses from mechanisms similar to (B) and (C) such as carcinogenesis and teratogenesis. Type (A), (C), and (D) toxicities are for the most part thought to follow classic dose–response relationships, which defines how screening studies are designed, whereas type (B) toxicities do not [14,29]. MacDonald and Robertson [14] reviewed specific reasons for the withdrawal of drugs from the US, Japanese, and European markets during 1998–2008 after finding that the two major reasons were hepato- and cardiotoxicity. Other reasons included psychiatric problems and addiction, gastrointestinal toxicity, muscle toxicity, renal dysfunction, accelerated carcinogenicity or death, mutagenesis, drug–drug interactions, hypersensitivity, and pulmonary hypertension. Mechanisms and molecular events associated with liver toxicities included mitochondrial toxicity, cholestasis, cytotoxicity from reactive metabolite, and unknown mechanisms for rare idiosyncratic hepatotoxicity. Cardiotoxicity was a result of alterations in ion channel function leading to QTc prolongation, altered valvular function, and arrhythmias resulting in fatalities [14]. Not surprisingly, screening programs within

the industry key in on these findings, with the majority of programs screening for genotoxicity, cardiotoxicity, and hepatotoxicity [11]. This generally includes all small-molecule programs regardless of the intended indication, and results from screening can be used repeatedly to generate “safer” SAR libraries either virtually or synthetically.

The other important assessments are generated from clinical trial data when this information is accessible. Considering small-molecule and biologic targeted therapies, Roberts and colleagues [30] assessed issues emerging during phase I in cancer patients with data gathered from journal articles and abstracts submitted to annual meetings of the American Society of Clinical Oncology (ASCO) presented from 1991 through 2002. The database included 213 trials with 6474 patients, where 47% of the trials involved targeted small molecules and biologics (combined together by the authors). There were approximately 4% objective responses in all patients and 137 deaths (~2%) of which 35 were classified as treatment related. The overall toxic death rate was estimated to be ~0.5%. Targeted agents had a lower death rate than cytotoxics. The overall serious toxicity rate was ~10%, with major categories from small molecules being hepatic and neurological. During the early period of the study (1991–1994), the odds of a patient dying was 10 times that of the latest period (1999–2002), and over time, there was an increase in safety but a decline in response rates in phase 1 trials. The authors discussed several theories for these findings, which included the opinion that more targeted small molecules and biologics appeared in the later time periods, which supports the rationale that targeted therapies are better tolerated than cytotoxics. It has become apparent over the last 10 years that toxicities associated with targeted therapies in cancer patients are, in fact, different from those seen with standard chemotherapeutic agents and several can be predicted from effects of modulating a specific biological target or multiple targets.

2.9 IMMUNOMODULATORY THERAPEUTICS

Immunomodulatory therapies are widely used in a number of diseases including cancer, autoimmune disorders, multiple sclerosis, and organ transplantation. During the years 1995 to 2008, 174 biotherapeutics (excluding vaccines) were approved for marketing; 136 in the United States and 105 in the European Union, with 67 of these approved in both regions [31]. The majority of safety-related regulatory actions taken in the United States and European Union were related to immunomodulatory effects, which lead to an increase in the risk of secondary and opportunistic infections during long-term treatments. These types of reactions are primarily detected post approval and not well predicted from animal studies. As an example, patients on immunosuppressant therapies display an increased incidence and severity of community-acquired pneumonias and viral infections [16]. The type of infection depends on the underlying defect in the immune system. Cell-mediated immunity involves the activation of phagocytic components of the immune system, natural killer cells, antigen-specific cytotoxic T lymphocytes, and the release of cytokines. Abnormalities in cell-mediated immunity often result in infections and illness called *opportunistic infections*. Opportunistic infections are caused by microorganisms that are usually subclinical or latent in individuals with normal immune function. These types of infections are those normally seen in patients with HIV/AIDS and/or who have undergone organ and stem cell transplant [16].

2.10 MOLECULAR CHALLENGES IN SCREENING TARGETED THERAPIES

This raises the questions pertinent to the title of this chapter: are there molecular challenges to the standard practice of front-loading toxicity screening and testing in the discovery and development of targeted therapies? If so, what are the challenges? Three distinct development approaches, which affect “front loading,” are currently used in the biopharmaceutical industry [12]. The major difference is when the decision is made to pursue full development of the lead compound or development candidate, which triggers full-scale process chemistry and development expenditures. In one scheme, full development starts when the development candidate is selected. This scheme is considered the fastest path to the market and follows the assumption that the drug will succeed. In other schemes, full development starts when all Investigational Drug Application (IND) work has been completed or at least when pivotal toxicology studies have been reported. This scheme imposes a risk-benefit decision based on preclinical data. With some exceptions, a hybrid of these two is the usual approach in the pharmaceutical industry, and this practice imposes the front loading of toxicity screening and testing in order to make the informed decision faster. In the third approach, full development starts when preliminary clinical information suggests an increased probability of success. This is the approach used by smaller companies in the biotechnology field, which then becomes the driving force behind additional funding or collaborative transactions with larger partners.

2.11 SIGNALING PATHWAYS IN CANCER RELATED TO TOXICITIES OF THERAPEUTICS

Because of the prominent role the EGF and VEGF pathways play in cancers and the continued information developing on toxicities seen with agents inhibiting these signaling pathways, these processes and drugs are highlighted in this chapter. Both targets and signaling pathways include small-molecule and biotherapeutic inhibitors representing both single- and multiple-target approaches. These signaling pathways are shown in Figs. 2.1 and 2.2.

2.11.1 Epidermal Growth Factor

EGFR is a transmembrane tyrosine kinase receptor, which maintains a pivotal role in regulating cell division and death. EGFR belongs to the HER family of receptors composed of four related proteins (EGFR(HER1/ErbB1), ERBB2(HER2), ERBB3(HER3), and ERBB4(HER4)). The HER receptors are known to be activated by binding to different ligands, including EGF, TGFA (transforming growth factor α), heparin-binding EGF-like growth factor, amphiregulin, betacellulin, and epiregulin. After a ligand binds to the extracellular domain of the receptor, the receptor forms functionally active dimers [EGFR–EGFR (homodimer) or EGFR–HER2, EGFR–HER3, EGFR–HER4 (heterodimer)]. Dimerization leads to the activation of the tyrosine kinase domain, which results in autophosphorylation of the receptor involving multiple tyrosine residues. This leads to recruitment of a range of adaptor proteins (such as Shc, GRB2) and activates a series of intracellular signaling

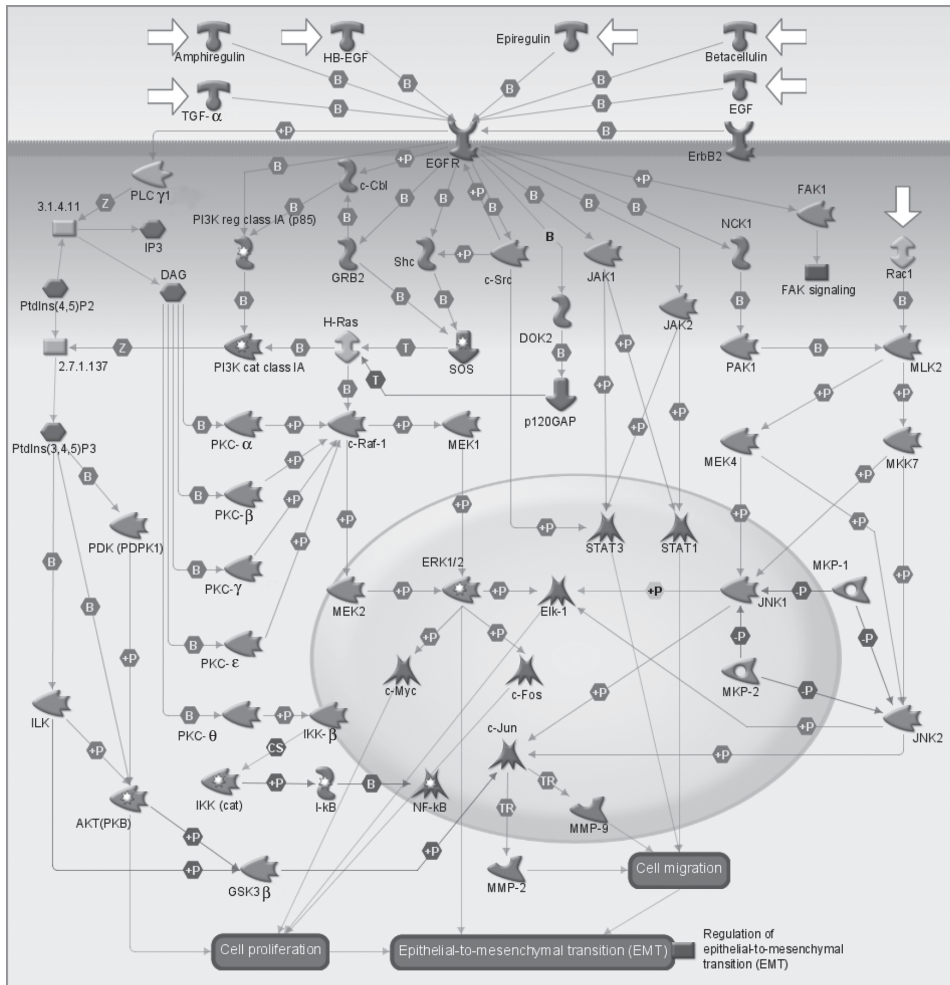


Figure 2.1 EGFR signaling pathway. Binding of ligands such as epiregulin and EGF dimerizes EGFR and activates it. ErbB2 (HER2) does not have to bind to any ligand with high affinity but is a preferred dimerization partner for other ErbB receptors. One component of EGFR signaling acts through the adaptor protein Shc, resulting in activation of RAS-RAF pathway and MAPK cascade. Another component acts through PLC γ and PI3K-Akt axis leading to cell survival. *Source:* Pathway map provided by Genego, Inc. (See color insert.)

cascades, which ultimately affect gene transcription. Gene transcription results in reduced apoptosis, cancer cell proliferation, invasion, and metastasis. These events also stimulate angiogenesis that reacts in relation to stimuli to create new blood supply (neovascularization) to developing or expanding tumors [32]. Major biological pathways mediate downstream effects of EGFR. The first pathway involves the RAS-RAF-MAPK pathway, where phosphorylated EGFR recruits the guanine nucleotide exchange factor via the GRB2 and Shc adaptor proteins, activating RAS and subsequently stimulating RAF and the MAP kinase pathway to affect cell proliferation, tumor invasion, and metastasis. The second pathway involves the PI3K/AKT

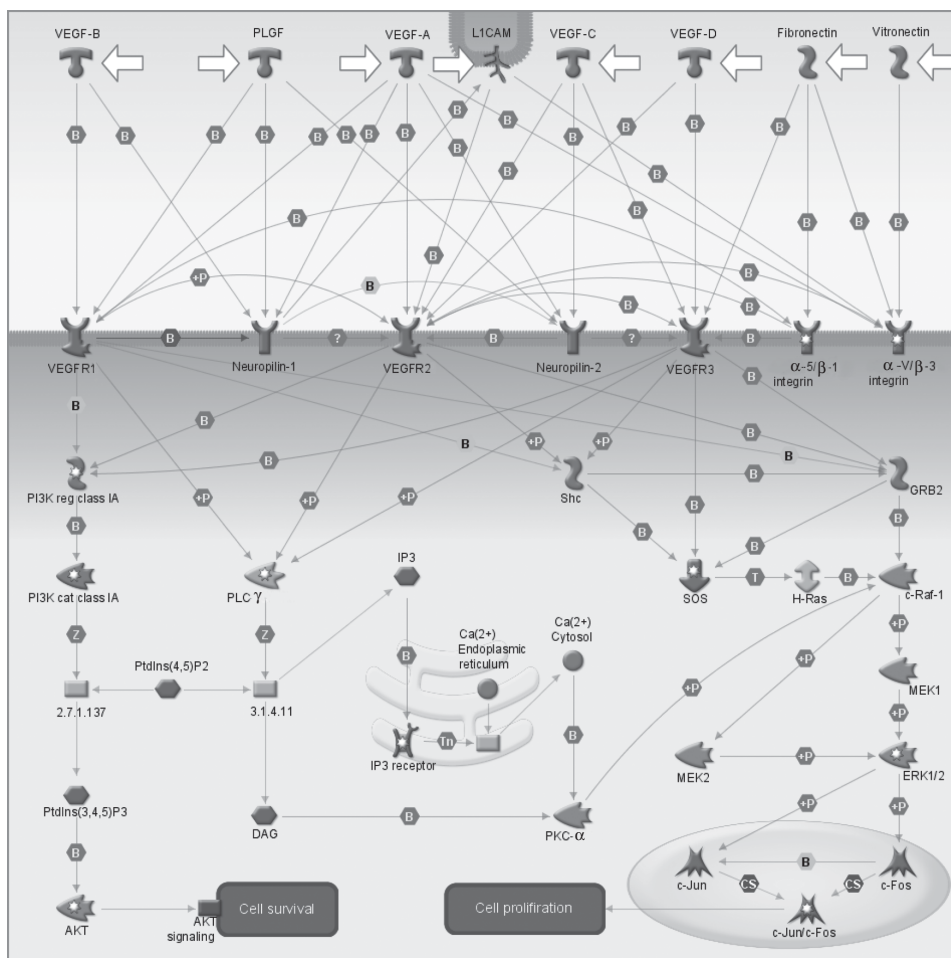


Figure 2.2 VEGF signaling pathway. VEGF-A binding to VEGFR2, a tyrosine kinase, initiates a cascade of signaling including phosphorylation of PLC γ and adaptor protein Shc. PLC γ in turn catalyzes the hydrolysis of phosphatidylinositol ultimately leading to the release of stored Ca. Signaling through Shc activates RAS pathway resulting in MAPK cascade and cell proliferation. Another component of the VEGF signaling pathway acts through the PI3K-Akt axis leading to cell survival. *Source:* Pathway map provided by Genego, Inc. (See color insert.)

pathway, which activates the major cellular survival and antiapoptosis signals by activating various nuclear transcription factors, including NF κ B (nuclear factor κ B). The third pathway, JAK/STAT, is also implicated in activating gene transcription associated with cell survival [32]. Anti-EGFR antibodies (e.g., cetuximab) bind to the extracellular domain of EGFR monomer and therefore block ligand-induced receptor activation by competing for receptor binding by endogenous ligands. Small-molecule EGFR inhibitors (e.g., gefitinib) compete with ATP to bind the catalytic domain of the kinase, which inhibits EGFR autophosphorylation and subsequent downstream signaling [32].

EGFR inhibitors cause certain types of skin toxicity, typically seen as a papulopustular rash in ~45–100% of patients given these drugs [33,34]. Interestingly, the skin effects have also been used as a biomarker of effect and potential efficacy of these drugs in the clinic. Other toxicities noted with EGFR inhibitors include ingrown nails, paronychia, xerosis [35], and ocular toxicities including changes in eyelids such as squamous blepharitis and in the tear film [36]. Understanding the molecular mechanism of dermal toxicity has led to the design of compounds that potentially rescue the downstream effects of signal inhibition. One such approach is the use of a potent phosphatase inhibitor, menadione, applied topically [33]. Other nonrash toxicities, including skin hyperpigmentation, xerosis, pruritus, hair growth, and color changes have been reported with targeted therapies [37].

2.11.2 Vascular Endothelial Growth Factor

VEGF plays a key role in new blood vessel formation and pathological angiogenesis such as in tumor growth, ischemic diseases, and macular degeneration. The increase in secreted biologically active VEGF protein from cells exposed to hypoxia, a potent inducer of VEGF, is linked to an increased transcription rate, mediated by binding of hypoxia-inducible factor-1 (Hif1) to a hypoxia responsive element in the 5'-flanking region of the VEGF gene. Secreted VEGF protein is a major angiogenic factor that regulates multiple endothelial cell functions, including mitogenesis. Cellular and circulating levels of VEGF are elevated in hematologic malignancies and are adversely associated with prognosis. Angiogenesis is a very complex, tightly regulated, multistep process. Current therapeutic approaches include the inhibition of angiogenesis stimulants (e.g., VEGF) or their receptors, blockade of endothelial cell activation, inhibition of matrix metalloproteinases, and inhibition of tumor vasculature [37].

Compounds that inhibit VEGF affect multiple signaling pathways in cells that regulate and maintain the microvasculature [38]. Side effects from inhibitors of VEGF signaling have ranged from overt cardiac effects to hypertension [15,24,38,39]. Molecules with varying degrees of cardiorelated effects include those with both single and multiple targets. Single-target agents include Avastin and trastuzumab (Herceptin; Genentech/Roche). Multiple-target agents include Nexavar (c/B Raf, VEGFR2/3, PDGFR α/β , KIT, FLT3) and Sutent (VEGFR1/2/3, PDGFR α/β , KIT, FLT3, RET, CSF-1) [22]. Although the mechanism of cardiotoxicity has not been fully elucidated, Fig. 2.3 shows a diagram where three anti-VEGF therapeutics, trastuzumab, imatinib, and sunitinib, share a similar signaling event, translocation of BCL2-associated X protein (BAX) to mitochondria in cardiomyocytes, which can lead to apoptosis [39].

2.12 OTHER TOXICITIES

Targeted therapies can also lead to renal toxicity since most of the biological pathways involved are expressed in the kidney. Related toxicities reported include hypertension and proteinuria [40]. Adverse effects can be manifested through single-target therapies primarily through pathway inhibition such as the p38 mitogen-activated protein kinase (p38MAPK) inhibitors [41] and the type 1 insulinlike growth factor receptor (IGF1R) inhibitors [42]. Multikinase inhibitors induce a number of toxicities

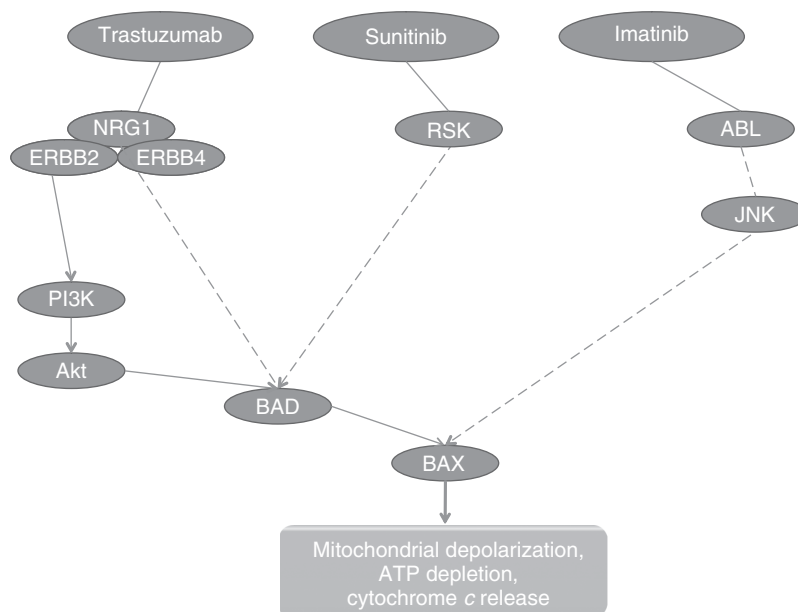


Figure 2.3 Schematic representation of putative molecular mechanisms of cardiotoxicity of three tyrosine kinase inhibitors in cardiomyocytes. A key signaling event common to all three inhibitors is the translocation of BAX to mitochondria, which is known to be involved in mitochondrial depolarization, ATP depletion, and cytochrome *c* release, which is a common mechanism in apoptosis. But in cardiomyocytes, the same mechanism could lead to toxicity. Detailed molecular events of cardiotoxicity due to kinase inhibitors remain to be classified. Trastuzumab is an ERBB2 antibody that reduces oncogenic signaling in breast cancer because of the overexpression of ERBB2(HER2) via PI3K-Akt pathways, ultimately leading to apoptosis. But in cardiomyocytes, pro-apoptotic signals may lead to cardiotoxicity. Imatinib is an inhibitor of ABL and blocks oncogenic signaling in CML through RAF-ERK and PI3K-Akt pathways, leading to apoptosis. But in cardiomyocytes, ABL, Abelson tyrosine kinase may have a survival function, and inhibition of ABL leads to activation of prodeath JNK pathway, ultimately leading to translocation of BAX and apoptosis. Sunitinib is primarily a KDR, kinase insert domain receptor (VEGFR2) inhibitor but also has several off-target effects such as inhibition of ribosomal kinase RSK, perhaps leading to inhibition of BAD, BCL2-associated agonist of cell death phosphorylation and eventual translocation of BAX to mitochondrion. *Source:* Adapted from Force T, Krause DS, Van Etten RA. Molecular mechanisms of the cardiotoxicity of tyrosine kinase inhibitors. *Nat Rev Cancer* 2007;7:332–344. (See color insert.)

where the commonality of toxicities between certain drugs is associated with common targets within a multikinase profile [43–46]. For instance, sorafenib and sunitinib target PDGFR, FLT3, VEGFR, as well as other kinases (see above), and share certain toxicities including cardiotoxicity. Toxicities of specific drugs are also influenced by the status of the patient and the type of cancer being treated. Several anecdotal reports appear in the literature that involve specific patient characteristics linked to toxicity, such as a case of plantar erythrodysesthesia in a patient being treated for non small-cell lung cancer, who was given low doses of sorafenib [47]. Although toxicities can be broadly based, the multikinase inhibitors are reasonably well tolerated as a class [43] and toxicities are becoming more predictable as use becomes more widespread.

2.13 DO CURRENT PRECLINICAL STUDIES PREDICT HUMAN ADVERSE EVENTS FROM MOLECULAR TARGETED THERAPIES?

Summaries of documents for four drugs submitted to the US FDA for registration purposes were examined to see if cardiovascular toxicity associated with VEGFR inhibitors and/or dermal effects from EGFR inhibitors were identified in *in vitro* systems or animal studies before clinical development [48–51]. Nonclinical studies on sorafenib (Nexavar) indicated a potential for cardiotoxicity primarily detected in safety pharmacology studies. Positive findings were seen in the *in vitro* hERG and action potential assays; however, there were no clear findings in dog telemetry studies or in a chronic one year study in which elevated CK (creatinine kinase) levels were observed. Limited histopathological findings in cardiac tissue were present in some animals in toxicology studies. In clinical trials, there was an increased incidence in hypertension versus control arms and, although rare, valvular disease and heart failure were reported as a cause of death in sorafenib arms. In addition, the drug was found to affect thyroid and parathyroid function and electrolyte imbalance from GI toxicity and diarrhea, both conditions that could increase the risk for cardiovascular toxicity. Force speculates that the overall rate of cardiomyopathy for sorafenib is low. On the other hand, the rate for cardiomyopathy with sunitinib (Sutent) is rated as moderate with hypertension and hypothyroidism seen as contributing factors. In preclinical studies, sunitinib caused both *in vitro* and *in vivo* cardiac effects including increased action potential duration in canine Purkinje fibers, blockage in hERG assays, and QTc prolongation in monkeys. In three- and nine-month studies on monkeys, reductions in heart rate and changes in ECHO parameters were seen, including left ventricular ejection time. There were also histopathological findings in individual animals, including capillary proliferation, myocardium vacuolization, or inflammation of the pericardium. Evidence of hemorrhage in various tissues was seen in both rats and monkeys. In clinical trials, decreases in left ventricular ejection fraction were also seen, which directly correlated with preclinical findings, as did incidences of hemorrhage in several tissues.

Preclinical studies with the EGFR inhibitors erlotinib (Tarceva) and gefitinib (Iressa) showed mechanistic evidence of dermal toxicity. With erlotinib, dermal toxicity was noted in mouse cancer xenograft models and in multiple dose studies in rats and dogs. In addition, ocular toxicity including corneal ulcerations was seen in longer-term dog studies. Dermal toxicity with gefitinib was seen in multiple dose studies in mice and dogs. Other toxicities seen with these compounds were almost universally related to the molecular mechanisms associated with the pharmacology of the drugs. This includes the generalized gastric toxicity leading to diarrhea seen clinically.

A number of other toxicities have been seen in animal studies with both single- and multitargeted compounds, including findings in the hematopoietic system, liver, kidneys, both male and female reproductive systems, bone, teeth, adrenal glands, pancreas, and changes in coagulation parameters. Common findings other than cardiovascular and dermal toxicities include bone marrow hypocellularity, incomplete epiphyseal closing and/or thickening of growth plates particularly in dogs, dentin alteration in juvenile animals, and osteodystrophy of the jaw in rats.

One conclusion that can be drawn from about 10 years of experience with targeted therapies is that rather than front-loading general toxicology studies, it would be more appropriate to design toxicology studies where the molecular mechanisms could be studied and related to potential adverse effects. For the most part, these toxicities

per se can be monitored effectively in early clinical trials, particularly when adequate pharmacokinetic/pharmacodynamic (PK/PD) correlations can be made. If general toxicology studies are not predictive of these molecular mechanisms because of the lack of definitive molecular assays, then additional time should be spent on developing biomarkers to monitor mechanisms in relation to adverse effects. In the ideal situation, assays developed for animal studies can be translated into assays used for clinical trials [5], but as noted earlier, these assays and direct links to toxicity are only starting to emerge.

2.14 EXPLORATORY AND PHASE 0 TRIALS IN CANCER PATIENTS

Several exploratory and phase 0 trial designs have been proposed and issued in a guidance by the US FDA [52]. In addition, a new ICH Guideline S9 is proposing a more abbreviated preclinical resting scheme for FIH oncology trials [10], and several exploratory approaches are currently in use. These include microdosing for preliminary PK information [53], screening studies for more than one compound to judge comparative bioavailability [52], radiolabeled studies at very low doses for distribution and elimination [53], and heavy-water labeling to probe fluxes through various biological pathways [54]. What distinguishes anticancer therapeutics in cancer patients is the ethical consideration that cancer patients should receive doses that have a chance to deliver an efficacious response [55]. Therefore, using low doses that are several multiples below the estimated therapeutic dose is not a preferred approach for FIH dosing in cancer patients but is, in fact, included in the phase 0 oncology designs [55]. In the NCI (National Cancer Institute) proposal [56], the essential rationale is the integration of PD assays into the first trial as a way to explore target modulation as evaluated with PK correlates [56]. These trials have been proposed to be conducted under an Exploratory Investigational New Drug (xIND) application. Since these trials are to be conducted in late-stage cancer patients, standard genotoxicity studies are not required; however, assessment of vital organ function, including cardiovascular, respiratory, and central nervous systems, should be done in animals and would normally be included in the general toxicology studies. Stand-alone safety pharmacology studies are optional under this proposal; however, because of the cardiotoxicity noted with several targeted therapies, cardiovascular safety pharmacology studies would normally be included. In the ICH S9 proposal, the animal toxicology studies essentially mirror the clinical dosing schedule. In the shortest clinical dosing scheme where the drug is given once every three weeks, single-dose toxicology studies will support the FIH trial. When the drug is given once every two weeks, toxicology studies can be designed as two-dose, 14 days apart, studies. The standard determination of an NOAEL is not considered essential in the preclinical toxicology studies, and demonstration of complete reversibility from all adverse effects is not considered an essential part of the supporting data. However, an assessment of reversibility up to a point is always included in supporting toxicology studies. Toxicology studies include rodent and nonrodent species, and a common approach for small-molecule therapeutics, including most targeted therapeutics, is to set a starting dose in humans at one-tenth the severely toxic dose in 10% of the rodents studied. If the nonrodent species (e.g., dog, monkey) is more sensitive to the drug than the rodent model, then the starting clinical dose is one-sixth of the highest nonseverely toxic dose. Since this new guidance is expected to be viewed on a case-by-case basis,

there are potential examples where the toxicology program to support the FIH trial could consist of rodent species only [10,52].

Changes in the requirements needed to initiate clinical trials with oncology therapeutics have occurred for a number of well-publicized reasons. Of major concern is the low success rate in this therapeutic category; only about 5% of INDs for new molecular entities in oncology make it past the investigational phase, which compares to ~10% in other therapeutic areas [56,57]. At issue is certainly the prolonged time line and costs of clinical trials, but at the heart of the problem is the lack of validated preclinical systems that accurately predict the efficacy and toxicity of new therapeutic agents and modalities [6]. These deficiencies include both *in vitro* and *in vivo* assays and models and have led both regulators and investigators to initiate procedures to move new drugs into clinical trials in cancer patients faster and develop both PK and PD endpoints in humans rather than rely on imperfect systems.

2.15 CONCLUSION AND FUTURE PERSPECTIVES

With the changing regulatory view on requirements for FIH dose selection for anticancer drugs, and the increase in discovery and development of targeted therapeutics, the concept of front-loading toxicology studies into the discovery and development programs with the intent on accelerating the development time line can be questioned from a scientific/molecular standpoint. This question is based on the belief that the modulation of specific targets, the goal of targeted therapies, directly relates to both efficacy and toxicity. Therefore, measuring markers of these events as related to exposure via PK/PD relationship modeling may in fact be a more rational way to accelerate a program to phase II, in particular, for phase II dose selection. However, maximizing benefit from this approach will require increased efforts to define and create assays for relevant biomarkers of biological activity and safety as PD endpoints. Currently, this has not been realized to the extent that this model can be applied to a majority of anticancer discovery and development programs. It is possible, however, that current programs could save and preserve (i.e., biobank) samples from nonclinical studies and correlate findings from these samples when molecular markers become established. This approach would create the molecular underpinnings of future anticancer programs related to the same molecular targets. It is tempting to speculate that there will be increased efforts on identifying and qualifying markers seen in preclinical studies with the aim of translating these to humans to provide rational estimates for FIH dose selection. A greater emphasis is expected to be placed on translating effects in animal models into patients in the first trials, and these trials are expected to be initiated with less toxicity testing than seen in the past. The increased use of PK/PD modeling to estimate exposure–response relationship in animal models and clinical trials will take on added importance with targeted anticancer therapeutics. The practice of front-loading toxicity testing to accelerate programs must be tempered with the realization that many toxicities of targeted therapeutics relate to the actual mechanism of action for efficacy. Whether mechanistic assays can be directly linked to toxicity in nonclinical studies has yet to be fully elucidated. Presently, the best approach is to make use of PK/PD relationship modeling across species and biobanking blood and tissue specimens of preclinical as well as clinical studies for future analysis. Early screening for chemical-based toxicity will continue to be performed as part of the

lead optimization process, and cardiotoxicity—other than QTc prolongation—should be incorporated early on with targeted therapeutics because of the complex cross talk apparent in multiple signaling pathways.

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3

Role of ADME Studies in Selecting Drug Candidates: Dependence of ADME Parameters on Physicochemical Properties

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3.1	Summary	1
3.2	The role of ADME studies in drug discovery	2
3.3	Drug design and the importance of physicochemical properties: Is there life beyond the “rule of five”?	5
3.4	<i>In vitro</i> ADME studies	12
3.5	<i>In vivo</i> pharmacokinetic studies	18
3.6	Tissue distribution	19
3.7	Metabolite identification	20
3.8	<i>In silico</i> ADME modeling	23
3.9	Multiparameter optimization	28
3.10	Incorporation of ADME data in drug discovery to improve pharmacokinetics	29
3.11	Conclusions	39
	References	39

3.1 SUMMARY

Drug discovery is a time-consuming and expensive endeavor fraught with many glaring failures in clinical trials. Optimization of drug absorption, distribution, metabolism, and excretion (ADME) parameters continues to play an important role to ensure that the exposure is sufficient to achieve proof of concept, and ultimately efficacy, safely in clinical trials to address unmet medical need. This chapter provides an overview of various *in vitro* and *in vivo* ADME studies commonly employed in drug discovery. Emphasis will be placed on the dependence of ADME parameters on physicochemical properties. The literature has been reviewed to highlight key relationships. However, literature reviews mainly focus on retrospective analyses. Therefore, examples are

included to illustrate how these principles can be embedded in drug discovery and used in a prospective drug design fashion with an emphasis on finding the physicochemical “sweet spot” that balances various properties. *In silico* (ADME) models should be an integral part of design as well and the value of various models has been illustrated.

3.2 THE ROLE OF ADME STUDIES IN DRUG DISCOVERY

Determining drug ADME properties of drug candidates early on in the drug discovery process is critical for achieving drugs with optimal properties and, in particular, an acceptable dose and dosing regimen. Many people refer to the data presented in Fig. 3.1—the reasons for attrition in development in 1991 and 2000—to highlight the impact of early ADME studies and their positive impact on a significant reduction in attrition due to pharmacokinetic (PK) reasons. In the 1980s, inappropriate ADME characteristics were a leading cause of attrition in drug development (about 40% of drugs failed due to drug metabolism and PK reasons) and this was reduced to <10% in the 1990s [1]. The latter was confirmed by another analysis of drug development project terminations from 1992 until 2002 with ADME being cited as the reason for attrition in 14%, 17%, and 4% in phase I, II, and III, respectively [2]. Although this trend is undoubtedly real, it is important not to overinterpret these data and assume that ADME sciences are sufficiently mature. First, there were many anti-infectives in development in the 1980s with many of them failing due to poor PK and contributing to the statistics presented in Fig. 3.1. If anti-infectives are taken out of the equation, the reduction in attrition due to ADME reasons is less pronounced; the attrition due to PK reasons was 39% from 1964 until 1985, but this was reduced to 7% if anti-infectives

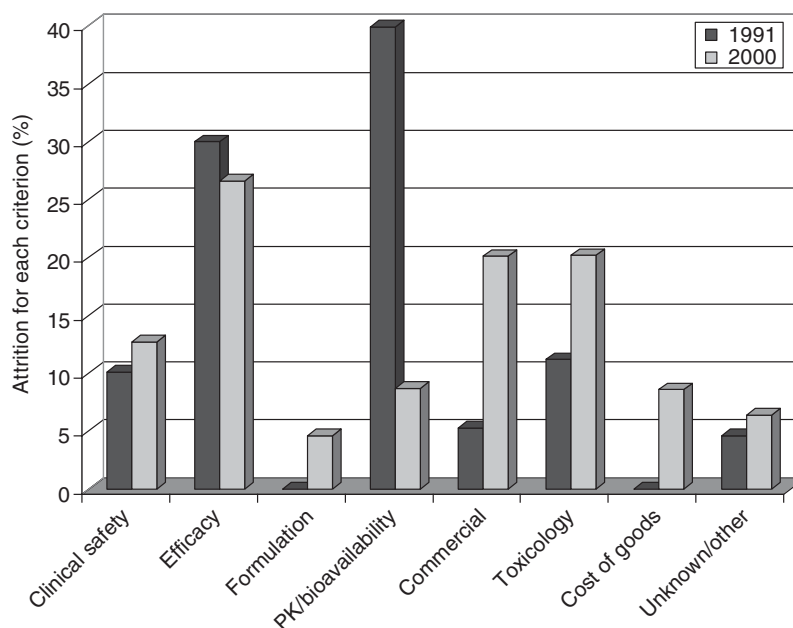


Figure 3.1 Reasons for attrition of drugs in development. *Source:* Data from Ref. 1.

were excluded [3]. Second, many compounds fail in development because they fail to illicit efficacy. Indeed, a key concern in the pharmaceutical industry is the very high attrition in phase 2 (>60% is quite common among the leading pharmaceutical companies and it is getting worse) and this is largely due to lack of efficacy or insufficient efficacy compared with standard of care [4,5]. However, in some cases, the lack of efficacy may be due to insufficient exposure and it is not inconceivable that efficacy may have been achieved if the clearance had been lower or absorption better. The latter is particularly worrisome because the clinical validity of the target may not have been properly assessed and, therefore, the decision to nominate a backup compound or not cannot be made in a reliable manner. Indeed, 12 of 44 phase 2 programs failed at Pfizer because no downstream pharmacological effect was observed in human and confidence that sufficient exposure was achieved to test the mechanism was low [6]. Third, some of the attrition due to toxicity may be metabolically mediated and, therefore, a greater understanding of the metabolic fate of drug candidates could influence survival in drug development. Moreover, the regulatory standards keep increasing with an increasing demand for ADME studies and a reduced tolerance for ADME-related issues such as drug–drug interaction and reactive metabolite formation. Thus, although the trend is very encouraging, it is critically important to continue to advocate early incorporation of ADME properties in the complex multiparameter optimization process in drug discovery and do so on an equal footing with potency, selectivity, toxicity, and so on. The important role of ADME properties is nicely illustrated by the “*stringent selection criteria*” presented by AstraZeneca in 2011 [7] and reproduced in Table 3.1 with the parameters having a direct bearing on ADME highlighted.

In vitro and *in vivo* ADME studies serve multiple roles in drug discovery:

- Determination of *in vitro* ADME parameters using cell lines and subcellular fractions from rodents, dogs, or monkeys to identify the best compounds for preclinical PK studies or *in vivo* proof of concept studies.
- Determination of plasma or tissue exposure to prepare for or to support interpretation of preclinical efficacy and toxicity studies.
- Determination of *in vitro* ADME parameters using human cell lines and subcellular fractions and *in vivo* ADME parameters from studies in rodents, dogs, or monkeys to predict human PK parameters and the propensity for drug–drug interaction.

TABLE 3.1 Stringent Selection Criteria Presented by AstraZeneca in 2011 [7]

Right Target Engagement	Link Between Target/Disease Predictive Biomarkers
Right tissue exposure	<i>Bioavailability and tissue exposure</i> <i>Human PK/PD prediction</i>
Right safety	Differentiating safety <i>Reactive metabolites</i>
Right patients	Scientific evidence in lead indication Stratification of patient population
Right commercial	Differentiated value proposition Embedded payer perspective

- Determination of the anticipated human efficacious dose using a combination of predicted human PK parameters and preclinical pharmacodynamic (PD) and/or efficacy studies.

Considerable progress has been made with the suitable incorporation of preclinical *in vitro* and *in vivo* ADME studies, but understanding the ADME structure-activity relationship (SAR) and the ability to effectively manipulate it in drug discovery is still problematic. Indeed, in many cases, it is easier to optimize potency and selectivity than the ADME properties, which provides further impetus to incorporate ADME in drug design early on. The following DMPK-centric strategies can have a positive impact on identification of compounds with superior properties and result in a reduction in the time spent in drug discovery:

- (continued) emphasis on physicochemical properties in drug design
- use of *in silico* ADME models to enable assessment of ADME properties before synthesis
- early incorporation of *in vitro* and *in vivo* ADME studies in drug discovery to identify compounds with optimal preclinical and ultimately clinical ADME attributes as well as potency, selectivity, toxicity, and so on; *in vitro* ADME data should be considered right after identification of hits from the biochemical high throughput screen (HTS) to facilitate identification of chemical series that are easier to optimize
- integration of all preclinical *in vitro* and *in vivo* data to improve the confidence in prediction of the human PK
- exposure–effect modeling with appropriate PD biomarkers in preclinical models to get a quantitative understanding of target and pathway modulation and the anticipated human exposure for sufficient target coverage and, subsequently, efficacy in humans; combination of these data with anticipated human PK will provide the expected efficacious dose
- detailed analysis of clinical PK and PD data to influence design of backup compounds

Interestingly, some have questioned the need for substantial ADME involvement in early drug discovery if the target is both clinically and preclinically unvalidated. Although it is understandable that certain ADME properties [such as reversible or time-dependent cytochrome P450 (CYP) inhibition] do not need to be considered if achieving preclinical proof of concept in an appropriate animal model is the main objective, having sufficient exposure at a reasonable dose will still require substantial involvement of ADME scientists.

In this chapter, a number of strategies have been outlined and examples included to illustrate the importance of ADME in drug discovery. Particular attention has been paid to physicochemical parameters because manipulation of these parameters is something medicinal chemists can relate to easily and, moreover, these parameters have a direct bearing on various ADME attributes. The ultimate goal is to find the right balance of physicochemical properties because some parameters have an orthogonal dependence on physicochemical parameters. For example, a low log *D* value may adversely influence absorption, but a high log *D* value may lead to poor solubility and, hence, poor

absorption and poor metabolic stability. Thus, proper incorporation and balancing of physicochemical parameters will have a positive impact on various ADME parameters and, therefore, increases the probability of success in drug discovery and development, although it does not guarantee it.

3.3 DRUG DESIGN AND THE IMPORTANCE OF PHYSICOCHEMICAL PROPERTIES: IS THERE LIFE BEYOND THE “RULE OF FIVE”?

The first thorough analysis of the impact of physicochemical parameters was performed by Christopher Lipinski at Pfizer in the late 1990s [8]. Lipinski proposed the “rule of five” and he argued that poor absorption is more likely if

- $\log P > 5$
- molecular weight (MW) > 500
- number of hydrogen bond donors (HBDs) > 5
- number of hydrogen bond acceptors (HBAs) > 10

Fortunately, all these parameters can be calculated easily and incorporated in compound design. A recent analysis (Table 3.2) has shown that phase 1 oral drugs have a considerably higher MW and more HBAs and rotatable bonds than marketed oral drugs, which suggests that “rule of five” violations are associated with higher attrition in drug development. The latter has been confirmed by an independent analysis of a large corporate database showing that relatively speaking a higher percentage of compounds with “rule of five” violations attrite for PK reasons (Hop, unpublished data). In addition, an analysis of a database of oral drugs and the ChEMBL database of “hits” indicated that the mean MWs of drugs and “hits” are 333 and 430 Da, respectively, and the mean $\log P$ values are 2.5 and 3.5, respectively [9]. On the other hand, the chemical complexity of oral drugs launched between 1983 and 2002 versus those launched before 1983 has increased with a significant increase in MW and the number of HBAs and rotatable bonds (Table 3.2). The latter is not surprising because the introduction of high throughput screening has increased the emphasis on potency and it has been shown that potency increases with MW and $\log P$ in a statistically significant manner

TABLE 3.2 Average Physicochemical Properties of Oral Drugs in Phase I and Marketed Oral Drugs in Comparison with Lipinski’s “Rule of Five”

	Phase 1 Oral Drugs	Marketed Oral Drugs	Oral Drugs Launched Pre-1983	Oral Drugs Launched 1983–2002	Lipinski’s Rule of Five
Molecular weight (Da)	423	337	331	377	≤ 500
$c\log P$	2.6	2.5	2.3	2.5	≤ 5
$c\log D_{7.4}$	1.3	1.0	—	—	—
Hydrogen bond donors	2.5	2.1	1.8	1.8	≤ 5
Hydrogen bond acceptors	6.4	4.9	3.0	3.7	≤ 10
Rotatable bonds	7.8	5.9	5.0	6.4	—
Rings	—	—	2.6	2.9	—

Source: Data from Wenlock *et al.* [11] and Leeson and Davis [12].

[9]. Nevertheless, the average properties of drugs launched between 1983 and 2002 are well within Lipinski's "rule of five." Analysis of a data set of 108 compounds with clinical data provided by multiple companies corroborates the trend toward greater molecular complexity and lipophilicity with an average MW of 443 Da (range: 171 to 725 Da) and an average log P of 3.75 (range: -5.95 to 12.1) [10]. Unfortunately, it is not known which of these 108 drug candidates—if any—were approved as new drug applications (NDAs).

Most of these analyses refer to clinical candidates nominated before Lipinski's "rule of five" was presented and, therefore, may not reflect current practices and the extent of incorporation of the "rule of five" and associated principles in drug design. Leeson and St-Galley [13] performed a comprehensive analysis of compounds patented by large pharmaceutical companies between 2000 and 2005, and 2006 and 2010, which could potentially reflect the degree of incorporation of the "rule of five" in compound design as it is practiced at this moment. Several interesting conclusions can be drawn.

1. The authors comment on the influence of the "organizational factor" on physicochemical parameters. The differences in clog P and MW across companies are striking with a one log unit difference in clog P between Wyeth (average clog P 4.5) and Pfizer and AstraZeneca (average clog P 3.5) and 60-Da difference in MW between Boehringer Ingelheim (average MW 495 Da) and Pfizer and Vertex (average MW 435 Da). Moreover, large organizational differences in clog P and MW are maintained if only shared targets are taken into consideration. For example, data have been presented for CCR5 antagonists showing a 2 log unit difference in mean log P and 100-Da difference in mean MW between leads from AstraZeneca, GlaxoSmithKline, Merck, and Pfizer [14]. Organizational differences were also obtained for other physicochemical properties such as the number of HBAs and HBDs, the number of rotatable bonds, and the number of sp^3 carbon atoms. An in-house analysis of the physicochemical properties of both the Genentech and Roche compound libraries showed significant differences with the median MW and clog P about 50 Da and close to 0.5 log unit, respectively, higher in the Genentech library than the Roche library. It is likely that the composition of the library has a direct bearing on the properties of the leads and ultimately drug candidates, although fragment-based approaches could have a positive influence, but its full impact may not yet be visible.
2. The mean clog P values decreased for many companies when comparing data from 2000 to 2005 and 2006 to 2010. Statistically significant reductions were noticed for Takeda, Lilly, Schering-Plough, and AstraZeneca. In contrast, the mean MW did not decrease significantly for most companies and even increased for some companies.
3. In agreement with observations by Morphy [15] and Paolini *et al.* [16], significant differences in clog P and MW were observed across targets, but in many cases, the mean values still did not violate the "rule of five." For example, for oral central nervous system (CNS) drugs, the "cutoffs" for parameters such as MW, number of HBAs and rotatable bonds, and polar surface area (PSA) have to be reduced to ensure that the drug can cross the blood–brain barrier [12,17] and this is illustrated in Table 3.3. This chapter focuses on oral drugs, but it is worth noting that different criteria apply for non-oral routes of administration, such as intravenous, topical, and inhaled.

TABLE 3.3 “Pajouhseh’s Rules” for CNS Compounds

Molecular weight (Da)	<450
clog <i>P</i>	<5
Hydrogen bond donors	<3
Hydrogen bond acceptors	<7
Rotatable bonds	<8
PSA (Å ²)	60–90
p <i>K</i> _a	7.5–10.5

Source: Data from Pajouhesh and Lenz [17].

Several companies have confirmed that attrition of compounds breaking the “rule of five” is higher than those abiding by the “rule of five.” Price *et al.* [18] have shown that physicochemical properties are not only important for superior ADME and pharmaceutical properties, there is also a statistically significant relationship with attrition due to toxicity. Specifically, they showed that attrition due to toxicity is about six- to seven-fold higher if clog *P* > 3 and topological polar surface area (TPSA) < 75 Å² versus clog *P* < 3 and TPSA > 75 Å². Although not confirmed, this could be partially due to increased off-target activity of these compounds. Indeed, Gleeson *et al.* [9] have shown that the number of off-target hits increases with increasing log *P* and MW. Alternatively, lipophilic compounds with clog *P* > 3 and TPSA < 75 Å² could be less metabolically stable and (reactive) metabolites could contribute to toxicity.

Although most drug discovery scientists recognize the importance of good physicochemical properties, the degree of incorporation in drug design still varies and many claim that their target (e.g., disruption of protein–protein interaction) necessitates exploration of chemical space beyond traditional drug-like chemical space [19]. Indeed, very successful drugs that violate the “rule of five,” such as atorvastatin, ritonavir, alendronic acid, and montelukast, are used as examples to justify their decisions. Although it is beyond doubt that successful drugs exist beyond the “rule of five,” it is worthwhile to look at the attributes of some of these compounds in relation to their physicochemical properties. An exhaustive analysis is beyond the scope of this chapter, but a more detailed look at several examples from an internal analysis performed in 2006 provides valuable context; the structures of the drugs in question are presented in Fig. 3.2.

3.3.1 Alendronic Acid

Alendronic acid—a synthetic analog of pyrophosphate—has seven HBDs, which is a potential liability. Indeed, absorption is quite low with a bioavailability of about 1% and there is a substantial effect of food and beverages (in particular those rich in multivalent cations such as calcium) on absorption. However, alendronic acid is taken up in the bone—the site of action—and has a half-life of >10 years, which reflects the rate of bone turnover rendering the poor bioavailability less relevant.

3.3.2 Atorvastatin

The MW of the cholesterol-lowering drug atorvastatin is 558.6 Da and this violates the MW “limit” of 500 Da by 12%. The solubility of the calcium salt is very low

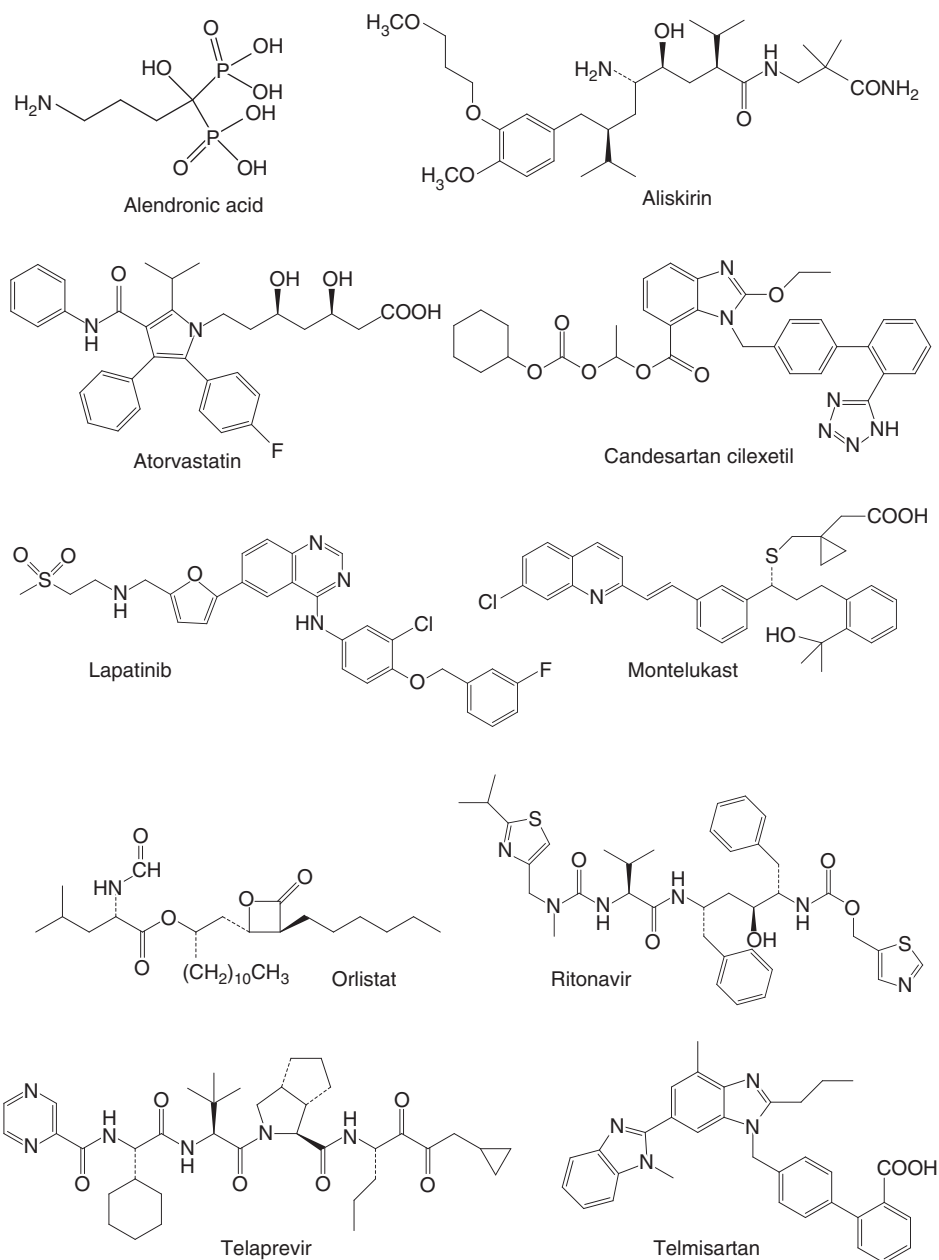


Figure 3.2 Structures of drugs referred to in the text.

and the intestinal and hepatic first-pass extraction ratios are high, 0.76 and 0.42, respectively [20]. Not surprisingly, the systemic exposure of the acid form of atorvastatin is relatively low and that may be perceived as a flaw. However, the acid form of atorvastatin is taken up in the liver by organic anion transporting proteins (OATPs) and H^+ -monocarboxylic acid cotransporters—albeit not by design—where it is responsible for most of its dramatic cholesterol-lowering activity. This is nicely illustrated

by Maede *et al.* [21] who administered a microdose of atorvastatin (about 20 mg) to healthy volunteers with or without coadministration of rifampicin (600 mg; an OATP inhibitor) or itraconazole (200 mg; a CYP3A4 inhibitor). The PK plasma area under the curve of atorvastatin increased 12-fold with rifampicin co-administration and stayed the same with itraconazole. In addition, two of atorvastatin's metabolites, 2-hydroxy-atorvastatin and 4-hydroxy-atorvastatin, are active as well. Thus, there is a systemic PK/PD disconnect due to fortuitous hepatic uptake, and neither the relatively low systemic exposure nor the high MW is a liability. Indeed, Lennernäs pointed out that *“The effect of statins is not closely related to the plasma concentration of the drug and its active metabolites. Instead, several reports have shown that the pharmacological response was better correlated with the daily dose of statins”* [20].

3.3.3 Candesartan Cilexetil

Candesartan cilexetil breaks the “rule of five” on several fronts: MW, log *P*, and HBAs. However, candesartan cilexetil is a prodrug by design, and it is completely and rapidly converted to candesartan in the gastrointestinal tract during absorption. Candesartan itself—an angiotensin II receptor antagonist for the treatment of hypertension—is a polar dianionic compound (it contains tetrazole and carboxylic acid functional groups) that would not be absorbed appreciably if it had not been for generation of a prodrug.

3.3.4 Montelukast

Montelukast breaks both the MW and the log *P* rule. The latter is not surprising because the endogenous substrates of the target, the leukotriene D4 (cysLT1) receptor, are very lipophilic as well. However, montelukast has sub-nanomolar potency and this effectively counters, among others, the high plasma protein binding driven by its lipophilicity and being a carboxylic acid.

3.3.5 Orlistat

Orlistat is a reversible covalent lipase inhibitor for weight loss and has a log *P* of 8.6. Not surprisingly, orlistat is practically insoluble in water and the bioavailability is <1%. However, the target is in the intestine and systemic exposure is not required for efficacy.

3.3.6 Ritonavir

Ritonavir is a HIV protease inhibitor and breaks the MW rule by a considerable margin with a MW of 720.9 Da. The log *P*, 4.9, is relatively high as well. Overall, ritonavir has quite undesirable properties and the development was encumbered with significant physical form and formulation issues [22]. Originally, ritonavir was marketed as an oral liquid and a semi-solid capsule. Both formulations contained ethanol to aid solubility because ritonavir is not bioavailable from the solid state. However, after a few years, problems arose because a different conformational polymorph appeared in the production process with even lower solubility and, therefore, greatly reduced bioavailability. This threatened supplies and necessitated immediate reformulation. To solve these issues, the original capsules were replaced with refrigerated gelcaps. Clearly,

the physicochemical properties were a serious liability, but ritonavir was a life-saving treatment for AIDS and the benefits outweighed its distinct disadvantages.

3.3.7 Telmisartan

Telmisartan—another angiotensin II receptor antagonist for the treatment of hypertension—is part of a large group of compounds, which breaks the “rule of five” based on its log *P* value, but the log *D* value is much lower. The log *P* value of telmisartan—a carboxylic acid—is 7.3, but the log *D* value at pH = 7.4 is between 3 and 4. The solubility at neutral pH is relatively low, but the permeability is acceptable and the absolute bioavailability in humans is 43%, which is higher than most other angiotensin II receptor antagonists.

3.3.8 Intrinsic and Extrinsic Attenuating Circumstances

An appropriate term—also used in social sciences—to describe these compounds (3.3.1–3.3.7) is “positive deviants.” This analysis is not meant to be complete, but it does highlight a few valuable points. Many positive deviant drugs that break the “rule of five” are associated with specific attenuating circumstances.

Intrinsic attenuating circumstances:

- The MW rule may be broken because of the presence of a large number of halogen atoms (e.g., amiodarone and levothyroxine). Some argue that the molecular volume is a better measure for absorption than MW and halogen atoms have a disproportionately high atomic weight compared with their volume. This, has resulted in the proposal to calculate the “corrected” atomic weight with fluorine, chlorine, bromine, and iodine counting for 5.2, 19.2, 26.3, and 37.4 Da, respectively, instead of 19.0, 35.5, 79.9, and 126.9 Da, respectively [23].
- Some drugs with a high MW are prodrugs that are cleaved presystemically (e.g., candesartan cilexetil and valganciclovir). Note that several drugs on the market are prodrugs, but not by design (e.g., sibutramine and terfenadine).
- Several acidic and basic drugs break the “rule of five” based on their log *P* value, but the more physiologically relevant log *D* value at the intestinal pH is much lower and compatible with absorption (e.g., gefitinib and telmisartan). Unfortunately, calculation of the log *D* at a specific pH requires calculation of the pK_a , which can be problematic.
- Some drugs with multiple HBDs and HBAs form internal hydrogen bonds, which reduces the number of HBDs and HBAs able to interact with water. See the following sections for more details.

Extrinsic Attenuating Circumstances:

- Some drugs act locally in the gastrointestinal tracts (e.g., miconazole and orlistat).
- Some of drugs that break the “rule of five” display a significant systemic PK/PD mismatch (e.g., alendronic acid and atorvastatin) and the low systemic exposure is not a liability. This determination requires an in-depth knowledge of the biological mechanism and site of action.

- Many drugs that violate the “rule of five” are for life-threatening diseases and the pharmacological benefits outweigh the disadvantages (e.g., ritonavir and saquinavir).

Setting aside the positive deviant drugs described above, the number of compounds that break the “rule of five” without attenuating circumstances and are commercially successful is quite limited and they usually distinguish themselves by being extremely potent (e.g., montelukast). An analysis of drugs that have been launched between 2006 and 2008 comes up with similar conclusions [24]. For example, lapatinib has an MW of 581.1 Da and a $\text{clog } D$ of 6.0. Not surprisingly, the absorption of lapatinib is incomplete and variable and the human daily dose is 1.25 g. However, lapatinib targets a life-threatening disease: breast cancer and other solid tumors. Another example is aliskiren, which is a direct renin inhibitor for primary hypertension. The MW of the free base is 551.8 Da and it has six HBDs and a PSA of $157 \text{ \AA}^2$. Although the solubility of aliskiren is very good, the absolute bioavailability is only 2.6% and the absorbed fraction is estimated to be 5% [25]. In addition, the interindividual variability in human aliskiren PK is large and there is a large, negative food effect [26]; apple juice and orange juice impact the exposure negatively as well [27]. The human PK of aliskiren are governed by a combination of (i) limited passive permeability, (ii) active intestinal efflux mediated by P-glycoprotein (P-gp) and uptake by OATPs, and (iii) a significant hepatic first-pass effect mediated by hepatic uptake and active biliary secretion of the parent compound. However, aliskiren can be detected in the kidney—the site of action—weeks after administration, even though the drug is undetectable in the plasma [24]. Fortuitously, aliskiren accumulates in renin granules, which explains the long-lasting renin angiotensin blockade beyond the half-life of aliskiren [28]. Thus, aliskiren is another classical example of a drug with poor PK driven by specific “rule of five” violations, but a PK/PD disconnect renders the systemic exposure less relevant. These examples and several others led Teague to conclude,

“Where there is a choice in a therapeutic area between a lipophilic agent that violates the rules and one with a better balance of properties, senior management would still be best advised to back the latter. Imperfect agents might simply validate a target and encourage competitors to enter with a drug having the improved properties [24].”

In summary, although differences in physicochemical properties exist across targets (and companies), the majority of existing drugs for most target types follow the “rule of five.” Thus, the conclusion is that extreme physicochemical properties are more likely to be accompanied by poor pharmaceutical and PK properties and, to be a successful drug, other advantageous properties—such as potency, a long off-rate from the receptor, or active distribution to the site of action—have to make up for those deficits and/or the drug should target a life-threatening disease with few therapeutic alternatives. However, as research progresses and more drugs reach the markets, the bar will be raised for drugs targeting very serious disorders as well. A good example is telaprevir (MW is 679.9 Da and the $\log P$ is 4.0), a very promising drug for the treatment of hepatitis C virus (HCV) infection [29]. The bioavailability in preclinical and clinical studies with the initial form was poor (2% or less) and variable. Indeed, scientists from Vertex commented that marble was more soluble than telaprevir. Extensive formulation development was required to come up with a stable amorphous dispersion

with good bioavailability, 20–40%. Despite these heroic and laudable efforts, the dose and the daily pill burden are still remarkably high: 750 mg three times a day. It is likely that in this highly competitive therapeutic area [30], new drugs will appear on the market with a superior regimen and lower cost of goods. Indeed, Merck focused on improving the PK properties and hence the dose in their second generation HCV NS3 protease inhibitor, narlaprevir, which is currently in clinical trials [31]. In conclusion, the nature of the target and its tissue distribution, the therapeutic area, and the competitive landscape—and therefore the target candidate profile—should define *up front* the acceptable physicochemical space and whether potential liabilities associated with breaking the “rule of five” are acceptable or not.

One class of drugs that deserve special recognition is natural products [32]. Despite pronounced “rule of five” violations, many natural products and in particular macrocyclic natural products such as cyclosporine, erythromycin, and tacrolimus display acceptable PK properties, including bioavailability. Several reasons have been proposed for this such as involvement of uptake transporters and extensive internal hydrogen bond formation and reduced conformational flexibility [33]. A recent nuclear magnetic resonance (NMR) and gas phase computational analysis [34] has shown that cyclosporine forms four internal hydrogen bonds, which reduces the number of HBDs from 5 to 1. The internal hydrogen bonds also reduce the effective PSA (not predicted by the TPSA) and lead to a more compact conformation with a lower molecular volume. These factors could explain the remarkably good oral bioavailability of cyclosporine in humans, 30% [34]. Unfortunately, natural product research has been scaled back by most pharmaceutical companies.

Overall, these data reemphasize the importance of physicochemical parameters. However, physicochemical parameters not only influence absorption, they also affect other ADME parameters such as clearance and distribution and the appropriate physicochemical “sweet spot” should be defined for each parameter and each project. This dependence will be illustrated in Section 3.10 after a brief outline of ADME assays deployed in drug discovery to guide design. In addition, in Section 3.10, the dependence of ADME properties on physicochemical parameters will be explored in an *integrated* and *prospective* manner, whereas the relationships highlighted in this section relied mostly on a *retrospective* analysis.

3.4 *IN VITRO* ADME STUDIES

With advances in bioanalysis and the availability of both specific cell lines and subcellular fractions, it has become possible to incorporate a range of *in vitro* ADME end points more effectively in drug discovery with the hope of steering projects towards superior ADME properties. Of course, as will be illustrated below, the ADME properties have to be weighed against other attributes such as potency and selectivity. This has resulted in complex screening cascades incorporating end points that reflect potency, efficacy, selectivity, toxicity, and ADME properties. The following *in vitro* ADME end points are routinely assessed in drug discovery:

- Permeability in Caco-2 or Madin–Darby canine kidney (MDCK) cells or parallel artificial membrane permeability assay (PAMPA)

- Efflux in MDCK or Lilly Laboratories cell-porcine kidney (LLC-PK) cells over-expressing transporters such as P-gp and breast cancer resistance protein (BCRP)
- Metabolic stability in microsomes, S9, cytosol, hepatocytes, or recombinant CYP enzymes
- Reversible CYP inhibition
- Mechanism-based or time-dependent CYP inhibition
- CYP induction
- Plasma protein binding or drug binding in other matrices such as microsomes or brain
- Blood to plasma partitioning

Each of these *in vitro* ADME assays should be incorporated in the overall screening cascade for discovery projects, although the order and extent may vary from project to project. An example of an effective screening cascade for a CNS target is presented in Fig. 3.3.

3.4.1 Permeability

Absorption across the epithelial cell layer in the gastrointestinal tract is the first hurdle a drug has to overcome to reach a systemic site of action. Caco-2 cells, a well-differentiated intestinal cell line derived from human colorectal carcinoma, display many of the morphological and functional properties of the intestinal epithelial cell layer and, consequently, have been used extensively as an *in vitro* model to study absorption. Indeed, a correlation between flux across Caco-2 cells and the fraction absorbed has been established. However, growing these cells is time consuming. Therefore, other cell lines that take less time to grow to confluence, such as MDCK and LLC-PK cells, have been introduced. In these experiments, the bidirectional flux, from apical to basolateral and from basolateral to apical, is determined. If there is asymmetry, this suggests the involvement of uptake or efflux transporters (see below). Non cell-based assays, such as the PAMPA, are used as well. PAMPA employs a barrier made of phospholipids (e.g., phosphatidyl choline and egg lecithin) solubilized in a long-chain hydrocarbon. Despite the experimental convenience of PAMPA, most companies prefer using cell-based assays, because they are a more realistic reflection of the *in vivo* absorption barrier.

3.4.2 Metabolic Stability

Most of the *in vivo* clearance of drugs is driven by metabolism and the most important drug-metabolizing enzymes belong to the cytochrome P450 (CYP) family. The liver is the most prominent site of metabolism and, therefore, hepatocytes and/or hepatic subcellular fractions are used to study metabolism. CYP enzymes and flavin-containing monooxygenases (FMOs) are present on the cytoplasmic side of the endoplasmatic reticulum and as such are active in liver microsomes. Liver microsomes from various species are ubiquitously available and are used in a higher throughput manner to determine the propensity of drugs to be metabolized by CYP enzymes and FMOs. Other enzymes contribute to metabolism as well, but a different subcellular fraction

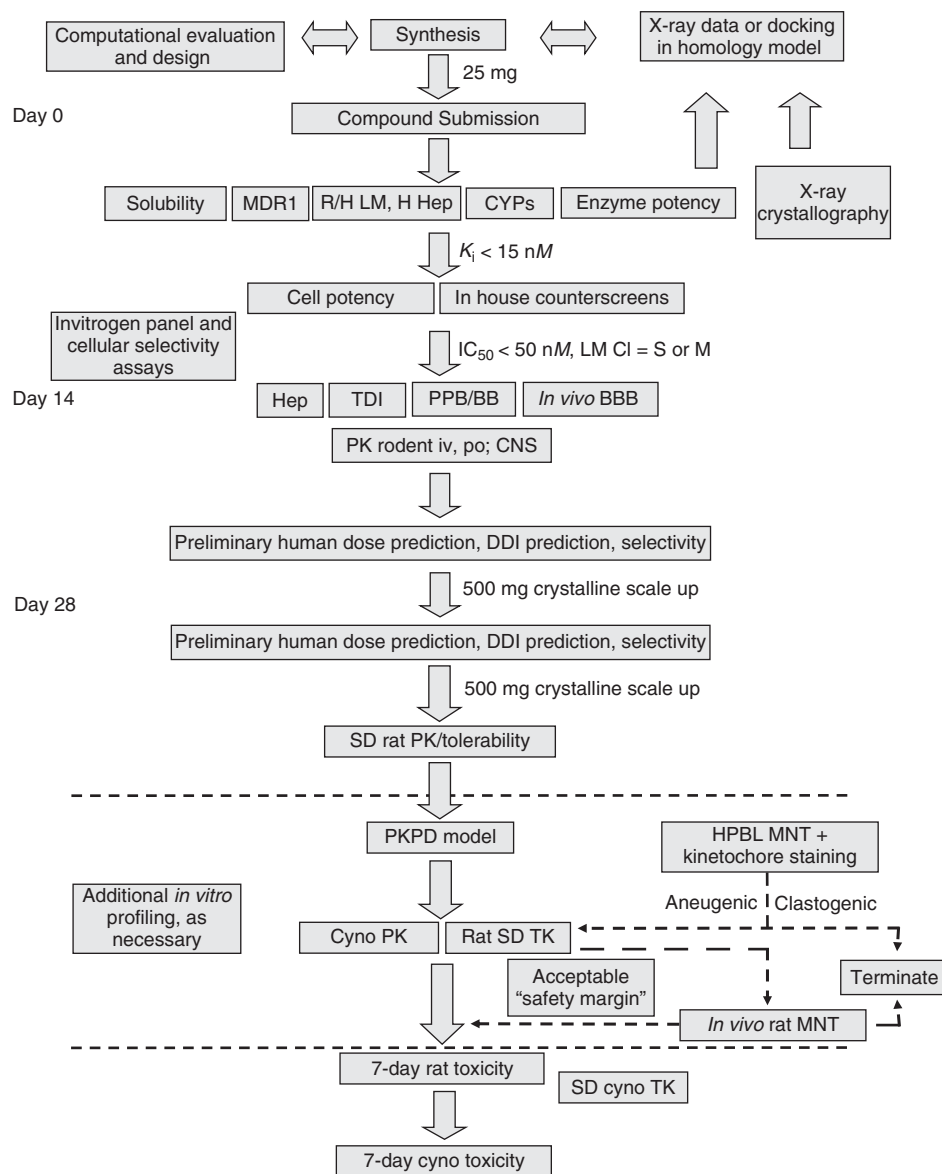


Figure 3.3 Screening cascade for a CNS target.

may be required. For example, aldehyde oxidases, alcohol dehydrogenases, and aldehyde dehydrogenases are present in cytosol. To get the drug exposed to the full hepatic complement of drug-metabolizing enzymes, including phase 2 enzymes such as uridine diphosphate glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), hepatocytes can be used and the availability of cryopreserved hepatocytes has advanced the field considerably. Thus, it is important to look at different matrices to make sure that specific metabolic pathways are not missed. In the metabolic stability experiments, the compounds are incubated for a short period (20–60 min for microsomes and 1–4 h with

hepatocytes) with appropriate cofactors and the abundance of the parent compound is monitored as a function of the incubation time. Sometimes, the percentage parent compound remaining is reported, but the data are usually converted to a half-life, $t_{1/2}$, and CL_{int} or CL_{hep} values using appropriate scaling factors.

In most cases, microsomes are used as the tier 1 assay with hepatocytes as a downstream, lower capacity tier 2 assay. However, as projects progress and more is known about the metabolic fate of compounds, the cascade can and should be revised accordingly. For example, if direct glucuronidation appears to be the major metabolic pathway, hepatocytes should be used as the tier 1 assay. Metabolism is not limited to the liver and can occur in other organs, such as the intestine, kidneys, and lungs, as well. Microsomes of those tissues are commercially available. Usually, most attention is paid to metabolism in the liver, but if it becomes clear that hepatic metabolism cannot explain the observed *in vivo* clearance, other tissue should be considered and microsomes of those tissues can be incorporated in screening cascades.

3.4.3 Phenotyping

To better understand the human PK and to assess the likelihood of a compound being the “victim” of a drug–drug interaction, it is important to identify the individual enzymes responsible for the metabolism of a compound. This is commonly done for CYP and/or UGT enzymes. The experiments involve incubating the compound with recombinant enzymes or incubating the compound with microsomes and using specific antibodies or chemical inhibitors.

3.4.4 Drug–Drug Interaction

Several drugs, such as cerivastatin, cisapride, terfenadine, and mibefradil, have been withdrawn because of severe drug–drug interaction, and therefore, this should be addressed early on in the screening cascade. The most ubiquitous drug–drug interactions are the result of inhibition of CYP enzymes. Reversible CYP inhibition can be determined by looking at the inhibition of metabolism of probe substrates to specific metabolites mediated exclusively by a specific CYP enzyme. Examples of commonly used probe substrates are provided in Table 3.4.

If throughput is an issue, the percent inhibition of turnover of the probe substrates at a specific concentration of the drug candidates can be determined. Alternatively, multiple concentrations of the drug candidates can be used and an IC_{50} value can be determined. The IC_{50} value provides value information to assess the risk of a clinical drug–drug interaction, but quantitative predictions also require information about the anticipated exposure of the “perpetrator” and the fraction of the “victim” drug being metabolized by the inhibited CYP.

Mechanism-based CYP inhibition should be explored as well, because it inactivates the enzyme either in a covalent manner (heme or apoprotein adduct formation) or via metabolite–intermediate complex formation and requires *de novo* synthesis of the enzyme. Indeed, many of the most pronounced drug–drug interactions are due to mechanism-based CYP inhibition [35] and it is remarkably prevalent these days; an analysis by Zimmerlin *et al.* [36] indicated that 4% of 400 marketed drugs were positive, whereas 23% of 4000 randomly selected new chemical entities from Novartis

TABLE 3.4 Probe Substrates and LC-MS/MS Detection Method Used for Inhibition of Specific Cytochrome P450 Enzymes

Enzyme	Reaction	Ionization Mode	MRM Transition
CYP1A2	Phenacetin- <i>O</i> -deethylation	+	152 → 110
CYP2B6	Bupropion hydroxylation	+	256 → 238, 139
CYP2C8	Paclitaxel-6 α -hydroxylation	+	870 → 286, 105
CYP2C9	<i>S</i> -Warfarin-7'-hydroxylation	+	325 → 179
CYP2C9	<i>S</i> -Warfarin-7'-hydroxylation	—	323 → 177
CYP2C9	Tolbutamide-4'-hydroxylation	+	287 → 171, 89
CYP2C9	Tolbutamide-4'-hydroxylation	—	285 → 186
CYP2C19	<i>S</i> -Mephenytoin-4'-hydroxylation	+	235 → 150
CYP2C19	<i>S</i> -Mephenytoin-4'-hydroxylation	—	233 → 190
CYP2D6	Dextrometorphan- <i>O</i> -demethylation	+	258 → 199, 157
CYP2E1	Chlorzoxazone-6-hydroxylation	+	186 → 130
CYP2E1	Chlorzoxazone-6-hydroxylation	—	184 → 120
CYP3A4/5	Midazolam-1'-hydroxylation	+	342 → 324, 297, 203
CYP3A4/5	Testosterone-6 β -hydroxylation	+	305 → 269

drug discovery projects were positive. Mechanism-based inhibition is characterized by the following:

- NADPH-dependent inactivation;
- concentration-dependent inactivation;
- time-dependent inactivation;
- enzyme activity not restored by dialysis;
- inactivation rate diminished in the presence of competing substrate;
- spectral shift from 450 nm.

The most common assay format involves preincubation of the drug candidate in microsomes followed by dilution and incubation with an appropriate probe substrate (Table 3.4). The preincubation may give rise to a marked decrease in activity of the CYP enzyme and, hence, reduced turnover of the probe substrate specific for that enzyme. Because the experiment is carried out in a time-dependent manner, this phenomenon is usually called *time-dependent inhibition* (TDI), but it is important to realize that the observed inhibition can be due to both mechanism-based inhibition and the formation of metabolites that are potent CYP inhibitors. To increase throughput, the experiment can be performed with a single preincubation period (usually 30 min) with multiple concentrations of the investigative drug in the absence and presence of NADPH. The extent of TDI is reflected by the shift in the IC₅₀ value, hence the name “IC₅₀ shift assay” [37]. To enable clinical prediction, multiple concentrations and multiple preincubation times are desirable, and K_I and k_{inact} values can be obtained after mathematical transformation of the data. As explained for reversible CYP inhibition, quantitative predictions also require information about the anticipated exposure of the “perpetrator” and the fraction of the “victim” drug being metabolized by the inhibited CYP.

Both reversible and mechanism-based CYP inhibition have been performed in hepatocytes, but the use of microsomes is most ubiquitous.

3.4.5 CYP Induction

CYP induction assays are usually not tier 1 assays in drug discovery projects because the prevalence of induction is limited. However, for certain classes of drugs, induction is quite common. The gold standard assay is incubation of the drug candidates with multiple batches of fresh hepatocytes for 24–48 h. After removal of the supernatant and washing of the cells, the hepatocytes are incubated with specific probe substrates (Table 3.4) to determine via functional activity if the expression of specific CYP enzymes is up-regulated. Alternatively, mRNA expression can be monitored. Cell viability should be monitored as well, because it could circumvent detection of CYP induction. This is clearly a low throughput assay and for projects with specific induction issues pregnane X receptor (PXR) binding is usually employed to enable sufficient throughput and an adequate turnaround time.

3.4.6 Plasma Protein Binding and Tissue Binding

Knowing the non-covalent binding of a drug to plasma proteins or lipids in tissue is important because the free concentration is the presumed driver of efficacy (the “free drug hypothesis” or “free drug concept”) [38,39]. However, it is not a parameter that should be “optimized” (see below). In plasma, most drugs bind extensively to plasma proteins such as albumin (neutral compounds and acids) and α -acid glycoprotein (basic compounds). In tissues, binding is much less specific and mainly reflects affinity for interaction with phospholipids. Several assays are available to determine the free or unbound drug concentration. Ultrafiltration and ultracentrifugation are used, but the gold standard is considered to be dialysis. Dialysis can be performed with individual tubes or using a 96-well block with buffer on one side and plasma or tissue homogenate on the other side. After incubation, both sides are sampled. If binding is high (plasma protein binding in excess of 99% is not uncommon for higher MW and/or lipophilic drugs), a very sensitive LC-MS/MS assay has to be used. For proper PK/PD modeling, it is important to know the free tissue concentration at the site of action and this can be obtained by multiplying the total concentration and the free fraction in the relevant tissue. In the absence of the involvement of uptake or efflux transporters, the free drug concentration in plasma and tissue should be the same, although the free fraction in plasma and tissue, and hence the total drug concentration in plasma and tissue, may be quite different.

3.4.7 Drug Transport Assays

Studying drug transport has become more common because many drugs are to some extent substrates or inhibitors of uptake or efflux transporters [40]. The most extensively studied drug transporter is P-gp (also called *MDR1*) because it can (i) limit intestinal absorption, (ii) enhance biliary excretion, and (iii) limit brain and/or tumor penetration. Thus, for central targets requiring sufficient brain exposure, it is essential to incorporate a P-gp assay early on in the screening cascade. Efflux in Caco-2 or MDCK cells may be indicative of the compound being a P-gp substrate, but the evidence is not conclusive until confirmed by efflux reduction with a specific P-gp inhibitor. A more common assay involves cells overexpressing P-gp. Cell lines for other transporters such as BCRP, OATs, OCTs, OATPs, and MRPs are available as well, but are usually not used

extensively in drug discovery, because they do not seem to play a major role in drug disposition with the exception of specific classes of drugs (such as hepatic uptake of statins via OATPs).

Many pharmaceutical companies have higher throughput ADME screening facilities for these assays (3.4.1–3.4.7). The assays are heavily automated and use 96- or 384-well format [41,42]. The end points usually involve sample analysis via LC-MS/MS. A capacity of 200–1500 compounds per week through the tier 1 assays (usually metabolic stability in microsomes and reversible CYP inhibition) is not uncommon [43]. Although these data can and should be used to influence design in drug discovery, they are not rigorous enough for regulatory submissions and more customized assays are required for that purpose.

3.5 *IN VIVO* PHARMACOKINETIC STUDIES

In vivo PK studies continue to play an important role in drug discovery and development. The purpose of these studies is threefold:

- determination of exposure in preclinical species to inform subsequent PD or efficacy studies
- determination of exposure in preclinical species to aid in the interpretation of toxicology studies
- determination of PK parameters in preclinical species to aid in the identification of compounds with appropriate human PK.

The availability of sensitive and selective mass spectrometers interfaced with LC systems has greatly facilitated the incorporation of *in vivo* PK studies in drug discovery. In most cases, exposure in plasma is measured, but other matrices such as blood, urine, feces, or specific tissues of pharmacological or toxicological interest may be sampled as well. Some companies rely heavily on *in vivo* PK data in their screening cascades necessitating higher throughput approaches. The most common approaches are the following.

3.5.1 Cassette or “n-in-one” Dosing

A mixture of drugs is administered to the same animal resulting in a significant reduction in the number of animals used for PK studies. With the advent of highly selective and sensitive triple quadrupole mass spectrometers operating in the multiple reaction monitoring (MRM) mode (Fig. 3.4), it is feasible to determine the exposures of all drugs in the cassette [44,45]. Nevertheless, it is important to select the drug candidates in the cassette such that common metabolic pathways do not interfere with the detection of the parent compounds. For example, drug candidates that differ in MW by 16 or 32 Da should be avoided. Drug–drug interaction via inhibition of CYPs or other metabolic enzymes is not inconceivable either [46]. However, this would generally result in a higher exposure and these “false positive” compounds would be captured in subsequent experiments. Thus, the risk of “false negatives” (i.e., compounds with lower exposure in a cassette than as a single agent) is low. Nevertheless, the dose is usually lowered in cassette studies to reduce the risk of drug–drug interaction. In

Triple Quadrupole Scan Modes					
	Ionization		Fragmentation		Detection
Product ion spectrum	ABC ⁺	→	A ⁺ + BC	→	A ⁺
			AB + C ⁺		C ⁺
	ABD ⁺	→	A ⁺ + BD	→	A ⁺
			AB + D ⁺		D ⁺
Precursor ion spectrum	ABC ⁺	→	A ⁺ + BC	→	A ⁺
			AB + C ⁺		A ⁺
	ABD ⁺	→	A ⁺ + BD	→	A ⁺
			AB + D ⁺		A ⁺
Constant neutral loss spectrum	ABC ⁺	→	A ⁺ + BC	→	C ⁺
			AB + C ⁺		C ⁺
	ABD ⁺	→	A ⁺ + BD	→	D ⁺
			AB + D ⁺		D ⁺

Figure 3.4 Principles of the product ion, precursor ion, and constant neutral scan modes feasible with a triple quadrupole mass spectrometer.

addition, a reference compound with well-established PK and a close analog of the drug candidates under investigation is usually included in every cassette. Generally, the substantial increase in output outweighs the (manageable) risks.

3.5.2 Sample Pooling

To avoid the complications associated with cassette dosing as described above, some companies employ sample pooling. In this approach, individual compounds are dosed and plasma samples are subsequently pooled and analyzed with a triple quadrupole mass spectrometer (e.g., cassette-accelerated rapid rat screen [47]). This technique eliminates the risk of PK drug–drug interaction, but the risk of bioanalytical interference due to metabolites remains.

In addition, equipment such as automated blood sampling, multiplexing, and column switching with staggered parallel analysis is routinely used to enable higher throughput for *in vivo* PK studies. Although MRM on a triple quadrupole mass spectrometer is the mainstay in bioanalysis, there is a recent push to use high resolution MS [48]. The data are gathered with a single stage of mass analysis, which would result in a loss of selectivity, but the high resolution makes up for that. Moreover, it is now possible to monitor both the parent compound and metabolites without prior knowledge about the nature of the metabolites.

3.6 TISSUE DISTRIBUTION

Knowledge of tissue distribution can be valuable to relate to efficacy or toxicity in a particular tissue. Some compounds are taken up in tissues and are present in much higher concentrations than in plasma either due to uptake transporters, preferential binding to cellular components (e.g., lipids or melanin), or pH-mediated trapping of the charged form of the drug (e.g., lysosomal trapping). Alternatively, there can be

active efflux of drug candidates out of tissues such as the brain. The latter can be either desirable (e.g., limited brain penetration of cytotoxic oncology drugs that target non-CNS tumors) or undesirable (e.g., limited brain penetration for CNS agents). A comprehensive view of tissue distribution can be obtained by quantitative whole body autoradiography (QWBA), but this requires radiolabeled material, which is usually not available in drug discovery. Moreover, QWBA cannot distinguish the parent compound from metabolites. A more convenient, but time-consuming, approach is excision of individual tissues, followed by homogenization and quantitation by LC-MS/MS. This technique does not require radiolabeled material and is very sensitive; the MRM mode prevents significant interference by endogenous components. However, each tissue must be examined separately and the distribution within each tissue is lost.

Imaging via matrix-assisted laser desorption/ionization (MALDI) was introduced in the early twenty-first century and it has been successfully used to detect drugs and their metabolites in tissues [49]. Tissue or whole body slices are obtained in the same manner as for QWBA, but the slices are subsequently coated with a finely dispersed matrix solution (such as sinapinic acid in methanol). The solvent solution extracts the analytes out of the tissue and the matrix itself allows ionization after irradiation with a laser. In most cases, detection in the MS/MS mode is preferred to prevent interference by the abundant matrix ions. The tissue distribution of either the parent drug and/or its metabolites is obtained by scanning the laser across the tissue with a spatial resolution of about 50 μm . Although significant progress has been made, this technique still suffers from limited sensitivity and lengthy data acquisition. The latter can be addressed by acquiring data in the MS mode with a high resolution mass spectrometer and extracting the images for the parent drug and its metabolites post-acquisition [50].

3.7 METABOLITE IDENTIFICATION

LC-MS/MS is a very powerful tool for metabolite identification. Metabolites generated from *in vitro* incubations or *in vivo* studies can be detected with relative ease and this information can be communicated to medicinal chemists to address specific metabolic liabilities in the next generation of compounds. Several mass spectrometric scan modes are available to aid in the assignment of structures of metabolites (Fig. 3.4). The most common scan modes are as follows:

- *Product Ion Scan.* A protonated or deprotonated metabolite is selected with the first mass analyzer and subsequently fragmented by collision with an inert gas. Finally, the fragments are analyzed according to their mass or, to be precise, their mass-to-charge (m/z) ratio in the second mass analyzer.
- *Constant Neutral Loss Scan.* In this scan mode, the first and second mass analyzers are scanned with a fixed offset that corresponds to a common fragment lost from the parent drug and/or its metabolites in the collision cell. For example, glucuronide metabolites can be detected by performing a 176-Da constant neutral loss scan in the positive ion mode and glutathione adducts can be detected with a 129-Da constant neutral loss scan.
- *Precursor Ion Scan.* A precursor ion scan allows monitoring of ions (corresponding with the parent drug and/or its metabolites) that generate a common fragment ion. In this scan mode, the first mass analyzer is scanned and the second mass

analyzer is held constant at the mass-to-charge ratio of the common fragment ion. For example, glutathione adducts can be detected by a m/z 272 precursor ion scan in the negative ion mode [51].

Note that not all scan modes are available on all types of mass spectrometers due to design limitations. However, the most common type of mass spectrometer, the triple quadrupole instrument, allows all three of these scan modes. With the availability of high resolution time-of-flight, orbitrap, and Fourier-transform mass spectrometers, it is possible to obtain accurate mass values in either the MS or the MS/MS modes, which greatly facilitates interpretation of spectra and assignment of metabolite structures. In the MS mode, an accurate mass provides—with a reasonable degree of confidence—the molecular formula of the metabolite and thereby the type of biotransformation involved. In the MS/MS mode, accurate mass values of the fragment ions facilitate interpretation of the MS/MS spectrum and, consequently, assignment of the structure of the metabolite. An additional feature of a high resolution mass spectrometer is the ability to apply a mass defect filter to the data file to remove endogenous matrix-related ions [52,53]. The mass defect refers to the fractional mass of the parent compound and its metabolites obtained using the accurate mass for each atom. For example, the exact mass of atorvastatin is 558.2521 Da and the corresponding mass defect is 0.2521 Da. Metabolites with minimal structural changes relative to the parent compound (i.e., +O, +2O, and -CH₂) can be detected with a mass defect filter similar to that used for the parent compound because the change in fractional mass is minimal (-0.0051, -0.0102, and -0.0156 Da for +O, +2O, and -CH₂, respectively). For conjugates such as sulfates or glucuronides, the mass defect filter should reflect the change in the fractional mass of the conjugates.

Despite these advances in the field of MS, it is still very hard to identify the exact site of metabolism, in particular for the most common metabolic process: aliphatic and aromatic hydroxylation. Identification is usually limited to determination of the moiety of the molecule that was metabolically transformed and a Markush structure of the metabolite is obtained. Sometimes, the exact site of metabolism may be unambiguous (e.g., for simple N-dealkylations), but for common pathways, such as oxidation, it is frequently required to isolate the metabolite and obtain NMR data to identify the exact structure. Additional tools available to facilitate metabolite identification are as follows:

- isotope patterns of $[M + H]^+$ or $[M - H]^-$ ions; metabolite identification is greatly facilitated by the presence of atoms with a unique isotope pattern such as chlorine and bromine;
- presence of a radiolabel (usually ³H or ¹⁴C);
- chemical derivatization;
- hydrogen–deuterium exchange [54];
- studies with close in analogs.

More recently, it has been advocated to integrate quantitative bioanalysis and metabolite identification in one experiment. This can most easily be achieved using a high resolution instrument operating in the MS mode [48]. The high resolution ensures appropriate selectivity for detection and quantitation of the parent compound.

In addition, the data can be interrogated for the presence of metabolites and the accurate mass measurement can provide the molecular formula of the metabolite. A subsequent experiment may be required to obtain structural information via product ion scans. Alternatively, the instrument can automatically switch from MS to MS/MS mode (provided it has this capability) in a data-dependent manner. It remains to be seen whether the quality of the quantitative information is sacrificed.

Another area of metabolite identification that is receiving more and more attention is the detection of adducts that are indicative of bioactivation [55,56]. Many drugs are bioactivated and there is some (mostly circumstantial) evidence that reactive intermediates may give rise to (idiosyncratic) toxicity. Although the reactive intermediates cannot be detected, it is possible to trap them with agents such as glutathione (abundant in the liver and an effective nucleophile by virtue of its cysteine sulfhydryl group), potassium cyanide (a “hard” nucleophile for detection of iminium species), and methoxylamine and semicarbazide (to detect aldehydes). LC-MS/MS assays have been implemented to facilitate detection of these adducts. For example, glutathione adducts can be detected by a 129-Da neutral loss scan in the positive ion mode or a m/z 272 precursor ion scan in the negative ion mode [51]. Some labs have taken this a step further and are examining covalent binding to cellular components (usually proteins). Nakayama *et al.* [57] looked at the correlation between covalent binding and glutathione adduct formation and TDI using a data set of 42 structurally diverse compounds. First, they found no statistically significant correlation between the formation rate of glutathione adduct formation and the extent of covalent binding; several compounds, including pioglitazone and ritonavir, showed extensive covalent binding (>50 pmol/min/mg protein as defined by Evans *et al.* [55]). It would be interesting to see whether a correlation would have appeared if other trapping agents, such as cyanide and methoxylamine, would have been incorporated in the assay. Second, there was no statistically significant relationship between the enzyme inactivation rate (as assessed by the TDI assay) and the extent of covalent binding. Third, the authors calculated the intrinsic clearance for forming reactive metabolites by summing (i) the intrinsic clearance for forming reactive metabolites based on the formation rate of glutathione adducts and (ii) the intrinsic clearance for forming reactive metabolites based on the enzyme inactivation rate in the TDI assay. Fortunately, there was a statistically significant correlation between the intrinsic clearance for forming reactive metabolites as defined above and the extent of covalent binding with no obvious false negatives (clean in the TDI assay and/or glutathione trapping assay, but more than 50 pmol/min/mg protein in the covalent binding assay). Thus, this suggests that the combination of the TDI and the glutathione trapping assays can serve as a surrogate to assess the risk for covalent binding. Many scientists have advocated the use of covalent binding as a surrogate for toxicity, but it has been shown that there is no clear relationship between the extent of covalent binding and hepatotoxicity [58]. Moreover, it requires radiolabeled material and, therefore, is not very practical in drug discovery. A most interesting finding in the analysis of Nakayama *et al.* [57] is that atorvastatin was determined to have covalent binding of 352 pmol/min/mg protein, which far exceeds the guideline of 50 pmole/min/mg protein [55]. Nevertheless, atorvastatin is remarkably safe and commercially successful and the low dose, 10–80 mg, may be the main reason for this. It has been well established that the vast majority of drugs withdrawn from the market due to idiosyncratic adverse drug reactions (IADRs) or having a black box warning due to IADRs have a dose of >100 mg [59].

In conclusion, the degree of concern about formation of reactive metabolites should be guided by the following parameters:

- clinical dose and exposure;
- duration of therapy (short vs long duration);
- therapeutic area (life threatening or not?);
- target population (young vs old, healthy vs diseased);
- first in disease/class or not;
- lead compound versus backup;
- species differences (bioactivation may be unique to one species).

3.8 IN SILICO ADME MODELING

A substantial benefit from higher throughput *in vitro* ADME screening is that there is now a wealth of data available that can be used to build sophisticated *in silico* ADME models. *In silico* models have been available for years and some of them are commercially available. However, most of the commercially available models are of limited value because the chemical diversity of the data used to build the model is limited in size and, sometimes, the data were obtained from the literature using different experimental protocols. High throughput ADME screening—generating in excess of 10,000 data points per year—has overcome these hurdles and, consequently, models have been built for most *in vitro* ADME endpoints: permeability, metabolic stability in microsomes and hepatocytes, microsomal binding, plasma protein binding, reversible and time-dependent CYP inhibition, and so on. [60]. Although global models are useful, in some cases, it may be better to build a local model that better defines the chemical space of interest. The models involve a range of chemical descriptors and/or the structures of the compounds in the training set. Different statistical methods (regression methods: partial least squares; Bayesian methods; supervised learning methods: decision trees, support vector machine; and neural networks) can be used as well. The output of the model can either be categorical or numerical. Besides the output, the confidence in the prediction is a critical parameter. The confidence in the prediction is usually derived from (i) the structural similarity of the compound under investigation with those in the training set and (ii) the number of structurally similar compounds (nearest neighbors). This, combined with an objective validation of the model using compounds not in the training set, guides the ultimate use and incorporation of the model in drug discovery. For example, Lee *et al.* [61] built an *in silico* model for metabolic stability in microsomes and showed that 72% of the compounds without close nearest neighbors were predicted correctly, whereas 88% of the compounds with more than 33 nearest neighbors were predicted correctly. The same phenomenon is also apparent from the data in Fig. 3.5 showing the correlation between the *in silico* and *in vitro* human liver microsomal extraction ratio. The color of the symbols characterizes the similarity with the compounds in the training set and the shape of the symbols reflects the number of nearest neighbors in the training set. Generally, the dark gray squares in Fig. 3.5 (similarity score ≤ 0.7 ; number of nearest neighbors ≤ 7.8) reflect compounds with less similarity and fewer nearest neighbors and, not surprisingly, the predictions are less

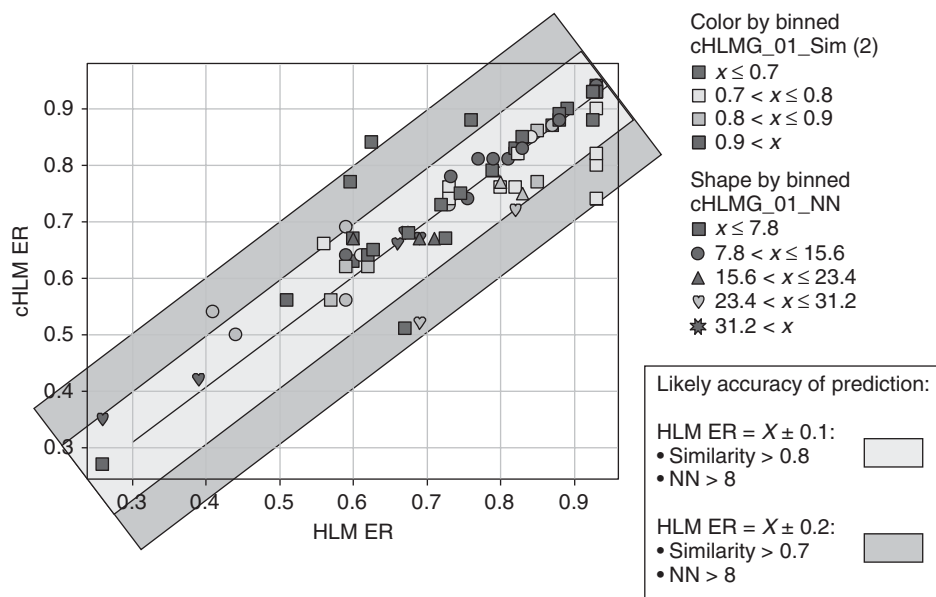


Figure 3.5 Extraction ratio (ER) measured in human liver microsomes (HLM) versus the calculated ER in HLMs derived from an *in silico* metabolic stability model. The color of the symbol is indicative of the similarity (Sim) and the shape is indicative of the number of nearest neighbors (NN). (See color insert.)

accurate. It is important that the data set to build the *in silico* models is regularly updated to reflect the evolving chemical space.

Although models serve a very useful purpose, most models are generally not meant to replace experimental data—or at least not at this stage. The main goal should be to increase the *probability* of identifying compounds with optimum properties, such as metabolic stability or permeability, and to identify the SAR of the ADME properties. Indeed, the models can be used to triage compounds *before* synthesis and speed up the lead optimization process. In-house data at Genentech show that the number of compounds with good microsomal stability (CL_{hep} in human liver microsomes $< 6.2 \text{ mL/min/kg}$) increased from $< 10\%$ of all new compounds synthesized to slightly more than 20% after implementation of an *in silico* metabolic stability model in 2010, but without dramatic changes in the portfolio. Of course, there are many reasons to synthesize particular compounds and if there is a good design hypothesis (e.g., based on specific interactions with the target derived from the crystal structure or a homology model), synthesis should proceed despite unfavorable ADME predictions. Ideally, multiple *in silico* models, including potency models, are available and used in a parallel manner to triage very large virtual libraries. Unfortunately, the progress with building reliable *in silico* models for potency appears to lag behind the advances with *in silico* ADME models. The *in silico* ADME model can also be used to triage compounds already synthesized before submission to *in vitro* ADME assays if the capacity of the assays is limited. For example, if the model is highly confident that a particular compound is metabolically unstable, there is no reason to submit it for experimental verification.

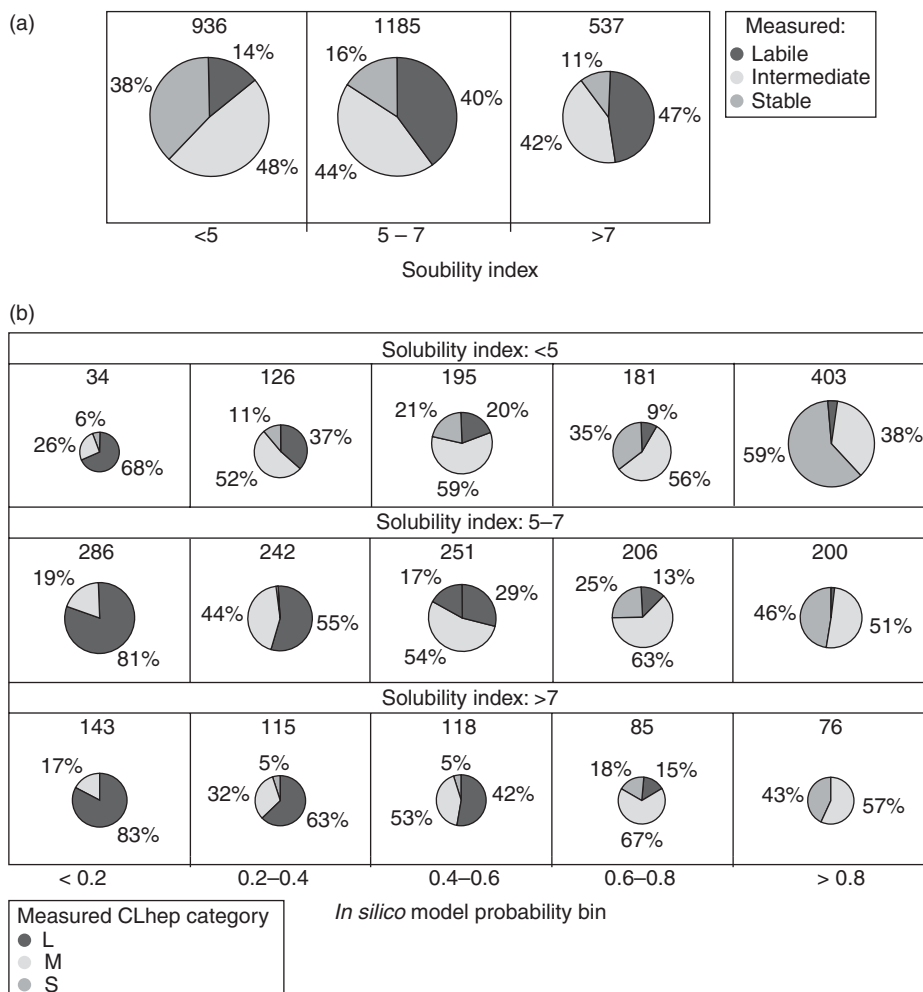


Figure 3.6 (a) Relationship between solubility index (sum of the log P and the number of aromatic rings) and the metabolic stability in human liver microsomes. The following definitions for metabolic stability are used: stable $CL_{hep} < 6.2$ mL/min/kg (gray), intermediate $6.2 \text{ mL/min/kg} < CL_{hep} < 15$ mL/min/kg (light gray), and unstable $CL_{hep} < 15$ mL/min/kg (dark gray). (b) Relationship between the predicted probability of being stable in the human liver microsomes metabolic stability *in silico* model (0, infinitely small probability of being stable; 1, highest probability of being stable) and the measured metabolic stability as a function of the solubility index. (None of the compounds examined here were in the training set of the model.) The definitions are the same as in (a). (See color insert.)

The value of *in silico* models is also illustrated by the data in Fig. 3.6. Figure 3.6 shows the predicted and experimental human liver microsomal metabolic stability data of more than 2600 compounds not in the training set as a function of the solubility index. The *in silico* metabolic stability model is built with a training set of *in vitro* data from close to 20,000 diverse compounds obtained under consistent experimental conditions. The solubility index is the sum of the log P and the number of aromatic

rings and the combination of these two parameters is a reasonably reliable indicator of the experimental solubility and can be routinely calculated [62,63]. A solubility index of 7 or more indicates a high *likelihood* of very low solubility, $\leq 1 \mu M$, and a solubility index of 5 or less is usually desirable. First, the experimental data in Fig. 3.6a show that compounds with a solubility index of <5 have a 3.5-fold greater probability of being *in vitro* metabolically stable than compounds with a solubility index of 7 or more (38% vs 11%). The latter probably reflect the increased likelihood of large lipophilic compounds with many aromatic rings to be both poorly soluble and extensively metabolized. Second, Fig. 3.6b shows that the ability of the *in silico* metabolic stability model to identify compounds that are experimentally stable in human liver microsomes is better for compounds with a low solubility index. For compounds that have a predicted probability of being stable >0.8 (0 = infinitely small probability of being stable; 1 = highest probability of being stable) close to 60% are indeed metabolically stable if the solubility index is <5 , whereas about 40% are experimentally stable if the solubility index is more than 7. Thus, both the solubility index and the *in silico* metabolic stability model can serve as a tier 0 screen to improve the *probability* of identifying compounds with better PK and pharmaceutical properties.

The output of an *in silico* CYP3A4 time-dependent CYP inhibition model is presented in Fig. 3.7. The TDI model is based on a training set of close to 2000 compounds with midazolam or testosterone as the probe substrates of 3A4 and using an “IC₅₀

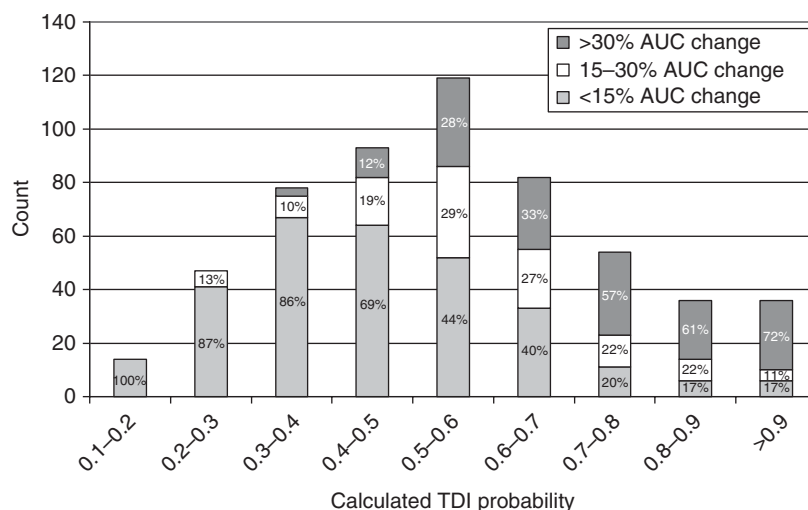


Figure 3.7 Relationship between the predicted probability of displaying time-dependent inhibition (TDI) of CYP3A4 using an in-house *in silico* model (0, very low probability for TDI; 1, high probability for TDI) and the measured extent of TDI using an “IC₅₀ shift” protocol with the fold-change in area under the percent activity remaining versus inhibitor concentration curve (i.e., AUC change) rather than the traditional fold-change in IC₅₀ value as the experimental endpoint to determine the magnitude of TDI [64]. (None of the compounds examined here were in the training set of the model.) The following definitions for TDI are used: low risk $< 15\%$ AUC change (light gray), intermediate risk between 15% and 30% AUC change (white), and high risk $> 30\%$ AUC change (dark gray).

shift” protocol with the fold-change in area under the percent activity remaining versus inhibitor concentration curve rather than the traditional fold-change in IC_{50} value as the experimental endpoint to determine the magnitude of TDI [64]. The output of the model is the probability of showing TDI with 1 representing a very high probability for TDI and 0 a very low probability. Although using hard “cutoff” values is not advisable, a probability of 0.7 or more indicates a high probability of displaying TDI, whereas below 0.4 the risk is much lower. Nevertheless, there is a finite probability for both “false negatives” and “false positives”—as is the case for any *in silico* model despite an effort to minimize them while building the model. “False negatives” are of limited concerns because compound synthesis will proceed and the experimental data will ultimately supersede the *in silico* data. “False positives” may greatly concern project teams because those compounds may be dropped from further pursuit. Although that may be alarming, other compounds that are presumably predicted to display superior properties are synthesized instead. Thus, the detrimental impact may be minimal and the overall positive impact of incorporating *in silico* models far outweighs this risk.

As mentioned before, some models are commercially available and models such as MetaSite and StarDrop have proven to be relatively reliable. Although the underlying computational algorithms are different for MetaSite and StarDrop, both are capable of predicting the site of metabolism considering both the intrinsic reactivity of each potential site to oxidative metabolism by CYP enzymes as well as the accessibility of the site of metabolism to the active oxy-heme species in the CYP enzymes. Using the literature and in-house compounds, it was shown that both MetaSite and StarDrop are capable of predicting the right site of metabolism about 60–90% of the time for CYP2C9, CYP2D6, and CYP3A4 [65]. Note that both software packages predict the site of metabolism, but not the exact metabolic pathway nor the rate of metabolism. An example of the use of MetaSite is presented in Fig. 3.8 (only partial structures are shown because the structures are still proprietary). A potent compound was identified, but its clearance in mice was very high, 157 mL/min/kg. MetaSite predicted the site of metabolism as the 4-position of the piperidine ring. Next, a compound was synthesized with a substituent at the 4-position and the clearance was much lower, 8.6 mL/min/kg, and the free fraction in plasma was slightly increased as well. Subsequent metabolite identification efforts with the first compound indicated that the piperidine ring was indeed the site of metabolism. Unfortunately, the potency of the second compounds was more than 1000-fold lower.

There is another inherent and implied advantage of using *in silico* ADME models and that is that it enables evaluation of hits from the HTS screen. In the past, the

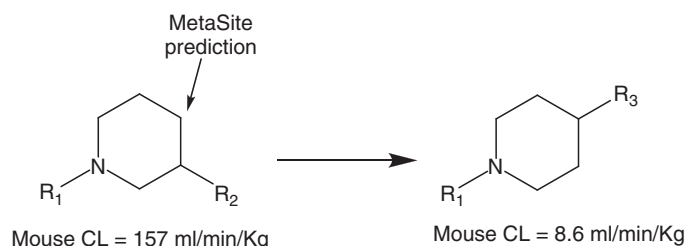


Figure 3.8 MetaSite prediction of the site of metabolism for a high clearance compound inspiring chemical modification that resulted in a low clearance compound.

decision to pursue a certain chemical series was based on its potency and chemical intuition. However, it has been shown that different medicinal chemists view attractiveness of a compound or series remarkably different [66]. The *in silico* ADME models allow early incorporation of appropriate ADME parameters and have them weighted as important as endpoints such as potency and selectivity. This may be particularly important because on many occasions, it is easier to optimize potency and selectivity than ADME properties. Thus, early and appropriate incorporation of *in silico* ADME models followed closely by experimental verification will facilitate identification of drugable chemical space and subsequently speed up lead optimization.

3.9 MULTIPARAMETER OPTIMIZATION

Currently, the lead optimization process is much more complex than it was even 10 years ago because the sheer quantity of parameters and data has grown almost exponentially. The ability of a human, even an experienced drug “hunter,” to optimize more than three parameters in a simultaneous manner is severely limited. Thus, computational tools are available that allow the user to incorporate multiple parameters and rank order the compounds. A simple model resembling a traffic light that can be used to assess absorption in the gastrointestinal tract is presented in Table 3.5 [23] and incorporates solubility, lipophilicity, the corrected MW (see above), PSA, and the number of rotatable bonds. The “cutoff” values for each property are presented in Table 3.5 and the overall score is a summation of those for the individual parameters with a score of 0 being ideal and 12 being highly undesirable. An analysis showed that 70% of the oral drugs have an overall score of 2 or less, whereas HTS hits from 20 screens showed a Gaussian-like distribution peaking at a score of 5. Additional parameters can be incorporated easily and these parameters can be either computational or experimental in origin.

Another example is the model used to assess whether a compound is likely to be able to cross the blood–brain barrier [67]. The parameters included are $\text{clog } P$, $\text{clog } D$, MW, TPSA, number of HBDs, and $\text{p}K_a$. Each parameter has a desirable and less desirable range and a transition resulting in a score of 0–1 with 1 being most desirable. Subsequently, the individual scores for the six parameters are summed with 6 being the highest possible score. Most CNS drugs have a score of 4 or more. Not surprisingly, experimental verification indicates that a high score is accompanied by a high passive permeability (P_{app} value in MDCK cells), low P-gp liability (efflux ratio

TABLE 3.5 Traffic Light Multiparameter Optimization Tool

Traffic Light Color	Traffic Light Value	Solubility (mg/L)	$\text{clog } P$	Corrected Molecular Weight	PSA ($\text{\AA}^2$)	Rotatable Bonds
Green	0	≥ 50	≤ 3	≤ 400	≤ 120	≤ 7
Yellow	1	10–50	3–5	400–500	120–140	8–10
Red	2	< 10	> 5	> 500	> 140	≥ 11

Source: Data from Lobell *et al.* [23].

in P-gp overexpressing MDCK cells), and low unbound intrinsic clearance ($CL_{int,u}$ in human liver microsomes).

The two examples described above were focused on physicochemical parameters and each input parameter had the same weight. It is also possible to include a range of experimental data and more sophisticated tools allow the user to adjust the weight of each parameter and to incorporate experimental uncertainty [68].

Human dose predictions in early drug discovery (in contrast to dose predictions performed during candidate nomination, which are based on more sophisticated human PK prediction and PK/PD analysis) is another form of multiparameter optimization, although it may not be perceived by many as such. For example, the dose can be predicted with Equation 3.1 using the following parameters:

$C_{ss, avg}$, cellular potency IC_{50} value;

CL , human clearance derived from human liver microsomes or hepatocytes or rat–human scaling;

τ , dosing interval (usually 12 or 24 h);

F , back-calculated bioavailability assuming that the clearance is solely hepatic ($1 - CL/Q$) or based on preclinical bioavailability data.

$$\text{Dose} = \frac{C_{ss, avg} \cdot CL \cdot \tau}{F} \quad (3.1)$$

It is important to emphasize that this is not meant to be an accurate dose prediction. It is merely meant to provide an indication about the order of magnitude and it can be used to categorize compounds/chemical series.

A more comprehensive overview of multiparameter optimization in drug discovery is presented in Ref. 69.

3.10 INCORPORATION OF ADME DATA IN DRUG DISCOVERY TO IMPROVE PHARMACOKINETICS

Incorporation of all relevant parameters in drug discovery in a *prospective* effort to find a drug with the “perfect” balance of properties is extremely challenging and time consuming and frequently it is necessary to compromise. Indeed, “... *the level of in vitro potency of a lead for its intended target(s) is the key parameter at the beginning of a drug discovery program. This idea is extended further in lead generation-optimization based on the assumption that the most potent molecules in vitro are generally more likely to translate into more effective therapies, possessing a greater overall safety profile and requiring lower therapeutic doses. However, with regard to the first assumption, from this study and many others in the literature, it seems that the safety profile (ADMET and off-target related effects) is more likely to be inversely correlated with the level of target potency given their opposing relationship with regard to key physicochemical properties*” [9]. The same authors showed that *in vitro* potency can only explain roughly 30% of the variation in dose. Having the right screening cascade to balance out the various properties is critical and prevents late stage surprises. A somewhat generic example for a CNS target is presented in Fig. 3.3.

Table 3.6 summarizes the key physicochemical parameters—both molecular and specific structural features—that influence various ADME properties listed in the third column. Note that many physicochemical parameters are not independent of each other. For example, if the MW increases, the number of rotatable bonds usually increases as well, and the PSA and the total number of HBDs and HBAs are also correlated. The interconnectivity between the physicochemical parameters and the ADME properties have been illustrated with several literature and in-house examples.

The first PK hurdle that oral drugs encounter is absorption. Absorption is influenced by both solubility and permeability. In general, solubility increases with increasing HBD and HBA count, PSA, and the fraction of the bonds that can freely rotate, but is negatively influenced by increasing $\log P$, $\log D$, and the number of aromatic rings [63,70]. However, the exact opposite is usually true for permeability and this necessitate identification of a physicochemical “sweet spot” for absorption. The situation is even more complex if absorption is limited by intestinal efflux. A nice example illustrating the level of complexity was presented by Lévesque *et al.* [71]. The authors were trying to identify a rennin inhibitor for blood pressure control. Earlier, lipophilic compounds with a basic amine functionality were potent and orally bioavailable but suffered from CYP 3A4 and hERG inhibition. To address the latter liabilities, while maintaining potency, the size and lipophilicity of the leads were reduced significantly, but this resulted in negligible bioavailability. Increasing the dose improved the bioavailability somewhat, but the clearance did not change. This, combined with the observation that the majority of the drug was detected in the feces after oral dosing in bile duct cannulated rats, suggested P-gp limited absorption. Indeed, P-gp KO (knockout) mice and rats codosed with elacridar (a P-gp inhibitor) showed improved bioavailability (but no change in clearance). All these compounds showed very poor permeability in LLC-PK cells and, therefore, it was impossible to determine the efflux ratio in LLC-PK cells overexpressing P-gp. Thus, the authors prepared more lipophilic compounds ($\log D_{7.4} > 1.7$) and this increased the passive permeability significantly ($P_{app} > 10 \times 10^{-6}$ cm/s). The latter did not eliminate the efflux as detected in the P-gp overexpressing

TABLE 3.6 Molecular and Structural Features that Influence ADME Properties

Molecular Features	Structural Features	ADME Properties
Molecular weight	Hydrogen bond donors	Solubility and dissolution rate
Molecular volume	Hydrogen bond acceptors	Permeability
pK_a	(Aromatic) rings	Absorption
$\log P$	% sp^3 carbon atoms	Plasma protein binding and tissue binding
$\log D_{7.4}$	Rotatable bonds	Red blood cell partitioning
Polar surface area		Clearance (metabolic, transporter-mediated, passive renal, or biliary)
% Polar surface area		Distribution (including transporter-mediated uptake or efflux)
		Inhibition and induction of drug-metabolizing enzymes and transporters

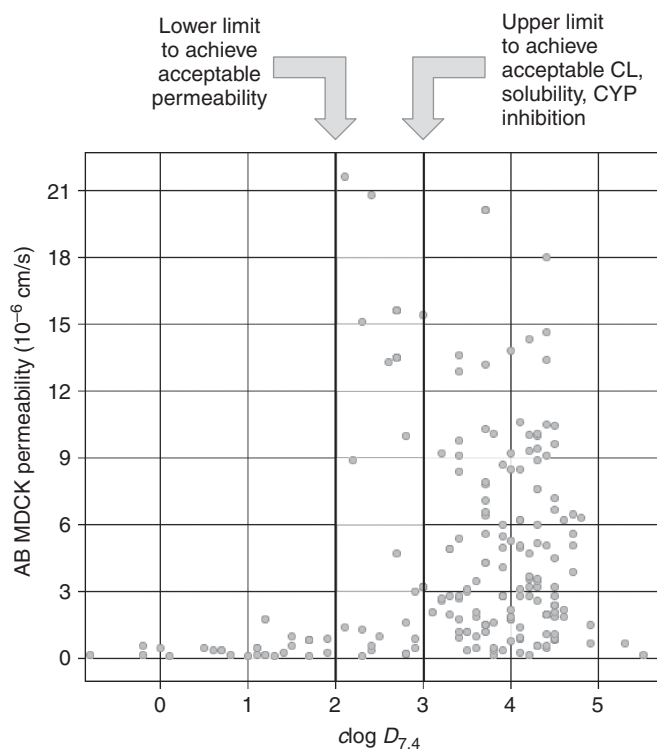


Figure 3.9 Relationship between $\text{clog } D_{7.4}$ and the measured A to B permeability in MDCK cells for a large number of compounds synthesized for an in-house project.

LLC-PK cells (efflux ratio ≈ 20), but the *in vivo* impact was drastically reduced and the bioavailability of many of the more lipophilic compounds exceeded 25%. However, not all the more lipophilic compounds showed good bioavailability and this was ascribed to a significant first-pass effect. Thus, the lipophilicity was modulated to balance potency, bioavailability, CYP inhibition, and hERG. The same phenomena are illustrated in Fig. 3.9 based on data from an in-house project. The AB permeability in MDCK cells is plotted as a function of the $\text{clog } D_{7.4}$ and illustrates that a $\text{clog } D_{7.4}$ of at least 2 is needed to achieve acceptable permeability, although it does not guarantee it. Data not presented here indicated that a $\text{clog } D_{7.4} > 3$ increased the probability of poor solubility and high clearance, resulting in a narrow “sweet spot.”

There appears to be a Gaussian or parabolic relationship between the $\log D_{7.4}$ and the extent of absorption and bioavailability [72]. At $\log D_{7.4} < 0$, a compound's solubility is usually good, but its permeability across membranes is poor resulting in limited absorption. In contrast, at $\log D_{7.4} > 5$, the membrane permeability is adequate, but the solubility is low, which significantly reduces absorption. In addition, metabolic clearance in the intestine and liver, which affects F_g and F_h , tends to be higher for compounds with $\log D_{7.4} > 5$. However, this effect appears to be modulated by MW with the $\log D$ range that leads to good absorption and metabolic stability being wider at low MW than at high MW [73]. The PSA is another important parameter for passive membrane permeability with a PSA in excess of $120 \text{ \AA}^2$ associated with poor absorption

[74] and in excess of $90 \text{ \AA}^2$ associated with poor brain penetration [17]. This effect can be ascribed to decreased membrane permeability for polar compounds. In contrast, a very low PSA ($<50 \text{ \AA}^2$) can lead to increased intestinal and hepatic extraction [72].

Two common approaches to identify absorption issues are the maximum absorbable dose (MAD) and the dose number (D_0).

$$\text{MAD} = k_a \cdot S \cdot V_{\text{intestine}} \cdot T \quad (3.2)$$

where k_a is the absorption rate constant (usually obtained from PK studies in preclinical species); S the solubility of the drug in a relevant medium; $V_{\text{intestine}}$ the volume of water in the small intestine available to dissolve the drug (usually 250 mL in humans); and T the small intestine transit time (usually 4 h for the small intestine in humans).

$$D_0 = \frac{D}{(V_{\text{intestine}} \cdot S)} \quad (3.3)$$

where D is the dose, $V_{\text{intestine}}$ the volume of water in the small intestine available to dissolve the drug (usually 250 mL in humans), and S the solubility of the drug in a relevant medium.

The parameters influencing the MAD and D_0 are fraught with experimental uncertainty, most of all the solubility. The solubility determined in discovery is usually kinetic in nature and the true thermodynamic solubility is not available until later. Moreover, the solubility is strongly influenced by the solvent system (buffered solvents or fed or fasted state simulated intestinal fluid) and its pH and the physical form of the drug (amorphous or crystalline, the most stable polymorph or not, presence of excipients, etc.). In addition to the extent of solubility, the dissolution rate should be taken into consideration as well and both the MAD and D_0 ignore this aspect. Nevertheless, the MAD is easy to calculate and provides a crude guide; if the MAD is $<10\%$ of the anticipated human dose, one should consider the possibility of dose-limited absorption. Similarly, if the dose number is >10 , absorption could be incomplete. Note that these numbers are merely indicators of absorption risk and should be used as impetus to explore absorption further.

Once absorbed, the clearance is usually the most important parameter. An analysis of the routes of elimination of the 200 most prescribed drugs indicated that for about 72% of the drugs, metabolism was the major route of drug elimination, whereas renal elimination and biliary clearance were prevalent for 25% and 3%, respectively, of the top 200 drugs [75]. For those drugs predominantly cleared via metabolism, the CYP enzymes were the major contributors to metabolism. Considering the trend toward higher MW compounds (as illustrated in Table 3.2), it is reasonable to assume that passive renal clearance will be low for most drugs and that the major component of clearance will be either metabolism or active excretion into the bile, urine, or intestine. It is well established that increasing the lipophilicity can lead to increased clearance. In addition, increasing the PSA and the HBDs and HBAs decreases the clearance [72]. However, it is imperative to focus on the unbound or free clearance. The unbound clearance can be obtained as follows:

$$\text{CL}_u = \frac{\text{CL}}{f_u} \quad (3.4)$$

where CL is the total clearance, CL_u the unbound or free clearance, and f_u the unbound fraction in plasma.

In keeping with the concept that in most cases, efficacy is driven by the amount of unbound drug available, it is imperative to convert the relevant PK parameters to their unbound equivalent (e.g., AUC_u and CL_u). Too often, drug discovery efforts lead to an increase in lipophilicity and, consequently, a smaller f_u , while maintaining or sometimes even improving CL. This could result in a false sense of success. Instead, teams should focus on the CL_u value (which reflects the free amount of drug to drive efficacy), which may have increased dramatically. It is also important not to optimize f_u or use it to prioritize compounds for further studies. There is no optimum f_u and, indeed, there are many successful drugs with small free fractions [38]. Interestingly, there is a difference between the effect of plasma protein binding *in vitro* and *in vivo* [39]. *In vitro* an increase in f_u from 0.1 to 0.5 by changing the structure of the compound increases the unbound concentration five-fold as the total concentration remains the same. *In vivo* after an oral dose, an increase in f_u from 0.1 to 0.5 will increase the unbound concentration only if the intrinsic hepatic clearance decreases at the same time. If the intrinsic hepatic clearance stays the same, an increase in f_u from 0.1 to 0.5 will not increase the unbound concentration and will reduce the total concentration. Thus, the goal should be to optimize the physicochemical parameters such that it leads to a lower intrinsic clearance. Obach *et al.* [76] performed a detailed analysis of 670 drugs administered to human intravenously, which allowed determination of the clearance and volume of distribution. Their analysis showed that the unbound clearance increases dramatically with the lipophilicity with hydrophilic compounds with a $\log P$ between 0 and 1 having a median unbound clearance of 8.5 mL/min/kg and very lipophilic compounds with a $\log P > 4$ having a median unbound clearance of 150 mL/min/kg. The relationship between $\text{clog } P$ and the free fraction for three chemical series in one project is illustrated in Fig. 3.10 and shows that the free fraction steadily decreases with increasing lipophilicity, although the three chemical series are offset from each other. Another in-house analysis involving more than 2200 compounds across all projects came to the same conclusion. Specifically, 54% of the compounds with a measured $\log D_{7.4} > 4$ have a free fraction smaller than 0.01, whereas only 6% of the compounds with a measured $\log D_{7.4}$ between 1 and 2 have a free fraction smaller than 0.01. Conversely, 66% of the compounds with a measured $\log D_{7.4}$ between 1 and 2 have a free fraction >0.1 , whereas only 3% of the compounds with a measured $\log D_{7.4} > 4$ have a free fraction >0.1 . A graph derived from the data compiled by Obach *et al.* [76] is presented in Fig. 3.11 and displays the correlation between f_u and CL_u . The implication of this graph is that lowering the lipophilicity may actually have a dual effect by lowering CL_u and increasing the free fraction. Of course, lowering the lipophilicity may reduce the potency (because of less interaction with the target) and decrease the permeability. An analysis (Fig. 3.12) based on rat PK data for more than 50 compounds in one project shows how sensitive the relationship can be. If the $\text{clog } P$ value is <1 , more than 90% of the compounds have an unbound clearance of <170 mL/min/kg, whereas with a $\text{clog } P$ value >2 , only 32% of the compounds have an unbound clearance of <170 mL/min/kg.

A nice example of optimization of physicochemical parameters is illustrated by the discovery of crizotinib. Initially, crizotinib was positioned as a c-MET inhibitor. Later it was also found to inhibit anaplastic lymphoma kinase (ALK) and it was launched as treatment for non-small-cell lung cancer in 2011. The original lead candidate, PHA-665752 (Fig. 3.13), demonstrated poor PK and pharmaceutical properties,

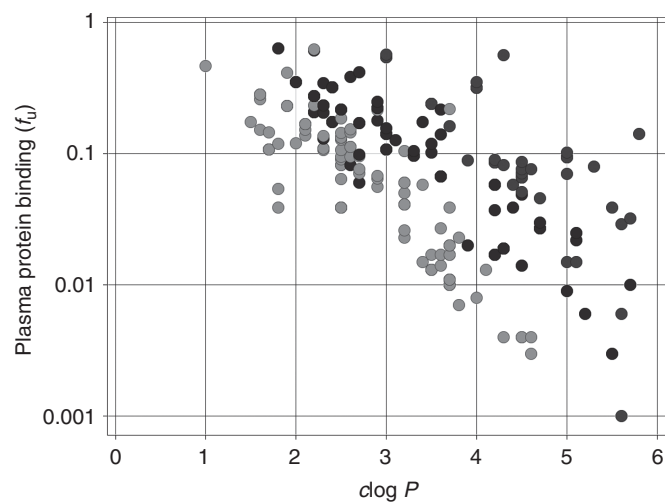


Figure 3.10 Relationship between $\text{clog } P$ and the measured free fraction (f_u) in plasma for a large number of compounds synthesized for an in-house project.

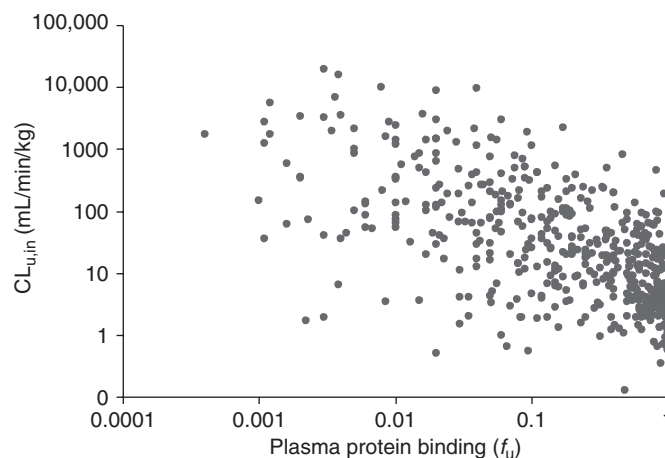


Figure 3.11 Relationship between the measured free fraction (f_u) in plasma and the unbound intrinsic clearance ($\text{CL}_{u,\text{in}}$) derived from data presented in Ref. 76.

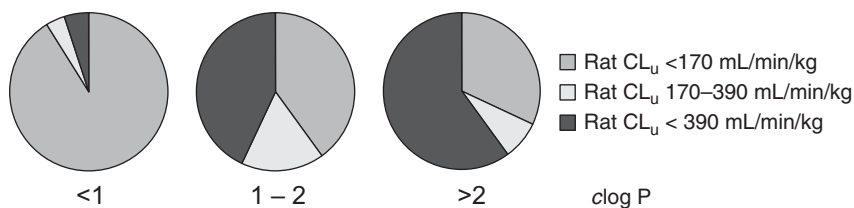


Figure 3.12 Relationship between $\text{clog } P$ and unbound clearance (CL_u) in rats for more than 50 compounds synthesized for an in-house project.

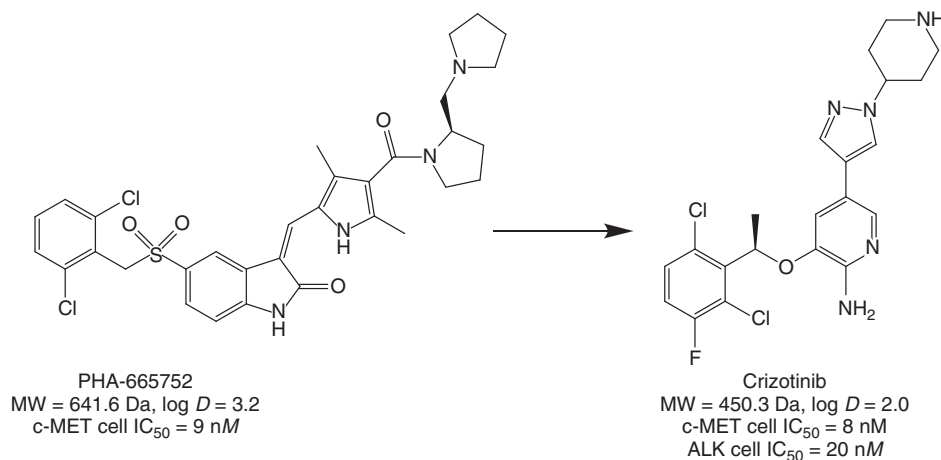
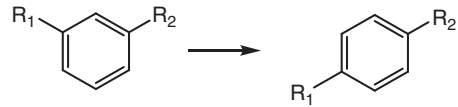


Figure 3.13 Optimization of the original lead c-MET inhibitor, PHA-665752, leading to the dual c-MET/ALK inhibitor, crizotinib.

which limited its development as a clinical candidate despite good cell potency (c-MET cell IC_{50} = 9 nM) and selectivity [77]. For example, the clearance of PHA-665752 in rats was higher than hepatic blood flow, 77 mL/min/kg [78], and it had low permeability and poor solubility at pH 7.4, 0.9 μ g/mL, which prevented nomination as a potential intravenous treatment [77]. PHA-665752 having a high MW, 641.6 Da, and being quite lipophilic (log $D_{7.4}$ = 3.2 and LipE = $pIC_{50} - \log D_{7.4}$ = 4.8), the goal was to identify equally potent compounds with improved physicochemical properties. Detailed SAR studies, fueled to a large extent by cocrystal structures of compound bound to c-MET, led to PF02341066, crizotinib. Crizotinib still makes the same key interaction with the c-MET protein, but is much smaller with a MW of 450.3 Da. Considering the lower lipophilicity, clog $D_{7.4}$ = 2.0, and a similar cellular potency in the c-MET assay, 8 nM, the LipE of crizotinib is improved to 6.1 [77]. Moreover, the solubility is improved and the same applies to the preclinical PK (e.g., the clearance values in rat and dog are 29 and 9 mL/min/kg, respectively, and the bioavailability values are 63% and 65%, respectively). The human dose is 250 mg twice daily (BID), the half-life is 43–51 h, and there is no food effect, which defines an attractive clinical profile [79].

An interesting computational approach to improve clearance was proposed by Keefer *et al.* [80]. They performed a very comprehensive pairwise analysis of a large database of compounds with metabolic stability data in human liver microsomes, permeability data in RRCK cells (MDCK cells with lower levels of endogenous transporters), and P-gp-mediated efflux in MDCK cells overexpressing P-gp. They determined the 20 most frequently occurring transformations in their database and their mean change and standard deviation associated with the three *in vitro* ADME parameters described above. A few examples and their impact on microsomal metabolic stability and RRCK permeability are presented in Table 3.7. They also showed that transformations that involve multiple steps are frequently additive. However, their analysis also showed that similar transformations in very different molecules may not always have the same net effect and sometimes they even have the opposite effect. Nevertheless, it provides a

TABLE 3.7 Statistical Changes in Metabolic Stability in Human Liver Microsomes and RRCK Permeability Associated with the Most Common Transformations Based on a Pairwise Analysis of a Very Large Database

Transformation	HLM Stability		RRCK Permeability	
	Mean Change	%Increase/% Same/% Decrease	Mean Change	%Increase/% Same/% Decrease
$R_1-H \longrightarrow R_1-CH_3$	21.9	45/41/12	-0.2	24/47/28
$R_1-H \longrightarrow R_1-F$	2.7	24/56/18	-0.5	17/55/26
$R_1-H \longrightarrow R_1-Cl$	10	38//42/19	-3.1	16/35/48
$R_1-H \longrightarrow R_1-CH_2CH_3$	20.5	45/45/9	-1.0	20/48/31
	-15.4	12/49/37	-0.1	10/60/19
$R_1-H \longrightarrow R_1-F$	-19.9	10/43/45	1.1	33/47/18
$R_1-H \longrightarrow R_1-OH$	-32.0	11/33/54	-3.1	23/28/47

Listed are the mean change of the property for each transformation and the percentage of the pairs with the property increasing, staying the same, or decreasing.

Source: Data are from Ref. 80.

simple (computational) rule of thumb to increase the likelihood to identify compounds with improved ADME properties.

Finally, it is important to recognize that multiple enzymes can contribute to the metabolism of compounds and, if the choice of *in vitro* studies and preclinical PK studies is not comprehensive enough, the data can be deceiving. A classical example is carbazeran (Fig. 3.14). Carbazeran is relatively stable in microsomes across species and the PK in dogs looks good with a CL of 11.5 mL/min/kg and a bioavailability of 68% [81]. In sharp contrast, the clearance in humans was 38 mL/min/kg, which is higher than hepatic blood flow, and carbazeran was not detected in the plasma after an oral dose of 350 mg. Subsequently, it was determined that human metabolism was mainly via aldehyde oxidase leading to oxidation of the phthalazine moiety. Moreover, dogs have very low levels of aldehyde oxidase, which explains the good PK in dogs. Had the investigators used either hepatocytes, S9 or cytosol, to examine the metabolic stability of carbazeran across species before nominating the compound for development, they would have identified the aldehyde oxidase liability in humans. Remarkably, compounds failed in the clinic as recent as 2011 due to extensive aldehyde oxidase metabolism in humans (e.g., FK3453 [82] and BIBX1382 [83]; see Fig. 3.14 for structures) despite the ability to identify the liability preclinically by doing the right *in vitro* studies or performing a monkey PK study.

Another important parameter to consider is the volume of distribution and, not surprisingly, Obach *et al.* [76] have shown that the volume of distribution, and in particular the unbound volume of distribution, increases with the lipophilicity. In addition, the volume of distribution decreases with the PSA and the number of HBDS and HBAs. The combined data for clearance and volume of distribution are summarized in Table 3.8 by charge state. Note that the V_d and CL are generally much lower for acids

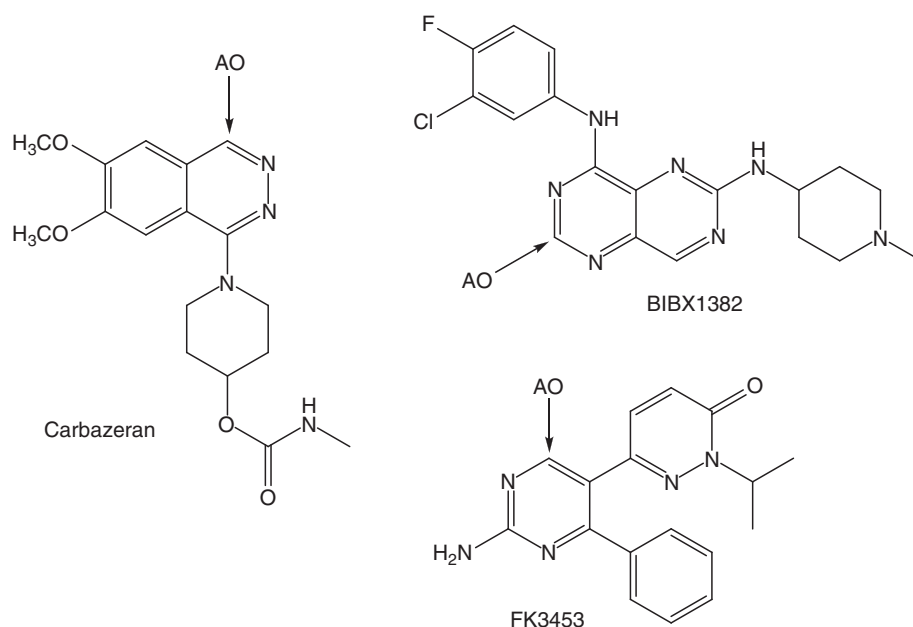


Figure 3.14 Structures of the aldehyde oxidase (AO) substrates carbazaran, FK3453, and BIBX1382. The sites of AO metabolism are indicated by arrows.

TABLE 3.8 Comparison of Median Values for Total and Unbound V_d and CL Values Relative to Charge Type

	Median V_d	Median $V_{d,u}$	Median CL	Median CL_u
Acids ($n = 159$)	0.22	2.2	2.1	17
Neutrals ($n = 173$)	1.1	5.5	4.0	21
Bases ($n = 267$)	2.3	9.2	7.6	23
Zwitterions ($n = 68$)	0.83	2.0	2.4	3.8

Source: Data from Obach *et al.* [76].

than bases, but the differences are reduced after correcting for plasma protein binding. If the drug target is in the brain, particular attention should be paid to CNS distribution. As described above, a TPSA in excess of $90 \text{ \AA}^2$ is associated with poor brain penetration [17]. This is partly due to poor passive permeability, and it also appears that the probability for P-gp-mediated efflux increases with a TPSA above $70\text{--}80 \text{ \AA}^2$, as illustrated in Fig. 3.15 for data from multiple chemical series for one particular in-house CNS target.

Obviously, there are numerous other parameters that play a role in drug design and ADME is only one of them. For example, the desire to increase potency frequently results in larger and more lipophilic compounds. This necessitates finding a “sweet spot” that represents the best overall balance. A generalization of the dependence of various ADME parameters on the key physiochemical properties is summarized in Table 3.9.

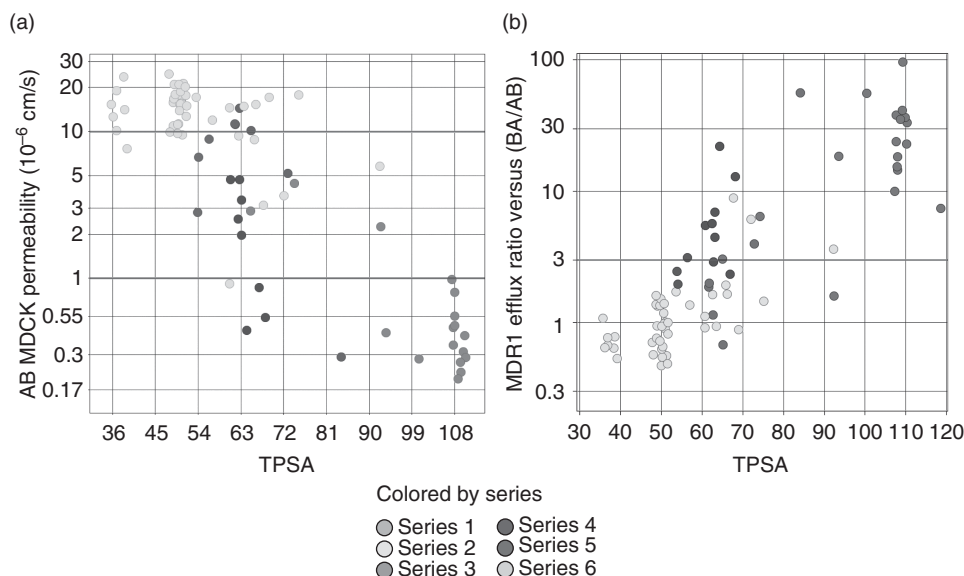


Figure 3.15 Relationship between TPSA and the A to B permeability in MDCK cells (A) and the B to A/A to B efflux ratio in MDCK cells overexpressing MDR1 for a large number of compounds synthesized for an in-house project. (a) Permeability versus TPSA and (b) MDR1 efflux ratio versus TPSA.

TABLE 3.9 Generalization of the Dependence of Various ADME Parameters on Physicochemical Properties

	$\log D$	MW	PSA	HBA + HBD
Solubility	↓	↓	↑	↑
Permeability	↑	↓	↓	↓
F_a	↑	↓	↓	↓
F_h	↓	=	↑	↑
CL	↑	—	↓	↓
F	Gaussian (↑ then ↓)	↓	Gaussian (↑ then ↓)	↓
V_d	↑	—	↓	↓
Plasma protein binding	↑	—	—	↓

^aThe arrows indicate an increase, no change, or decrease in the pharmacokinetic parameter in one of the rows as a function of an increase in the physicochemical parameter in one of the columns.

Finally, ADME scientists play a critical role in other aspects of drug discovery such as identification of drug–drug interaction liabilities, determination of exposure in PD and efficacy studies, PKPD analysis of preclinical data, and human PK and dose predictions. Detailed descriptions of these aspects are beyond the scope of this chapter and the readers are referred to others chapters in the Encyclopedia of Drug Metabolism for these components.

3.11 CONCLUSIONS

It is realistic to state that incorporation of ADME parameters early on drug discovery has had and will continue to have a positive impact on drug development. However, drug candidates have become larger and more lipophilic to meet potency criteria and this has in general a negative impact on ADME parameters and drug development in general. Before engaging in an extensive drug discovery effort, it is important to clearly define the acceptable target candidate profile and identify any attenuating circumstances that could influence the profile. Next, a thorough effort to establish the SAR of all key parameters (potency, selectivity, permeability, clearance, etc.) should be conducted, and finally, the physicochemical “sweet spot” that provides the best balance of properties should be identified.

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4 Effective Early Drug Development

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4.1	Introduction	1
4.2	Overview of drug development	3
4.3	Regulatory considerations	5
4.4	Ind-enabling development	6
4.5	Animals to humans	7
4.6	Clinical pharmacology	13
4.7	Summary and conclusions	29
	Acknowledgments	30
	Abbreviations	30
	References	31

4.1 INTRODUCTION

The process by which a new chemical entity (NCE) results in a new medical entity (NME) that, when administered to patients, results in a successful therapeutic intervention that improves the patients' quality of life is a long and costly process. This has remained so in spite of advances in all of the sciences that are contributors to and constitute the process. Even with the result of a greatly increased number of NMEs with promising pharmacological properties being routinely identified, the challenge remains: which molecule will be predictive of efficacy and safety in humans and what is the process that will support these outcomes?

In drug development, each pharmaceutical company has its own system, which (probably) is a reflection of previous successes. Although when "all is said and done," drug candidates become drugs by going through similar stepwise regulatory-driven process that demonstrates safety and efficacy. In this context, a faster and less costly process as a definition of "efficient drug development" is highly unlikely. This does not preclude that within the process, the attainment of conclusive Go/No-Go milestones can be attained effectively (efficiently) and thus result in an efficient process overall. In

this context, the addition of effective drug development should also be used in defining drug development.

Considering the number and diversity of molecules for potential development, some with multiple activities with active indication profiles, an all encompassing treatment of drug development is beyond the scope of this chapter. Thus, we have not attempted to be all inclusive or exhaustive in terms of a drug class or indication or to compile a drug development study checklist with the intent of “one size fits all.” Instead, we have attempted to present in outline concepts that define aspects/landmarks of early nonclinical and clinical development, as these concepts contribute to the traditional path to facilitate the first developmental outcome desired: an open investigative new drug (IND).

In one respect, drug development can be considered as a single path with a single outcome. However, it is not a line-driven process. Once started, the path forward quickly branches into the primary disciplines that will support safety and efficacy, which in turn develop subbranches to form a mosaic(s) to constitute total development as defined in the new drug application (NDA). Every path has to have a beginning: the path forward for a formal drug development is the IND. It should be stated that the IND, once opened, precedes the conduct of the multidisciplinary studies that continue mostly by paths that validate drug safety and efficacy. Once the initial studies are supportive of further development, continuation of full panels of studies are conducted on the way to the NDA. All of the studies that will define the content of the NDA are not addressed here in detail; however, for continuity of the development process, descriptions of IND-targeted studies that are germane to further development are presented. This theme will be defined and expanded further in some detail and is introduced here.

In early drug development, nonclinical studies are conducted in two to three species to determine a dose–toxicological profile in relationship to the proposed human dose (animal to human safety margins) in the first clinical trial(s), that is, phase 1.

It is important to state that phase 1 (and phase 2a) studies are the *learning phases* of drug development and, if appropriately coordinated and conducted, provide information that increases the efficiency/effectiveness for the rest of the drug development process [1]. In addition, phase 1 provides decision-making data and information for labeling. Some examples of such coordination and impacts on decisions will be presented later. In this context, the pivotal phase 3 studies (and phase 2) of drug development are *confirmatory studies* that define the safe and effective use of the drug in the targeted patient population. Careful consideration for the selection, design, and timing of phase 1 trials provide a solid foundation for the conduct of one or two proof of safety and efficacy studies in phase 3. The final approval process as entailed in phase 3 is outside the scope of the present report.

In addition, recent use of techniques such as biomarkers and surrogate markers [2–4], pharmacokinetic/pharmacodynamic (PK/PD) correlations [5], and microdosing [6], all of which can increase the understanding of the drug PK/PD properties in both early and later stages of development, are outside of the scope of this present report. These are mentioned as they are utilized in current drug development. The use of biomarkers and surrogate markers to define/predict clinical outcomes is an important consideration to an efficient drug development process [7]. As biomarkers and surrogate markers are developed, it must be kept in mind that the time to think about their selection should be early in the drug discovery and development process. Thus, a linkage(s) to clinical outcomes will transition the marker into a surrogate end point

and have a prospective value as the surrogate will predict an outcome. An essential component in the selection of biomarkers and surrogate markers is their validation. In this context, information must not be confused with knowledge and reliability with validity. The criteria for validity should be concurrent in that supporting evidence is obtained at the same time, predictive in that the evidence is also obtained at a later time, and prescriptive by providing the appropriate treatment.

More recently, the use of genotyping/phenotyping to provide information for predicting drug response and variation that are currently used with increasing regularity are also mentioned only in passing. These ultimately do contribute to the overall aspects of development, especially as polytherapy is the norm in today's aging population to provide effective patient management and contain the promise of individual therapy [8–12], but at present, this remains in the future.

To provide an adequate bibliography is prohibitive; thus, an abbreviated list of reference citations is provided for additional and in more depth insights for the process of drug development. Considering that drug development is essentially a regulatory process, FDA guidance documents are also included where applicable to provide a regulatory framework for the concepts presented.

4.2 OVERVIEW OF DRUG DEVELOPMENT

The basis for the marketing approval of a NME is safety and efficacy. To this end, the *road map* to develop a drug is both defined and regulated by FDA and ICH (International Committee for Harmonization) guidances. However, a road map primarily gives us the destination. To efficiently navigate, the road map requires both a plan and *road markers* that can ascertain progress as one moves forward. These road markers are studies and, even more importantly, data that are *needed* (not what can be generated) for Go/No-Go decisions. Even with these guides, there are few specifics provided that are “Not to Do”. However, the specifics of what to do, how to do it, and how to evaluate the validity of the results obtained during the drug development process remains time consuming and costly. This is true in spite of the very large number (e.g., 100,000) of new molecules that are synthesized yearly, the availability of high throughput screening for determining pharmacological activity, validated *in vitro* and *in vivo* disease models, the use of genomics and proteonomics, and other new and innovative technologies to expedite drug discovery and development. The expectation for new and numerous drugs available for therapeutic use have not been met, and in fact, there is a decline in the new drug discovery/development pipeline.

When all is said and done, only 1–2 of the 100,000 molecules actually reach the market for therapeutic use after a development time of 7–12 years (or more) and costs that can exceed \$700 million before an NDA is submitted to the FDA and filed for their review and evaluation. The review of the NDA adds additional time of 10–18 months until marketing approval. A goal during development is to obtain Go/No-Go milestones as quickly as possible to maintain the entry of drug dossiers for review by the regulatory agency. An outline of the development process (road map) is shown in Fig. 4.1 in which drug development is outlined in distinct phases. In addition, guidance documents issued by the FDA are available, which address specific studies/aspects of development, provide a wealth of specific information [13], and provide efficiency to a development process by resulting in an effective drug development.

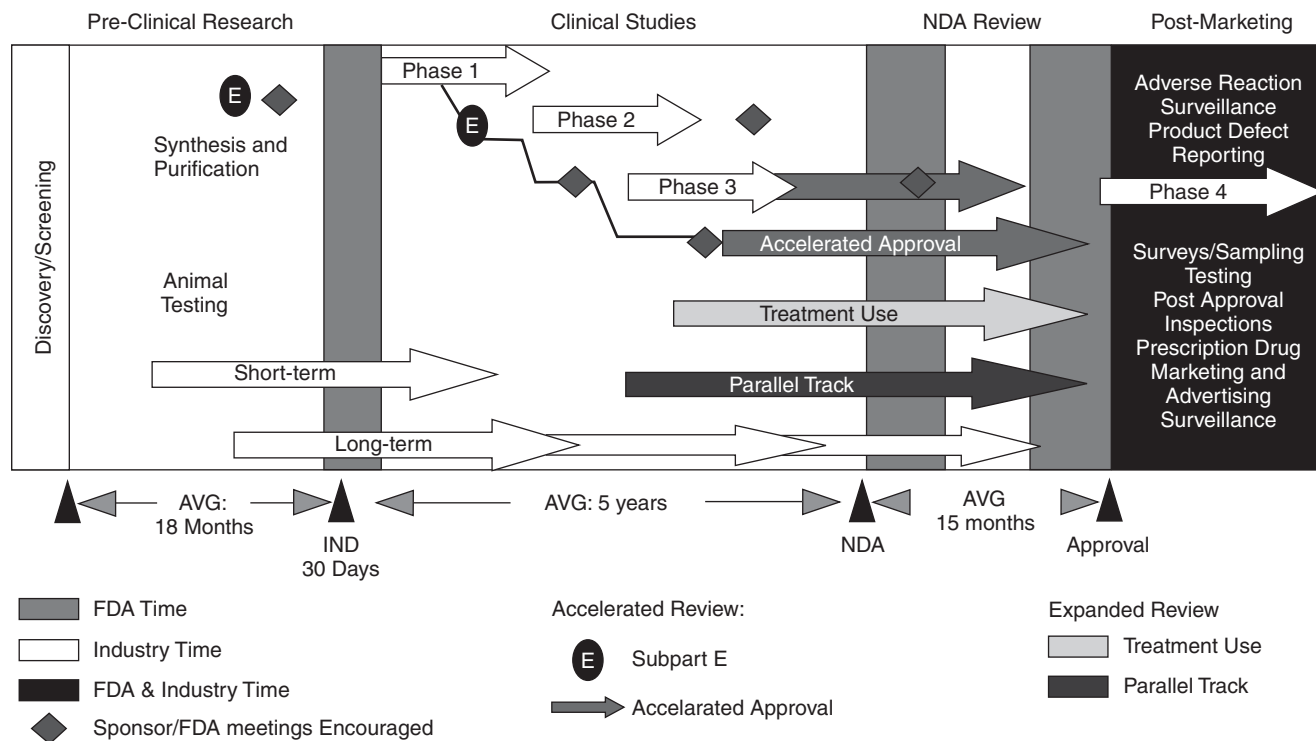


Figure 4.1 New drug development process.

Figure 4.1 outlines the distinct phases of drug development: discovery, preclinical studies (pharmacology and toxicology/safety, which lead to an open IND, enabling the first study(ies) in humans), the phase 1 studies (clinical pharmacology), followed by the phases 2 and 3 clinical studies that demonstrate and confirm safety and efficacy and thus provide the basis for marketing approval.

Discovery/screening studies are not included herein, as these are drug/indication specific and as such beyond the scope of the present report. Needless to say, those type of studies are carried out in order to provide a rationale for the development of the lead candidate(s). Usually, a relatively small number of such studies are conducted; the positive results from which support a rationale for further development. It is not uncommon that four to five animal pharmacology studies suffice, two or three of which hit the desired end point. Such an approach is alluded to in the *Exploratory IND Studies* guidance document [14] issued by the FDA as one of the steps to facilitate the entry into the ultimate model: humans. The phase 2 and pivotal phase 3 studies are also not addressed; the emphasis is on the traditional segment of drug development that is representative of what constitutes learning to understand the drug and thus allows development to move forward into the confirmatory aspects of drug development (phase 2b and phase 3) on which marketing approval is ultimately based.

The concepts that will be described are for the development of an oral drug, as this is the most desired commercially, and perhaps the most prevalent, route of administration from several perspectives. However, the development of a drug with vascular (or topical) administration would follow the same path (type of studies) to establish the safety profile of the intended route of administration for the management of patients.

4.3 REGULATORY CONSIDERATIONS

Drug development is subject to numerous the FDA and ICH guidances and regulations [Good Laboratory Practices (GLPs), Good Clinical Practices (GCPs), etc.]. These provide a wealth of information on both specific and general issues that contribute to any drug development process.

The need for more *better and cheaper* drugs is a widely used phrase. At a relatively recent national meeting, the FDA shared their concerns to this end by presenting data showing that new compounds entering phase 1 today have an 8% chance of reaching the market (vs. 14% chance 15 years ago); the failure rate in phase 3 is 50% (vs. 20% 10 years ago). In this context, the FDA is actively pursuing a critical path initiative (CPI) [15] in the attempt of modernizing the techniques and methods used to evaluate safety, efficacy, and quality of drugs as they move through the development and approval processes. The three areas of the CPI are (i) a new approach to clinical trials in terms of early proof of concept (POC) studies, computer modeling, microdose studies, and new and innovative methods of data analysis; (ii) the FDA will issue a guidance on the validation of biomarkers and surrogate markers; and (iii) the use of modeling and simulations. It is hoped that these will expedite the entry of drugs into the marketplace.

4.4 IND-ENABLING DEVELOPMENT

Drug development has its beginnings in pre-IND as the following factors are considered:

- understanding the disease process and therapeutic approach;
- determining the mechanism of action and establishing appropriate models;
- identifying and validating clinical safety and, if possible, efficacy markers for decision making;
- conducting *in vitro* metabolism screens using toxicology species and human liver microsomes to identify the cytochrome P450 (CYP450) isozymes involved;
- developing the appropriate toxicology–safety program (see ICH guidance).

At some time during the above development, it is advantageous to write a draft label for the indication sought, as this will provide focus to all aspects of drug development as it moves forward. It is also important to address the manufacturing of the drug substance and set the impurity limits/specifications and prepare the clinical supplies for the first human studies. At this stage, it is not necessary to have a final galenic formulation for the initial clinical studies, as these first such studies can easily be conducted with simple hand-packed capsules. It is given that the galenic formulation is ongoing for formal drug development. Also, in parallel, the impurity profile—qualitative and quantitative—is determined. This is important to ascertain that the drug substance used in the animal toxicity/safety studies that were conducted to open the IND did not have an impurity profile that exceeds that of the drug substance that is to be used in the initial clinical trials.

Using the FDA-recommended Exploratory IND, as mentioned previously, is an efficient and effective roadmap to an acceptable IND, in that a Go/No-Go decision can be reached relatively quickly and may include a pivotal first study in the ultimate model: humans. It is worth repeating that 15% of drug development programs fail at phase 1. In general, the Exploratory IND consists of a panel of general *in vitro* and *in vivo* safety pharmacology, toxicology/safety studies in two animal species, with the duration of such studies linked to the duration of the proposed post-IND trial. Safety pharmacology/toxicology (cardiac safety including a QTc prolongation in the telemetry dog), genotoxicity, and ADME (absorption, distribution, metabolism, and excretion) studies are normally required for the Exploratory IND before dosing in humans.

In this context, the current regulatory environment encourages INDs with minimum content, that is, pharmacology to support a rationale for drug development and appropriate animal toxicity–safety studies that allow development to move forward into the first studies in humans (exploratory INDs). Thus minimum as describing the requirement for the IND are a misnomer; the studies provide key nonclinical information for conduct of a human PK study. However, there are no shortcuts, and if development is to move forward, additional toxicology studies will be conducted at higher doses and with increased duration.

There is always value in stating (or restating) what has been (and will remain) the foundation of drug development: the IND. The IND consists of the following sections:

- Form FDA 1571
- Table of contents

- Introductory statement
 - List of references
- General investigational plan
 - Clinical development plan
 - Planned exposure
- Investigator's brochure (IB)
- Protocols
- Chemistry, manufacturing, and controls (CMC)
- Nonclinical pharmacology and toxicology
- Previous human experience
- Additional information.

It is important to have a clear objective(s) for the first human studies (phase 1) as this will have a direct impact on the nonclinical pharmacology and toxicology studies that will precede it. In this context, it is of value to utilize the FDA guidance document titled Exploratory IND Studies, which provides concepts that facilitate an early entry into clinical studies by focusing on the core nonclinical studies required. It should be stated, however, that should development move beyond the first phase 1 study and into formal development, it will be necessary to go back and conduct the studies that constitute a traditional IND/NDA.

4.5 ANIMALS TO HUMANS

Once a candidate for development has been chosen, the first goal is to obtain an open IND, thus allowing the first study in humans. As was outlined above, the dossier for the IND is started by conducting a series of nonclinical toxicology/safety studies that includes at least two animal species, usually rat and dog, that emerge as the primary species for the determination of a toxicologic and safety profile projected for human exposure.

Throughout the whole process (IND to NDA), measuring drug concentrations to determine the extent and duration of systemic exposure to the drug provides windows of understanding of both safety and efficacy (or the lack thereof). This is true of non-clinical toxicity/safety [toxicokinetics (TK)] and clinical studies (PK). The fundamental difference in animal studies and human studies can be outlined as follows:

Animals	Humans
Once/day dosing; defined regimen	qd, bid, tid, qid; widely varying regimens
Wide range of doses/dose strengths	Single, two to three strengths with narrow range
CMC suspensions, dietary admix (rats); neat drug in capsules (dog)	Elegant galenic formulation
Species differences	Ethnic and gender and/or age differences

4.5.1 Bioanalytical Considerations

As was stated above, the systemic exposure to the administered drug and the elucidation of its dispositional parameters is of importance. To this end, it is mandatory to develop and validate the analytical methods that would support both nonclinical and clinical studies. Usually, the method utilized for determining different aspects of the drug substance, for example, dose verification, purity, and stability is high performance liquid chromatography (HPLC). For determining drug concentrations in animal and human biofluids (blood, plasma, urine, tissues), the usual method(s) is LC/MS-MS (liquid chromatography/mass spectrometry/mass spectrometry) assay(s) for its sensitivity [low limit of quantitation (LOQ)] and specificity for drug (and metabolite). Validated bioanalytical methods for animal studies (TK, ADME) and humans studies characterize the biopharmaceutics (BP) and PK for phase 1/phase 2a clinical pharmacology studies. Thus, such bioanalytical methods are conducted under GLP regulations. The validation of such assays results in stand-alone reports that demonstrate selectivity, accuracy, precision, reproducibility, and recovery [16]. As most biological samples (animal/human) are stored frozen and bundled prior to assay, short- and long-term stabilities are required. The inclusion of BP and PK principles in the conduct and evaluations of animal toxicity/safety studies and GLP toxicity studies is virtually mandated. Using full or sparse sampling profiles over selected time points over the course of toxicology studies, TK provide drug systemic drug concentration data that aids in understanding the findings.

As development moves forward, a validated LC/MS-MS assay for drug and primary metabolite in human plasma (and urine) with a low LOQ should also be developed, the report(s) of which should be included in the IND in support of the conduct of the first phase 1 PK study.

4.5.2 Early Nonclinical Pharmacology and Toxicology

An IND-targeted program will start with nonclinical pharmacology and toxicology to provide the data from which safety assessment can be projected for safe use in the first human studies. The early nonclinical toxicology program to support an open IND is outlined below, although details of study design are not addressed. It should be stated that the success/failure of drug development program can depend on the early nonclinical toxicology/safety studies that provide a rationale/guidance for effective evaluation that provides rational safety margins [17]. Only selected studies are addressed, and specific guidance documents are referenced for additional information. As development continues, the nonclinical toxicology dossier ends up being one of the major portions of the NDA; a full in-depth review is not feasible within this report. However, in the guidance for the format and outline of the toxicology section of the NDA, one can obtain specific design information from the table of contents [18].

Acute toxicity studies [19] are the initial nonclinical studies conducted. These are usually conducted in rats, although a second species may be used. There are two basic study designs: escalating doses in the same animal with a 14-day observation period with incremental doses to ascertain any latent toxicity or escalating doses in parallel groups with a 14-day observation period. Doses are escalated until a clear toxicological outcome is reached. There is no requirement for conducting acute studies under the GLP regulations. However, most Sponsors do conduct these studies under GLP, in that

if a global marketing strategy is contemplated and proceeds forward, some overseas regulatory agencies require that acute toxicity be conducted under GLP regulations. As was stated above, acute toxicity studies are dose escalation studies, the primary objective is not to cause mortality per se but to have a clear concept of the dose–toxicity relationship (food consumption, body weight loss, clinical pathology findings, etc.) as an acute response and/or a delayed response after dose administration. In addition, the acute toxicity response may provide a window for investigating potential human overdose. It should also be mentioned that gross pathology, histopathology, or clinical pathology are not requirements; however, body weight loss, gross pathology, and the addition of key clinical chemistry indicators can be used in establishing a dose at which toxicity begins.

To ascertain, no potential for genotoxicity is a benchmark in early development. The core genotoxicity studies are the Ames test with and without S9 activation, chromosomal aberration (CHO), and *in vitro* mouse micronucleus test [20,21]. If there are no findings indicative of genotoxicity or mutagenicity, no further genotoxicity testing is required. If the genotoxicity results are positive in identifying a signal for mutagenicity, the relationship to dose relative to the first human dose should be carefully examined. It is possible that the program should be stopped as the genotoxic dose is too close to the proposed human dose.

The safety pharmacology studies in Refs 22,23 represent the *in vitro* and *in vivo* panel of nonclinical studies that support cardiac safety during an open IND. These are acute administrations at doses that result in systemic concentrations that are at least 100× the human plasma concentration of the projected clinical dose. These studies are conducted in animal models to identify undesirable PD properties of the NCE that may have a relevance to human safety. The core battery of safety pharmacology trials [22] addresses organs or systems toxicities and includes the following.

1. Effects on the central nervous system are assessed in terms of motor activity, behavior changes, coordination and sensory/motor reflex responses, and learning and memory.
2. Cardiovascular system studies assess cardiac output, ventricular contractility, blood pressure, heart rate, and the ECG (electrocardiography) *in vivo* (telemetry dog) and *in vitro* repolarization and conductance [23]. The effects of *in vitro* hERG potassium channels expressed in human embryonic kidney cells on cardiac ionic currents are assessed. Also, isolated rabbit Purkinje fibers assess the effects on a potential changes in the action potential duration (ADP). These *in vitro* studies are routinely conducted to be complementary to the human study.
3. Respiratory system assessed by measurements of the respiratory rate and function.

The duration of the proposed clinical studies are directly linked to the duration of the animal toxicity studies: two-week studies in two animal species (usually rat and dog) allow two-week studies in humans. It is usually more efficient to conduct four-week toxicity studies at this time, which will allow more flexibility for the first human study(ies). As early nonclinical trials indicate a signal in regard to the relationship to safety and an advantageous PK profile of the drug candidate, animal studies of longer duration (e.g., 4 and 13 weeks in rats and dogs, 6 month in rat, 9 month in dogs, 2-year carcinogenicity in mice and rats, the full panel of reproductive toxicity in rodents) will be conducted in sequence. These tabulated studies are needed to obtain an open IND

and thus allow the conduct of the first human studies (PK/tolerance) to initiate formal drug development [14,18].

In this context, the challenge in the early development program is to set the doses for the first animal toxicity study. It is not recommended to guess at the dose level because of either underdose or overdose and therefore, not allowing one to obtain an accurate picture of the dose–toxicity relationship. The early conduct of the acute toxicity studies (rats and dogs) can provide some information for setting the doses for the two-week toxicity studies, although conducting a 7-day dose range finding study with initial and terminal TK is more informative. The inclusion of TK into the acute toxicity studies is not a requirement; however, TK can focus on the drug plasma concentration relationship and the onset of toxicity.

Today, the inclusion of TK in toxicology studies is assumed [24]. In addition to ascertaining systemic absorption and a level of exposure of the drug in animals, it is also important to understand the systemic disposition of the drug in animals relative to human studies. Thus, the conduct of animal ADME studies is important in this transition. Animal studies with IV and oral dosing can be readily conducted to obtain a metric of bioavailability (BA), area under the curve (AUC), half-life, clearance, and volume of distribution. All of these parameters provide an understanding to the TK determinations that are routinely obtained from the toxicology studies performed.

It is prudent in early development to conduct studies that determine the metabolic profile of the drug using standard protocols and that utilize rat, dog, and human hepatocytes and microsomes (i.e., the metabolic stability of the drug). Drug metabolism studies are required for marketing approval, and metabolites must be identified and their pharmacological activity as well as their toxicological potential must be assessed [25]. In addition, the CYP450 isozymes involved will be identified, including whether the drug induces or inhibits their activity, and based on the collective information obtained, whether there is a potential for a drug–drug interaction in the therapeutic setting of the targeted population. Thus, the early elucidation of the metabolic pathway can be an important consideration to the overall development program.

The determination of the extent of plasma protein binding in rats, dogs, and humans is also important in these early drug dispositional studies. Only the free fraction of a drug is available for metabolism and excretion, pharmacological activity, and binding to tissues. The determination of the extent of protein binding (i.e., the free fraction of drug) over a concentration range that encompasses and exceeds the TK and clinical PK range is conducted in animal and human plasma. Whether binding is concentration dependent/independent can provide insights as to the activity and/or toxicity seen; determining the main carrier proteins of the drug [albumin, alpha-1 acid glycoprotein (AGP)] in the systemic circulation may have a relevance in certain disease states.

A tabulation of a nonclinical program in early development that supports continuing development is provided in the next two tables. The GLP status, the amount of drug needed, and a usual time from start to final report are presented. As development continues, an NDA-targeted toxicology program would include subchronic, reproductive, chronic toxicity studies including *in vivo* carcinogenic studies in two species.

Study	GLP status	Drug needed (g)	Time of start to final report
<i>Analytical/Bioanalytical</i>			
HPLC assay for dose verification	Y	10	One week for development and validation; Four weeks for report
LC/MS-MS for drug concentrations in rat plasma (for TK and PK)	Y	1	Four to six weeks for development and validation
LC/MS-MS for drug concentrations in dog plasma (for TK and PK)	Y	1	Four to six weeks for development and validation
LC/MS-MS for drug concentrations in human plasma	Y	5	Four to six weeks for development and validation
• Acute oral toxicity in rats	Y	50	Three to four months
• Acute oral toxicity in rats with necropsy and clinical path	Y	50	Three to four months
• Acute oral toxicity in dogs	Y	100	Three to four months
• Ames test with/without S9 activation	Y	2	Six weeks
• Chromosomal aberration (CHO test)	Y	5	One month
• <i>In vivo</i> mouse micronucleus	Y	10–15	One month
Study	GLP status	Drug needed	Time of start to final report
QT interval prolongation in conscious dog	Y	50 g	Four weeks in life, four weeks for report
<i>In vitro</i> hERG assay	Y	100 mg	Three to four weeks to QA'd draft report
Behavior in rats (Irwin test)	Y	1 g	One week in life, three to four weeks for QA'd draft report
Respiratory effects in rats	Y	1 g	One week in life, three to four weeks for QA'd draft report
Two-week oral toxicity/TK in rats	Y	300 g	Two weeks in life, six to seven weeks for QA'd draft report
2-Week oral toxicity/TK in dogs	Y	1 kg	Two weeks in life, eight week for QA'd draft report
Four-week oral toxicity/TK in rats	Y	500 g	4 weeks in life, 12 weeks for QA'd draft report
Four-week oral toxicity/TK in dogs	Y	2.2 kg	4 weeks in life, 12 weeks for QA'd draft report

(continued overleaf)

(continued)

Study	GLP status	Drug needed	Time of start to final report
IV/PO single-dose PK in rats	N	2 g	Five to six weeks
IV/PO single-dose PK in dogs	N	15 g	Five to six weeks
Protein binding in rat, dog, and human plasma	N	250 mg	Four weeks
Metabolic profile, rat, dog, and human liver microsomes	N	100 mg	Five to six weeks

4.5.3 Biopharmaceutical Considerations

BP, the use of PK to evaluate dosage forms, is an integral part of clinical development. In preclinical toxicology/safety studies, the dosage forms are not as complicated as they are for clinical use. However, the issue of dose selection is of importance, in that a dose–toxicological profile that, will hopefully, reflects what will be found in humans will be obtained. Thus, a good prospective projection regarding safety in humans based on preclinical systemic exposure would use both BA and BP principles in animal studies to ensure that the dosage/dose administered results in the maximum drug release profile [26].

Biopharmaceutical and BA studies are more complex for animal studies, and a maximal drug release profile should be determined in order to define the line between pharmacology and toxicology. To this end, the BA of the dosage form should be determined to confirm that the presence or absence of a toxic or pharmacologic effect is real and not a result of a low or no drug absorption (brick dust). In this context, ADME studies (including IV administration) and the use of *BP screens* in dogs and primates to evaluate the dosage forms for toxicology studies are very useful and have been presented earlier.

4.5.4 Go/No-Go

Usually, Go/No-Go decisions are used in the context of clinical studies. In the context of nonclinical development, toxicity in the animal safety studies in early development is the main reason for failure. Thus, it is important to ascertain a maximum drug release profile (TK) in early development. This is especially true if the toxicity found is organ specific or by an unknown mechanism. When a well-defined dosage form administered at 1×, 3×, and 10× of the projected human dose does not result in safety margins of $\geq 3\times$, the development should normally not go forward.

In early development, Go/No-Go decisions are not based on testing/evaluations of a single compound. In practice, depending on the resources, multiple NCEs are evaluated and the most advantageous are selected and brought forward for further development. It is usual that development proceeds for two to three candidates in parallel to expedite further development into the pre-IND stage. A low initial BA is not a *show stopper*, and BP considerations are evaluated to determine if the low BA is an intrinsic property or the physicochemical property of the drug substance. Thus, early development evaluates absorption as a function of dosage forms with micronized drug (reduced particle size),

various salts, or esters and routes of administration to obtain maximum absorption. Although pharmacological activity may not be uncovered and if shown not to be correlated with an extent of systemic exposure, further development could proceed at an accelerated pace.

Other factors that result in failure at the preclinical stage of development are summarized in the following table.

Toxicity	In one or more species Insufficient safety margins
Pharmacokinetics	Long or short half-life Nonlinear kinetics
Low bioavailability	Insufficient systemic exposure
Metabolism	High first pass CYP induction or inhibition
Instability	Short shelf life of product Instability of substance in biomatrices
Complex/costly synthesis	Low yield Difficult scale-up Unacceptable impurities in final product

It should be pointed out that there are caveats linked to the therapeutic target where some of the above factors are not pivotal. A long or short terminal elimination half-life may not be a show stopper: a long half-life would not be appropriate for a centrally acting drug but could be advantageous for an antiarrhythmic drug. Even if there is a high first-pass effect, depending on the targeted therapeutic area, this may not be problematic providing the drug does not have a very narrow therapeutic index (TI) and is not highly variable. However, if the first-pass effect is concomitant with a high systemic metabolism, it may be prudent not to go forward with development. Thus, detrimental results have to be evaluated and understood, and rather than a “No Go” point, it may be a “Slow Down” point, as in some cases, the detrimental results can be overcome with a good therapeutic agent brought ultimately to market. In this context, a phased approach has been proposed to facilitate a smart approach to development [27].

4.6 CLINICAL PHARMACOLOGY

It was stated earlier that basic research has used today’s technology to advance the understanding of disease(es) to new levels, which has resulted in a vast array of new NCEs to be translated into new drugs that improve patient health and management. However, in spite of all the advances on all fronts of the “medicinal sciences”, the successful transition/translation of discovery to a new therapeutic entity on the market is still not a routine process. It is our proposition that clinical pharmacology may be a foundation for the effective translation/transition of research and development to effective and safe medicines [28].

It goes without saying that drug development of a NME is not for animals but for humans; however, drug development starts with animal studies. Up to this point, an overview of animal nonclinical studies that have been outlined were presented in the context of determining a safety profile in animals that would support a first study in humans and thus continue development by conducting studies that collectively constitute clinical pharmacology essential for an NDA-targeted development.

In the simplest terms, clinical pharmacology can be defined as a qualitative and quantitative assessment of drug action once it enters the systemic circulation: an understanding of what the drug does to the body and what the body does to the drug. In the drug development process, these are studies in humans, single-dose and/or repeated-dose studies of short duration from which come the quantitative measurements of pivotal drug disposition. These first human studies are designated as phase 1 and are the learning phase for effective patient management, that is, safe and effective therapeutic use. These are usually conducted in normal subjects, although the use of patients may not be excluded. In general, exposure is limited to small numbers of subjects and safety/tolerance is always a looked-for outcome, whereas efficacy is not.

Clinical pharmacology has been a part of drug development for the past 30 years. In the recent past, there has been a trend to reduce the number of PK studies contained in an NDA submission to those considered to be essential, that is, BA, bioequivalence (BE), and dose proportionality, thus reducing unnecessary exposure to drug in healthy human volunteers. It was considered by some that these phase-1-type studies are not needed in depth as the NDA is approved based on the pivotal phase 3 safety and efficacy trials. In addition, PK studies not included in the NDA would/could be conducted as phase 4 commitments. More recently, the FDA has placed a new emphasis on PK/phase 1, as such studies are learning studies that provide understanding of drug disposition and thus being important to patient management. This can be attributed to the emergence of factors, such as race, gender, age, disease state, and administration with food, that can alter the PK–drug exposure profile and thus have an important impact on dosing regimens. This emphasis on clinical pharmacology/PK/BP and their importance to patient management is shown by the clinical pharmacology guidance documents issued by the FDA with recommendation that the results of clinical pharmacology studies should be included in the NDA and thus in the label. PK/BP studies are an integral part of the NDA and enhance an understanding of the drug in the therapeutic setting in addition to providing basic drug disposition information even those that may be conducted postmarketing (phase 4) to fill a gap.

It should be kept in mind that approval/approvability does not necessarily remove phase 4 commitments. Having some data that retrospectively addresses factors that affect drug disposition is not the same as formal studies designed to elucidate aspects that, today, are part and parcel of clinical pharmacology. Approval can be obtained, but what usually suffers is the label. In this context, it is worth remembering that a strong/complete label at approval will have a positive impact on marketing. The successful completion of phase 4 studies three to four years after approval has little impact other than fulfilling a regulatory requirement. In addition, at that time the argument may arise as to “why pay a \$200,000–\$300,000 fee for a labeling supplement in addition to the study cost.”

Often the value of phase-1-type studies is overlooked as emphasis is placed on phase 2 and phase 3 studies. It is certainly true that the data obtained from phase 1 studies does not approve an NDA. The issue falls back on *approvability* relative to having approval with a comprehensive label that provides important information for patient management. In other words, having a label that can be used by marketing and that differentiates the product from the competition. It should be mentioned that most of the negotiations/issues with FDA on the final label deal with the pivotal phase 3

studies, not with phase 1 data. This is not to say that a focused package of phase-1-type studies in early post-IND will not provide information for a better phase 2 program; additional phase 1 studies can, and should, be conducted on the way to the NDA.

4.6.1 First in Humans

The duration and safety margins obtained in the early animal toxicity/safety studies determine the entry of drug into human clinical pharmacology studies. However, before such evaluations, it is of value to estimate a maximum safe starting dose (MSSD) based on the safety profile obtained from the early animal toxicity studies. In this context, although not presented in detail, the algorithm to estimate the MSSD for first in human clinical trial has been issued by the FDA [29].

Such determinations are primarily dependent on observed preclinical toxicities and administered doses. In general, toxicity should be avoided at the initial doses. The doses chosen should allow a demonstration of the tolerability and the PK profile, which are the goals of early phase 1. The major elements in the animal species are the no observable adverse effect level (NOAEL); the level for each species tested should be converted to the human equivalent dose (HED) and converted by appropriate body surface area conversion factor (BSA-CF). This conversion factor converts animal dose in milligram per kilogram to the appropriate human dose in milligram per kilogram. In general, the resultant safety factor should be at least 10-fold. The guidance document presents tables of the factors to convert animal doses to HEDs based on the body surface area, which are to be used in the estimation of the first maximum human dose.

Usually, the first human study conducted is a single oral dose (SD) tolerance study that can also provide the first window on the clinical PK disposition of the drug: parameters such as $C_{\max}$, $T_{\max}$, AUC, $t_{1/2}$, Cl/F, and Vd/F. If an intravenous form is available, conducting this study as a PO/IV crossover would yield additional information such as absolute BA and a true estimate of Cl and Vd. In practice, a parenteral dosage form is not usually available; even if available, an IV toxicology study of at least one week duration has to be performed in at least one species (rat) to support the IV human study. There may also be resistance from management for using an IV dosage under the rationale that “we are not going to market an IV dosage form.” It should be pointed out that the rationale is not to develop an IV dosage form but to provide an important PK information, for example, BA to the overall development process. Therefore, drug is typically administered orally in hand-packed capsules.

Validated bioanalytical methods for measurements of drug concentrations are essential for the elucidation of drug disposition, and their importance has been presented earlier. The early validation of a very sensitive and robust bioanalytical method specific for drug substance can present an opportunity to conduct a microdose study in early development. Microdose studies administer doses resulting in suboptimal exposure and thus do not determine the safety or efficacy; the primary objective is to determine the drugs' PK profile. As such, they are used solely as a screen for ranking the ADME properties of drug candidates [14]. Such information early in development can contribute to better candidate selection for development. The advantage of microdose studies is thus readily discerned, obtaining a window on drug disposition before implementing

formal/traditional phase 1 clinical pharmacology studies. Every drug development program is not appropriate for microdose studies. However, if and when implemented, the quality of development decisions can be improved and thus decrease the drug failure rate.

Accelerated mass spectroscopy (AMS) is a new analytical technique that has much promise in the future [30]. AMS is really not mass spectroscopy as it is generally known. In short, ions are accelerated to extremely high kinetic energies before mass analysis. The special strength of AMS is that for techniques that measure decay, it separates a rare isotope from its abundant neighboring mass, for example, ^{14}C from ^{12}C and ^{14}N from ^{14}C . To a large extent, this is still experimental and not readily in use and thus primarily for macrodose studies, but its cost prohibits larger use. Considering the advances in MS/MS technology and highly improved detection limits, AMS may not be an added value as an analytical technique for drug disposition.

Multiple dosing (MD) studies for 7 days with dose escalation can follow until a maximal tolerated dose (MTD) is achieved, that is, an observed undesirable side effect or in rare instances, a measurable therapeutic effect is observed. In addition, a dynamic response may sometimes be measured, for example, diuresis; but a PD response has to be treated with some caution as normal volunteers (NVs), not patients are used.

4.6.2 Overview of Clinical Pharmacology

The text that outline studies that are examples of clinical pharmacology programs that give guidance for phase 2/3 dosing regimens. The conduct of these studies defines a comprehensive presentation of clinical pharmacology and, ultimately, become part of the product label as the guide for general therapeutic use. The figures show examples of clinical pharmacology studies that are conducted as regulatory requirements; the sequence of conduct is neither absolute nor important and is dependent on the individual drug, the route of administration, the targeted indication, and the information obtained to that date. It is a *menu* of studies required, the order of conduct can be *choose and pick* and thus provide a rational clinical pharmacology program with impact on the design of phases 2b and 3 that results in verifiable safety and efficacy leading to marketing approval. It should be stated that the content of the figures is not new or original to the authors, although they are arranged/grouped in an order that has been found to be advantageous in past experience.

In drug development, there always is a time-line-driven push to do more with less and faster. It was stated previously that such an approach may result in an undesired outcome and ultimately, in failure of the program. The research and development staff should thus focus on their goal and the desired outcome of the development program: a favorable package insert for filing. They need to decide how to get from where they are now to where they need to be (marketing approval) in the most the efficient, effective, and shortest time as possible.

In certain instances or places a Go/No-Go study may be suggested. These studies are conducted in NVs, although studies in patients in the targeted patient population are not excluded and used where/when warranted. Details of study designs are not given in detail; but some elements of design and the specific guidance to conduct the studies are provided.

- IV/PO single-dose PK in NVs provides the intrinsic PK following the administration of dosage forms (tablets, capsules, liquids) used in the pivotal phase 1 trial

to determine the relative BA and BE [31]. Usually, these studies are conducted as an open label; single-dose crossover design with a small number of subjects (usually, 18–32) determined statistically to meet the “80–125% rule.” However, if drug variability is high and requires very large subject numbers (60–80 or more) to meet the BA/BE criteria, use of a single-dose, four-period, four-sequence, randomized, crossover, replicate study design can be used to obtain BE [32].

- Effect of single doses of high fat food on the rate and/or extent of absorption relative to the fasted state studies are a requirement that should be conducted in early development [33,34]. Although the mechanism of the effects of food is not clear, food can also influence factors such as bile flow, gastric emptying, luminal metabolism, the first-pass, and complexity with excipients, all of which can affect drug absorption. If the effect of food is demonstrated, a study on the time of drug administration relative to food administration should be ascertained [34].
- If the drug is targeted for pediatric use, the development program includes the BP of oral solutions or suspensions, dosage forms targeted for the therapeutic use by children. However, if drug development is primarily for use in adults and is/can be used by children, oral dosage forms such as capsules and tablets may present children with difficulty in swallowing. In such case, studies should be designed to determine if the tablet can be crushed and administered with applesauce. These studies are considered *food studies* and are evaluated as described above.

The guidance documents mentioned earlier address the study design, the statistical evaluation for acceptance (90 CI, the 80–125% “rule”), and the number of subjects to be used. The main objective is the respective BA and/or BE study as directly applicable to clinical use. However, the study design generates the intrinsic PK properties of the drug ($C_{\max}$, $T_{\max}$, AUC, $t_{1/2}$), and these properties are included in the data presentation. For repeated-dose studies to steady state, escalating dose MTD/tolerance studies and dose linearity studies to ascertain if PK properties are linear are used. Although there are no specific guidance documents, those mentioned above provide the rationale, study design and statistical evaluation (if needed/appropriate) as above.

An understanding of the metabolism of the drug under development is a requirement for marketing approval. It is required to identify all metabolites formed and their relative concentration (percentage of total mass). The metabolic reaction types such as phase 1 transformations can be either by the introduction of a functional group (oxidation) or by the unmasking of functional groups (hydrolysis) and are the main steps of the metabolic disposition of drug. Phase 2 transformations are primarily the conjugation reactions that result in highly polar derivatives for excretion in urine, bile, or feces. Taken collectively, metabolism studies determine the mass balance of the drug (and drug-related material), characterize the routes of elimination and drug clearance, and identify and quantify the drug remaining in systemic circulation. An additional and more detailed treatment of drug metabolism is presented below in the role of drug interaction studies.

Single-dose metabolism studies using ^{14}C -radiolabeled drug(s) are conducted in early development to ascertain the information on the presystemic metabolism, the mass balance, and the routes of elimination. The use of ^{14}C -radiolabeled drug is a proven tool for drug metabolism; however, the radiosynthesis of the drug substance is required and may, depending on the synthetic route, be costly to obtain the specific

activity required. Replacing ^{14}C with stable isotopes for the use in drug metabolism may be more advantageous [35], and this approach has been used to determine an extensive metabolic disposition of drug in the rat [36].

An important *in vitro* study considered to be clinical pharmacology is the determination of the extent of plasma protein binding to determine the free drug concentration (not to be equated to the free fraction). Included is determining whether plasma protein binding is drug concentration dependent or not and the major carrier proteins, for example, albumin and AGPs.

Clinical pharmacology studies are regulatory requirements to determine drug disposition as affected by intrinsic factors (renal and hepatic insufficiency) that compare the PK of subjects with normal organ function to matched groups of the respective impaired organ [37,38]. This evaluation is important since the liver is the main organ of drug metabolism, whereas excretion of drug and metabolites are mediated by the kidneys.

It is generally recognized that numerous drugs are metabolized by the CYP450 enzymes located primarily in the liver. Most of the drug-metabolizing enzymes are in the CYP1, CYP2, and CYP3 families. The activation and metabolism of many drugs are dependent on the enzymes of the CYP450 system and can lead to wide interindividual differences in the clearance of drugs. These differences in metabolism can alter the dose–response relationship, leading to an increase in adverse events and toxicity as well as therapeutic failure [1,2].

Of the 50 CYP450 enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 are responsible for the metabolism of $\sim 90\%$ of currently marketed drugs [39]. These enzymes are predominantly located within the liver but may also occur in the small intestine, lungs, placenta, and kidneys [39]. The CYP3A4 isozyme is very abundant and important in the metabolism of many drugs. Its presence in the gastrointestinal (GI) tract is responsible for the low oral BA of many drugs. It should be noted that nondrug substances such as grapefruit juice and herbal supplements such as St John's wort can cause significant inhibitory and inducing effects, respectively, on CYP3A4 substrates.

Of the major isozymes identified above, three have shown genetic variance in their alleles (CYP2C9, CYP2C19, and CYP2D6), which can be expressed phenotypically in patients as poor, intermediate, extensive, or ultrarapid metabolizers. Therefore, drug metabolism via CYP450 isozymes exhibits variability (polymorphism) that can influence an individual patient's response to a drug.

Genotyping for patients who receive mono- or polytherapy medication that are metabolized by isozymes that show variable individual metabolism (CYP2C9, CYP2C19, and CYP2D6) may assist clinicians in avoiding adverse drug–drug interactions and to individualize treatment with new pharmaceutical agents better. In the future, the use of genotyping to determine metabolic status of polymorphic drug-metabolizing enzymes will be integral to efficient patient care and management.

Although not all inclusive, the above outline of clinical pharmacology reiterates and confirms the role of clinical pharmacology/phase 1 and represents the learning stage of development during which an understanding of the drug and how to use it is obtained, whereas, as was stated previously, phase 2/3 uses what has been learned and confirms safety and efficacy.

4.6.3 Decision-Making Studies

As mentioned above, for some indications, certain studies can (and should) be conducted in patients rather than in NVs. Studies on drug interactions and hepatic and renal dysfunction must be conducted according to their respective guidance's. Once a drug substance is targeted for development, the expeditious conduct of a core toxicology package allows the initiation of the early clinical studies: an effective development program. It should also be mentioned that, as indicated/suggested in the figures below, many of the studies can be conducted in parallel, resulting in the overall development time line on the way to the NDA.

It has been stated previously that today PK/BP are well-defined components of drug development. There has been a progressive and increased emphasis by the FDA on the understanding of the PK and PD of drugs not only in healthy subjects but also in special populations. In this context, there is a great emphasis to evaluate and understand the potential for *in vivo* drug interactions, especially as polytherapy is the norm with an aging population. Thus, the "Clinical Pharmacology" and "Dosing Information" sections that correspond to these sections of an NDA dossier can comprise the major part of the label as the information therein is targeted to guide patient management (as an example, the labels of the two marketed cyclooxygenase 2 (COX-2) inhibitors contain the results of 17–22 drug interaction studies). On the basis of the regulatory guidance documents, pharmacology studies will provide data that are decision making as the development program moves forward. In the following sections, examples of several scenarios that illustrate effective drug development in phase 1 with inclusion of the current emphasis on drug interactions are presented in some detail.

4.6.3.1 Decision Based on Early Phase 2. This development outline is based on an early understanding of the drug as to its therapeutic value and thus continuing development.

Figure 4.2 introduces a concept that is an important contributory to the Go or No-Go decision: POC. In its simplest definition, a POC is a demonstration that a certain method (or idea) is supported by data of its feasibility and whose purpose has the potential of being used. In drug development, POC is not synonymous to proof of principle as

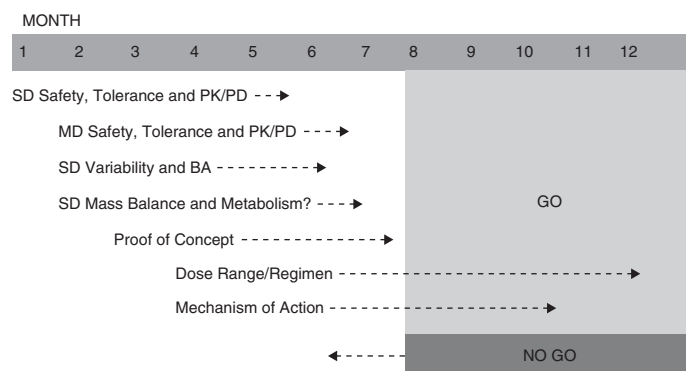


Figure 4.2 Phase 2a for Go/No-Go decision.

they lack clear definitions and they vary in usage between authors, investigators, and institutions and in usage over time.

In these early studies, it is usual that the preliminary dosage forms are used, for example, hand-packed capsules rather than galenic formulations. In many, perhaps, in most cases, the defined PK profile is the main objective. As/when development continues and a final dosage form is developed (for phase 1b or phase 2), a bridging BP study would be conducted relative to the initial form. The absorption PK would be compared as to the validity of the early study using a study design to determine whether the PK is linear over a projected dose range.

POC refers to early development, and the convention is to divide it into *phase 1* and *phase 2a* studies. Phase 1 studies are usually small ($n = 10\text{--}20$) single-dose studies in healthy volunteers; a short course of repeated doses (one to two weeks) can be conducted. A single-dose study determines whether the desired PK profile is achieved and the tolerance of the administered dose; with repeated doses, a signal for the desired clinical activity may be an outcome. At this stage, a Go decision implements the ADME studies required for complete development. Phase 2a is usually conducted in up to 100 patients to ascertain tolerance/safety and pharmacological activity and select a dose for the NDA-targeted studies.

The above panel outlines a series of studies that would allow the conduct of a POC study, the results of which determine the Go/No-Go decision.

The underlying principle for the POC concept is related to the use of biomarkers and surrogate end points in early clinical trials. This is a very extensive topic and, in fact, is the subject of not only research publications but also entire books; as such, only an introduction of the topic can be accommodated. In simple terms, a biomarker is a substance whose detection has been shown (validated) to correlate with the detection and/or progression and treatment of a disease. In clinical research, a clinical end point is a sign that defines a target outcome of a study. In this context, early drug development programs include investigations to identify and validate biomarkers to ascertain a Go/No-Go decision. In fact, the search for biomarkers is increasing and is now part of drug development in general. The scope and intent of this chapter can only be introductory in nature as a full treatment would warrant its own chapter.

However, it is necessary to distinguish between *disease-related* and *drug-related* biomarkers. Disease-related biomarkers indicate a probable effect of treatment in case(s) if/when the disease already exists. Drug-related biomarkers indicate whether a drug will be effective. At present, there is much effort in the discovery and development of innovative and effective biomarkers; these new biomarkers have become the basis of preventive medicine. However, it should be noted that even though a process can be repeated, it may not mean that it is true; thus, a new biomarker should undergo a rigorous validation.

Even when validated, in a clinical trial, meaningful outcomes based on biomarkers can be expensive or impossible to achieve. In this context, surrogate markers/end points that measure the effectiveness of treatment are used especially when these correlate with a real clinical end point. It is a measurement that is easier to assess than the true end point.

In essence, a surrogate marker (or end point) is a measure of effectiveness, as it is intended to substitute for a clinical end point. Surrogate end points often occur after an intervention in the disease process and can be an effective substitute. As with

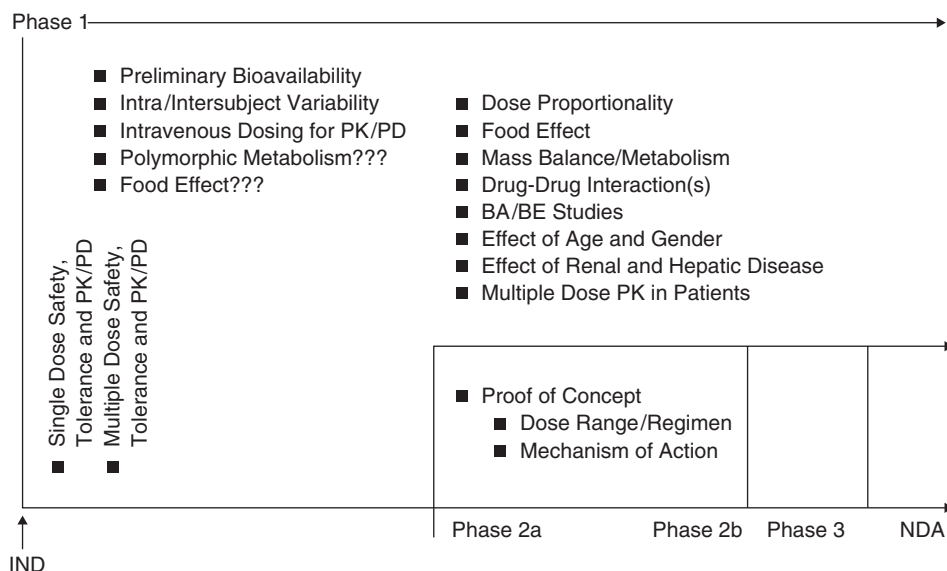


Figure 4.3 Efficient early drug development.

biomarkers, validation is a critical factor. For validation [40] as an end point, these conditions are to be met: (i) evidence that the surrogate predicts the true end point; (ii) end point must be specific, for example, the intervention is mediated via the surrogate end point; and (iii) all information on adverse effects associated with the intervention must be captured.

Thus, it is efficient to conduct a POC study as soon as possible, even though the animal toxicity studies indicate good safety margins for human studies; without efficacy, there is no need for further development.

Assuming that the POC study clearly indicates efficacy, development would enter phases 2a and 2b with studies in the targeted patient population in order to determine the dose(s) for the pivotal phase 3 studies.

Considering that conducting clinical pharmacology studies are not dependent on the final clinical dose, many studies can be conducted in parallel to phase 2. In this case, the sequence of conduct impacts efficiency. This is illustrated in Figs. 4.3 and 4.4, which present the sequence in a somewhat different manner.

There are occasions when the data in early development support a Go decision for further development, but for a variety of reasons, the decision is made to out-license the drug. Again, if there are no safety concerns from the animal toxicity panels conducted, interest to in-license the drug would depend on the clinical studies conducted. A suggested scenario with single-dose human data is shown in Fig. 4.5.

In the development program presented above, the goal was to obtain an open IND and to enter phase 1 clinical development. The above chart presents a program where the objective is to bring the development forward to the stage where out-licensing could be an option.

The above scheme would be greatly facilitated by the *in vitro* determination to determine how metabolism occurs using rat, dog (the toxicity species), and human liver

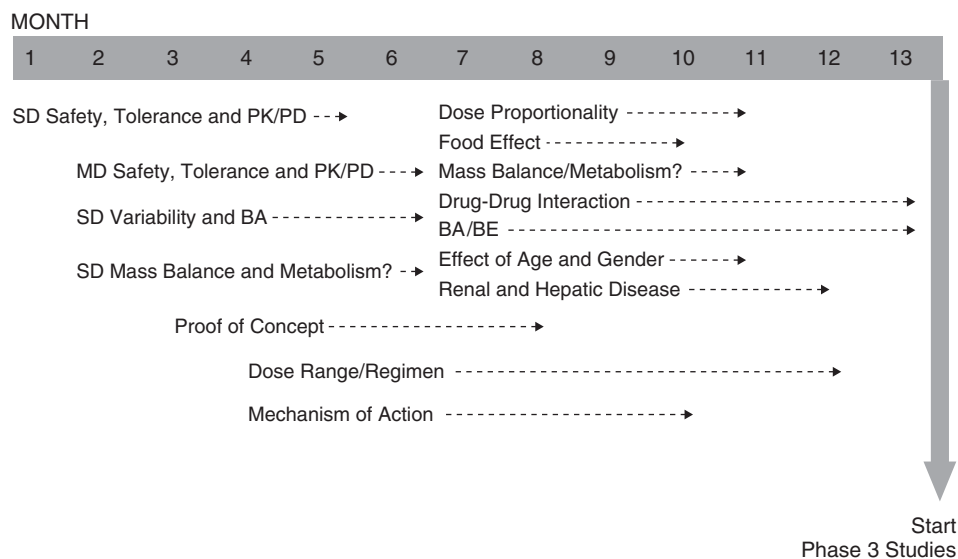


Figure 4.4 Efficient development to NDA.

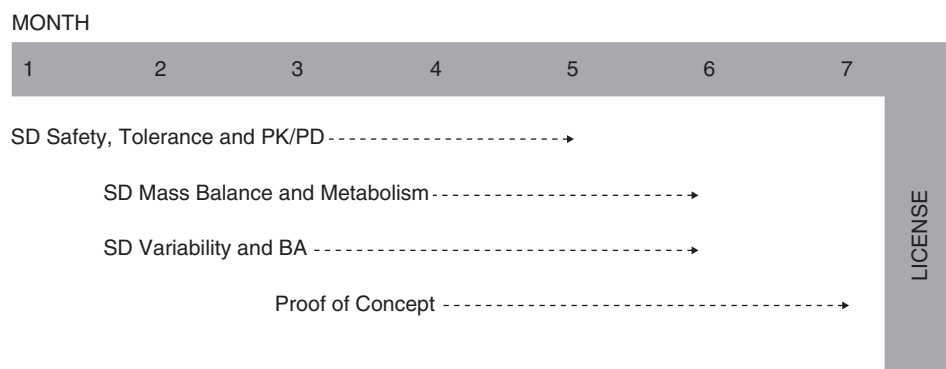


Figure 4.5 Single-dose proof of concept for licensing.

microsomes. This experiment would also identify the CYP450 isozyme(s) involved in metabolism and thus provide information as to a drug interaction potential if development continues. The availability of ^{14}C drug product at this time would greatly facilitate the determination of mass balance (and metabolism); however, using cold assays and collecting urine and feces in the SD study, in addition to plasma, would provide a reasonable preliminary determination of the mass balance without the additional expense of ^{14}C synthesis. However, this would need to be performed once a Go decision is reached after phase 2a.

Out-licensing can also be facilitated by the conduct of repeated-dose clinical pharmacology studies. An outline of such an approach is shown in Fig. 4.6.

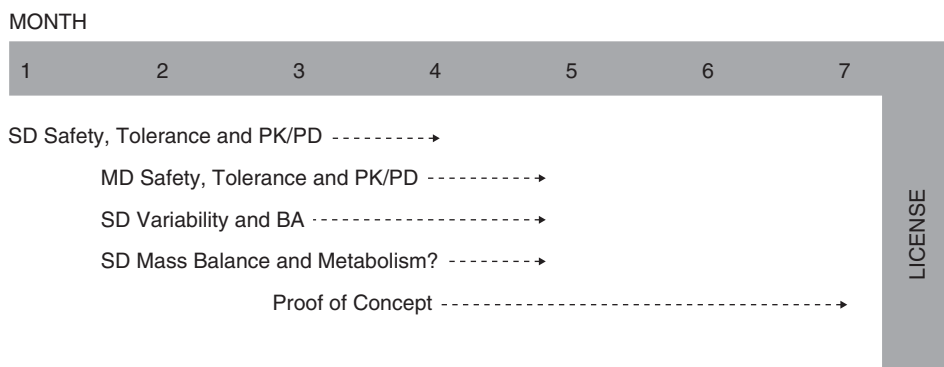


Figure 4.6 Proof of concept for licensing.

The advantage of a positive POC is self-evident. However, the availability of steady-state PK from the multiple-dose study would be an added attraction as such would have an impact on phase 2 studies in the targeted patient population.

4.6.3.2 Radiolabeled Studies (ADME). The conduct of a single-dose PK drug disposition study (ADME) is a powerful tool in development, especially when conducted early in the development process. A study with ^{14}C drug and the collection of serial plasma samples over a dosing interval can provide a qualitative window on the extent of metabolism from the respective slopes of total radioactivity relative to cold drug. Also, the AUCs of the total radioactivity relative to the cold drug can provide additional information, for example, elimination half-life of both moieties. In addition, from concentration–time data in urine and feces, the major routes of elimination and thus a mass balance can be obtained. Under ideal circumstances, a study in six NVs would be conducted using both IV and oral dosing in a crossover manner; the duration of sampling time would be monitored for radioactivity until the radioactivity is at LOQ. It must be stated that it is a regulation that a rat dosimetry study *must* be conducted to obtain an estimate of the residence time of the radiolabel and the conduct of human study must be approved by government of the state where the study will be conducted.

The challenge of an ADME study is the availability of the ^{14}C drug of sufficient purity and a high enough specific activity for accurate quantitation. There may be resistance to the cost of the synthesis, but it should be pointed out that once available, the radioactive drug can/will be used for required rat tissue distribution and plasma protein binding studies. In addition, in the case where the most appropriate animal species for the pivotal toxicology/safety evaluations is not evident, a radiolabeled study in several species (dog, monkey) would be conducted. By comparing radioactivity concentration in plasma, urine, and feces, it can be estimated which species approximates humans in metabolism and PK.

4.6.3.3 Role of Metabolic Disposition Studies. The role of metabolism in the overall process was mentioned previously. In general, drug metabolism (biotransformation) occurs as two categories: phase 1 and phase 2 reactions. Phase 1 reactions are nonsynthetic reactions and include oxidation, reduction, and hydrolysis. These reactions are

mediated by the CYP450 located primarily in phospholipid bilayer of the smooth endoplasmic reticulum and most of the organs in the body. Phase 2 reactions are synthetic biotransformation reactions that *link*/conjugate the parent drug or a phase 1 metabolite with an endogenous substance. These reactions include glucuronidation, sulfation, acetylation, methylation, and other conjugations. Taken collectively, the purpose of metabolism is to convert the exogenous lipophilic drug to water-soluble metabolites that are readily excreted from the body.

Formal studies to identify the major metabolite(s) in the systemic circulation—the number of metabolites formed *in vivo*, their structure, and whether any of the metabolites have a pharmacological activity; the routes of elimination; and a mass balance—constitute a more comprehensive role for metabolism in drug development. An important outcome of such evaluations is the elucidation of the metabolic pathway of a drug in humans and also in animals.

It is important to the understanding of the drug to determine the site of metabolism (hepatic, presystemic) and the specific member(s) of the CYP450 family of isozymes that mediates the metabolism. The identification of the specific CYP that is responsible for metabolism is virtually mandatory, especially as some isozymes have overlapping activity. The ability of the drug to inhibit or induce a member(s) of the CYP450 family *in vitro* is an important study that has downstream implications on drug–drug interactions potential *in vivo* in the clinical setting; specific protocols for such studies and when such studies need to be conducted can be found in the FDA and ICH guidance documents. It should be noted that these studies should be conducted earlier rather than later, especially if more than one isozyme contributes to the metabolic disposition of the drug. When metabolism occurs via multiple isozymes, clinically significant *in vivo* drug–drug interactions are less likely to occur (also see Section 4.6.3.4). What is more important to understand is the involvement of multiple isozymes rather than all possible outcomes.

In Figures 2–6, individual studies are presented as line items that, when taken in the collective, illustrate the mosaic defined as early drug development. As a collection of line item studies, it is natural to see the total, and thus easy to overlook one of the line items in terms of its importance to the whole. Thus, although drug–drug interactions are depicted in one line, their importance greatly exceeds their presence in drug development schemes. Today, the understanding of drug interactions with another drug administered concomitantly is an essential part of drug development, especially in polytherapy. It is prohibitive, and not the intention of this report, to recite drug–drug interaction presenting data of specific examples. The intention is to present key elements of drug interaction studies as a process, with study design based on the regulations as they apply to a specific drug and/or therapeutic area.

4.6.3.4 Drug Metabolism–Drug Interaction: *In Vitro* Studies. Once in systemic circulation, the process of a drug's elimination is initiated either by excretion intact in urine or feces or, primarily, by biotransformation (metabolism) [41]. If/when metabolism is the main process, the metabolic routes(s) are of importance as they can significantly affect the safety and efficacy of the drug. If elimination is via a single pathway, individual differences in the metabolic rate(s) can lead to significant differences in systemic concentrations of drug and/or metabolism as a consequence of activities of the CYP450-mediated metabolism. This metabolic pathway may be relatively simple or may be complicated by the involvement of multiple and

concomitant cytochrome isozymes with additional complexity added if these exhibit bimodal distribution because of a genetic polymorphism. For these reasons, it is important to learn the early stage of development in the metabolic route if the drug is eliminated primarily by metabolism and to ascertain its route of elimination. If metabolism is the primary route, it is important to identify the specific CYP450 isozyme(s) responsible for the drug's biotransformation.

The most prevalent site of drug metabolism is the liver. The most mature technology for the *in vitro* determination of hepatic drug metabolism is the use of human liver microsomes. Using selected commercially available CYP450 inhibitors for specific pathways, the metabolic pathway can be either demonstrated or ruled out. Once a pathway has been determined, the metabolic pathway can be confirmed using recombinant human proteins. Human liver microsomes are also used in assessment of a drug–drug interaction by concomitant incubation(s) using a concentration of the test drug and a standard test probe compound. A complete evaluation in detail is not the purpose here, and such a treatment is found in Ref. 41. It is also important to point out that *in vitro* studies do not adequately define the metabolic pathway and should be subsequently verified in a clinical study.

4.6.3.5 Drug Metabolism–Drug Interaction: *In Vivo* Studies. Current therapeutics seldom involve monotherapy, as the general population is older and whose treatment utilize multiple drugs on a daily basis [42]. The physiological changes in metabolism associated with aging increases a potential for an occurrence of drug interactions.

Not every drug–drug interaction is metabolism based. Changes in PK due to absorption, plasma protein binding, distribution, and excretion can be a mechanism for drug interaction. Thus, any drug interaction should be adequately assessed in the overall assessment of drug safety and efficacy. In this context, all drug–drug interactions are only relevant when a drug's metabolite(s) have been identified and their pharmacological properties elucidated. And only then, an understanding of the relation of specific drug(s) may emerge as a relationship between *in vitro* and *in vivo* is evaluated.

The potential for a drug–drug interaction is first evaluated *in vitro* by identifying the CYP450 isozyme(s) involved in its metabolism using proper studies designed for this purpose. Once identified, separate studies are conducted to assess the potential for its induction or inhibition and whether the outcome is a linear process. A clinical study is then used to determine the effect on disposition of the drug in question. Whether the uncovered PK changes are clinically significant is determined by the TI of the drug: if the TI is large numerically, changes of even 50% are not significant; however, if the TI is narrow, 10% changes can be significant in the clinical setting.

In general, *in vivo* drug–drug interaction studies compare concentrations of the drug to be evaluated (substrate, S) to determine if its exposure is changed relative to another drug that is interacting (interactant, I). A study can be conducted by several crossover designs and several combinations of single–repeated doses. The above mentioned guidance document provides various study designs, depending on the drug and study objective. One objective of the study design that needs to be kept in mind is that inhibition or induction needs to be evaluated as the end point. In general, induction takes longer to be ascertained, whereas inhibition is attained relatively more rapidly. In addition, the outcome sought may be concentration dependent: the induction of CYP3A4, the most prevalent hepatic isozyme accounting for ~ 40% of hepatic

mass, is both concentration- and time-dependent inductions [43]. In most cases, inhibition/induction drug interaction evaluations are conducted as single-dose studies; if done at steady state, data to document such is needed.

Clinical drug–drug interaction studies are/may be conducted using healthy volunteers. Safety considerations should always be carefully evaluated if the desired outcome may preclude their use because of not knowing if the drug’s metabolism is a result of polymorphism. In current drug development, it is not uncommon to determine a subject’s phenotype or genotype before study conduct. To characterize a drug as to its potential to be an inducer or inhibitor, the establishment of which CYP isozyme(s) is involved in its metabolism is desired as quickly as possible.

PK end points with studies at maximum or approved doses should be evaluated. Parameters to be assessed include AUC, $C_{\max}$, and time taken to attain $C_{\max}$; PK parameters are clearance, volume of distribution, and half-lives. The exposure parameters are reported as 90% confidence intervals of the geometric mean ratio of the PK measures with and without the interacting drug. The guidance documents [42] is important, as it presents not only specific aspects of study design but also data evaluation.

The importance of understanding drug–drug interactions and its role in therapeutic drug use has resulted in the development of surrogates using *Escherichia coli* for a rapid characterization of CYP450s 1A2, 2C9, 2C19, 2D6, and 3A4; these surrogates agree well with corresponding human hepatic isozymes in literature, and thus indicate that such recombinant isozymes may be of value as predictive human metabolism [44].

4.6.4 Drug–Drug Interactions: on the Way to the NDA

The drug–drug interaction studies in early development, by large, can be considered as monotherapy and as such determine the basic foundation of how/when interactions occur: the studies are designed to produce an interaction. A goal in development is and should be to learn the drug disposition, to understand the drug under development, and then to apply this knowledge for successful therapeutic interventions in a large diverse population. Today, monotherapy, although desired, is relatively rare. This is especially true as the general population is elderly and uses multiple therapeutic agents on a daily basis. Thus, as stated above, it is very important to evaluate and understand contraindications of concomitant drug use as the resultant adverse or serious adverse reactions can occur. If the prevalence of these adverse reactions is considered serious with some incidences of death, the drug may be *pulled* from the marketplace.

The identification of which CYP450 isozyme(s) is responsible for the drugs metabolism, whether it can be induced or inhibited, cannot be overemphasized, especially as several CYP isozymes, (e.g., 2D6, 2C9, and 2C19) show a bimodal distribution because of genetic polymorphism. Polymorphism can affect the metabolic route of elimination and alter the systemic plasma concentration profile of the drug, with a consequence requiring dose adjustments to maintain safety and/or efficacy.

The common use of pharmacogenetics (genotyping of patients) already influences therapeutics: for Caucasian patients, ~ 7% cannot metabolize drugs that are primarily metabolized by CYP2D6. Similar information is being uncovered for CYP2C9, CYP2C19, and *N*-acetyltransferase. CYP3A4 activity is involved as the primary isozyme in the metabolic disposition of many drugs, both systemically, as it is the most abundant hepatic enzyme, and pre-systemically (during the absorptive process).

When the role of CYP3A4 is established, a drug interaction with ketoconazole (a strong inhibitor of CYP3A4) is conducted to evaluate the effects on the $C_{\max}$ and AUC of the drug; effect–no effect is established by 90% confidence interval, the 80–125% rule.

A current understanding of all factors that impact on metabolism-based drug–drug interactions is available as a guidance document [45], although as a draft. This guidance combines the salient aspects of general factors for the evaluation of clinical drug–drug interaction but of more importance, presents a comprehensive and extensive treatment of all current knowledge of metabolic drug interactions. This information is presented in tabular form and includes current summary of data from recently conducted trials. Such a comprehensive treatment of drug interactions is not in the scope of the present report but is presented to as a continuum and references what can/should be used as the development process continues.

To this end, an overview of some new aspects of development, especially in drug–drug interactions are briefly presented since they are an indication of the direction of effective development being implemented now and refined in the future.

- The pivotal role of CYP3A4 as the most abundant hepatic and intestinal isozyme that metabolizes half of the drugs on the market will be evaluated in development programs of new drugs to determine if drugs are substrates, inhibitors, or inducers of this pathway [46].
- Substrate specificity and regulation of drugs that exhibit polymorphism of their expression, especially of isozymes that are considered to have a low level of their expression will be included in routine drug–drug interaction studies [47].
- The role of transporters will be elucidated to provide advancement to the critical path of drug development [48,49].
- The use of drug cocktails in the conduct of drug interaction trials assist in clearly defining drug interactions in the clinical setting [50–53].

The above represents current advances in technology that have been, and continue to be, incorporated into current drug development, resulting in better therapeutic outcomes.

4.6.5 Past “on the way to the NDA”

Most of the concepts previously presented were targeted for early drug development to support safety and efficacy, especially in drug disposition as the learning process to a successful marketing approval. As such, they are set pieces aimed to open an IND, contribute to the design of the phases 2 and 3 studies, and if/when a successful NDA is achieved, provide specific guidance to the safe and effective management of patients, which is the label. Thus, it is worthwhile to revisit the process as a whole presenting the end of drug development as it is condensed into a primer for patient management.

4.6.6 The Label

A general outline of the studies that would contribute to the final label (Package Insert) has been presented previously. The presentation order of the information in the label

can vary with the drug and the content of the development program. There is, however, a general format that is followed and this is (in part) given below.

Description of the drug product listing the dosage form(s), a brief galenic composition and available strength(s) is usually presented first, followed by the structure, molecular weight, chemical name, the empirical formula, and its appearance and solubility.

Clinical pharmacology: These are the phase 1 single-dose studies in NVs, although repeated-dose studies and using patients are not excluded. Studies dealing with BA; BE; food effects; effects of age, gender, and ethnicity; and the dosing regimen(s) are presented with the appropriate metrics.

Mechanism of Action: The targeted indication is then given with a short summary of the key elements that describe the activity of the drug in the indication.

Pharmacokinetics: The systemic moiety (parent drug and/or metabolite) that affects the activity is then stated. The key PK parameters ($C_{\max}$, $T_{\max}$, AUC, $t_{1/2}$, CL, and V) in NVs and targeted patient population following oral and, if conducted, intravenous administrations are presented. Intra- and intersubject variability is addressed in the context of a dosing regimen. The PK sampling compartment (blood or plasma) is given.

Absorption: The extent of absorption (percentage of relative and/or absolute BA) in the target patient population and NVs is given. If there are different strengths and dosage forms (e.g., 5 mg tablet and 10 mg capsule), the BE of these is determined. If the patient dosing regimen involves several doses, the linearity of the systemic exposure as based on the correlation of the doses to $C_{\max}$ and AUC is determined.

Food Effects: The rate and extent of absorption are tested under fasted conditions and compared to the same when the drug is administered in the presence of food. A high fat meal (850 kcal, 46% fat) is the meal tested. If significant, the effect of the high fat meal is compared to a high carbohydrate meal (680 kcal, 85% carbohydrate) relative to the fasted condition. The presented effects are significant relative to a clinical setting are thus described with the addition of the results of the time of food administration relative to drug administration (2 h before, with drug, or 1.5–2 h after the meal). Taken collectively, the food effects are an implicit recommendation for patient management.

Distribution: In very early development, the partitioning of the drug between blood and plasma should be established to ensure that the right compartment for PK is sampled. The extent of plasma protein binding of the parent drug and its major metabolites should be determined *in vitro* by equilibrium dialysis and compared to that in *ex vivo* plasma samples, especially in special populations (renal, hepatic insufficiency). The following should be ascertained: is binding concentration dependent, the contribution of carrier proteins (albumin, AGPs) as their concentration in plasma can change in disease states. If the extent of binding is high (>98%), it should be remembered that even small changes in the free fraction (Ff) can have a large effect on drug disposition.

Metabolism: The extent of metabolism as well as identifying the CYP450 isozymes is presented. The identity of the metabolites, especially the major metabolites, is given with *in vitro* results compared to *in vivo* results. A metabolic pathway is

proposed. The metabolic profile is important to considerations of a potential for drug–drug interactions as was presented above.

Excretion: The routes of elimination (urine, bile/feces) are determined and quantitated, and a mass balance estimation is given. Usually, this is conducted using the ^{14}C drug, although cold assay can be used but may present a challenge in terms of recovery and resolution of drug from noise.

Special Populations: The parameters of exposure (AUC and C_{max}) as well as $t_{1/2}$, V , and CL are determined in patients with renal and hepatic impairment and compared to subjects with the respective normal function.

Issues such as effects of race/ethnicity and gender on drug disposition are addressed as specific stand-alone studies, but such information can also be obtained from the phase 2/3 studies.

Nonclinical studies that impact on safety are also in the label: carcinogenesis, mutagenesis, and reproductive toxicity, which form the basis of the pregnancy category. These studies were stated previously in the outline of the nonclinical studies that are conducted as part of the toxicity/safety evaluations; the study design and resultant evaluations are given in their respective guidance documents.

4.7 SUMMARY AND CONCLUSIONS

When all is said and done, drug development is a costly and time-consuming process; there are no *shortcuts* in spite of a detailed road map and experience. Although the same outcome is desired in principle, each drug and its development process is different in specifics, cost, total development time, and more importantly, regulatory acceptance. If the development is successful, the end product is a dossier of study reports conducted that present a convincing conclusion that based on data, the drug is safe and efficacious and warrants approval to market. In this context, there is always pressure for efficient drug development implying the unspoken caveat meaning “do it quicker” or “less costly” or “be smarter.” However, the appropriate description should be effective for this characterization encompasses the process in terms of where it was, where it is, and where it is going, specifically, marketing approval.

It is not the intent of the present report to be all inclusive; in fact, this would not be possible because of pragmatic limitations. We have concentrated on segments in early development that present rationales to move development forward with the caveat that the bulk of development is conducted “on the way to the NDA.” Whatever variations in timing and procedures an individual development undergoes, safety and efficacy should never be lost in sight and should be demonstrated by data. Data using rational study designs and flexible study conduct sequences that provide information for Go/No-Go decisions are essential. In other words, an effective development program in which core nonclinical safety studies enable an open IND and is followed by the first human studies (phase 1, clinical pharmacology), which provide the learning tool for information on the drug. As development continues, what has been learned is confirmed in phase 2 and phase 3 and the safety and efficacy of the drug in the context of the dosage and dosing regimen established.

Figures 2–6 that outline several phase 1 scenarios are examples of an effective phase 1 process and are not singular in that these and their variations have been found to be of use by the authors (and others) and are thus given as examples. As shown, it is important to ascertain the drug release profile as an evaluation of the dosage form, the extent of systemic exposure and assess factors that can influence absorption (i.e., food). As a general consideration, mass balance, routes of elimination, major metabolite identification, and the elucidation of the metabolic pathway must be confirmed. Considering that today's polytherapy is the norm in an aging population, the identification of the key metabolites and the CYP450 isozyme(s) mediating such metabolism is essential. Whether these are induced or inhibited is mandated to understand the potential for a clinically meaningful drug–drug interaction(s) and is mandatory for effective patient management with safety and efficacy.

On the basis of today's knowledge, the role of polymorphism in determining drug interaction potential and role of transporters as substrates and/or their induction/inhibition potential are a major part of drug development. It is likely that the ability to understand the overall disposition of drugs, especially metabolism in current terms, will in the future result in individualized therapeutic regimens.

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ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism, and Excretion
CMC	Chemistry, Manufacturing, and Controls
CYP450	Cytochrome P450
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
IB	Investigator's Brochure
ICH	International Committee for Harmonization
IND	Investigative New Drug
LC/MS-MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
LOQ	Limit of Quantitation
NCE	New Chemical Entity
NDA	New Drug Application
NME	New Medical Entity
PD	Pharmacodynamics
Phase 1	Clinical Pharmacology Studied Primarily in Normal Volunteers
Phase 2	Clinical Studies in Patients; Early Dose Ranging
Phase 3	Pivotal Clinical Studies in Targeted Patient Population to Determine Safety and Efficacy
PK	Pharmacokinetics
POC	Proof of Concept
TK	Toxicokinetics

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5 Identification of Drug Metabolites

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5.1	Introduction	1
5.2	Sample generation and workup	6
5.3	HPLC-MS analysis	10
5.4	NMR spectroscopy	33
5.5	Quantitative aspects	44
5.6	Conclusions	47
	References	47

5.1 INTRODUCTION

Among the experimental activities carried out in pharmaceutical research and development organizations that are associated with absorption, distribution, metabolism, and excretion (ADME) sciences, the elucidation of the chemical structures of drug metabolites (addressing the “M” in ADME) is probably the most mature. In fact, many modern drug metabolism-pharmacokinetics (DM/PK) or ADME departments originated as drug metabolism groups assigned with the task of identifying the main metabolites present in excreta samples from laboratory animals and humans. Nevertheless, despite its maturity, the field of drug metabolism and metabolite identification has changed considerably and continues to be dynamic, not only in the techniques and technologies applied in conducting the laboratory work but also in the scope of questions being addressed in pharmacology, medicinal chemistry, and toxicology with drug metabolism information. In this chapter, modern techniques of structure elucidation of drug metabolites are described. These include mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Sample generation and workup procedures are also described, as well as some special techniques applied to elucidating structure. However, before describing these technical aspects, this section outlines the utility of metabolite structure elucidation in drug research.

5.1.1 Metabolite Structure Elucidation in Drug Discovery

The drug design process is an arduous one because it requires the simultaneous optimization of multiple properties, and in many cases, structural modifications that

improve one property will be to the detriment of other properties. For example, it is generally the case that target receptor affinity and selectivity can be increased by adding substituents to a structural backbone. However, it is also generally the case that the addition of these substituents will lead to declines in ADME properties, such as increasing metabolism or decreasing membrane penetrability. Thus, ADME scientists, as well as the subset of those scientists who specialize in the elucidation of metabolite structures, need to be working contemporaneously and in collaboration with medicinal chemists and pharmacologists charged with designing a new drug. In the drug discovery phase, the type of knowledge gained from metabolite structure elucidation can be leveraged to enhance the design of new drugs by

1. aiding in decreasing metabolism, which can yield drugs with lower clearance, longer half-lives, and increased oral bioavailability;
2. determining the main routes of metabolic clearance and, by identifying the type of metabolic modification, suggest which types of enzymes may be involved in these metabolic routes;
3. identifying routes of metabolism that yield chemically reactive intermediates that could cause toxicity; and
4. identifying metabolites that may possess pharmacological activity similar to the parent molecule and contribute to pharmacological effect.

These are described below.

5.1.1.1 Decreasing Metabolic Lability. In many drug discovery programs, the problem of high clearance is encountered. If one considers that the existence of xenobiotic-metabolizing enzymes in organisms is to prevent foreign materials encountered in nature (through purposeful or accidental ingestion) from entering and residing within the body and its vital organs, then it follows that a challenge will exist in trying to achieve bioavailability of foreign drug molecules. Most of the high metabolic lability of drugs is because of cytochrome P450 and glucuronyl transferase enzymes, which are highly abundant in liver and intestine. Many drug research programs now routinely test newly synthesized compounds in screens of metabolic lability in human liver microsomes supplemented with cofactors necessary for P450 catalytic activity. From screening, some insights to structure–activity relationships regarding metabolic lability can be gleaned in some cases; however, this can be challenging since multiple P450 enzymes could be responsible for lability observations.

To reduce lability caused by P450 enzymes, the determination of structures of metabolites can be highly informative. This can permit the introduction of chemical substituents associated with greater resistance to metabolism at positions of the chemical scaffold that are identified to be susceptible to metabolism. Small, directed modifications will also be less likely to disrupt the affinity for the target and they will not substantially change the physicochemical properties that direct the overall distribution properties of the compound. If the actual labile substituent cannot be removed because of its importance in target-binding activity, there are other approaches to reducing metabolic lability, which include sterically disrupting the interaction between the labile site and the active center of the heme of P450 or introducing substituents that alter the electronics of the metabolically labile site. Depending on the type

of metabolic reaction being catalyzed, P450 enzymes act by abstracting hydrogen atoms (e.g., aliphatic hydroxylation and *O*-dealkylation reactions) or electrons (e.g., *N*-dealkylations and aromatic hydroxylations) from their substrates. For hydrogen atom abstractions, replacement of the hydrogen with a nonabstractable atom, such as fluorine, is a common strategy to introduce metabolic stability. Electron abstractions are usually more difficult to reduce, and alterations of the electronics of the substituent or building steric bulk around it are most frequently attempted. Placement of electronically linked halogen atoms or replacing methyl/ethyl substituents with *t*-butyl are commonly employed strategies. To propose any of these strategies, the site of oxidation must first be identified. In most instances, this can be accomplished by identifying the major metabolites in extracts of *in vitro* incubation mixtures using high performance liquid chromatography-mass spectrometry (HPLC-MS) and NMR as needed. These are described in later sections.

5.1.1.2 Identifying Major Routes of Clearance. In drug design, a sense of what types of clearance mechanism will remove the new drug in humans is important. Since it is desirable to engineer the clearance to permit good bioavailability and suitable half-lives, which in turn permit facile dosing regimens, the mechanism(s) of this clearance must be known. Furthermore, different types of clearance pathways possess differing degrees of interpatient variability; thus, knowledge of the main clearance pathway will aid in predicting variability in pharmacokinetics. For example, in Section 5.1.1.1, it was stated that reduction of P450-mediated metabolic lability is important. However, if the compounds being studied are actually more readily cleared by another drug-metabolizing enzyme, such an endeavor will be fruitless in addressing the real problem. Thus, the identification of drug metabolites, using either *in vivo* samples (i.e., excreta samples from laboratory animals) or an *in vitro* system that contains a nearly complete complement of drug-metabolizing enzymes (e.g., human hepatocytes) can aid in generating the knowledge of the main clearance mechanisms. In drug discovery programs, the observation of inconsistency between the clearance predicted from data generated using animal-derived *in vitro* systems and the clearance observed *in vivo* in these species will suggest that there may be clearance pathways contributing, which are unaccounted for. The elucidation of metabolite structures will lead to the proposal of such pathways.

5.1.1.3 Decreasing the Generation of Chemically Reactive Metabolites. Although there are very few cases of bona fide proven examples of drug toxicities caused by the generation of chemically reactive electrophilic metabolites, it is generally held that metabolism of a drug to a reactive intermediate is a potentially deleterious property. In the drug discovery process, it has become commonplace to test new compounds for their potential to generate reactive electrophiles, using a variety of assay types [e.g., glutathione (GSH)-trapping assays, high throughput mutagenesis assays, and covalent binding assays]. Such data can be used as a mere screen, with an attempt to remove this property by blindly testing enough compounds so as to find one that lacks the property. However, the elucidation of the structure of nucleophile adducts as well as other metabolites downstream from a reactive intermediate is a more rational approach to solving such an issue. By identifying the substituent undergoing adduction with a nucleophile, such as GSH, advice can be provided to the medicinal chemist as to where the bioactivation is occurring and how it can be abated with structural modifications.

5.1.1.4 Identification of Pharmacologically Active Metabolites. When a drug undergoes metabolism, there is no *a priori* reason to assume that the metabolite will not possess a similar pharmacologic activity profile. In fact, there are numerous drugs known to possess metabolites that also contribute to the desired pharmacological effect (e.g., fluoxetine, loratadine, and atorvastatin). Thus, the identification of metabolites with pharmacological activity is important for two reasons. (i) To understand the relationship between the pharmacokinetics and pharmacodynamics, the concentrations, plasma protein binding, and target tissue penetrability of not only the parent drug but also all active metabolites must be known. This permits the setting of appropriate dosing regimens. (ii) To protect the intellectual property rights of an inventor of a novel drug, knowledge of metabolites that contribute to activity must also be known; otherwise, others could investigate this and claim the metabolites as potential new drugs in their own patents.

A first clue to the existence of active metabolites *in vivo* can be discerned from disparities between the potency of the parent drug measured *in vitro* and the concentrations *in vivo* associated with activity. Also, if the pharmacological effect is prolonged relative to the pharmacokinetics of the parent drug, it is possible that a long-lived active metabolite could be responsible. Of course, these observations could be due to other factors, such as inherent properties of the systems biology underlying the pharmacological target, but if observed, an investigation of the possibility of active metabolites is warranted. The other means by which active metabolites are identified is via a simultaneous examination of the structures of metabolites and the known structure-activity relationship (SAR) of the series. If small modifications (e.g., *N*-demethylations and hydroxylations) are introduced by metabolism at portions of the chemical scaffold known to be areas where the target receptor SAR is relatively loose, the possibility that the metabolite possesses pharmacological activity is heightened. It should be appreciated that there are even rare cases where a metabolite possessing substantial alterations in structure relative to the parent can possess target receptor potency (e.g., morphine glucuronide).

From these four aspects, it is apparent that metabolite structure elucidation can play an important role in drug discovery activities, aiding in drug design to optimize pharmacokinetics and reduce the likelihood of toxicity, as well as providing a clear understanding of the pharmacology of the target.

5.1.2 Metabolite Structure Elucidation in Drug Development

The characterization of the metabolic profile of a new drug is well recognized as an essential and required element in the introduction of a new drug to clinical use. This has been a mainstay of ADME/PK groups in the pharmaceutical R&D industry for decades. Unlike the targeted metabolite structure elucidation activities used in support of problem solving in drug discovery, the structure elucidation activities in drug development tend to be similar from compound to compound as they are done to provide a comprehensive, quantitative, and thorough picture of the total metabolism of a new compound. In drug development, metabolite structure elucidation is done for the following two main purposes:

1. To provide a comparison of metabolites in human to those in the laboratory animal species used to assess the safety and

2. To provide a complete picture of the total disposition of the drug in human to enable gathering needed knowledge of the main clearance pathways and the enzymes involved.

5.1.2.1 Cross-Species Comparison of Metabolite Profile. In the development of new drugs, thorough testing of safety is carried out using laboratory animal species. Testing for general target organ toxicity, reproductive toxicity/teratogenicity, genetic toxicity, carcinogenicity, and safety pharmacology is done using various animal species for each test (mostly mouse, rat, rabbit, dog, and monkey). When these safety studies are done, the parent molecule is being tested and metabolites are also being tested by virtue of their being generated *in situ*. Thus, the determination of the structures and abundances of metabolites in humans and a comparison of these to that in laboratory animals is important in determining whether the animal species were exposed to the metabolites present in humans. If there are metabolites present in humans and no animal species generated these metabolites, then the extrapolation of conclusions made from animal safety tests to the human situation would not necessarily be as valid. The same would be true for metabolites unique to an animal species, since it would be possible that the toxicity observed in that species could be due to the metabolite and not anything to which humans are exposed. Granted, it is highly unlikely that any given drug will yield a profile of metabolites identical in structure and prevalence across humans and animal species. Nevertheless, there is a reasonable expectation that these profiles will be similar and that the exposure to any given metabolite will be greater in at least one of the animal species as compared to humans, largely due to the fact that doses (per body weight) given to animals in toxicology studies are considerably greater than those given to humans in clinical trials.

For these reasons, a comprehensive comparison of metabolites across species is important and is an essential element to the data set describing the properties of a new drug to physicians and government regulatory agencies.

5.1.2.2 Determination of Human Clearance Pathways. Knowledge of how a drug is cleared from the body is important because this can be a major factor in defining the interpatient variability of pharmacokinetics. Drug-metabolizing enzymes and transporters are expressed at different levels in different people. This can be due to inherent factors (e.g., genetics, age, and disease) or environmental factors (e.g., drug interactions and diet). The clinical pharmacology literature is rife with examples of investigations of the determinants of interpatient variability in pharmacokinetics. Genetic polymorphisms of important drug-metabolizing enzymes, such as CYP2D6, CYP2C19, CYP2C9, FMO3, UGT1A1, and others, have been described, in some cases, with prevalence of genotypes linked to geographical or ethnic origins, and their impact on drug pharmacokinetics well established. Drug–drug interactions with all the major cytochrome P450 enzymes have been described, with some at greater frequency (e.g., CYP3A) than others.

The identification of the total metabolic pathways of a new drug provides the first critical foundation on which the knowledge of mechanisms of interpatient variability can be built. An examination of the profile of metabolites in urine and feces of human after administration of a dose containing a radioactive atom in the molecule (typically carbon-14) allows the entire dose to be accounted for. The metabolites are identified such that the pathways can be logically constructed from the types of reactions that

have occurred to generate each metabolite. Then the fraction that each metabolite contributes to the whole dose can be calculated from the quantitation done using the radioactivity. This information is combined with the metabolic pathway scheme, and the percentages that each of the initial pathways from the parent drug contributes to its clearance can be calculated. From this knowledge, appropriate *in vitro* studies can be designed to elucidate the enzyme(s) involved in each initial pathway. When that information is obtained, it can be used to inform clinical pharmacologists of the types of investigations needed to determine the reasons behind interpatient variability, which types of drug–drug interaction studies should be done, whether drug pharmacokinetics in renally or hepatically impaired patients should be studied, and so on. Thus, while the studies needed to define interpatient variability extend far beyond the identification of metabolites, the latter is a fundamental first step in this process.

5.2 SAMPLE GENERATION AND WORKUP

Before any structure elucidation work can be accomplished using modern spectroscopic methods, an appropriate sample workup procedure needs to be applied. In the past, the procedures needed could be rather arduous because the main spectroscopic tool used was gas chromatography-mass spectrometry (GC-MS), which required considerable cleanup of samples and derivatization reactions. With the advent of HPLC-MS in the 1980s and 1990s, sample workup has become considerably simpler. Nevertheless, there are still some procedures required for many of the types of samples in which metabolites need to be identified. Fundamental elements of any sample workup procedure are as follows:

1. The procedure needs to be “comprehensive”—metabolites must be recovered through the procedure with efficiencies equivalent to each other.
2. The procedure must be gentle enough so as to not chemically modify metabolites.
3. The procedure must strike a balance between maximizing metabolite recovery while adequately removing endogenous interferences from the sample matrix.
4. The procedure must result in a mixture that can be analyzed by chromatography and spectroscopy (e.g., for standard reversed-phase HPLC, the sample must be miscible with aqueous solvents, not too viscous, and devoid of particulates that can clog an injector).

5.2.1 *In Vitro* Samples

The generation of *in vitro* samples for metabolite identification has become a very important procedure for gaining an understanding of metabolite profiles in drug research. It offers the advantages of permitting the use of human-derived reagents before clinical research to gain a prediction of human metabolite profile as well as reductions in the use of animals in research and the associated costs. *In vitro* metabolite profiles generated with human-derived reagents (e.g., hepatocytes, liver subcellular fractions, and expressed human drug-metabolizing enzymes) can fairly reliably identify the main clearance pathways of a new drug, albeit they are not perfect in generating a complete match to the *in vivo* metabolite profile [1]. Nevertheless, *in vitro* metabolite profiles are very valuable in addressing the challenges in drug discovery described in Section 5.1.1.

Selection of the best *in vitro* system for the task at hand is critical in order to generate a metabolite profile most appropriate to the *in vivo* situation. This is largely based on the substituents present in the molecule (i.e., what types of common drug metabolism reactions could occur and which enzymes would catalyze these reactions) and the question being addressed (i.e., main initial clearance pathways or a comprehensive total metabolite profile). If the structure of the molecule is such that only oxidative types of reactions could occur, the use of liver microsomes supplemented with NADPH may be all that is needed to ensure the activity of the cytochrome P450 enzymes. If substituents are present on the molecule that can undergo conjugative metabolism, a more complete *in vitro* system such as human hepatocytes or liver S-9 fraction supplemented with multiple cofactors needed by the various drug-metabolizing enzymes (e.g., uridine diphosphoglucuronic acid (UDPGA), S-adenosyl methionine (SAM), Adenosine 5'-phosphosulfate 3'-phosphate (PAPS), and acetyl coenzyme A) will be needed. And if the possible generation of a reactive metabolite is being investigated, inclusion of GSH in the *in vitro* system will also be important. The concentration of the substrate is also important as a balance must be achieved between using those concentrations predicted to be relevant to *in vivo* and those offering adequate quantities of metabolites for spectroscopic characterization. Since the compound will have different enzyme kinetics with the various drug-metabolizing enzymes, different metabolite profiles will be obtained with different substrate concentrations. A concentration between 1 and 20 μM is typically high enough to provide robust spectroscopic data while not being so high as to generate an unrealistic metabolite profile. Lower concentrations can be accommodated if the *in vitro* incubation volume is relatively high ($>2\text{ mL}$). Incubation times can vary, depending on the enzymes involved and the lability of the substrate. Liver microsomal incubations for P450 enzymes are usually run for 10–60 min, whereas for suspended hepatocyte incubations for 0.25–4 h.

Relative to *in vivo* samples, *in vitro* samples are “clean” of endogenous materials, and thus relatively simple sample workup procedures can be applied. In almost all cases, a simple procedure in which the incubation is terminated by addition of a miscible organic solvent such as acetonitrile or methanol is applied. This serves to precipitate the proteins while still maintaining the drug-related materials in solution. The precipitated protein can be removed by spinning the sample in a centrifuge. If enough solvent is added, buffers and salts present in the incubation will also be precipitated. The solvent chosen should be one that is miscible with water and that has relatively low boiling point for ease of evaporation. In most cases, acetonitrile is preferred since it is aprotic, while alcohols can react with some metabolites (e.g., form esters with carboxylic acid metabolites) to form metabonates.

After the precipitated proteins and salts are spun down, the supernatant is transferred to a new tube for evaporation of the remaining mixture of solvent and water. This is done for two reasons: to concentrate the sample for maximization of the response in the subsequent spectroscopic analysis and to ensure that excessive solvent will not confound the chromatography. Evaporation can be accomplished in several manners, but it is important to not apply excessive heating of the sample in the case that the metabolites are temperature sensitive. Commonly used practices include evaporation under a stream of inert gas such as nitrogen, evaporation under vacuum (e.g., vacuum centrifugation), and lyophilization. The latter is the most gentle process but takes the longest. Vacuum centrifugation offers the advantage that the sample will be concentrated into a small surface at the bottom of the tube, whereas evaporation under flowing gas will

tend to spread the sample out over a larger surface in the tube. (This can be important for effective reconstitution of the sample.) The evaporated sample is reconstituted in a small volume of mobile phase used for HPLC-MS analysis. To aid in reconstitution, the residue can be subjected to vigorous mixing on a vortex mixer and/or sonication. Depending on the amount of *in vitro* reagent used, there may be some cloudiness (due to lipids) or particulates in the reconstitution mixture; these should be removed by centrifugation before injection on HPLC or one runs the risk of clogging the injector and/or column.

5.2.2 *In Vivo* Samples

The analysis of *in vivo* samples for metabolite profiles is considerably more challenging than *in vitro* samples. This is due to the large number of endogenous interferences present, the generally lower concentration of drug-related materials, and for fecal and tissue samples, the need to homogenize the samples before extraction. For *in vitro* investigations of drug metabolism, the drug metabolism scientist has more control over “engineering” the sample to meet the need. However, for *in vivo* drug metabolism investigation, the nature and quality of the samples obtained is dictated by how the body handles the drug and the limitations on the dose that can be administered. For example, when analyzing plasma samples for metabolite profile, the concentrations can be so low as to prohibit gathering rich spectral data, and these low concentrations can be due to either a low dose administered or a proclivity of drug-related materials to readily distribute into tissues, or both. These types of phenomena create challenges for scientists engaged in the identification of metabolites *in vivo* and drive aspects of the sample workup procedures employed.

5.2.2.1 Urine and Bile Samples. Among the *in vivo* samples that are typically analyzed for metabolite profiles, urine and bile samples generally have the simplest sample workup procedures. These are excretory fluids, and thus the body excretes foreign materials such as drugs and their metabolites into these fluids, thereby concentrating them. In particular, for small animals (rats and mice), the concentrations of drug metabolites in urine and bile are generally high enough that no concentration step is needed, and the samples can be directly injected onto HPLC-MS without any sample workup beyond simply removing particulates by centrifugation and adjusting the pH of the sample to match that of the HPLC mobile phase. Such a simple procedure assures that there are no selective losses of individual metabolites. However, because of the large amounts of endogenous materials, it yields the challenge of sifting through HPLC-MS chromatograms to distinguish drug-related entities from endogenous compounds. It is thus critically important to compare the data to control samples. The best control samples are collected from the same animals/humans before administration of the drug, since the identities and quantities of some endogenous interfering materials will be specific to the individual and the environmental conditions of the study site (e.g., diet components and collection vessels). For larger species, the concentrations of drug-related material in urine and bile will be lower and may require a concentration step, such as lyophilization, vacuum centrifugation, or evaporation under nitrogen. Of course, in addition to the drug-related materials of interest, the endogenous interferences will also become more concentrated.

Urine and bile samples can also be subjected to solid-phase or liquid extraction techniques. The risk of these is that recoveries of the different metabolites may begin to differ and thus yield a metabolite profile that has been altered. For liquid extraction, water soluble and very polar metabolites such as the glucuronide, sulfate, or GSH conjugates are not extractable from aqueous solutions. When using samples from a radiolabel study, total recovery through the process can be readily assessed with liquid scintillation counting, and if recoveries are >90%, it is reasonable to assume that recoveries of drug-related materials through the process are equal. Selection of a solid-phase sorbent can be done such that the principles used to retain and elute the drug-related material can be orthogonal to the principles used in the chromatography of the HPLC-MS method. For example, if the HPLC-MS analysis is done using a reversed-phase C18 column, then the solid-phase extraction (SPE) can use an ion exchange resin. For liquid extraction, recoveries for some polar metabolites can suffer. Adjustments of pH can be employed to enhance recoveries, but extremes of pH (e.g., <2 or >12) should be avoided because some metabolites may degrade. Commonly used liquid extraction solvents include methyl tertiary butyl ether (MTBE), ethyl acetate, chloroform, dichloromethane, or polar solvents such as *t*-butyl alcohol or a mixture of isopropyl alcohol and toluene. Isopropyl chloride is another solvent that can be considered for extraction of drug components. Although not often used, this can be an excellent solvent for extraction purposes considering its properties (boiling point 36°C, dielectric constant 9.82, and water solubility 1.3%).

Bile samples have the added complexity of possessing high concentrations of bile salts. These can act in a detergentlike manner to alter the chromatography and cause small shifts in retention times of metabolites. They can also elute on reversed-phase HPLC columns at retention times of interest, when metabolites elute, and suppress the ionization of the metabolites. When analyzing the metabolite profile in bile samples, one must be aware of this possibility and limit the volume of bile injected as much as possible.

5.2.2.2 Fecal Samples. Fecal samples offer unique challenges in profiling metabolites because unlike urine and bile, they are not homogeneous liquids. Thus, they must be adequately diluted (with water or a water-miscible solvent) to permit homogenization as well as an ability to pipette discrete volumes. Sample consistency can vary considerably across species, individuals, and with diet; thus, there is no single best method of handling such samples and the investigator must qualitatively assess the process in real time in each instance. When analyzing fecal samples from a radiolabel study (which is almost always the case for this matrix), recovery through the homogenization and extraction process can be measured. Typically, solid samples are homogenized with water, at 2–4× the weight, using a stomacher mixer or probe homogenizer. This yields a slurry that can be used for subsequent extractions. The key to success is to physically remove the drug-related material away from the particulate matter. The slurry can be spun in a centrifuge, the supernatant removed, the particulates resuspended in solvent (water or a mixture of water and miscible solvent), followed by vigorous mixing to extract drug-related materials. Mixing can be done using a vortex mixer, shaking mixer, or sonicator, and the use of sequential combinations of these techniques with relatively long times (e.g., hours) can increase yields. Once a good recovery (>85%) is obtained in a liquid phase combined from the series of extractions, it is evaporated as described above. In general, the solid residue obtained from fecal extracts can be difficult to

work with, depending on the quantity extracted and the techniques used. Reconstitution into HPLC mobile phase may also require physically vigorous conditions such as sonication. As with other samples, a final removal of particulates from reconstituted samples should be done to avoid clogging the HPLC.

5.2.2.3 Plasma and Tissue Samples. The analysis of plasma samples for metabolite profile generally offers the challenge of low concentrations of metabolites relative to excreta samples. Thus, sample workup of plasma has two aims—to remove endogenous materials, especially protein, and to concentrate the sample. For example, to generate high enough amounts of metabolites from plasma to get rich spectral data, it is not uncommon to need to workup samples in excess of 1 mL for a single injection.

Workup techniques for plasma almost always involve a deproteination step. This is frequently done with addition of water-miscible organic solvents (such as 3–5× volumes of acetonitrile). Alternately, deproteination can be done by lowering the pH with trichloroacetic or trifluoroacetic acid, although one must be careful that potential metabolites are not acid labile. The proteins are removed by spinning in a centrifuge, and the supernatant is evaporated either by lyophilization, under vacuum, or under flowing nitrogen gas. The residue is reconstituted in HPLC mobile phase, and particulates are removed by centrifugation before injection on HPLC. In some cases, depending on the amount of plasma analyzed, there can be lipids present in the reconstituted mixture. These can be removed by centrifugation, although completely removing them can be challenging because they will float at the top of the supernatant. As with urine and bile samples, plasma samples can be analyzed by SPE or liquid extraction to yield samples with fewer endogenous interfering materials, albeit with the same caveats regarding recovery of analytes. Use of radiolabel can permit recovery to be tracked through such procedures. When using SPE of plasma samples, interactions between metabolites and plasma proteins (e.g., albumin and acid glycoprotein) must be disrupted before application to the extraction cartridge. Otherwise, the analytes may preferentially associate with the plasma proteins over the solid-phase matrix and be carried through the cartridge when the sample is applied.

To address some questions, it may be necessary to assess metabolite profiles in specific tissues. Tissue samples are best treated like plasma samples, with the added complexity that a homogenate must first be made to provide a liquid for processing. If the tissue is one possessing a high capability to metabolize drugs, such as liver, precautions will be needed to assure that further metabolism does not occur during homogenization, such as keeping the sample cold until protein is removed. Some tissues, such as brain and adipose, will possess a high amount of lipid such that the homogenates may need a prewash with hexane or petroleum ether to help remove excess lipids before further processing.

5.3 HPLC-MS ANALYSIS

Metabolite identification and profiling consist of three essential steps, namely separation, detection, and structural analysis of metabolites. With the advances made in each of these categories, the identification of drug-related components has become routine in a drug development setting and can even be accomplished rapidly enough in a drug discovery setting to help influence chemical design.

5.3.1 Chromatographic Separation

Chromatographic separation of metabolites from endogenous components has a vital role to play in metabolite identification. For identification of all metabolites, it is important that the separation be of high resolution and in high capacity. Several chromatographic tools have been used to accomplish this, and all operate under a common principle that compounds in a mixture interact differently with the stationary and mobile phases employed by a specific chromatographic method. Currently, the most commonly used chromatographic technique is HPLC. Some techniques less frequently employed include GC and thin layer chromatography (TLC). Other more sophisticated methods of separating drug-related components have also been tried (albeit sparsely), including supercritical fluid chromatography (SFC) and capillary zone electrophoresis. Although the separation of metabolites in older metabolite identification studies was conducted using TLC [2], it was quickly superseded by other separation methods that offered both high accuracy and sensitivity for the analysis of highly complex mixtures of compounds. GC was one such method that used inert carrier gases such as nitrogen and helium as the mobile phase and was highly efficient and sensitive in resolving the constituents in a mixture that were volatile enough to be vaporized [3–5]. Most drugs and their metabolites are nonvolatile and required derivatization before analysis, and several derivatization techniques have been reported. Several examples of use of GC in drug metabolism studies have been reported. One application of GC in drug metabolism studies is its use in determining which enantiomers are formed when the metabolic process produces a chiral center in the metabolite. For instance, the use of GC has been demonstrated in determining the abundance of the reduced metabolite of diethylpropion. Reduction of the carbonyl group in diethylpropion to an alcohol introduces a chiral center in the molecule, resulting in the formation of two enantiomers. The amount of the reduced metabolites was determined by derivatizing the alcoholic group with *N*-trifluoroacetyl-*L*-propyl chloride, and the diastereomers obtained were analyzed by GC [6]. Despite its high efficiency, GC suffers several disadvantages that have made the process of identifying metabolites cumbersome. First, the compounds have to be volatile enough to vaporize for analysis and detection. Alternatively, the analytes have to be derivatized before analysis. This results in increase in analysis time, which is not amenable to the high throughput that is required in discovery. Second, the compounds/analytes should be stable at high temperatures. Last, the highly soluble phase II conjugates of the drug are difficult to analyze by GC and cannot be detected intact. Most often, these require enzymatic digestion before analysis. Thus, GC has generally fallen out of favor among scientists engaged in metabolite structure elucidation; however, there are some instances where its use is advantageous.

In the past few decades, HPLC has replaced the use of GC methods in the conduct of drug metabolism studies [7,8]. The need for analytical methods with increased sensitivity, stability, and speed to separate these polar components has made HPLC a routine method for drug metabolism studies. The three particular advantages of HPLC over GC are (i) water-soluble compounds can be analyzed without extraction and derivatization, (ii) all analysis can be performed at room temperature, and (iii) it is nondestructive. In drug metabolism studies, the use of HPLC is dictated primarily by the fact that metabolites are typically more polar than the parent drug (and thus more water soluble). In HPLC, the separation of various components of the mixture depends on their distribution between the stationary and liquid mobile phase and the relative

affinities for the two phases. The instrumental and theoretical aspects of HPLC are well documented and are not discussed further.

Advances in this separation technology have greatly enhanced the capabilities of liquid chromatography (LC) in separating complex mixtures, not only in terms of resolution and associated speed of separations but also with regard to the capacity and robustness afforded by the current generation of columns, autosamplers, pumping systems, and so forth. Traditional 2.1- and 4.6-mm reversed-phase columns have filled this need for some time and their use in HPLC is a mature technique. However, there have been significant advances in column technology, including decreased particle size, increased porosity, chemical stability, and bonded ligands. In recent years, development and commercial availability of porous stationary phases with particle sizes $<2\ \mu\text{m}$ has enabled better separation with increased sensitivity and faster resolution. Although the default technique for metabolite identification is reversed-phase chromatography, related techniques such as hydrophilic interaction chromatography (HILIC), which use low aqueous/high organic mobile phase, is emerging as a valuable supplement to the reversed phase HPLC and might offer advantages for the retention of very polar metabolites [9,10]. HILIC is based on the hydrophilic interactions between ionic analytes and zwitterionic groups covalently bonded to silica or polymer beads, for example, $-\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$, which provides a separation mechanism orthogonal to that of reversed-phase HPLC. This technology separates compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase. The highly volatile organic mobile phases such as methanol and acetonitrile used in HILIC provide an increased ionization efficiency for MS/MS detection (discussed later) and low column backpressure to perform fast chromatography. Other approaches such as the monolithic and microcolumn technologies have also seen increasing use in achieving chromatographic separation of metabolites. Monolithic columns are advantageous because of their high permeability, which allows the use of higher flow rates and therefore shorter chromatographic runs. High porosity of these columns render less backpressure than conventional columns at similar flow rates and which make higher speed separation possible without disruption of chromatographic resolution [11,12]. Microcolumn technique on the other hand adopts a shorter ($<5\ \text{cm}$ length) narrow-bore ($\sim 2\ \text{mm}$ i.d.) column packed with small particles and operated at higher-than-optimal flow rates to provide for fast chromatographic separations while still maintaining satisfactory chromatographic resolution. The reduced inner diameter of these columns is advantageous in the metabolite identification field because of its capability to use limited sample amount compared to conventional columns [13,14]. The most recent developments deal with the use of separation on a chip, which may be easily coupled with nanoelectrospray ionization with extremely low sample and solvent consumption and enormous sensitivity [15].

The latest chromatographic technique is ultraperformance liquid chromatography (UPLC), which is based on the same working principle as HPLC while utilizing smaller particles ($\sim 1.7\ \mu\text{m}$) as stationary phase and is able to perform under much higher liquid pressures (up to 15,000 psi). This technique allows the liquid handling system to handle the high backpressure resulting from the stationary phase with $<2\ \mu\text{m}$ particles. UPLC has generated considerable interest in many areas of research and has been applied to metabolite identification for a variety of sample types [16–18]. The increased chromatographic resolution of the technique, achieved via reduced particle size packing materials and high pressure LC systems, has resulted in dramatic

improvements in chromatographic resolution. This offers significant advantages for metabolite characterization and has led to the technique being commonly coupled to MS, where the rapid scanning speed allows the acquisition of sufficient data points across very narrow peaks [19,20].

The modifications caused by metabolic reactions can have major effects on the chromatographic behavior of metabolites. In most cases, the metabolic changes lead to an increased polarity of the metabolites and therefore decreased retention in reversed-phase HPLC systems in relation to the parent drug. The polarity increase and simultaneous retention decrease is particularly relevant for high polar metabolites, such as sulfates, glucuronides, and other polar conjugates. The separation of the parent drug and polar metabolites in one chromatographic run usually requires the use of gradient elution and often the addition of ionic modifiers into the mobile phase to increase the retention of polar conjugates. The type of mobile phase used in the separation depends on the detector that is used in the identifying the metabolites. Common HPLC methods for the analysis of drugs and their metabolites frequently rely on the use of nonvolatile buffers and additives, such as phosphate buffers and other inorganic additives. Since the use of an MS detector for identifying metabolites has become the common practice (see below), nonvolatile buffers have been replaced by mobile phases containing volatile modifiers. Organic acids such as formic or acetic acid (0.1% concentration) or their ammonium salts (2–10 mM) are typically used as aqueous solvents when performing LC-MS experiments [21,22]. Although trifluoroacetic acid is a favorite ionic additive in HPLC assays for biological compounds such as peptides, it may cause serious ion suppression effects in HPLC-MS [23,24]. For an alkaline environment, ammonium hydroxide in a similar concentration range has been used with HPLC columns with an extended stability at alkaline pH [25]. If ion-pairing HPLC is needed for the successful separation, dialkylammonium or trialkylammonium acetates or formates are recommended for negatively charged analytes and perfluorated carboxylic acids for positively charged analytes [21,26].

5.3.2 Detection

Generally, the chromatographic separation techniques are coupled with appropriate detectors by which the separated drug-related components can be readily detected. Some common detectors used are ultraviolet–visible (UV–vis), fluorescence, or electrochemical detectors. However, in case a radiolabel analyte is available, radioactivity detectors including radioflow detection, microplate scintillation counting, stop-flow/dynamic flow radioflow detection, and microplate radioactivity imaging techniques are often used for detection purposes. In the past two decades, MS and NMR (discussed later in the chapter) have emerged as ideal detectors for identifying structurally diverse metabolites in the incubation mixtures or biological samples obtained from animals or humans.

Both UV–vis as well as fluorescence spectroscopy find widespread use in the early stages of metabolic studies. However, the UV–vis detectors are more commonly used in early drug discovery setting, when radiolabeled materials are generally not available. These techniques are sensitive and informative enough to be applied to a range of metabolic problems. Both fixed (254 nm) or variable wavelength detectors as well as photodiode array (PDA) detectors have been used in line with HPLC or UPLC separation systems. Since many metabolic transformations do not generally alter the

UV chromophore of the drug to a great degree, the UV chromatogram allows detection of most drug-related peaks that are present in the biological sample, and a crude comparison of abundances among the metabolites can be made. However, sometimes, a metabolic transformation of a compound (e.g., aromatic oxidation) can modify the UV chromophore and as a consequence, the relative quantitative importance of a metabolite may be over- or underestimated. The PDA detectors can sometimes overcome this problem since array detectors are especially useful for recording the full UV–vis absorption spectra of samples that are rapidly passing through a sample flow cell. This characteristic of the diode array detectors makes it particularly useful in drug metabolism studies and for identification of unknown compounds. A general strategy used is to compare the UV chromatogram of the biological sample containing the drug-related material with that of the control (devoid of the drug or drug-related material). This allows one to distinguish the drug-related material from the endogenous peaks in the biological sample. While UV–vis can be extremely valuable for samples from *in vitro* incubations, its application to *in vivo* samples can be compromised by the vast array of endogenous chemical entities that will also have UV–vis absorbance. Most software allows subtraction of the UV chromatogram obtained from the control sample and hence allows the detection of most drug-related peaks in the mixture. The successful application of UV–vis is highly dependent on the absorbance characteristics of the compound of interest. If substantial absorbance occurs at wavelengths of 250 nm or greater, the UV–vis chromatogram will be of use (provided that the concentrations of test compound and metabolites are not too low). However, the amount of structural information that can be derived from a UV or a fluorescence spectrum is not sufficient enough to characterize the structure of the metabolite solely by these means. The best use of UV in identification is when these detectors are used in conjunction with mass analyzers and are placed between the chromatographic separating system and the mass spectrometer. The current practice in the pharmaceutical industries is to place a PDA detector between the HPLC and MS.

5.3.3 Mass Spectrometry

5.3.3.1 Instrumentation and Scanning Techniques. In the past 20 or more years, MS has emerged as a standard technique for identification and characterization of metabolites in incubation mixtures or biological samples. It can be easily coupled with both gas- and liquid-phase separation techniques in the analysis of complex biological samples; it has an extremely high sensitivity and low sample consumption, and the required information may be obtained relatively easily by a proper selection of ionization technique and mass analyzer. The advantage of MS resides in its ability of being able to not only serve as a detector but also provide structural information for the compound of interest. This information provided by MS detectors comprises a molecular ion of a compound and a mass spectrum that provides fragment ions specific to a molecule. As a result, this technique has revolutionized the process of metabolite identification. Hyphenated techniques in which a chemical separation technique such as GC, HPLC, or SFC is interfaced with an MS system have become standard practice in drug metabolism studies. Owing to their inherent sensitivity and selectivity, these hyphenated systems normally do not require labor-intensive sample preparation procedures or prolonged chromatographic run times. Further, the technique is extremely robust and rapid and can be readily automated.

The first popular hyphenated technique to be used for drug metabolism studies was GC-MS [27–29]. In this system, the highly efficient capillary GC was coupled to MS analyzer that employed electron impact (EI) or chemical ionization (CI) as a mode of ionization. Because the output from the GC column is in the vaporized form, it was readily amenable to MS analysis. Further, the MS analyzer was able to produce structural information within the peak width obtained from GC separation. Hence, this combination was the best mode for identifying and getting structural information of drug-related components. However, as described previously, the application of GC-MS is limited to volatile, nonpolar, easily extractable organic compounds and highly volatile compounds with low vapor pressure [30]. Since most drug metabolites are more polar than the drug from which they are derived, they can have considerably less volatility. Thus, the detection of these metabolites from biological samples requires analyte extraction into an organic solvent before analysis frequently followed by chemical derivatization of the analytes [31,32]. Although this is feasible, the extra work-up required for this analysis not only reduces the speed in metabolite identification but also does not give proper structural information, especially of phase II metabolites. Further, some metabolites are prone to undergo “on-column degradation” because of the high temperatures that are employed in the GC methods. Despite these limitations, GC-MS remains a useful analytical tool for metabolic profiling in various biofluids, such as urine and blood, due to its high sensitivity, peak resolution, and reproducibility [5]. Many GC-MS approaches to metabolite structure elucidation rely on derivatization with a silylation reagent before analysis to convert the polar functional groups that are problematic in GC-MS analysis to less polar groups. In addition to one-dimensional GC-MS techniques, methods in which a comprehensive two-dimensional GC coupled with a time-of-flight (TOF) mass spectrometer are used to identify a broad range of small, moderately polar to polar metabolites. Recently, an analytical method using in-line silylation coupled to GC-MS, which is suitable for metabolic profiling in ultrasmall sample volumes of 2 μ L down to 10 nL, has been reported [33].

The need to detect polar compounds in aqueous media directly without the limitations imposed by derivatization and/or extraction for GC-MS analysis led to the interfacing of HPLC with MS. HPLC-tandem mass spectrometry (LC-MS/MS), in which the HPLC system and the tandem mass spectrometers (MS/MS) are in line, is by far the most used technique for structural characterization of metabolites and a cornerstone in metabolite identification in the past 20 years. Two types of tandem mass spectrometers are used in the biotransformation studies. These are the triple quadrupole tandem mass spectrometer [34] and the 3D and linear ion traps [35,36]. Like the GC-MS, the LC-MS/MS technique is highly sensitive in detecting drug-related material in biological samples. Its ability to separate, detect, and identify many diverse metabolites with limited workup, especially in the presence of endogenous material, makes it a technique of choice for drug metabolism studies. The first generation of robust triple quadrupole LC-MS/MS systems became commercially available only in the early 1990s with the introduction of the Sciex API 3 mass spectrometer. Since then, advances in MS technologies have led to new mass analyzers such as the hybrid triple quadrupole–linear ion-trap (Q-trap) [37]. Further, commercialization of high resolution mass spectrometers, such as the TOF and quadrupole time of flight (QTOF) and the advent of Fourier-transform mass spectrometers (FTMS) or linear trap quadrupole orbitrap (LTQ-Orbitrap), have further improved the efficiency of metabolite profiling in pharmaceutical research. The latter instruments have added accurate mass capabilities

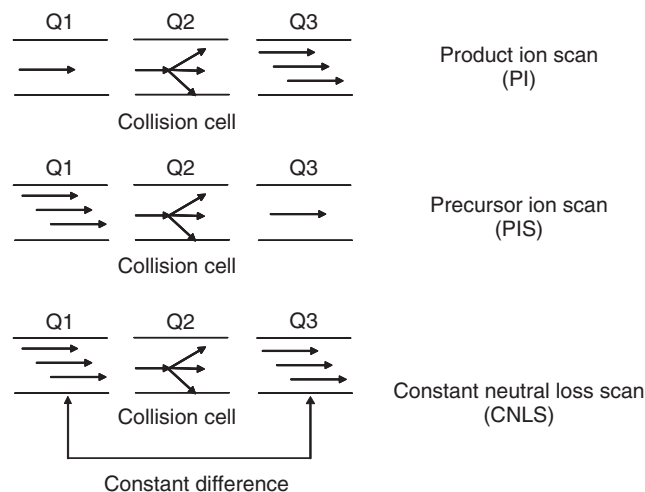


Figure 5.1 Scheme describing the product ion (PI) scan, precursor ion scan (PIS), and (CNLS) [44].

and help in providing accurate mass data for all ions in the spectrum, resulting in elemental composition information that is immensely valuable in the structure elucidation of drug metabolites in complex mixtures of biological origin [9,27,38–41].

Tandem mass spectrometers use two stages of mass analysis, the first to preselect an ion and the second to analyze the product ions (PIs) produced most commonly by collision activation with an inert gas such as argon, helium, or nitrogen. In addition to the full scan mass spectra that allow assignment of molecular weight, triple quadrupole mass spectrometers offer three scanning functions that can be used to detect metabolites and assign structures to them. The three scanning functions include PI scan, precursor ion scan (PIS), and constant neutral loss scan (CNLS) [42–44]. The schematics of the three modes of scanning are depicted in Fig. 5.1. In PI scanning mode, an ion of a given m/z value is selected in the first quadrupole, fragmented in the collision chamber that is positioned between the first and the third quadrupole, and the resulting mass-to-charge ratios of the fragment ions are analyzed in the third quadrupole. The spectrum thus obtained can be used to provide structural information for the compound or the metabolite. In PIS mode, the second mass analyzer is set to pass only the ions with a selected m/z value, while the first mass analyzer is scanned over a defined m/z range. In CNLS experiments, both the first and the third quadrupoles are operational in a way that the m/z difference between the mass spectrometers is kept constant, hence the term *constant neutral loss scan*. In both these scanning modes, the fragment ion or the neutral loss is generated in the collision cell.

Since the MS/MS scan modes are very specific in the ions that they are monitoring, the signal from the remaining endogenous material present in the sample is eliminated thus increasing the signal-to-noise ratio of a particular analyte. The PIS and CNLS modes of scanning have been very valuable in identifying conjugates of drugs in complex biomatrices. Thus, the triple quadrupole provides information that is more discriminatory for a particular compound and a data set that can be readily used for metabolite structure elucidation and biotransformation studies.

Ion-trap mass spectrometers have greatly influenced metabolite identification and its importance in early discovery. Because of the ease of use and the low cost, these mass spectrometers have become the “workhorses” to conduct biotransformation studies in early discovery. In addition to simplicity, the ion traps have improved sensitivity, specificity, and scan speed relative to TSQ mass spectrometers. The main advantage of the ion-trap mass spectrometer over the triple quadrupole mass spectrometers is its ability to collect sequential stages of ion fragmentation data (MS^n). Such fragmentation pathway data often simplify structure elucidation. However, unlike the TSQs, they are incapable of conducting experiments with PIS or CNLS. The LTQ is similar to the LCQ in terms of the experiments that can be performed; however, its capability to store more ions increases its sensitivity significantly over the conventional LCQs. A comparison of the triple quadrupole mass spectrometer and the ion traps has been described by King and Fernandez-Metzler [37]. An interesting aspect of this comparison is that the limitations of one instrument are the advantages of the other. In complex metabolite structure elucidation challenges, both types of instrument platforms can be leveraged in a complementary manner.

The high utility of the ion-trap mass spectrometers but the deficiency of these analyzers to conduct selective PIS and CNLS studies has led to further novel MS instruments that combine ion traps with TSQ, such as the MDS Sciex/Applied Biosystems 4000 Q trap [37]. The Q-trap mass spectrometers combine the functions of both triple quadrupole and linear ion-trap instruments and are capable of performing all the MS/MS experiments, including CNLS, PIS, multiple reaction monitoring (MRM), full scan MS-dependent MS/MS analysis, and the acquisition of MS^3 spectra. Additionally, these MS/MS experiments can initiate the so-called information-dependent acquisition of an enhanced product ion (EPI) spectrum, enabling detection of drug metabolites and acquisition of their PI spectra in a single LC-MS run. One of the unique scanning functions of the Q-trap is the EPI scanning mode. In this experiment, a metabolite ion is isolated in Q1, fragmented in Q2, and then, all fragments are trapped in Q3 with a subsequent trap scan. MS^2 spectra generated from EPI are similar to those recorded by triple quadrupole mass spectrometers in the PI scan mode.

The task of metabolite identification has been greatly facilitated by recent developments in high resolution LC-MS technology [e.g., Waters time of flight (QTOF, ThermoElectron Fourier transform (FT), and Orbitrap mass spectrometers]. As a result, a combination of high resolution MS and other types of LC-MS instruments has been recommended for metabolite identification. The hybrid QTOF accurate mass spectrometer has an additional quadrupole in front of a TOF analyzer, enabling the collision-induced dissociation (CID) of molecular ions [45,46]. This adds the ability of obtaining accurate mass PI spectra from the LC-MS/MS analysis and therefore allows the confirmation of molecular formula for proposed fragment ion structures [47]. This also simplifies the PI spectral interpretation for metabolite identification as demonstrated by Hop *et al.* [48]. When coupled with UPLC, it could significantly increase the analytical throughput and sensitivity for metabolite identification and profiling as a result of improved chromatographic resolution. Higher sensitivity lowers sample concentration requirements, whereas high resolution mass measurement ($\sim 5\text{--}10$ ppm) provides exact masses affording molecular formulae that are helpful for the identification of unknown metabolites. Even though the QTOF instruments can conduct fast data-dependent scanning for MS and MS^2 , they are unable to conduct MS^n experiments like the ion traps. Another limitation of the current LC/QTOF tandem instruments is

the inability to select the precursor ion with high resolution. The low resolution precursor ion selection may allow more than one species to be transferred to the collision cell where fragmentation occurs.

The more powerful hybrid LTQ-FTMS [49] and LTQ-Orbitrap mass spectrometer [50] combines the speed and sensitivity of the linear ion traps with the high resolution and accurate mass capabilities of a Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer or the Orbitrap technologies [51]. These instruments are more sensitive than QTOF and provide mass accuracies better than 1 ppm with external mass calibration at resolutions as high as 100,000 for an acquisition rate of one scan per second. Both instruments have the potential to make metabolite identification as simple as a mathematical calculation based on accurate mass measurements and theoretical atomic weights of the elements known to compose the parent drug under study. LTQ-Orbitrap MS was introduced recently and is capable of high resolution full MS scan with improved signal-to-noise ratios. Like the ion traps, this analyzer can perform MSⁿ structural elucidation experiments. The practical limitation is the ability to rapidly store, retrieve, and process the large volumes of data the system can generate. The current version of LTQ-Orbitrap lacks ability of performing true neutral loss (NL), PI, and MRM scans that are available readily with a triple quadrupole or a triple Q-trap instruments. To overcome this type of drawback, several postacquisition data manipulation techniques, including mass defect filtering (MDF), background subtraction, neutral loss filtering (NLF), product ion filtering (PIF), and isotope pattern filtering (IPF), have been developed for mining data for metabolites.

5.3.3.2 Ionization. The most significant development to convert the aqueous HPLC effluent to gas phase was the advent and commercialization of atmospheric pressure ionization (API) techniques [52]. These techniques provide efficient ionization for various molecules, including polar, labile, and high molecular mass drugs and metabolites. In contrast to EI, which operates in vacuum and substantially fragments the analyte (i.e., rarely observe a molecular ion), the API interfaces operate at atmospheric pressure and cause very little fragmentation of the molecule. These ionization techniques are therefore referred to as *soft ionization techniques* and have become extremely popular in metabolite detection, identification, and quantitation because of their ability to couple easily to reversed- and normal-phase chromatography and generate intact molecular ions at very high sensitivity. Three different API sources are available. These are electrospray ionization (ESI) [53,54], atmospheric pressure chemical ionization (APCI) [55,56], and, the more recently introduced, atmospheric pressure photoionization (APPI) [57]. All three sources can ionize a variety of polar drug components in the aqueous media and can be operated in either a positive or negative ionization mode, which is governed by the voltage polarity of the capillary probe. The ESI technique is the most frequently chosen method in drug metabolism studies since it can be used to ionize compounds with a range of polarities and sizes. It is especially valued in the identification of metabolites since it enables the soft ionization of phase II metabolites, providing reliable information on the molecular weights of these conjugates without extensive fragmentation, unlike other ionization techniques. The mass spectra obtained from ESI fragmentation are relatively simple and yield intact analyte ion intensities. One characteristic of ESI is that the intensity of the ions is affected by the flow rate at which the matrix is introduced into the ion source. Higher sensitivity is generally achieved at lower flow rates. For instance, a flow rate in nanoliter per minute (the

nanospray technique) has resulted in even increased sensitivity compared to the conventional ESI where a flow rate of $\sim 50\text{--}500\ \mu\text{L}/\text{min}$ is introduced into the source. Despite the numerous benefits of ESI, this ionization technique is susceptible to ion suppression effects from high concentrations of buffer, salt, and other endogenous materials in matrix solutions.

The APCI source is a better ionization tool for the analysis of molecules that are nonpolar, relative to the ESI. This technique provides better ionization efficiency and sensitivity for these compounds. Although APCI also generates ions at atmospheric pressure like ESI, the ionization in APCI occurs mainly in the gas phase. This reduces the ion suppression and provides a wider dynamic detection range than ESI. A better tolerance to salts and matrix effects has been reported for APCI and APPI in comparison with ESI. Typically, a higher flow rate is used with APCI ($1\text{--}2\ \text{mL}/\text{min}$) than that in conventional ESI, and therefore this technique requires thermal desolvation. Because of this, extremely polar and large molecules ($\text{MW} > 1000\ \text{Da}$) are not well suited for APCI, where thermal degradation may reduce sensitivity. Both ESI and APCI can be used for small molecule ($\text{MW} < 600\ \text{Da}$) pharmaceuticals and drug metabolism studies. APPI also has a similar application range like APCI, but slightly extended toward nonpolar compounds. APPI has produced equal or better detection limits compared to APCI in selected applications; however, this technique still needs to be evaluated further before its broader implementation.

5.3.3.3 Strategies for Identification of Metabolites in Biological Matrices. As described previously, MS has evolved as an essential component of strategies used for fast metabolite identification. Several approaches using a variety of different mass analyzers have been described in the literature [27,40,41]. Regardless of the MS platform, drug metabolite identification by LC-MS techniques involves two steps: (i) detection of drug metabolite ions in a biological matrix and (ii) acquisition of their PI spectra for structural characterization. This can be supplemented by non-MS techniques, such as chemical derivatization, hydrogen/deuterium (H/D)-exchange, and NMR (see later), to provide specific structural information in cases where MS data alone are not sufficient to determine metabolite structure.

The most simple approach used in detecting and identifying metabolites in a biological matrix employs a full scan as a survey scan for searching for metabolites in the matrix [58,59]. This procedure involves analysis of test and control samples over the full mass range (typically scanning over a mass range that exceeds the m/z value for the parent drug by 400 mass units). After acquiring total ion chromatogram (TIC) data of the control samples (generally produced by a similar incubation lacking the study compound or *in vivo* matrix from an untreated subject) and study samples, the drug-related peaks are usually scouted by comparing the TIC of both these runs. When LC-MS is used in line with UV-vis (PDA) or radioactivity detectors, the detection of the peaks in the TIC is accomplished by matching the peaks and retention times in the UV or radiochromatogram with that in the TIC (Fig. 5.2).

The main advantage of this method is that all drug-related components that are present in the matrix can be detected and will generate an MS response as long as they are ionizable in the polarity that is used in the experiment. However, the method is only useful in detecting metabolites that are generated via known/expected biotransformation reactions such as hydroxylation and *N*-dealkylation and whose molecular masses can be readily predicted (predictable metabolites). Uncommon and novel metabolites

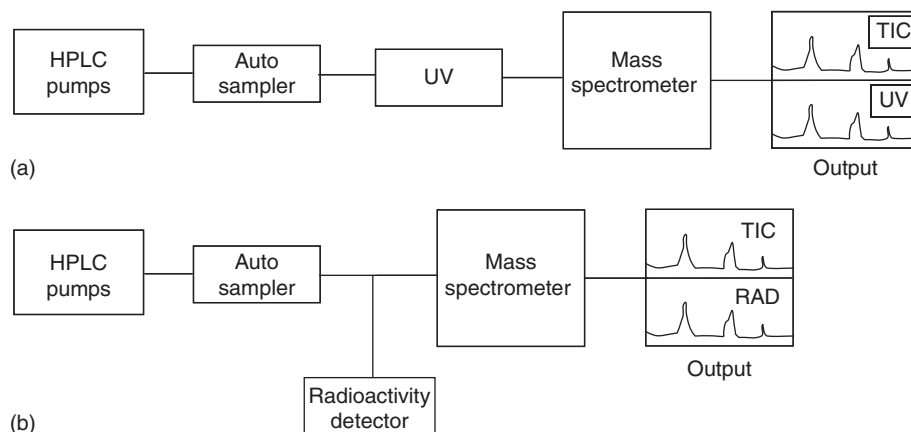


Figure 5.2 (a,b) Schematic of the arrangement of mass analyzers and UV or radioactivity detectors.

(unpredictable) that are formed via unconventional or multiple-step biotransformation reactions are difficult to detect using this method. Hence, when using this method, it is important to have a proper knowledge of all the biotransformation pathways. Further, the analysis can be confounded by the interfering ions from the endogenous material that are present in the biological sample.

The modification on the drug is generally determined by comparing the molecular ions of the peaks with those of the parent drug after background subtraction. The mass shift between the parent and the metabolite is the first indication of the type of biotransformation that the parent drug has undergone. The mass shifts occurring due to common metabolic pathways and predictable metabolites are shown in Table 5.1. Mass shifts of uncommon metabolites are generally difficult to predict from such a comparison with parent drugs. It is also worthwhile to apply the so-called nitrogen rule, which states that molecules of an odd MW possess an odd number of nitrogen atoms, while those with an even MW possess either an even number of nitrogen atoms or no nitrogen atoms. The formation of nitrogen-containing metabolites (e.g., amino acid conjugates or GSH adducts) or the loss of a portion of the molecule possessing a nitrogen will cause a characteristic change in the odd/even character of MWs. While this approach is likely to be reliable for common biotransformation pathways, more unusual metabolic reactions may sometimes be overlooked.

The most effective comparison of data acquired from the sample and the negative control is using software developed for this purpose. A number of software packages exist to enable targeted detection of potential drug-related components from complex data [60]. The information of the parent compound (molecular formula and some additional parameters restricting the screen) is fed into the software, and the chromatograms are screened automatically to show peaks present in the sample but absent in the negative control. These software packages have built-in intelligence to suggest identification of the detected metabolites. Extracted ion chromatograms for expected metabolites based on predicted molecular mass changes relative to the parent compound's molecular weight can therefore be constructed. Predictive software may improve the coverage of metabolite detection and can help expedite the detection

TABLE 5.1 Possible Metabolic Reactions and Related Changes in Mass of the Product [10,40,41,148,149]

Phase I		Phase II	
Metabolic Reaction	Mass Change	Metabolic Reaction	Mass Change
Monohydroxylation	+16	Glucuronidation	+176
Dihydroxylation	+32	<i>N</i> -carbamoyl glucuronidation	+220
N/S-oxidation	+16	Sulfation	+80
S-dioxidation	+32	Acetylation	+42
Epoxidation	+16	Glycosylation	+162
N-hydroxylation	+16	Methylation	+14
Demethylation	-14	Amino acid conjugations	
Alcohol to acid	+14	Glycine	+57
Methyl to acid	+30	Taurine	+107
Unsaturation (dehydrogenation)	-2	Glutamine	+128
Dethylation	-28	Proline	+97
Hydration	+18	Serine	+87
Dihydrodiol formation	+34	Arginine	+156
Cyclic amine to carbinol	+18	Histidine	+137
Imine formation	-1	Aspartic acid	+115
Alcohol to ketone	-2	<i>N</i> -acetylcysteine	+163
Lactam formation	+14	Glutathione conjugation	+305 or 307
Cyclic amine to amino acid	+32		
Oxidative dehalogenation	-18		
Oxidative dehalogenation	-2		
Amides to acids	+1		
$\text{RCH}_2\text{NH}_2 \rightarrow \text{RCO}_2\text{H}$	+15		
$\text{RCH}_2\text{NH}_2 \rightarrow \text{RCH}_2\text{OH}$	+1		
Decarboxylation	-44		
Reductive displacement of chlorine	-34		
Dehydration	-18		
Reduction	+2		

process of common drug-related metabolites. However, the approach is not guaranteed to detect all the significant metabolites in biological samples and is not applicable to the detection of uncommon metabolites.

The structure of detected metabolites is generally elucidated in the following run in which the PI spectra (MS^2) of the molecular ions are acquired [10]. In cases where an ion trap is used, data-dependent scanning experiments are triggered, which enable the immediate generation of PI or MS^n data that can supplement PI scanning data via elucidation of additional fragmentation pathways. Therefore, both metabolite detection and MS/MS spectral acquisition can be accomplished in a single LC-MS run. The MS^2 and MS^3 spectrum obtained gives a fragmentation pattern that is characteristic of a metabolite. Interpretation of spectral patterns using basic interpretation rules allows one to propose a structure for the drug-related peak. For basic interpretation rules of the mass spectrum, reference to the specialized literature for electron ionization and soft ionization techniques is recommended. Although the fragmentation pattern can give

- Get a mass spectrum (CID spectrum) of the parent drug.
- Interpret the spectrum.
- Select specific product ions from the spectrum for precursor ion scanning experiment.
- Determine the specific neutral losses (CNL) in the fragmentation pattern to run a constant neutral loss scan experiment.
- Determine the mass spectra of the molecular ions that are observed in the PIS and CNLS.
- The complete process can be repeated using the product ions and the neutral losses from the spectra of each of the metabolites.

Figure 5.3 General strategy for metabolite identification using tandem mass spectrometry.

some idea of the site of metabolism, it should be kept in mind that MS is generally unsuitable for distinguishing positional isomers (except for isomers with functional groups on different rings), stereoisomers, and enantiomers, so the support of other separation and spectral techniques is essential.

The MS/MS capability, the precursor ion scanning mode, and the constant neutral loss scanning mode of a triple quadrupole mass spectrometer are frequently used for metabolite identification in addition to the full scan [61–63]. As previously mentioned, this approach makes use of the fragmentation pattern of the parent molecule and its fragment ions or neutral losses to detect metabolites. Hence, it is more selective in metabolite scouting than the full (LC-MS) scan approach and is useful in detecting some unknown metabolites. The process to identify metabolites by precursor ion or constant neutral loss scanning is depicted in Fig. 5.3.

In such an analysis, the first step is to get an MS/MS spectrum of the parent drug and select appropriate precursor ions, or neutral losses are into the acquisition methods. In order to cover a variety of metabolites, multiple LC-MS runs are often conducted, each of which follow few specific precursor ions or neutral losses. Like in the full scan approach, the first identification of the metabolite is made by comparison of the masses of drug-related peaks in the ion chromatograms with the mass of the parent. In order to confirm the structures of these metabolites, these runs are always followed by an MS² product scan for each metabolite for further confirmation. The uncommon metabolites that are detected by this method are directly subjected to PI scan for further identification. The method is very useful in detecting certain commonly encountered conjugative biotransformation pathways (glucuronide and sulfate conjugates) that often undergo common cleavages to generate specific neutral fragments, notably the CNL of 176 mass units and the loss of 80 mass units for glucuronide and sulfate conjugates, respectively, under CID conditions. The method has also been commonly used in the detection of GSH conjugates (CNL of 129 in a positive ion mode, formed by cleavage of the pyroglutamic moiety from the GSH conjugate or PIS of m/z 272 in a negative ion mode) of reactive metabolites that are formed via bioactivation of the parent drug or its metabolites. Although precursor ion and constant neutral loss scanning methodology is undoubtedly more selective, its success in increasing the number of detected metabolites still relies to some extent on the ability of the analyst to predict the metabolic pathways of the compound under analysis. Although advantageous over the full scan, the multiple steps of these processes can hamper the throughput, which is sometimes required in the drug discovery arena, especially when there is limited sample available. To detect both common and uncommon metabolites, the use

of a combination of ion-trap instruments and triple quadrupole instruments has been recommended.

Owing to scanning capabilities of triple Q-trap instruments into a single platform, the Q-trap mass spectrometer enables one to use CNLS, PIS, or MRM scan as a survey scan to trigger several new data-dependent scanning modes in the third quadrupole [64]. These include enhanced full mass scan (EMS), EPI scan, enhanced resolution scan, enhanced multiply charged scan, MS³, and time-delayed fragmentation. The term *enhanced* is used to indicate that the data are generated in the ion-trap mode with improved performance. Thus, these hybrid triple Q-trap mass spectrometers clearly provide increased metabolite screening capability compared to traditional ion-trap or triple quadrupole instruments. No follow-up PI experiments are required. This not only increases the throughput but also generates more information from limited sample. The MS/MS survey-scanning capabilities have been utilized for rapid detection and characterization of several phase I and II metabolites [37]. Owing to superior sensitivity and selectivity of the MRM method and automatic acquisition of high quality MS² spectra by EPI, the MRM-EPI scanning method has been successfully applied to rapid screening and characterization of oxidative metabolites. However, the design of this experiment depends on the ability of a scientist to predict biotransformation pathways in advance and also predict the PIs of these metabolites using the fragmentation pattern of the parent. The effectiveness of oxidative metabolite screening by MRM is dependent on the right selection of theoretical metabolite. More recently, to overcome some of the limitations of the MRM method, the multiple ion monitoring coupled to the EPI mode (MIM-EPI scanning mode) has been applied to oxidative metabolite profiling [65]. In an MIM experiment, the collision energy is greatly reduced to the minimal to avoid fragmentation. The MIM experiments offer several advantages over MRM in that it allows the identification of metabolites that would have been missed due to incorrect prediction of an MRM. It is also evident that MIM-EPI had comparable sensitivity and selectivity to those of MRM-EPI.

The task of metabolite identification has been greatly facilitated by the advent of high resolution LC-MS technology. These instruments allow for the determination of molecular formulae and PI formulae with minimal uncertainty. Recently, an MDF technique (also referred to as *fractional mass filtering*) was developed for detecting drug metabolites via postacquisition processing of high resolution LC-MS data [66]. The approach attempts to discriminate metabolite ions from matrix ions based on the similarity of the mass defect values of a drug and its metabolites. This capability to detect predictable and unpredictable metabolites avoids the need for the biotransformation list that is otherwise needed to assure if the ion detected is in fact due to a drug-related peak and has changed the role of high resolution MS in drug metabolite profiling.

Mass defect is defined as the difference between the exact and nominal molecular weights of a molecule. The concept of MDF is based on the fact that the majority of drug substances exhibit a negative mass defect relative to endogenous background constituents of biological samples with a similar molecular weight. This is because the fractional mass of a molecule (the figures after the decimal point) is strongly influenced by the number of hydrogen atoms in that molecule. Drug substances tend to be relatively deficient in hydrogen atoms due to the prevalence of heterocycles and other centers of unsaturation and therefore have different fractional mass defect values than endogenous substances. The mass defect values of most metabolites will be in the similar range (<50 mDa) to that of the parent. For example, hydroxylation changes the

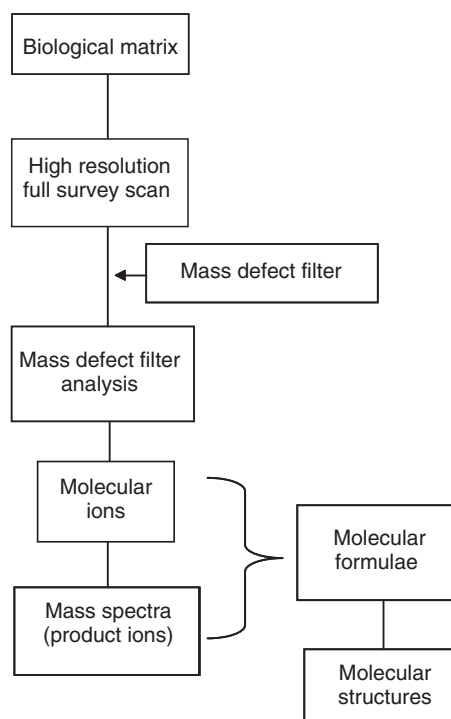


Figure 5.4 Schematic for detection and characterization of metabolites using mass defect filter technique following high resolution mass spectrometry [66].

mass defect by -5 mDa, dehydrogenation by -16 mDa, demethylation by -23 mDa, glucuronidation by $+32$ mDa, and sulfation by -43 mDa. Therefore, this method can quickly filter out masses of drug-related peaks from a complex medium.

Since most phase I and II biotransformation reactions result in shifts in mass defects by <50 mDa, a mass defect window of ± 50 mDa is generally used in this method. The general strategy used for detecting metabolites using the MDF approach is depicted in Fig. 5.4.

There are cases, however, where the molecular ions of the metabolites undergo changes of >50 Da. These include metabolites with significantly different molecular masses than the parent drug that are generally formed via cleavage of the parent drug (such as *N*- or *O*-dealkylation). For example, the mass defect values of nefazodone (NEF; MH^+ 470.2323) and its major metabolite, 1-*meta*-chlorophenylpiperazine (*m*-CPP; MH^+ 197.0845), are 148 mDa apart. Similarly, conjugation with polar molecules such as GSH results in molecular masses that are sometimes significantly greater than the parent drug. Likewise, a dehalogenation reaction of a multihalogenated parent drug can also result in significant mass shifts, especially since the presence of one or more halogen atoms in the parent drug further enhances the negative mass defect of metabolites relative to endogenous materials. Such biotransformation pathways will result in mass defect differences that are $>\pm 50$ mDa and are likely to be missed when a mass defect filter of ± 50 mDa is used. To overcome this obvious deficiency, an improved strategy has been developed in which templates of MDFs

have been generated based on (i) the structure of the drug, (ii) the core substructure of the parent drug, and (iii) the conjugates of the drug [67]. While the MDF template of the parent drug helps detect metabolites that have undergone relatively minor changes in their molecular masses, the substructure filter template, which uses one or a few core substructures of a drug as filter templates, can be applied to detect metabolites that are significantly smaller than the parent drug. Most of the metabolites that can be readily detected using the parent drug MDF template are formed via oxidation, reduction, and dealkylation of a small aliphatic group (<50 Da). While metabolites that are generated via hydrolysis, dealkylation, and other biotransformation reactions that cleave the parent drug into two smaller molecules can be detected by the substructure filter template. The conjugate filter template uses typical drug conjugates as filter templates and is designed to detect different classes of conjugated metabolites. For example, the glucuronide conjugates of the parent and/or hydroxylated metabolites can be detected using a filter based on the glucuronide of the drug. In addition to the three common templates of MDFs described above, some atypical filter templates can be designed for analyzing components such as an active moiety of a prodrug [67]. Additionally, filters that can detect oxidatively dehalogenated metabolites have also been designed. Following detection of these potential metabolites, information on their molecular structures may be extracted from MS/MS spectra with the benefit of the elemental composition information encoded in the accurate masses of their respective parent and fragment ions. More recently, a mass defect-triggered data-dependent MS/MS scanning technique has been developed for selective acquisition of metabolite data with a high resolution mass spectrometer [67].

Several other postacquisition data filtering methods such as NLF or PIF based on metabolite fragmentation patterns obtained from MS/MS spectral data that are acquired using data-dependent MS/MS acquisition methods with linear ion-trap, triple Q-trap, Orbitrap, and QTOF instruments have been developed for finding both common and novel metabolites. For example, LTQs have recently been employed for screening GSH adducts by processing the acquired MS/MS data with NLF of 129 Da in the positive mode and PIF of m/z 272 in the negative mode [68,69]. To increase the detection sensitivity of low levels of metabolites in some complex biological samples, the detection of metabolites is sometimes facilitated through postacquisition data processing with either MDF alone or a combination of MDF and other data mining techniques. For instance, multiple postacquisition data mining techniques were used in parallel for the detection and characterization of *in vitro* metabolites of indinavir.

A so-called all-in-one analytical procedure has been proposed recently for metabolite identification, in which analytes that are separated by UPLC are introduced into QTOF for structural analysis [70–72]. A typical setup utilizes two different data high mass range acquisition functions one with low and one with high collision energy settings and both without MS/MS precursor ion selection. Using this so-called MS^E (where E represents collision energy) approach, both molecular ion and nonselective MS/MS fragment ion data are obtained for all detected compounds, each to separate data acquisition files, and the molecular ions and their fragment ion data can be linked by their retention times. The major advantage of the MS^E methodology is its suitability for coupling with UPLC for short run times and better chromatographic separation, which has been proved to be very useful in high throughput profiling of *in vitro* metabolites in lead optimization. Another promising strategy for parallel use of multiple postacquisition data mining techniques is the application of MDF in combination with

MS^E experiments for fast metabolite analysis. In this approach, full scan accurate mass data sets are processed both with MDF and with narrow and wide EIC combined with control sample comparison, respectively, for the discovery of both common and uncommon metabolites. The MS/MS data acquired with the MS^E scanning can also be utilized for data mining via PIF and NLF as well as for reconstruction of PI spectra of metabolites.

5.3.4 Other Strategies for Metabolite Identification

Most often, MS alone is insufficient to provide the precise structure of metabolites. First, an excess of endogenous material in biological samples often suppresses the ionization of drug-related material complicating metabolite identification by MS. Second, the metabolite can be too polar or too small in size, which can make its identification difficult. In these cases, multiple analytical and wet chemistry techniques, such as chemical derivatization, enzymatic hydrolysis, and hydrogen/deuterium exchange (H/D exchange) combined with MS, are helpful to probe novel and isomeric metabolites of drug candidates [27,44].

5.3.4.1 Chemical Derivatization. Derivatization techniques combined with LC-MS/MS have proved to be very powerful tools for the characterization and quantification of novel and unusual metabolites. This technique can supplement metabolite identification by increasing sensitivity (especially those metabolites that are poorly ionized) and lipophilicity (improvement in retention and separation) of the metabolites. Tremendous structural information can also be obtained for drug metabolites by analyzing MS/MS data before and after chemical intervention. Most importantly, chemical modification of these metabolites can lead to derivatives with characteristic fragmentation. Further, the use of chemical derivatization of low molecular weight compounds to improve their detection characteristics for chromatographic analysis is also well documented [44]. For example, derivatization of the phenolic OH of ethinylestradiol with dansyl chloride by introducing a basic nitrogen significantly increases the ionization efficiency in ESI/MS for pharmacokinetics studies.

Several chemical reactions have been used in identifying metabolites of drugs when the mass spectrum does not offer further confirmation of the sites [44,73–75]. Some reactions include oxidation of alcohols with mild oxidizing agents such as potassium dichromate and sulfuric acid (Jones reagent), pyridinium chlorochromate (PCC), pyridinium dichromate (PDC, Cornforth reagent), reduction of ketones with sodium borohydride and *N*- and *S*-oxides with $TiCl_3$, alkylation with diazomethane; and esterification with methanolic hydrochloric acid. These relatively simple chemical modifications can confirm the site of metabolism especially in molecules where regioisomers and isobaric metabolites are possible. For instance, the site of metabolism on the secondary versus the primary carbon center of an aliphatic ethyl chain in pioglitazone was easily determined by oxidation with Jones reagent (Fig. 5.5) [76]. Such a reaction converted the secondary alcohol to a ketone and the primary alcohol to a carboxylic acid.

Analysis of products of these reactions can help differentiate the regioisomers and pinpoint the site of modification since the formation of a ketone will reduce the molecular ion by 2 amu, while the formation of the carboxylic acid will increase the mass of the molecule by 14 amu. Similarly, selectivity of reagents toward a particular functional

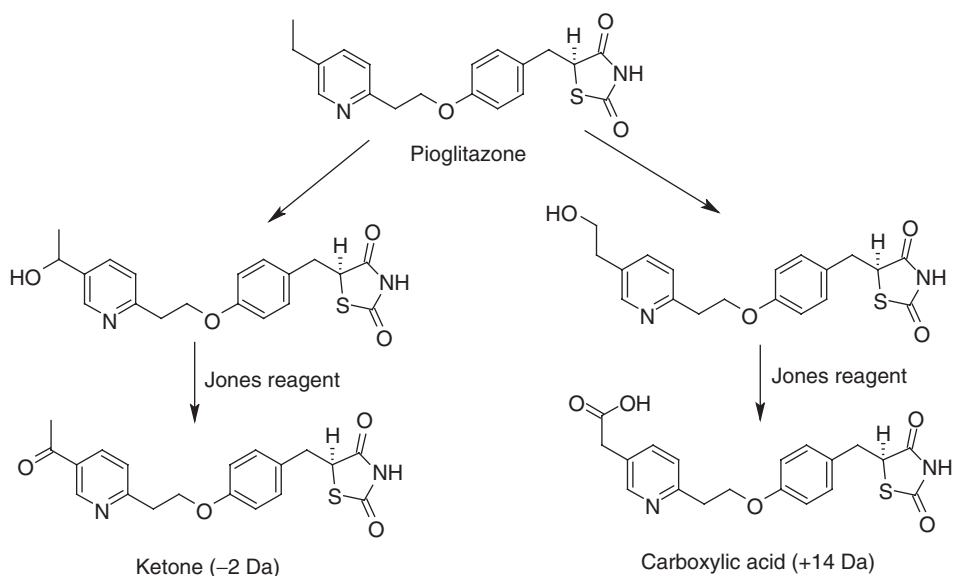


Figure 5.5 Oxidation of pioglitazone metabolites with Jones reagent [76].

group can be used to differentiate and identify the type of modification. For instance, acyclic amine moieties such as piperidines or pyrrolidines in a molecule can undergo P450-mediated oxidation to piperidones or lactams. Both these pathways result in addition of 14 amu to the motif. A differentiation of a ketone formation versus a lactam (addition of 14 amu to the molecular ion of the parent drug) formation following oxidation of an alicyclic amine can be made by treatment of the sample with sodium borohydride (Fig. 5.6). Treatment with sodium borohydride can reduce ketones to alcohols, thus increasing the molecular ion of the product by 2 Da. On the other hand, there is no change in the molecular weight of the lactam since these functionalities are not affected by these reducing reagents. Alternatively, the two functionalities can be distinguished by treatment of the mixture with hydrazine or methoxylamine. Conversion of the carbonyl functionality in the ketone to a hydrazone can readily differentiate the lactam from the ketone.

Methods to distinguish formation of *N*-oxides from sulfoxides and *C*-hydroxylations could have a significant impact on the rapid metabolite identification/profiling of drug molecules by MS. Kulanthaivel *et al.* have developed an efficient and a simple method to selectively convert *N*-oxides to their corresponding amines [77]. Their work demonstrated TiCl_3 to be a facile and easy-to-use reagent in the presence of biological matrices that could selectively reduce *N*-oxides in the presence of other labile groups. Prakash *et al.* [78] have also used aqueous titanium chloride to reduce a sulfoxide metabolite of a sulfur-containing drug. This led to the disappearance of the sulfoxide peak and appearance of another metabolite peak, the methyl sulfide (Fig. 5.7). This led the group to conclude that the former was a sulfur oxidation product of the latter.

Alkylation with diazomethane or diazoethane to the corresponding ether has been used to characterize the phenolic hydroxyl and carboxylic acid functionalities from the aliphatic hydroxyl groups. The use of diazoethane for derivatization proved to be

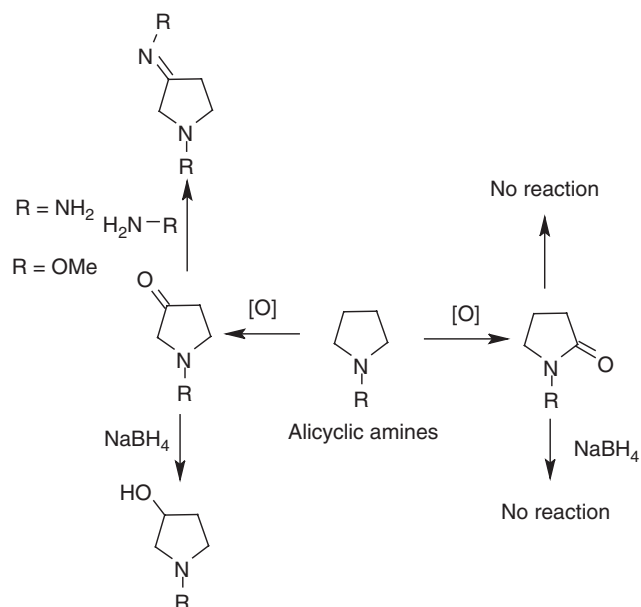


Figure 5.6 Derivatization of the ketone with hydrazine or methoxylamine.

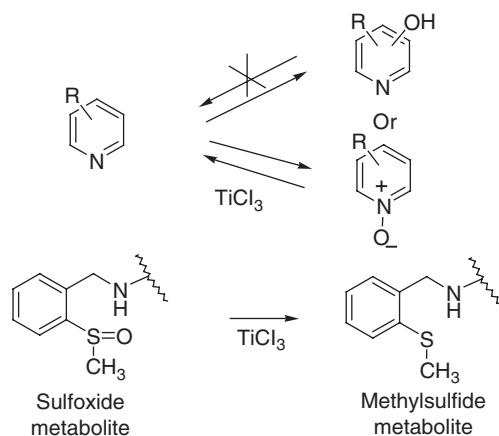


Figure 5.7 Reduction of *N*-oxides and sulfoxides with titanium trichloride.

useful in elucidation of the locality of the phenolic OH group in structures of the metabolites, for instance, RWJ-26240 [79]. Introduction of a methyl moiety into a carboxylic acid can also be accomplished by treatment of the sample with methanolic hydrochloric acid ($\text{CH}_3\text{OH}/\text{HCl}$). However, this method cannot methylate phenolic or aliphatic hydroxyl groups in the molecule. This selectivity can be used to differentiate between a carboxylic acid functionality and a phenolic or aliphatic hydroxyl groups in case isobaric metabolites are formed. The conversion of the molecule to a nonpolar methyl ester also changes its retention time in the chromatogram in addition to the

increase of 14 amu in the mass spectra. Methylation was also used to identify the site of glucuronidation for traxopodil [80]. On the same lines, Schaefer *et al.* [81] have demonstrated the use of a selective acetylation strategy to elucidate the positions of glucuronidation of carvedilol. The approach relies on the selective acetylation of hydroxyl and amine groups under different conditions. Nucleophilic groups such as amines and hydroxyls are readily acetylated in nonaqueous solution by acetic anhydride in the presence of a base (such as pyridine). In aqueous solution, however, the more nucleophilic amine groups are rapidly acetylated by acetic anhydride, while acetylation of hydroxyl groups is prohibited due to the presence of water. The site of modification can therefore be identified from the number of acetyl groups that are added into the molecule. Thus, addition of number of acetyl groups in carvedilol in the nonaqueous media indicated the site of glucuronidation of this molecule.

Differentiation of aromatic hydroxyl group from an aliphatic hydroxyl group can also be made by treating the biological sample with acetic anhydride [44]. Under these conditions, the sample is treated with sodium bicarbonate solution and acetic anhydride. Phenolic hydroxyl groups can be acetylated selectively in the presence of aliphatic hydroxyl groups by acetic anhydride in water at pH 9–10. Hence, an addition of 42 amu to the molecule and a subsequent shift in the retention time can readily differentiate between the two hydroxy groups. An alternative derivatization that is specific for phenols (also useful for amines) is the reaction with 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride), which forms a sulfonate in the aqueous alkali [73]. Given the ability of the derivative to be protonated in the acidic medium, this reaction has been useful in enhancing the ionization of otherwise nonionizable metabolites in addition to discriminating the aliphatic and aromatic hydroxyl groups.

The hydrolytic cleavage of ester under alkaline conditions is a reaction that is commonly used to distinguish an acyl glucuronide from an ether glucuronide (Fig. 5.8). The use of this reaction is based on the fact that esters are readily hydrolyzed by aqueous alkali, while the aliphatic and/or aromatic ethers are resistant to hydrolytic cleavage in the alkaline medium and require harsh conditions. Thus, regeneration of the parent drug on treatment of aqueous alkali confirms that the conjugate is an acyl glucuronide. Thomas *et al.* [82] described the differentiation of acyl- and *O*-glucuronides by hydrolysis with β -glucuronidase and sodium hydroxide in studies on metabolism of gemfibrozil.

A chemical reaction is regularly employed to identify reactive metabolites formed via bioactivation of xenobiotics [83–85]. This helps in the elucidation of plausible

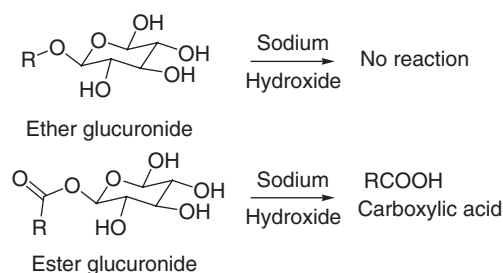


Figure 5.8 Saponification of ester versus ether glucuronide conjugates.

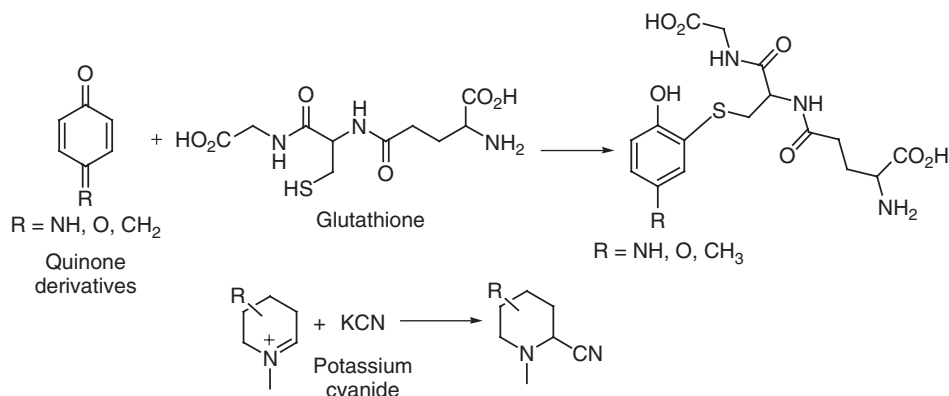


Figure 5.9 Reaction of reactive intermediates with glutathione and cyanide.

intermediates and their potential mechanism of formation and helps in designing new compounds that are devoid of this liability. Several nucleophilic trapping agents have been employed to scavenge these intermediates (Fig. 5.9). Addition of thiol reagents such as GSH or *N*-acetylcysteine in *in vitro* incubation is most commonly used for trapping reactive intermediates, making them amenable to characterization by MS. In cases where reactive metabolites cannot be trapped by GSH, other means have been used in the characterization of these intermediates. For instance, cyclic amines are known to undergo two electron oxidation to imines or iminium ions. These intermediates can also modify proteins via a covalent reaction between the intermediate and the macromolecule. Despite their electrophilicity, these intermediates do not react with GSH (because of their potential instability). However, these intermediates are readily trapped by cyanide anion following addition of potassium or sodium cyanide [86]. The corresponding aminonitrile adduct formed by the reaction between the iminium intermediate and the cyanide anion results in addition of 26 mass units to the molecule, which can be readily characterized by MS.

Similarly, the ring opening of heteroaromatic rings such as the furans to dienols has been determined by treatment of the incubation mixture with semicarbazide or methoxylamine [87,88]. For instance, metabolism of L-739,010, a 5-lipoxygenase inhibitor, containing a furan ring, undergoes oxidative ring opening to form a reactive 2-butene-1,4-dialdehyde intermediate [89] (Fig. 5.10). This intermediate can covalently bind to proteins via nucleophilic addition to the aldehyde groups or Michael addition to the carbon–carbon double bond. Indeed, addition of methoxylamine dramatically reduced the extent of covalent binding. In the presence of methoxylamine, several *O*-methyloximes were generated and characterized by LC-MS/MS and ^1H NMR analysis. Many of the chemical derivatization methods documented for chromatographic analysis are suitable for ESI MS analysis, and readers should not be limited to the types of reaction discussed in this review.

5.3.4.2 Hydrogen/Deuterium Exchange. Hydrogen/deuterium exchange is a strategy widely used in studying protein structures or used for elucidation of mass spectral fragmentation mechanisms. However, in recent times, it has proved itself to be useful in metabolite identification studies, especially in cases where alternative structures

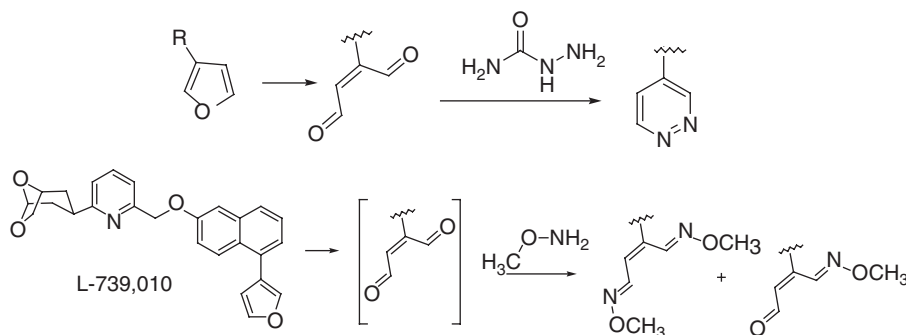


Figure 5.10 Derivatization and trapping of reactive metabolites following oxidation of furan-containing compounds.

for an observed biotransformation are possible [44,90–92]. As described previously, metabolism of xenobiotics involves functionalization of polar groups (e.g., $-\text{OH}$, $-\text{SH}$, $-\text{NH}$, $-\text{NH}_2$, and $-\text{CO}_2\text{H}$), thus increasing the number of exchangeable hydrogen atoms. These labile, exchangeable protons can be replaced with deuterium (D) isotope in the presence of deuterated solvents or reagents. The resulting deuterated molecule shows an increase in its m/z value in comparison to a nondeuterated molecule. The difference in the m/z value of the deuterated and a nondeuterated compound facilitates the determination of the presence, number, and position of H/D exchangeable functional groups within a metabolite and serves as an aid in its structure elucidation. In addition, H/D exchange experiments facilitate the interpretation of MS/MS fragmentation processes. For example, this approach was applied successfully to differentiate N -oxides, sulfoxides, and epoxides from monohydroxylated metabolites and sulfones from dihydroxylated metabolites. Even though the increase in mass of N -oxide, a sulfoxide, or the epoxide is similar to that of a hydroxylated metabolite, the former functionalities do not contain an exchangeable proton like the hydroxyl group. Although some information is obtained from the mass spectrum of the three metabolites, treatment with a deuterated solvent provides proper confirmation of the functionality that is incorporated. This is because such a treatment with result in a mass shift of the hydroxylated compound by 2 amu, sulfoxide by 1 amu, while no change in the mass shift of N -oxide is observed. H/D exchange experiments can be performed online, where either a deuterated solvent is used as the LC mobile phase or a deuterated reagent gas is used or by postcolumn infusion of deuterium oxide into a normal LC-MS run. The use of deuterium oxide as the regular chromatographic LC-MS mobile phase for studying drug metabolism has been reviewed recently [93].

5.3.4.3 Enzymatic Hydrolysis. Enzymatic hydrolyses of metabolites especially phase II conjugates have also proved useful in their identification/confirmation of these conjugates, especially when the conjugates have poor ionization efficiency. The presence of any phase II metabolite in the biomatrix can also be verified using xenobiochemical experiments with β -glucuronidase and/or arylsulfatase [94,95]. These two enzymes are able to quite selectively and quantitatively cleave the ether or ester bond between glucuronic or sulfuric acid and the xenobiotic part of the phase II drug metabolite under the back-formation of the phase I drug metabolite. Comparative

HPLC analyses of the sample incubated without an enzyme and the same sample after enzymatic treatment using β -glucuronidase and/or arylsulfatase afford qualitative and quantitative information about phase I and II drug metabolites in the biological sample. In some instances, the enzymatic hydrolysis of the conjugates proves difficult. For example, when a compound containing a carboxylic acid undergoes glucuronidation, it tends to undergo acyl migration. These rearranged products are very weak substrates of β -glucuronidase and therefore do not undergo the desired cleavage. In such cases, the advantage of susceptibility of the site of glucuronidation to a chemical reaction (described above) can be helpful in further confirming the presence of an acyl glucuronide in the molecule.

5.3.4.4 Stable Isotope Labeling. Stable isotope-labeled compounds have been synthesized and effectively utilized by drug metabolism scientists and toxicologists to gain better understanding of drugs' disposition and their potential role in target organ toxicities [96–98]. The combination of stable isotope-labeling techniques with MS and NMR spectroscopy allows rapid acquisition and interpretation of data and has promoted greater use of these stable isotope-labeled compounds in ADME studies. MS has a unique capability to separate stable isotopes, which can be utilized in a large number of applications. Some stable isotopes that have been utilized for identification purposes are ^1H and ^2H (D), ^{12}C and ^{13}C , ^{14}N and ^{15}N , ^{16}O and ^{18}O , and ^{32}S and ^{34}S . In cases where the compound includes one or more Cl or Br atom, an incorporation of a stable isotope is not necessary. The principal isotopes of ^{35}Cl and ^{79}Br are ^{37}Cl and ^{81}Br . These two atoms will display abundant $M + 2$ ions in the mass spectra with known abundance ratios ($\sim 25\%$ for ^{37}Cl and $\sim 50\%$ for ^{81}Br). In metabolism studies, a method called *isotope cluster* technique where a 1:1 mixture of labeled and unlabeled drug is administered and analyzed by LC-MS is generally employed to detect metabolites. Following processing and analysis of the samples, the peaks that show an isotope cluster with known mass difference are detected. Usually, a separation of >2 amu between the two ions is desirable, which leads to the appearance of a twin ion pair pattern for the parent compound and its metabolites in the mass spectra of the biological extracts. The identification of a metabolite should fulfill the following criteria:

1. Two peaks with identical shapes and retention times must be recorded in the ion chromatograms.
2. The mass difference of the two peaks must be the same as the mass difference between the labeled and the unlabeled parent drug.
3. The relative abundance ratio of the peaks must be the same as the concentration ratio of the labeled and the unlabeled parent drug.

Computer programs have been developed to identify potential metabolites by searching each scan of a chromatographic run for the characteristic isotope cluster of the parent compound mixture. Numerous examples exist in the literature where investigators have used this technique to study *in vitro* and *in vivo* metabolic disposition of compounds [98].

Besides locating metabolites in biological matrices, the stable isotopes can be particularly useful in determining the structures of unusual or unpredictable metabolites formed from compound. The benefit of isotopic labeling here is fragmentation pattern

recognition. This exercise can give information about the potential site of modification, especially when the molecule produces a same fragment ion following cleavage of the molecule. When the labeled part of the molecule is retained during the fragmentation, the fragment ion maintains the isotopic difference introduced by labeling. However, if the labeled moiety is lost, the m/z value of both the labeled and the nonlabeled fragments are identical.

The incorporation of stable isotopes into compounds also permits delineation of possible mechanisms and the enzymes responsible for the formation of particular metabolites. Isotopically labeled oxygen or water, $^{18}\text{O}_2$, or H_2^{18}O , have been frequently used for this purpose. For example, replacement of $^{16}\text{O}_2$ with $^{18}\text{O}_2$ in an *in vitro* incubation allows the incorporation of ^{18}O in the metabolites that are formed oxidatively. Conversely, in some cases, H_2^{18}O has been used in the incubation mixtures to assess whether the oxygen incorporated into the metabolite is derived from water. This type of experiment not only allows for assessing the fate of oxygen incorporated into the molecule (from air or a water molecule) but also helps delineate the enzyme that can catalyze this oxidation. For instance, enzymes such as monoamine oxidase (MAO), flavin monooxygenase (FMO), or P450 use air as a source of oxygen to oxidize the compound, while the source of oxygen in aldehyde-oxidase-catalyzed reactions is water. Such mechanistic information about metabolite formation and the enzymes involved is very valuable in the early discovery studies.

Stable isotope labeling does not necessarily have to be performed on the compound whose metabolism is being studied. At times, moieties (e.g., GSH) that are eventually incorporated into the compound of interest may be labeled with stable isotopes so that the products can be identified as metabolic products. The use of stable isotope-labeled GSH to trap and characterize *in vitro*-generated reactive metabolites is an example of this approach [98].

5.4 NMR SPECTROSCOPY

As stated earlier, the structure of a metabolite is critical to the development of a pharmaceutical compound and can play an important role in understanding its toxicology, enzymology, or biotransformation pathways. Beyond the normal challenges of structure elucidation, metabolites are typically only available at low levels ($\mu\text{g/mL}$ or less) and are contained in very complex matrices such as blood, excreta, or tissue. While MS is very valuable in structure elucidation, there are situations where MS data alone can fail to definitively elucidate a metabolites structure. In these instances, other spectroscopic techniques need to be employed. One of the most powerful of these techniques is NMR spectroscopy.

Since its inception in the late 1940s, NMR spectroscopy has grown to be arguably the most valuable analytical tool for unambiguous structural characterization. NMR data provides information on the number of nuclei within a compound as well as the microchemical environment of each nuclei through chemical shift and connectivity information through coupling constants [99]. This diversity of information makes NMR data, when used in conjunction with MS data, a very useful structural elucidation tool.

While NMR is an incredibly powerful technique, it does have limitations, largely in the area of sensitivity. Only specific nuclei are NMR active, those with a quantum spin of $1/2$ [100,101]. Hydrogen (^1H), carbon (^{13}C), nitrogen (^{15}N), and fluorine (^{19}F)

are perhaps the most useful NMR-active nuclei to a drug metabolism scientist. The sensitivity of experiments targeting these nuclei, in some cases, is further limited by the lack of an abundant active isotope. As an example, the NMR-active nucleus of carbon is the ^{13}C isotopic form, which is only 1.1% of the total carbon pool. Obviously, this limits the sensitivity of experiments utilizing this nucleus.

In the past 15 years, many advances have been made to overcome this relative lack of sensitivity. New hardware incorporating cryoprobes, NMR probes with small cell volumes, and higher field instruments have increased NMR sensitivity dramatically [102,103]. Additionally, new more sensitive pulse sequences have enhanced the ability to establish structural connectivity through long-range heteronuclear (^1H - ^{13}C and ^1H - ^{15}N) correlations [104–106].

Another limitation of NMR, when performing experiments for metabolite structural elucidation, is the need for pure sample. The need for an isolated sample has long been a stumbling block for drug metabolism scientist. Although there has been significant improvement in preparative chromatography columns and HPLC systems, it is still a considerable effort to isolate a pure metabolite from biological matrix. An alternative to preparative isolation is LC-NMR, where in much the same manner as LC-MS, an NMR spectrometer is interfaced with a chromatography system. While this has great potential, it is not a panacea. Solvent suppression, column loading, and computer automation can all be problematic. However, when a metabolite is unstable or recalcitrant to isolation, HPLC-NMR may be the best option for acquisition of meaningful NMR data [107,108].

Discussed in this section is the use of NMR in structural elucidation of drug metabolites. It is important to distinguish NMR application to drug metabolite identification from its application to metabonomics. Metabonomics is the study of whole system's biology using biomarkers, and although NMR and LC-NMR may be used in support of biomarker identification, metabonomics is a separate discipline from metabolite structure elucidation [109–111].

5.4.1 Sample Preparation for NMR Spectroscopy

The successful structural elucidation of a metabolite starts well before the acquisition of NMR data; it begins with the design of the sample generation experiment. Obviously, the amount of metabolite generated in these experiments is critical, and also important is the matrix and the subsequent ease of metabolite isolation. If the compound of interest can be generated in sufficient quantities using *in vitro* systems such as liver microsomes, other subcellular fractions, hepatocytes, or individually expressed recombinant drug-metabolizing enzymes the isolation is typically more efficient. When *in vitro* generation systems are used, off-line isolation can sometimes be eliminated with the use of LC-NMR (see section below). In other cases, where the *in vitro* turnover rate of the parent compound is low, dosing an animal of appropriate species may generate metabolite quantities in sufficient amount such that using urine or bile as a starting matrix may be the most expeditious route to a pure sample. Excipients used in the dose formulation or *in vitro* incubation should also be considered. Glycolic polymers, which are commonly used in dosing solutions and enzymatic preparations, chromatographically separate into individual components with a wide variety of polarities. Frequently, these compounds have no UV chromophore making them invisible, until either MS or NMR are used to analyze the sample. The combination of these two traits makes the isolation of pure low

level metabolites difficult when these types of compounds are present. Consequently, if there are alternatives to the use of these polymers, they should be pursued.

In the case of an *in vivo* study, sample collection strategies can strongly influence the amount and quality of the metabolites generated. Which matrix to use, whole blood, plasma, urine, bile, or feces, should be considered. Clearly, the metabolite concentration will vary depending on the matrix and the time of collection. Some matrixes may contain endogenous materials that interfere with the chromatographic separation of the metabolite of interest. Sample stabilization may also be critical. Some metabolites are unstable under certain pH conditions, and the collection should be made to optimize the stability of the isolate. As an example, acyl glucuronides, to varying degrees, are prone to migration under neutral and basic conditions. In order to stabilize these metabolites, samples should be made acidic as soon as collected to ensure the highest concentration of the O-1 β -glucuronide [112–114].

The isolation of metabolites for NMR analysis using SPE and preparative chromatography has been the standard for many years. In general, the procedures are relatively similar (Fig. 5.11). The matrix is clarified through either protein precipitation and/or centrifugation. Portions of the clarified solution are loaded onto a reversed-phase SPE column, and fractions are eluted using increasing percentages of organic solvent, in general, acetonitrile or methanol. Aliquots from these SPE fractions are profiled via LC-MS. Once the fractions containing the metabolite of interest are identified, they are evaporated to dryness and reconstituted in a mixture of aqueous and organic in preparation for chromatographic separation [115,116]. Chromatographic isolation of a metabolite can be performed on any size HPLC column; typically, this is performed on a column with a 10-mm diameter using flow rates of approximately 5 mL/min. The chromatographic analysis may be either isocratic or gradient depending on the complexity of the separation. Fractions are collected based on UV or MS output from the

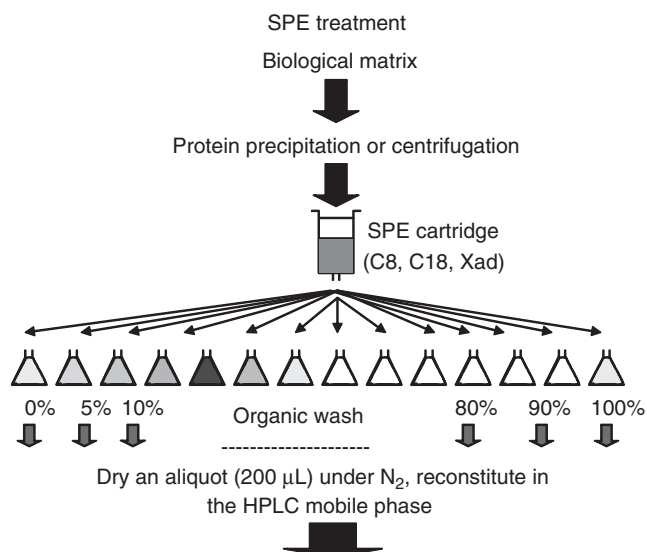


Figure 5.11 General scheme illustrating the isolation of metabolites from biological matrices for NMR analysis. (See color insert.)

TABLE 5.2 Resonances From Residual Protonated Solvents Typically Used in NMR Spectroscopy

Solvent	¹ H		¹³ C		H ₂ O	Comments
	Chemical Shift	Multiplicity, Coupling Constant	Chemical Shift	Multiplicity, Coupling Constant	Chemical Shift	
D ₂ O	4.80	s	—	—	4.80	Contains exchangeable hydrogens
DMSO	2.50	5, <i>j</i> = 1.9 Hz	39.5	7, <i>j</i> = 21.0 Hz	3.30	Contains exchangeable hydrogens
Methanol	3.31	5, <i>j</i> = 1.7 Hz	49.2	7, <i>j</i> = 21.4 Hz	4.78	
Acetonitrile	4.78	s	—	—	2.1	
	1.94	5, <i>j</i> = 2.5 Hz	1.4 118.7	7, <i>j</i> = 21.0 Hz s		
Chloroform	7.24	s	77.2	3, <i>j</i> = 32.0 Hz	1.5	
Acetone	2.05	5, <i>j</i> = 2.2 Hz	29.2 206.7	7, <i>j</i> = 19.4 Hz s	2.8	

preparative system. Final fractions should be checked for purity and then the sample exhaustively dried before NMR analysis. This method produces a high concentration and quality sample. Even if the final purification step is in line with LC-NMR, this SPE sample preparation is highly recommended.

The final consideration in sample preparation is solvent choice. For NMR operational reasons, all solvents used must be deuterated. The isolated metabolite must be soluble in the chosen solvent. Generally, if the parent compound is readily soluble in a given solvent, this solvent will also be a suitable choice for the isolated metabolite. Dimethyl sulfoxide, methanol, and acetonitrile are usually adequate for solubilizing most metabolites. Impurities in the solvent should also be considered. Resonances from residual protonated solvents (e.g., CD₂HOD in CD₃OD) will be the largest nonsample-related resonance in the spectrum (Table 5.2). To avoid these impurities, the best available solvent should be used. Most vendors offer deuterated solvents of “100.0% grade.” This grade should be used for any critical NMR analysis. Last, the locations of the residual solvent and water lines should be considered. If the molecules of interest, parent and metabolite, have resonances critical to the structure solution that could be interfered with by either the residual solvent or water resonances, a different solvent may make the data interpretation more straightforward.

5.4.2 Nuclei

While most other spectroscopic techniques evaluate the response of an entire molecule (MS, IR, and UV), NMR response is centered around individual classes of nuclei. Each family of nuclei will have different physical and quantum characteristics that make them unique. These differences dictate the class of experiment that can be performed and the type of information that can be gleaned from each experiment. For the drug metabolism scientist, there are a limited number of relevant elements that are NMR active.

5.4.2.1 ^1H -Hydrogen. Without question, the most useful nuclei for metabolic studies is ^1H . It is present in almost all organic compounds of interest; endogenous or xenobiotic. The NMR-active form is essentially 100% naturally abundant and is the most sensitive of all common nuclei. Limits of detection for most modern high field instruments are in the low nanomole range for directly observed experiments. The chemical shift range is relatively small (0–13 ppm), which can lead to overlapping resonances. ^1H - ^1H coupling can cause further complication of the spectra, but may also be used to delineate the structure of unknown metabolites through the judicious interpretation of coupling constants and patterns. One of the largest difficulties with ^1H NMR is the ever-present interference from endogenous compounds, which necessitates the isolation of the metabolite for spectroscopic characterization. This may be done before NMR analysis using a variety of separation science techniques (see above). Alternately, this isolation may be performed in line with these same separation techniques directly coupled to the NMR system.

5.4.2.2 ^{13}C -Carbon. ^{13}C NMR data can be critical to the elucidation of a metabolite structure. Establishing the number of carbon atoms and their multiplicity (C, CH, CH_2 , and CH_3) in an unknown metabolite, in conjunction with ^1H data, can provide key information in the structural elucidation process. Directly observed ^{13}C data are usually acquired with ^1H decoupling resulting in single resonances for each carbon atom. The spectra for these experiments are relatively clean and easy to interpret. ^{13}C has a wide range of useful chemical shifts, 0–220 ppm, limiting the opportunity for overlapping resonances. However, the low native sensitivity (approximately 25% that of ^1H) and the low natural abundance (1.1%) usually preclude the direct acquisition of meaningful ^{13}C data on isolated metabolites. These sensitivity issues can be surmounted by the use of indirect heteronuclear 2D experiments, which are discussed in detail below.

5.4.2.3 ^{19}F -Fluorine. ^{19}F NMR data is very useful as a tool to determine the distribution, both quantitatively and qualitatively, of metabolites in biological matrixes. As is carbon, direct observed ^{19}F data are usually acquired with ^1H decoupling typically resulting in a single resonance for each individual ^{19}F atom. If the data are acquired appropriately (using a power-gated decoupling pulse sequence), the responses for the resonances within the spectra are quantitative [117–120]. A major advantage of ^{19}F NMR is the lack of interference from endogenous compounds. The spectra width of ^{19}F is in the 200–250 ppm range, providing a broad spectral width for data acquisition. Additionally, the relative sensitivity of ^{19}F is quite high (approximately 83% that of ^1H) and the natural abundance of the NMR-active nuclei is 100%. While there are many advantages of ^{19}F NMR, the obvious requirement of a fluorine atom in the molecule of interest can limit the utility of these experiments.

5.4.2.4 ^{15}N -Nitrogen. Historically, the acquisition of meaningful ^{15}N NMR data from metabolites has been precluded due to the low sensitivity and natural abundance of the nuclei. However, over the past 5 years, with the advent of cryoprobes and indirectly detected experiments, sensitivity levels of ^{15}N experiments have come within reach of the metabolism scientist [121]. ^{15}N data are particularly useful with two classes of metabolites, nitrogen oxidation and nitrogen conjugation. In simple cases, both these types of metabolites can be easily classified by other means. *N*-oxides can be delineated with deuterium exchange experiments or through the use of titanium trichloride

reduction. *N*-glucuronides have characteristic molecular weights and MS/MS fragmentation patterns. Additionally, they are susceptible to enzymatic cleavage. However, in situations where there are multiple N sites available for oxidation or conjugation, these techniques are not sufficient for definitive structural elucidation. This information can be acquired using ^1H - ^{15}N heteronuclear NMR experiments. ^1H - ^{15}N heteronuclear multiple quantum coherence (HMQC)-type experiments (directly bonded H-N) can be used to observe changes in nitrogen chemical shifts and ^1H - ^{15}N heteronuclear multiple bond correlation (HMBC) can be used to establish location through long-range coupling.

5.4.2.5 Other Nuclei. ^2H NMR is limited by negligible natural abundance (0.015%), small spectral width, and that ^2H is a quadrupolar nuclei, which results in broad line shape except for low MW compounds. Despite these drawbacks, in instances where a ^2H label can be inserted in the parent molecule, deuterium NMR can be utilized as an analytical tool. Nicholls *et al.* [122,123] have used ^2H NMR extensively to study futile deacetylation of acetanilides.

Tritium is a particularly sensitive nucleus with an operational NMR field strength at 6% greater than that of ^1H . It also has a chemical shift range and coupling constants close to that of hydrogen making data interpretation familiar. However, this is a radioactive isotope which requires special precautions and monitoring making its ease of use significantly more difficult. Additionally, the natural abundance of ^3H is negligible, hence, requiring a synthetically labeled parent molecule. Despite these obstacles, there are several studies where tritium NMR data have been successfully used to address metabolic questions [124].

5.4.3 NMR Experiments

The theoretical basis of NMR experiments has been discussed elsewhere at length including text for a cursory understanding as well as advanced treatment of the physics of NMR [99,101]. Briefly, certain nuclei, when placed in a magnetic field, behave as individual gyroscopes precessing at a frequency unique to each class of nuclei (^1H , ^{13}C , ^{19}F , etc.). As a pulse of RF energy is introduced into this system, the nuclei achieve an excited state. When the excited nuclei relax to ground state, they generate a decaying sinusoidal signal. This signal is measured in time versus magnitude and is referred to as the *free induction decay* (FID). These experiments are controlled via a computer program called a *pulse sequence* that contains specific instructions that regulate the excitation RF and data acquisition. After acquisition, the FID can then be transformed mathematically from the time domain to the frequency domain using a fast Fourier transformation (FFT). This transformation results in the recognizable and interpretable 1D NMR spectrum.

When 1D experiments fail to provide the structure of a metabolite, data from 2D experiments may grant additional insight. The basic 2D NMR experiment involves the acquisition of multiple 1D data sets with a systematic variation of parameters such that a second dimension of FID is developed. As with 1D data, a fast FT can be applied to the second dimension of data. The Fourier transformation of data in both dimensions results in a 2D data set. The detected dimension (referred to as *F2*) typically contains 1–2k data points. The indirect dimension (referred to as *F1*) typically contains 128–512 data points. In each case, there is dramatically less digital resolution than that in a 1D data set. This limitation in digital resolution should always be considered in experimental selection. There are many excellent reviews of the acquisition of 2D NMR [101,125].

5.4.3.1 1D Pulse Sequences. As reviewed above, the most popular of all NMR experiments is the one-dimensional ^1H experiment. One-dimensional NMR experiments, as the name implies, collect data in a single dimension from a single class of nuclei. The data from this experiment provide information on the number of hydrogen atoms, the individual microchemical environment of each hydrogen, and the proximity of the hydrogen atoms to each other. This information alone is very powerful and in many instances may be sufficient to identify the structure of a metabolite. In Hutzler's work on (1-[(2-ethyl-4-methyl-1H-imidazol-5-yl)methyl]-4-[4-(trifluoromethyl)-2-pyridinyl]piperazine (EMTPP), the α -hydroxyl metabolite was easily characterized by ^1H data (Fig. 5.12) [126]. The ethyl moiety of the parent compound had two resonances: a triplet at 1.25 ppm integrating to three ^1H with a coupling constant of 7.6 Hz (B) and a quartet at 2.64 ppm integrating to two ^1H with a coupling constant of 7.6 Hz (A). In the isolated metabolite (M2), both these resonances have changed, the B to a doublet with a chemical shift of 1.37 ppm integrating to three ^1H with a coupling constant of 6.6 Hz and the A to a quartet with a chemical shift of 4.62 ppm with a coupling constant of 6.6 Hz. These changes can only be accounted for by the oxidation of the α -carbon. There are many other examples in the literature where 1D ^1H NMR data provided critical information to the structure proof of a metabolite [127–130].

Similar 1D data can be collected from other nuclei, most notably ^{13}C . Unlike ^1H spectra, ^{13}C spectra are acquired decoupled. Each carbon atom may be coupled to one or more hydrogen atoms in a given molecule. Because of this heteronuclear coupling, individual resonances within ^{13}C spectra have very complex structure. In order to simplify the 1D ^{13}C spectra, ^{13}C acquisitions are usually performed with broadband decoupling of all protons [99]. The decoupling removes the scalar coupling of the ^1H to the ^{13}C resonances and produces a singlet for each carbon resonance. The condensation of the multiplets to a single resonance also increases the signal-to-noise ratio. Because of the broad range of ^{13}C resonances (~ 230 ppm), one line is typically observed for each carbon atom in the molecule with resonances rarely overlapping. As with ^1H spectra, the chemical shift of each ^{13}C resonance is indicative of its microchemical environment. Because of this, it is possible to identify certain functional groups within a molecule for which there is no direct evidence in the proton spectrum, for example, carbonyls.

There is a wide range of relaxation time for ^{13}C atoms. This makes integration of a typical ^{13}C spectrum inappropriate. However, even with this limitation, the determination of the total number of carbons within a molecule from 1D ^{13}C spectra is easily done. While carbon chemical shift information is often critical to the solution of a metabolite structure, the amount of material required for the acquisition of a directly observed ^{13}C spectra can preclude this type of experiment. Instead, ^{13}C chemical shift data are usually acquired through the much more sensitive indirect ^1H - ^{13}C 2D experiments (see below).

The nuclear Overhauser effect (nOe) experiment is also a one-dimensional experiment. It is unique from other 1D experiments because its data is used to establish through-space interaction between nuclei. This information can be very valuable in establishing either the stereochemistry or regiochemistry of a metabolite. The experiment applies a radio-frequency to a single resonance in the ^1H spectrum, such that the hydrogens at that resonance become saturated. Like any chemical system that has been disturbed, it will attempt to return to a steady state. For the nOe experiment, this adjustment takes the form of redistribution of energy from the saturated nuclei to

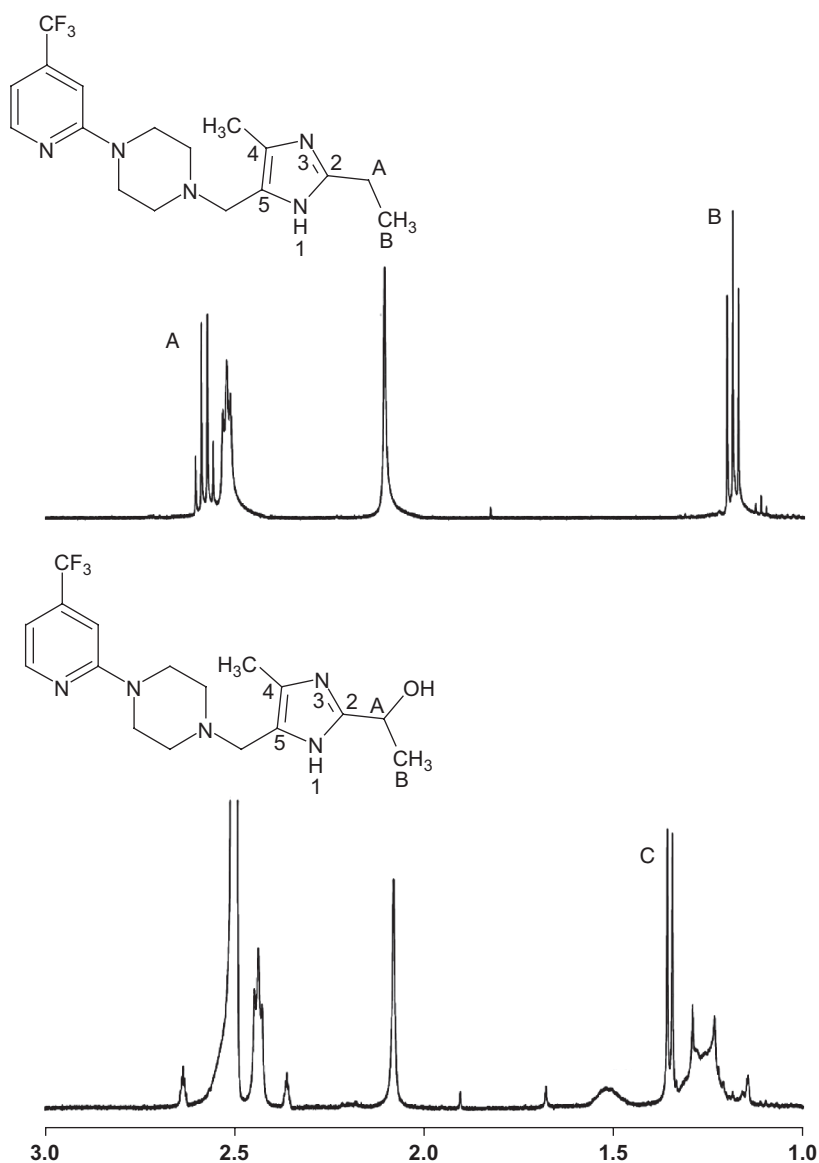


Figure 5.12 ^1H NMR data for EMTTP and its metabolite.

nuclei that are spatially close to the irradiated ^1H . An acquired ^1H nOe spectrum can show changes in signal intensities for those ^1H s close in space (usually less than $3 \text{ \AA}$) to the saturated proton.

Data from nOe experiments are particularly useful in connecting ^1H spin systems that are isolated by heteroatoms. In drug metabolism, the classic example is establishing the site of glucuronidation [131–133]. Here, the nOe enhancement from the irradiation of the anomeric ^1H provides information connecting the glucuronic acid to specific moieties of the parent compound [131].

5.4.3.2 2D Pulse Sequences. The most sensitive 2D experiments, and therefore the most useful, are ^1H - ^1H homonuclear pulse sequences. The ^1H - ^1H correlation spectroscopy (COSY) and ^1H - ^1H total correlation spectroscopy (TOCSY) are the first strata of 2D NMR experiments to be performed in the structural elucidation of a metabolite. Each of these methods is based on scalar coupling between ^1H atoms within the same molecule. COSY experiments are optimized for ^1H - ^1H couplings that are 5–12 Hz; typically 2–4 bonds remote from each other. TOCSY experiments are typically optimized for longer range ^1H - ^1H interactions (1–4 Hz) that are three to five bonds remote. Both these experiments provided “nearest neighbor” information through scalar coupling [99,134,135].

A nuclear Overhauser effect spectroscopy (NOESY) also is a homonuclear ^1H - ^1H 2D experiment that is predicated on dipolar coupling (through space). Like its analogous 1D experiment, a NOESY can provide information about a molecule's three dimensional structure and/or its stereochemistry [136,137].

Heteronuclear 2D experiments fall into two categories: one-bond correlation or multiple bond correlation experiments. Within these groups, there are several variations on each theme. One-bond correlation experiments link a given hydrogen resonance with the specific heteroatom resonance (either ^{13}C or ^{15}N) to which it is bonded. There are numerous versions of this type of experiment. The more sensitive ones are ^1H -detected. That is the nucleus that is monitored is the ^1H and the chemical shift of the Carbon is inferred. The ^1H -detected versions are heteronuclear single quantum coherence (HSQC), and heteronuclear multiple quantum coherence (HMQC), each with gradient and/or multiplicity-edited variations. There are also carbon detected heteronuclear experiments, heteronuclear correlation (HETCOR), which are ^{13}C detected. Carbon-13 detected experiments are less useful because of their lack of sensitivity stemming from the low natural abundance (1.1%) of the active carbon form, ^{13}C . However, when there is insufficient resolution in the carbon dimension, a HETCOR may be preferable to either an HSQC or HMQC because of the increased resolution in the ^{13}C dimension. The direct correlation between ^1H and ^{13}C is useful when there are carbon resonances with unusual chemical shifts that could be diagnostic. For example, a methine attached directly to a carbon with a chemical shift greater than 185 ppm indicates an aldehyde function. Another more common example is the metabolic oxidation of a methylene ($\text{X-CH}_2\text{-X}$) to a methine with a hydroxyl (X-HCOH-X). Methylene carbon resonances typically have chemical shift ranges from 20 to 40 ppm, while a methine carbon with an attached oxygen has a chemical shift of 60–75 ppm. Consequently, establishing that the HSQC spectrum of an isolated metabolite is minus one methylene resonance that was observed in the parent molecule and has a new methine resonance with a chemical shift between 60 and 75 ppm can be key in the structural assignment of a metabolite that has undergone an aliphatic oxidation.

Multiple bond correlation experiments provide correlation between ^1H and an X nucleus (either ^{13}C or ^{15}N) that are two, three, and sometimes four bonds removed. As with the one bond experiments, there are several varieties of multiple bond experiments. The HMBC, a ^1H detected experiment, is the most sensitive. Data from all these types of experiments enable the assignment of ^{13}C chemical shift to quaternary carbons. Additionally, these experiments provide data that “skips over” heteroatoms to provide correlation between isolated spin systems. One example of the utility of this experiment is defining the location of GSH addition. Cross peaks from the methylene resonance of the GSH cysteine through the sulfur to a carbon resonance on the parent

molecule can definitively establish the site of conjugation [138]. Beyond this, in conjunction with the HMQC/HSQC data, the HMBC data enable the assignment of ^{13}C chemical shifts which in turn establishes the carbon backbone of the molecule. This is particularly advantageous when the structure of a metabolite is beyond the expected biotransformation.

5.4.4 Strategy for NMR Characterization of Metabolite Structure

Having reviewed the NMR-active nuclei, sample preparation, and common experiments, it is also appropriate to examine the overall strategy of how to use this information in assigning the structure of a metabolite. The first step is deciding if NMR data are needed. There are many situations where MS or MS/MS data are sufficient to unequivocally identify the chemical structure of a metabolite. For example, a parent molecule that has a single methoxy moiety and the MS data indicate a difference of 14 amu between the metabolite and the parent: in this situation, NMR analysis would not typically be necessary. Once it is decided that NMR analysis is required, the type of NMR data that are needed should be considered. This will factor into the decision about the amount of material needed for analysis. Can the structure of the metabolite be determined with 1D ^1H NMR? This is often a function of the complexity of the parent molecule. Molecules with multiple aromatic systems and redundant functional groups will likely require more complex experimentation to provide an unambiguous structural assignment.

If it is suspected that 1D ^1H analysis would be sufficient, would it be easier to isolate the material or pursue LC-NMR? If 1D ^1H analysis is not going to provide the answer, can a COSY or TOCSY experiment suffice? Often, in molecules with rich ^1H spin systems (e.g., steroids), 2D ^1H - ^1H data can provide adequate insight for definitive structural characterization even in molecules with complex spin systems. In circumstances where there is novel or unprecedented metabolism suspected, a full suite of 1D $^1\text{H}/^{13}\text{C}$ and 2D data may be required. It is a situation like this where the interplay between the COSY, HSQC, and HMBC data is critical. All these data must support the assignment of the metabolite structure. Once the ^1H chemical shifts are assigned and the carbon backbone of the metabolite is established, stereochemistry may be considered. For these situations, nOe or NOESY experiments may have to be explored.

In any of the scenarios described above, two things are absolutely required before the NMR analysis of a metabolite. First, a comprehensive analysis of the parent molecule should be performed where all ^1H and ^{13}C resonances are carefully assigned. The complete assignment of the parent makes the structural assignment of the metabolite a much easier process. Secondly, all other data, spectroscopic, chromatographic, and biological, should be incorporated into the assignment of a metabolites structure. The melding of all this information should paint a complete picture.

5.4.5 HPLC-NMR

Since its inception over 30 years ago, the coupling of liquid chromatography and NMR instrumentation has grown to become a common laboratory technique within the pharmaceutical industry. Much of the early LC-NMR work paralleled LC-MS using “on flow” techniques to interface the two instruments, in which NMR data

was collected in real time as the chromatographic system is constantly flowing. This work lacked sufficient sensitivity for routine practical application in drug metabolism studies and eliminated the possibility of two dimensional experiments. Since that time, several strategies have been developed to resolve these issues and are discussed in detail below. Proponents of LC-NMR claim the advantage of eliminating the step of a separate chromatographic isolation. However, the advantages of directly coupling NMR and HPLC instrumentation must be considered against compromises in performance made to each technique to achieve a hyphenated system. In LC-NMR, chromatographic fidelity is often compromised because the NMR cell volume is typically large (100 μL or greater) when compared to the cell volume of other detectors (UV 10 μL or less). Additionally, the presence of two large residual solvent lines from the mobile phase in the ^1H NMR spectrum can mask structurally informative analyte resonances. Despite these challenges, LC-NMR has proved to be a popular technique for the structure elucidation of metabolites.

Three common approaches to the acquisition of LC-NMR data are on-flow, stop-flow, and peak storage. Early LC-NMR experiments used direct acquisition of NMR data during chromatographic separation. These on-flow techniques were conceptually simple and required minimal hardware development. However, they were limited by the small number of scans that could be collected over a chromatographic peak as it elutes through the NMR flow cell. Each scan has a fixed duration, typically 2–3 s. If a given chromatographic peak is 30–60 s wide, this only allows 10–30 scans per peak. In the case of metabolites, this is generally insufficient time to collect data with adequate signal to noise for confident interpretation. To address this issue, several techniques have been designed to automatically isolate the peak of interest from the flow stream and direct it to the NMR flow cell where acquisition of data can be performed for extended periods of time. This enables one-dimensional spectra to be acquired with greater signal to noise and potential acquisition of two-dimensional spectra. One commonly used approach is stop-flow, where the chromatographic peak of interest is centered in the NMR flow cell by halting the flow of mobile phase [139]. Once the NMR data is acquired, flow is resumed. While this approach allows for the collection of a greater number of scans, it adversely affects the chromatographic separation of later eluting analytes. A second challenge with this approach is chromatographic fidelity. As stated above, the relatively large volume of the NMR flow cell can decrease the resolution of two closely eluting peaks. This problem is accentuated if the peak of interest is preceded by a peak of much greater abundance, as is common with metabolites and parent compounds. Another common approach is loop storage, where the chromatographic peaks are channeled from the mobile phase flow to storage loops and later pumped into the NMR cell after the chromatographic analysis is completed [140]. This technique allows analysis of multiple components of interest from a single chromatogram, while retaining chromatographic fidelity. However, loop storage may reduce NMR sensitivity because of postcolumn band broadening. The band broadening is the result of additional post column dead volume caused by extra valves and capillaries required to divert the chromatographic peaks. Obviously, the degree of loss in sensitivity will be variable and depend on the system configuration.

A variation on the loop collection strategy is in-line LC-SPE-NMR. LC-SPE-NMR adds an additional automated in-line sample handling step, which traps the chromatographic peak on a SPE cartridge [141]. In this method, the chromatographic eluent is diluted with aqueous mobile phase after the analytical separation to make the solvent

system less retentive. The mobile phase is then channeled to an SPE cartridge when a peak of interest is detected. Because of the now lower elutrophic strength of the mobile phase, the peak is trapped onto the cartridge. The cartridge is dried and the analyte subsequently eluted in a much smaller amount of deuterated solvent (typically deuterated acetonitrile). This procedure significantly increases the concentration of the analyte in the NMR cell and hence sensitivity of the overall system. Additionally, because the chromatographic peak is eluted with a single solvent, it reduces the need for solvent suppression as well as helps maintain maximal chromatographic fidelity. The primary disadvantage of the technique is the need to customize each analysis with the appropriate trapping cartridges and solvent conditions to achieve efficient retention and release of analyte.

5.5 QUANTITATIVE ASPECTS

As described above, the identification of metabolites is important in drug research. Additionally, in many cases, it is also important to determine the concentrations of metabolites in *in vivo* and *in vitro* samples. The degree of accuracy needed in quantitating metabolites can differ with the question being addressed and the stage in research/development. Some questions require high accuracy (e.g., measurement of pharmacologically active metabolites and comparison of metabolite exposures across species), while other questions can be adequately addressed with less precise estimates (e.g., estimates of the contribution of a major route of metabolism and which metabolites appear to be predominant in the profile in circulation). The selection of the quantitation method is driven by a balance of the need and the resources required. The use of synthetic standards of metabolites and standard bioanalytical methods is done when precise measurements are needed and is beyond the scope of this section. However, determination of metabolite concentrations in which authentic synthetic standards can also be done using radiometric approaches and NMR spectroscopy, and these approaches are described below.

5.5.1 Radiometric Quantitation

Radiometric quantitation of metabolites offers the advantage that authentic standards of metabolites are not required in order to quantitate them. Since the radioactivity response is strictly determined by the number of radioactive atoms within the molecule, and not dependent on spectral properties of the molecule, metabolite and parent responses will be equivalent to each other. (This is of course provided that the radionuclide is not lost on metabolism. A judicious selection of the position of incorporation of the radioactive atom within the parent drug molecule is essential to avoid loss of the atom through metabolism.)

Radiometric quantitation requires the resolution of metabolites by HPLC. A small portion of effluent is split to go to the mass spectrometer and the remaining large portion is diverted for radiometric detection. The disproportionate split is necessary because MS detection is vastly more sensitive than radiometric detection of carbon-14 or tritium. The effluent can be radiometrically quantitated by three methods (in order of sensitivity): flow detection, stop-flow detection, and off-line quantitation of collected fractions.

In radiometric flow detection, the HPLC effluent is diverted to a flow detector into which a liquid scintillation fluid is pumped, typically at a ratio of 2:1 to 4:1. The scintillations are detected in a flow cell, the volume of which determines the sensitivity and the resolution of the peaks. Large flow cells will increase sensitivity because the residence time of the radioactive peak within the cell is greater, but there is a greater chance that closely eluting metabolites will lose baseline resolution. Small cells will do the opposite. Flow cell volumes are usually around one-fifth to one-tenth of the volume compared to the total flow rate per minute (e.g., for a 1 mL/min HPLC flow and 3:1 ratio of scintillant, a flow cell of 0.5 mL would be appropriate). The total amount of radioactivity injected onto the column and the number of radioactive peaks is very important for radiometric flow detection. In general, injection of 10,000–20,000 dpm represents a lower limit, unless there are very few metabolite peaks. However, injection of too much radioactivity can cause a loss of resolution of peaks. In some instances, determining the correct amount to inject on column needs to be optimized experimentally to obtain data suitable for quantitation.

The sensitivity and signal-to-noise ratio of radiometric flow detection can be increased using stop-flow techniques. The technology for this has emerged over the past 10 years or so. It requires connectivity between the radiometric flow detector and the HPLC pumps such that when radioactivity in the detector exceeds a user-defined threshold, the detector will trigger the pumps to slow down or stop. The radioactive peak will stay in the detector cell for a long enough time to permit more accurate counting. When the accuracy of the reading has been attained, the pumps are reactivated and the flow restarts, until the next radioactive peak is detected. While this approach is suitable for accurate determinations of radiometric HPLC profiles, it can lengthen the total HPLC run time for a single injection to several hours. Thus, it is not suitable for real-time analysis needs such as optimization of chromatography or active, iterative analysis of mass spectral data.

Finally, radiometric detection can be done by collecting fractions from the HPLC and individually counting the samples with liquid scintillation counting. This is considerably more tedious and labor-intensive; however, it offers the most sensitive quantitation, and total radioactivity injections as low as 500 dpm can still yield good data. As with radiometric flow detectors, sensitivity is increased with larger fractions which causes decreases in resolution and vice versa. Fractions can be collected into standard liquid scintillation vials or using newer 24- and 96-well plates for scintillation counters that can measure radioactivity in plates. In the latter case, there are plates available in which the scintillant is embedded into the clear plastic on the plates such that scintillation fluid does not need to be added to the samples. Such fractions can be recovered for further spectral analysis as needed.

The greatest advantage offered by radiometric quantitation methods, as mentioned above, is the ability to provide quantitation of individual metabolites without requiring authentic standards of these metabolites for construction of calibration curves. It also offers the ability to readily identify where the drug-related materials elute in the HPLC in a highly complex array of materials in a biological matrix, which permits the analyst to thus focus on the mass spectral data coincident with these retention times. However, radiometric approaches have several distinct disadvantages which cause them to be generally reserved for use in later stages of drug research. First, a large initial investment is needed to carry out the custom radiosynthesis of new compounds, with the label in an acceptable, metabolically inert position. There are additional costs

involved in the maintenance of a laboratory in which radioactivity is used (registration with government nuclear regulatory authorities, extensive training of personnel, careful management of inventories, security issues, and costs of proper disposal of radioactive wastes).

Finally, a technique for measuring carbon-14 that has recently emerged for use in quantitatively measuring metabolite profiles is accelerator mass spectrometry (AMS). While this technique also uses carbon-14, it is important to note that it is not radioactive decay that is being measured, but rather it is carbon-14 atoms themselves. Thus, this method is far more sensitive and can permit the use of carbon-14 at levels of 1000-fold less than when using radiometric measurements. Such sensitivity can offer an option for conducting quantitative metabolism studies in special circumstances where a typical radioactive dose may not be able to be given to human study subjects (i.e., when estimations of tissue dosimetry exceed allowable limits). Because doses of carbon-14 can be extremely low, the AMS approach can be applied in human carbon-14 metabolism studies without requiring prerequisite animal carbon-14 studies and therefore could be done earlier in clinical development. The technique suffers the disadvantages of requiring off-line analysis of HPLC effluents as well as expensive and sophisticated instrumentation. Nevertheless, it is reasonable to expect that this technology will increase in use in the future.

Radiometric quantitation can also be used as a bridging method to MS quantitation. In this approach, a biological sample generated using radiolabeled parent drug is used as the mother stock of a metabolite(s). The biological sample will be analyzed using HPLC with radiometric detection, and using the specific activity of the parent compound, the total concentration of drug-related material in the sample, the percentage which is comprised by a metabolite of interest, and the concentration of the metabolite in this biological sample can be calculated. Then, the MS response for this metabolite can be calibrated using this concentration value. Thus, samples containing unknown concentrations of the metabolite can be measured using the biological sample of known concentration in the construction of standard curves [142].

5.5.2 Quantitation Using NMR Spectroscopy

In the absence of a synthetic standard of a metabolite, and in the absence of a radiolabeled standard of the parent drug, NMR spectroscopy can be used as a quantitative tool for metabolites [143,144]. A critical step in the definitive structural characterization of a metabolite is its isolation for NMR analysis. As stated earlier, isolated samples for NMR analysis can have concentrations of approximately 0.5 mM (~40 µg of isolated material). The material isolated for NMR experiments could also easily be utilized as a quantitative standard for assessment of metabolite concentration if the concentration of the NMR solution were determined. However, the microgram-level mass of the isolated material precludes gravimetric analysis. Quantitative NMR can fill this void.

Unlike many other spectroscopies, NMR response is independent of the structural characteristic of the molecular moiety. A resonance from single hydrogen in a 0.1 M carbohydrate solution will have the same response as single hydrogen from a 0.1 M solution of a steroid, provided the data are acquired appropriately. This enables the calibration of NMR systems to provide quantitative information without authentic standards.

Like other quantitative systems, quantitative nuclear magnetic resonance (qNMR) requires the calibration of the acquired signal against a known standard. This is performed either through the use of an internal standard that is added to the isolated sample or by the calibration of an external signal that is electronically or mathematically added to the sample's NMR signal. In the first case, the internal standard may be added as a separate chemical entity or the signal from the residual deuterated solvent used. In either situation, the internal reference signal must be free from interference, stable, and nonvolatile. It is surprising how limiting these few simple restrictions can be.

In an effort to surmount these restrictions, an electronic referencing method was developed [(electronic reference to access *in vivo* concentrations (ERETIC))] [145]. Conceptually, this method places an artificial signal in the data of a 1D ^1H NMR spectrum as it is being acquired. Because of the synthetic nature of the signal, this method has the advantage of being able to control both the frequency and the magnitude based on the needs of the sample. However, in order to produce this artificial signal, specialized hardware and pulse sequences are needed for the acquisition of ERETIC data.

An alternative to ERETIC is the arithmetic merging of an artificially generated NMR signal with the NMR signal of a metabolite [artificial signal incorporation for calculation of concentration observed (aSICCO)] [146,147]. In this method, a mathematically generated FID is merged with a standard of known concentration. This allows the calculation of the "concentration" of the aSICCO signal. In turn, if the aSICCO signal is merged with the acquired NMR signal of a metabolite, the concentration of the metabolite can be calculated. In all the methods described above, the concentration of the isolated metabolite can be determined with precision and accuracy of $\pm 10\%$.

5.6 CONCLUSIONS

Metabolite structure elucidation represents an important activity in the design of new drugs as well as a required activity in the characterization of drug candidates during the development process. The identification of drug metabolites is a complex process that utilizes several types of sophisticated high technology instruments. However, the successful employment of these tools and technologies requires scientists knowledgeable of xenobiotic metabolism, the types of reactions that molecules can undergo in the body, the drug-metabolizing enzymes, as well as insight into fundamental organic chemical reactions. Furthermore, the strategy regarding when metabolite identification is needed to address questions regarding the dispositional, pharmacological, and toxicological aspects of drugs remains the domain of the individual scientists engaged in the discovery and development of new drugs.

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6 Drug Bioactivation and Oxidative Stress

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6.1	Bioactivation and oxidative stress summary	1
6.2	Drug metabolism and bioactivation	2
6.3	Reactive metabolites of drug molecules and initiation of toxicity	3
6.4	Adverse drug reactions	4
6.5	Adverse drug reactions, bioactivation, and oxidative stress	6
6.6	Biomarkers of drug bioactivation	12
6.7	Case studies	16
6.8	Summary points	31
	References	31

6.1 BIOACTIVATION AND OXIDATIVE STRESS SUMMARY

Drug metabolism generally renders drug molecules less reactive and increases their hydrophilicity, assisting the drug, and its metabolites' elimination from the body. Drug metabolism can also uncover more pharmacologically reactive groups, thereby making the metabolite more reactive than the parent compound (prodrug). Reactive metabolites (RMs) can also be created by the metabolism of certain drugs. RMs are generally short lived and are *bioinactivated* by conjugation reactions with glutathione (GSH). If cellular stores of GSH become depleted or its production has been impaired, RMs, created by this bioactivation of the parent compound, can covalently bind with cellular macromolecules and/or induce oxidative stress, which are important initiators of toxicity.

The propensity of a drug molecule to be bioactivated is a function of its chemistry. A number of functional groups have been identified that are frequently associated with bioactivation and subsequent drug-induced toxicity (Table 6.1). In some instances, these structural fragments are crucial to the potency and pharmacological selectivity of the compound. However, the mere presence of these moieties in a drug or compound

TABLE 6.1 Examples of Drugs Causing Off-target Clinical Adverse Drug Reactions and the Reactive Intermediates Involved

Drug	Adverse Reaction	RM
Amodiaquine	Hepatotoxicity	Quinone imine
Paracetamol	Hepatotoxicity	Quinone Imine
Halothane	Hepatotoxicity	Acyl halide
Diclofenac	Hepatotoxicity	Quinone imine/acyl glucuronide
Tacrine	Hepatotoxicity	Quinone methide
Indomethacin	Hepatotoxicity	Quinone imine/chloro-indole
Valproic acid	Hepatotoxicity	α , β -Unsaturated carbonyl
Vesnarinone	Hepatotoxicity	Iminium ion
Phenacetin	Hepatotoxicity	Quinone imine
Phenytoin	Teratogenicity/hepatotoxicity	Free radical
Clozapine	Agranulocytosis	Nitrenium ion
Aminopyrene	Agranulocytosis	iminium
Ticlopidine	Agranulocytosis	S-oxide
Sulfamethoxazole	Toxic epidermal necrolysis	Hydroxylamine/nitroso
Lamotrigine	Toxic epidermal necrolysis	epoxide
Carbamazepine	Hypersensitivity	Quinone imine/epoxide
Tienilic acid	Hypersensitivity	S-oxide
Felbamate	Aplastic anemia	Atropaldehyde
Remoxipride	Aplastic anemia	Hydroquinone

does not automatically result in bioactivation and toxicity [1]. A combinatory effect of a number of other factors will ultimately determine whether a RM will be either detoxified and eliminated or cause toxicity. Dose and duration of therapy of a drug will control the levels of exposure to the drug a patient receives; genetic factors may also affect rates of RM formation, cellular defense mechanisms, and susceptibility to immune-mediated toxicities; and environmental factors and disease state of a patient can influence their ability to detoxify and/or eliminate RMs.

This chapter is focused on the role of drug metabolism and bioactivation in oxidative stress and adverse drug reactions (ADRs). These main themes are discussed in a general manner and then further explored, as case studies, to demonstrate these principles.

6.2 DRUG METABOLISM AND BIOACTIVATION

Drug metabolism and bioactivation are catalyzed by a range of enzymes that occur naturally in the body and have endogenous substrates. Metabolism is divided into two phases. Phase I involves the functionalization of the drug; functional groups are added or revealed, thereby making the metabolite more water soluble and available for conjugation (phase II metabolism). Phase II metabolism involves conjugation of the phase I metabolite, at the functional groups, rendering them less reactive and more easily eliminated.

6.2.1 Involvement of Cytochrome P450 Enzymes in Bioactivation

The cytochrome P450 (CYP) superfamily of enzymes is responsible for the majority of phase I metabolism and is, therefore, responsible for the majority of RM formation

[2,3]. Within this enzyme superfamily exist a number of isoforms that differ in substrate specificity. Expression of different CYP isoforms varies between species, sex, and individual and can be altered through environmental changes, diet, and disease state [4]. Each organ, in a single individual, will have a different complement of CYP isoforms, making them differentially susceptible to drug-induced toxicity [2]. The CYP enzymes are found predominantly in the liver and most drug biotransformation occurs here, making the liver a frequent target of drug-induced toxicity [5].

Quantitatively, the CYP isoforms in the endoplasmic reticulum are the most important group of enzymes involved in this process. However, products of phase II metabolism can also lead to toxicity [6]. Additionally, non-CYP oxidative enzymes, such as myeloperoxidase and prostaglandin H synthetase, have been implicated in the bioactivation of drugs and other chemicals [7], the metabolites of which are thought to be responsible for observed toxicity, for example, clozapine and agranulocytosis, benzene, and aplastic anemia [8–11].

6.3 REACTIVE METABOLITES OF DRUG MOLECULES AND INITIATION OF TOXICITY

The concept that small organic molecules can undergo bioactivation to electrophiles and free radicals, and elicit toxicity by covalent modification of cellular macromolecules, has its basis in chemical carcinogenicity and the pioneering work of the Millers [12,13], who studied the hepatotoxic effects of *p*-dimethylaminoazobenzene in the rat and found that aminoazo dyes become tightly bound to the protein constituents of liver tissue. The application of such concepts to human drug-induced hepatotoxicity was established through the studies of Brodie, Gillette, and Mitchell on the covalent binding to hepatic proteins of toxic doses (samples were obtained from overdose patients) of the widely used analgesic acetaminophen (APAP) [14,15].

6.3.1 Chemical Nature of Reactive Drug Metabolites

RMs may be broadly classified as either electrophiles or free radicals [16]. In the vast majority of cases, the ultimate reactive species is electrophilic in nature [1], for example, epoxides and quinones. Electrophiles are reactive because they are electron deficient and have either a high positive charge density (hard electrophiles) or a low positive charge density (soft electrophiles) at the electrophilic center [17]. RMs that possess unpaired electrons can be described as free radicals. Free radicals usually abstract a hydrogen atom from other molecules rather than becoming covalently bound; however, they can also add to double bonds [6]. Free radical reactions can be self-propagating: abstraction of a hydrogen atom from a lipid can initiate a chain reaction leading to lipid peroxidation, oxidative stress, or modification of other types of biological molecules by free radicals [7].

6.3.2 Mechanisms Underlying Drug-Induced Toxicity

RMs have the ability to interact with cellular proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress (Fig. 6.1)

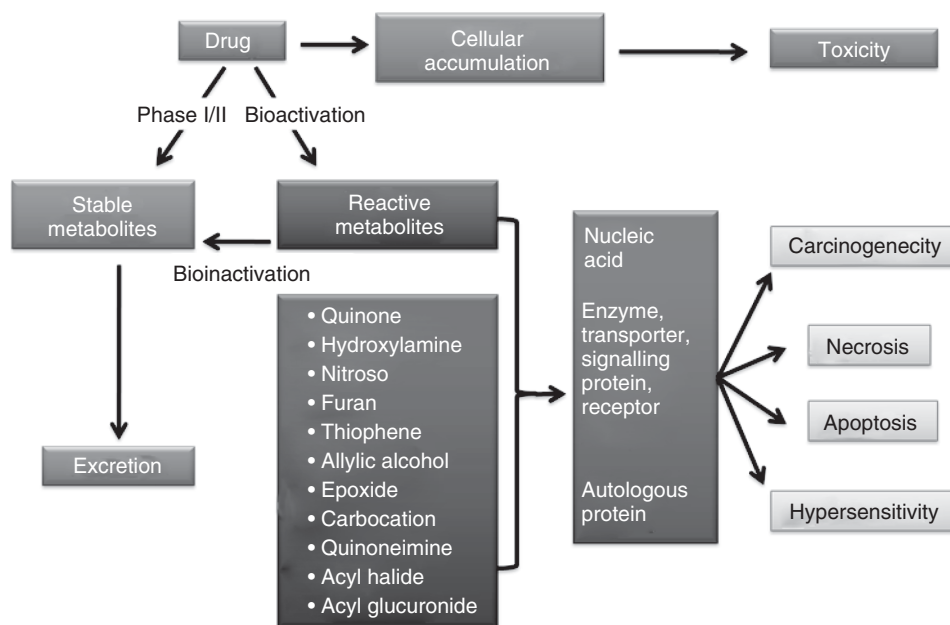


Figure 6.1 Relationship between drug metabolism and toxicity. Toxicity may accrue through accumulation of parent drug or, via metabolic activation, through the formation of a chemically reactive metabolite, which, if not detoxified, can effect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequence of its modification will dictate the resulting toxicological response [18]. (See color insert.)

[18]. Additionally, the metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can result in cell death and possible liver failure [18].

6.4 ADVERSE DRUG REACTIONS

ADRs are defined as a response to a drug that is noxious, unintended, or undesired occurring at doses normally used for the prevention, diagnosis, or treatment of disease [19]. Despite intensive investigation in the fields of chemical toxicology and molecular biology, ADRs remain a major complication of drug therapy [20,21]. ADRs account for a significant number of hospital admissions each year and contribute to patient morbidity and mortality [21]. A pilot study investigating hospital in-patient ADR development found that 19% of patients suffered an ADR, the majority of which were avoidable [22]. In addition, ADRs represent a major issue for pharmaceutical industries, accounting for 30% of compound attrition during the drug development process [23]. Drug toxicity can mimic natural disease and almost any body system can be adversely affected by drugs [24].

6.4.1 Classification of Adverse Drug Reactions

ADRs can be classified as “on-target” or “off-target/idiosyncratic” reactions [25–27]. On-target reactions can be predicted from the known primary or secondary pharmacology of the drug and often represent an exaggeration of the pharmacological effect of the drug. They show simple and clear dose–response relationships and, therefore, can usually be avoided by dose reduction and are only rarely life threatening [26]. In contrast, idiosyncratic adverse reactions cannot be predicted from knowledge of the basic pharmacology of the drug. They are extremely host-dependent and uncommon reactions [28].

Drug–drug interactions can influence both pharmacodynamic (PD) and pharmacokinetic (PK) properties of coadministered drugs. An understanding of the role of DDIs in on-target toxicity requires an understanding of PKPD properties of all coadministered agents. Pharmacodynamic interactions can produce synergistic and additive effects, leading to toxicology. For example, consumption of alcohol while taking antihistamines causing drowsiness can lead to impaired psychomotor skills [29]. PK interactions influence concentration of drug by alteration in absorption, distribution, metabolism, and elimination of one agent by another. Coadministration of a drug with an agent that inhibits its clearance mechanisms leads to an increase in plasma levels, potentially hitting a toxic threshold. The anticoagulant warfarin is metabolized by CYP enzymes in the liver, specifically CYP2C9 and CYP3A4. Therefore, agents that inhibit either of these two enzymes, such as fluconazole [30], will lead to an increase in prothrombin time and possible bleeding.

6.4.2 Idiosyncratic Adverse Drug Reactions

Idiosyncratic drug reactions represent a major problem for the pharmaceutical industry because they add significant uncertainty to the process of drug development and can ultimately lead to drug withdrawal or warnings in drug labeling [31,32]. They are particularly difficult to deal with because they are likely to be discovered late in development or after the drug has been approved; this has important implications as the latter a drug fails, the more expensive is the failure [33,34]. Drug attrition rates are at their highest; the overall success rate for approval of a new chemical entity (NCE) is 11% and with 62% of attrition occurring at phase II clinical trials, the financial cost is huge [32]. During 1975–2000, over 10% of newly approved drugs in the United States were withdrawn or given black-box warnings [31]. In 1991, adverse PK and bioavailability results contributed to a total of 40% of attrition. In 2000, this had been reduced to 10%, but lack of efficacy and safety each contribute to 30% of drug attrition [32].

The exact mechanisms of idiosyncratic drug reactions are still unclear; however, it is believed that some idiosyncratic reactions are initiated by RM [28,35], which bind covalently to macromolecules and either cause direct cell damage or trigger an immune response leading to cell death [34,36,37]. Therefore, the propensity of a NCE to undergo bioactivation needs to be determined at an early stage of the drug development process. Considerable efforts have been made to develop screening systems for RM based on subcellular liver fractions [38,39] or isolated hepatocytes [39]. However, a noninvasive, generic, and quantitative method for assessing bioactivation of drugs in experimental animals, human volunteers, and patients is not currently available.

6.5 ADVERSE DRUG REACTIONS, BIOACTIVATION, AND OXIDATIVE STRESS

It is a widely accepted hypothesis that some idiosyncratic reactions are initiated by RM [28,35], which bind covalently to macromolecules and either cause direct cell damage or trigger an immune response leading to cell death [34,36,37]. Metabolic activation of drug molecules to electrophilic RM can contribute to stress in the cell and interfere with normal cellular homeostasis. Drug-induced oxidative stress can be defined as an imbalance between prooxidants and antioxidants in a cell caused by either parent drug or a reactive intermediate of metabolism [40,41]. Oxidative stress is most commonly mediated by reactive oxygen species (ROS); these include the oxygen free radicals, superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), and nonradical hydrogen peroxide (H_2O_2) [42]. Other species and processes that can mediate oxidative stress include reactive nitrogen species (RNS), RMs of xenobiotics, reduced transition metals, γ -radiation in the presence of oxygen, activated neutrophils, UV light, and by-products of lipid peroxidation [43]. Oxidative stress can lead to cell damage through a number of mechanisms including lipid peroxidation, protein oxidation, DNA oxidation, and mitochondrial damage [44]. Products of oxidative damage can also have prooxidative effects, thereby propagating oxidative damage in the cell [45].

6.5.1 Generation of Reactive Oxygen Species

ROS are generated, in small amounts, in the cell by normal physiological processes [46]. The mitochondria serves as a source of ROS through the electron transport chain, which has redox centers that can leak electrons to molecular oxygen generating $O_2^{\cdot-}$ [47–49]. Peroxisomes contain high amounts of H_2O_2 that can leak out into the cytosol if degradation via catalase is inefficient [50–52]. A number of enzymatic processes can increase the amount of oxidants in the cell, including the CYP monooxygenase system [53]. This enzyme system oxidizes xenobiotics in phase I metabolism; however, incomplete oxidation of a substrate can lead to reduction of O_2 [54]. Electrons can also escape from flavins in the NADPH/P450 reductase enzyme [55]. The cell has an extensive antioxidant defense system that is put into play when an imbalance in pro- and antioxidant species is detected [41,56,57]. These systems can degrade ROS, provide ROS scavengers, prevent/repair oxidation of lipids, and maintain the thiol status of the cell [44].

6.5.2 Cellular Defense against Drug-Induced Oxidative Stress

Once cellular stress has been detected, the cell can activate an immediate adaptive defense response via the upregulation of gene expression that is mediated by transcription factors such as activator protein-1 (AP-1) [58], nuclear factor κ B (NF κ B) [59], and nuclear factor erythroid-2 p45-related factor 2 (Nrf2) [60,61]. These are regulatory proteins that often reside in the cytoplasm and translocate to the nucleus on activation where they bind to specific DNA sequences or response elements within gene promoter regions. They can then recruit RNA polymerase and other binding proteins to initiate gene transcription [62]. Activation and translocation of a transcription factor is often oxidant and thiol status sensitive. GSH synthesis is highly inducible on conditions of oxidative stress and GSH depletion. Transcription factor controlling the expression of

enzymes involved in GSH synthesis can be directly activated by electrophilic species that conjugate to GSH depletion as a positive feedback mechanism of the adaptive response [63,64].

Although oxidative stress alone is not enough to kill a cell, all, the ability of RM to bind to cellular macromolecules, initiate oxidative stress, and promote GSH depletion, contribute to damage to key intracellular organelles and can subsequently lead to cell death.

6.5.3 The Role of Glutathione in Cellular Defense

GSH is a tripeptide, derived from the amino acids cysteine, glutamate, and glycine. It is a major endogenous antioxidant within hepatocytes owing to its free thiol group and its relatively high abundance (~ 10 mM in hepatocytes). It is present in two forms: reduced (GSH) and oxidized (GSSG). The change in the ratio between the two forms is directly reflective of intracellular redox alterations [65]. GSH homeostasis within hepatocytes is maintained through GSH synthesis and utilization. Synthesis occurs in the cytosol through a two-step ATP consuming reaction. The first step comprises the synthesis of γ -glutamylcysteine. This occurs through the condensation of cysteine and glutamate, catalyzed by γ -glutamylcysteinyl ligase. GSH synthetase then further catalyzes the formation of GSH from γ -glutamylcysteine and glycine [66,67]. It is then used either as an enzyme cosubstrate or as a scavenger molecule in the presence of an oxidized substrate, resulting in the formation of GSSG. Under normal physiological conditions, GSSG can then be recycled back to GSH by the enzyme GSH reductase (Fig. 6.2) [67].

GSH is critical for cellular function and cell survival, playing a major determinant of redox potential. The antioxidant role of GSH is to quench endogenous oxidative species and to overcome exogenous oxidative stress. Increased levels of ROS occur as a result of infection, inflammation, xenobiotics, and intracellular stress. This is potentially hazardous for the cell as ROS can oxidize proteins, lipids, and DNA, causing oxidative damage. GSH also acts as a hydrogen donor for GSH peroxidase. This enzyme is responsible for the subsequent conversion of H_2O_2 to water in parallel with catalase. The level of GSH consequently has a direct impact on the function of GSH peroxidase. GSH peroxidase also plays a role in the repair of lipid peroxidation. Additionally, GSH acts as a scavenger of RNS such as peroxynitrite ($ONOO^-$). An imbalance of endogenous oxidants and antioxidants could result in oxidative stress, emphasizing the crucial role GSH plays in cellular defense. Furthermore, GSH can act as a cofactor substrate in conjugation reactions of xenobiotics under the catalysis of GSH transferases. This acts as a detoxifying mechanism enabling the exportation of drugs and xenobiotics through the multidrug-resistance-associated transporter family [67]. As GSH plays a fundamental role in cellular defense, perturbation of cellular levels can lead to oxidative stress and/or potential irreversible binding of RMs to cellular macromolecules, resulting in direct cell toxicity or an immunological reaction.

6.5.4 Reactive Metabolites and Mechanisms and Consequences of Oxidative Stress

The formation of RM through bioactivation of drug molecules can push the balance toward a more oxidative environment in the cell by stimulating overproduction of ROS,

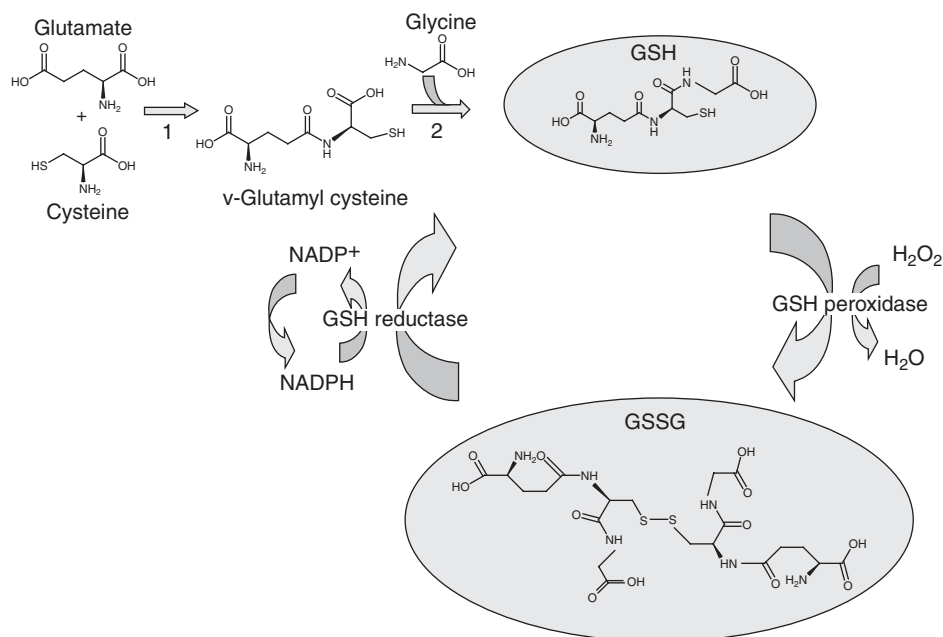


Figure 6.2 Enzyme regulation of GSH synthesis and recycling pathway. GSH is synthesized by a two-step ATP-dependent pathway. γ -Glutamylcysteine is formed from L-glutamate and cysteine, which is catalyzed by Glutamylcysteine ligase γ -GCL (1). Glycine is then added at the C-terminal of γ -glutamylcysteine through the catalysis of GSH synthetase (2). Redox cycling between GSH and GSSG is mediated by GSH reductase and GSH peroxidase [68]. *Source:* Adapted from DeLeve *et al.*, 1991.

introduction of electrophilic species, and disruption of thiol status. Some mechanisms by which ROS and other prooxidant species can be generated and how they cause oxidative damage to cellular organelles and macromolecules are outlined below; often, these mechanisms propagate and amplify the oxidative insult.

6.5.4.1 Redox Cycling of Drug Metabolites and Their Role in ROS Generation.

RM of xenobiotics can increase ROS by redox cycling, as in the classic case of paraquat [69,70]. Oxidative stress arises when the xenobiotic is reduced by reductases to a semiquinone radical that reduces molecular oxygen to superoxide radicals and reforms the drug or metabolite; repeated reduction and oxidation of a xenobiotic can generate cytotoxic levels of hydrogen peroxide and GSSG [44,71]. Structures capable of redox cycling include catechols and other quinone compounds, iron chelates, and aromatic nitro compounds [72]. Iron chelators, administered in cases of iron overload following blood transfusion, are associated with redox-cycling-mediated toxicity [73]. Drug-induced redox cycling is often associated with quinone moieties in the drug molecules. Cytotoxicity of quinones has been attributed to free radical generation and to arylation of cellular nucleophiles. For redox-cycling quinones, cell injury is associated with mitochondrial permeability transition, whereas aryating quinones directly depolarize the mitochondrial membrane and deplete ATP [74]. The quinone containing anticancer agent doxorubicin (Adriamycin) induced a dose-limiting cardiotoxicity by

inducing reactive species in the heart via redox cycling of the drug at complex I of the electron transport chain [75]. Diclofenac (DCF) quinone-imine-related redox cycling has been implicated in DCF-induced hepatotoxicity [76]. Redox cycling can instigate lipid peroxidation through the generation of ROS.

6.5.4.2 Role of Lipid Peroxidation in Generation and Propagation of ROS and Involvement in Cellular Damage. RM can initiate lipid peroxidation either directly if the RM is electrophilic or indirectly by increasing the generation of free radicals [16,77]. Lipid peroxidation can damage biological membranes [78], leading to further damage to the cell through increased permeability of the plasma membrane or the membranes of organelles [79]. Hepatotoxic mechanisms of carbon tetrachloride are thought to be mediated through lipid peroxidation and disruption of cellular homeostasis, which can lead to disruption of the cytoskeleton, cell signaling, and gene expression pathways [80,81]. Incubation of paraquat with liver microsomes or a system containing NADPH-cytochrome *c* reductase, NADPH, and microsomal lipid greatly increases the formation of malonaldehyde; the amount of malonaldehyde formed in these studies was dependent on the concentration of paraquat in the incubation mixture [82,83]. Oxidative stress has been implicated in the hepatotoxicity associated with the aromatic antiepileptic drugs, carbamazepine, phenytoin, and phenobarbital, as their metabolites increased malondialdehyde levels, oxidation of cardiolipin, oxidation of sulfhydryl proteins, and alteration of the cellular redox status [84].

6.5.4.3 Mitochondrial Dysfunction as a Consequence of Drug-induced Oxidative Stress. Products of lipid peroxidation, ROS, and electrophilic RMs can disrupt mitochondrial function through membrane damage, uncoupling oxidative phosphorylation [85,86] and critical protein oxidation [87], leading to depletion of cellular ATP levels that could ultimately lead to apoptosis and necrosis [49]. The anti-HIV drug class of nucleoside reverse transcriptase inhibitors (NRTIs), used to inhibit viral replication in host cells, induces a variety of serious ADRs including lactic acidosis and cardio and skeletal myopathies [88]. These prodrugs are phosphorylated into the active state and are believed to induce ADR through mitochondrial dysfunction and altered mitochondrial DNA [88]. Nefazodone is an antidepressant that was withdrawn from the market in 2004 owing to severe cases of hepatotoxicity resulting in 1 case of liver failure resulting in death or transplant per 250,000–300,000 patient years. The effect of nefazodone on the mitochondria and resulting hepatotoxicity has been investigated in isolated oxidative phosphorylation (OXPHOS) complexes, HepG2 cells, and sandwich human hepatocytes [89], in which it has been demonstrated that in isolated OXPHOS complexes nefazodone inhibits complex I.

6.5.4.4 Glutathione and Thiol Status Disruption in Drug-Induced Oxidative Stress. As discussed previously, drug metabolites are generally electrophilic species and can form GSH conjugates either enzymatically or nonenzymatically [90]. During alular GSH depletion, such as that seen during high RM exposure, other thiol groups, especially those on critical proteins, can become adducted or vulnerable to oxidation, cross-linking, and formation of mixed disulfides. APAP provides a good example of how thiol status is critical for the successful handling of electrophilic drug intermediates (Section 6.7.1).

6.5.4.5 Irreversible Binding of Reactive Metabolites to Cellular Macromolecules.

RMs usually have a low electron density (electrophilic) and as a result are capable of reacting with nucleophiles, molecular centers of high electron density. Nucleophilic targets are dependent on whether the electrophile is chemically defined as hard or soft. Hard electrophiles tend to react with nucleophilic N, O, and C in biological molecules. Hard electrophiles are likely to be genotoxic as they have the potential to react with DNA, forming DNA adducts [91]. This acts as the initiatory stage of carcinogenesis when the mechanism is genotoxic. Aflatoxin B₁ is a genotoxic carcinogen, which is bioactivated to 8,9 epoxide metabolite, which is then able to bind to DNA, forming B₁-N₇-guanine adducts [92,93]. This emphasizes both the importance of drug bioactivation and covalent binding in aflatoxin B₁ causation of drug-induced liver injury (DILI).

Soft electrophiles react more readily with sulfur and target cellular protein. Target proteins for adduction usually contain strong nucleophilic sites such as cysteine thiols, lysine amines, histidine imidazoles, and protein N-terminal amines [94–96]. As discussed previously, GSH plays a crucial role in protecting the thiol status of the cell by quenching endogenous oxidative species such as some drug RMs. However, in some cases, RM can overwhelm the GSH store and cellular GSH levels can become depleted, as in the case of APAP. The depletion of hepatic GSH has been shown to be an obligatory step to enable *N*-acetyl-*p*-benzoquinone imine (NAPQI) to covalently modify and potentially inhibit the function of a number of critical proteins within cells, leading to cell death and has been discussed previously [97]. A number of therapeutic drugs have been reported to undergo irreversible binding to various target proteins despite having different chemical structures and pharmacological actions. Metabolic bioactivation is usually a prerequisite, resulting in the formation of RMs, which are able to bind to proteins by nucleophilic substitution and/or Schiff base formation [98–100]. Irreversible binding with cellular protein has been well documented in retrospective association with drug toxicity, with a number of drugs incriminated, including APAP, amodiaquine, and clozapine [101–103]. However, protein adduct formation does not always lead to toxicity. This concept is best illustrated by APAP and its structural isomer 3'-hydroxyacetanilide (*N*-acetyl-*m*-aminophenol, 3-acetamidophenol; AMAP), where AMAP at the same molar dose as APAP was found to covalently bind to cellular protein and yet did not result in toxicity. The RM of APAP and AMAP, a quinone imine (NAPQI) and quinone, respectively, are chemically similar species, yet there is an intrinsic difference between them that accounts for the difference in ability to cause hepatotoxicity [104]. This introduced the concept of the critical protein hypothesis, delineating certain proteins as critical targets in hepatotoxicity [18,105]. Supporting this, the RM of APAP, NAPQI binds to a number of proteins. In mice, one of the major targets of NAPQI is urate oxidase, an enzyme lost during primate evolution. As a result, it is thought unlikely that irreversible binding to urate oxidase represents a role in APAP-induced hepatotoxicity in mice, as both humans and mice develop centrilobular necrosis with similar per kilogram doses [106]. This highlights the need to identify the critical protein adducts involved in the mechanism of DILI.

Drug-protein adducts can act as neoantigens and induce immune-mediated toxicity. Halothane is the best-studied drug with respect to immunoallergic hepatitis. A significant proportion of patients exposed to this inhalation anesthetic develop asymptomatic rises in transaminases. Fulminant irreversible hepatitis is a rare but life-threatening phenomenon. Most of the patients recorded in the literature with immunoallergic hepatitis had more than one exposure [107]. Antibodies have been detected in such patients

that recognize autoantigens and neoantigens created by trifluoroacetylation of hepatic proteins. Preincubation of halothane pretreated, but not control, rabbit hepatocytes with sera from patients with halothane-induced fulminant hepatic failure rendered the hepatocytes susceptible to the cytotoxic effects of normal lymphocytes *in vitro* [108]. This phenomenon was not observed in control hepatocytes. It is, thus, likely that drug-specific T cells may play a role in the pathogenesis of hepatocyte injury, but direct evidence for this lacks.

Halothane is metabolized by CYP2E1, such as APAP, but forms a chemically reactive acyl halide and bioactivation to the acyl halide is substantial as it is the only metabolic route available. It is hypothesized that the formation of the acyl halide triggers an immune response. Evidence for this concept of an immune trigger is both direct and indirect, with drug-specific antibodies showing that the drug has initiated an immune response in affected patients and newer metabolically inert inhaled anesthetics, such as enflurane and isoflurane, being rarely associated with hepatotoxicity (Fig. 6.3). Target proteins modified by the acyl halide have been identified with the principal chemical modification trifluoroacetylation of lysine residue [109–112]. Chemical modification of protein(s) is associated with the immune response observed with halothane hepatitis; however, there is no conclusive evidence that the antibodies mediate liver injury. Antidrug antibodies have also been observed with idiosyncratic drug

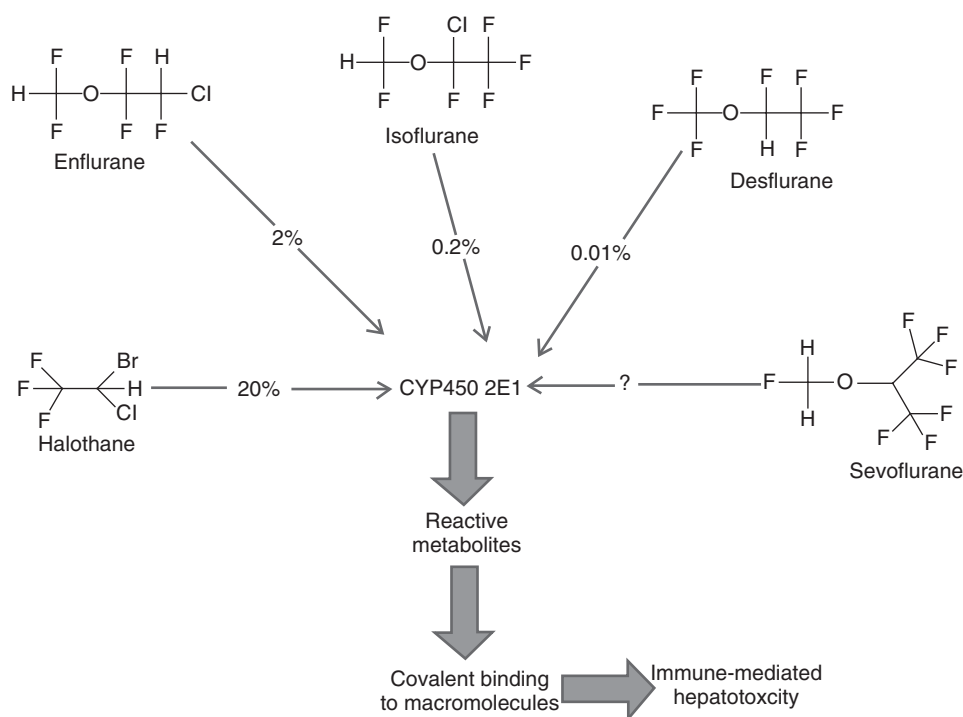


Figure 6.3 Metabolic bioactivation of inhaled anesthetics potential of new inhaled anesthetics to cause immune-mediated hepatotoxicity in man correlates with extent of metabolic bioactivation and liver protein adduct formation, which can be quantified experimentally (*in vivo/in vitro*).

TABLE 6.2 Idiosyncratic Drug Reactions with Evidence of Immune Involvement

Drug Class	Drug Name	Is There Evidence to Support Immune Involvement?
Anesthetic	Halothane	Yes
Analgesic	Diclofenac	Yes/no
	Sulindac	Yes
Antibiotic	Amoxicillin	Yes
	Erythromycins	Yes
	Minocycline	Yes
	Nitrofluantoin	Yes
	Rifampicin	Yes/no
Anticonvulsant	Phenytoin	Yes
	Phenothiazines	Yes
Antiepileptic	Carbamazepine	Yes/no
Antihypertensive	Dihydralazine	Yes
	Methyldopa	Yes
	Tienilic acid	Yes
Antithyroid	Propylthiouracil	Yes
Depression	Tricyclic antidepressants	Yes
Gout	Allopurinol	Yes
Hypertension	ACE inhibitors	Yes

Source: Amended from Ref. 115.

reactions to tienilic acid [113] and dihydralazine [114], but there is equivocal evidence that DILI such as that caused by halothane is immune mediated. Most of the information available is compatible with the hapten hypothesis where the drug undergoes bioactivation in hepatocytes leading to drug–protein conjugate formation in the liver. The current theory suggests that the occurrence of idiosyncratic drug hypersensitivity in man represents interplay between a number of factors, which include the chemistry of the drug, drug metabolism, and the immune system of the patient (Table 6.2). Whether cytokines may be useful biomarkers in such idiosyncratic cases is currently unknown, as is the mechanistic details. However, if the hapten hypothesis is correct it is clear that the inflammatory response induced by chemical insult such as APAP overdose compared to that of idiosyncratic DILI may be very different.

6.6 BIOMARKERS OF DRUG BIOACTIVATION

Clearly, drug metabolism can play an important initiating step in the development of ADRs, and RMs have been correlated with the toxicity of several drugs. Therefore, a major interest in biological and analytical techniques has been to minimize metabolic activation of drug candidates during the drug discovery process [1,116]. A variety of experimental approaches have been developed and applied to early optimization phases through to development stages of lead candidates. These studies are largely based on the discovery and quantitation of drug conjugates with endogenous molecules and *in vitro* trapping agents that serve as indicators of drug safety [117]. By allowing for

improved decision making, these indicators, referred to as *biomarkers*, can significantly improve the efficiency of drug discovery and development. However, these studies are time consuming and, in some cases, may delay the process of drug discovery without sufficient improvement in the safety of NCE.

Generally, chemically RMs are detected and estimated indirectly through the irreversible binding of uncharacterized radiolabeled material to hepatic protein [118] and/or the formation of stable metabolites such as GSH conjugates [119]. Until recently, most assays of RM formation *in vivo* and *in vitro* depended on measuring irreversible binding of radioactivity, using relatively laborious methods of exhaustive solvent extraction [118–120].

6.6.1 Trapping Agents and *In Vitro* Biomarkers of Bioactivation

Most RMs are electrophilic in nature because of the inherent reactivity and instability and can readily be trapped with a suitable nucleophile to form stable adducts; the strategy of trapping these intermediates *in vitro* is widely used [121,122]. The *in vitro* assay most widely used to screen for RMs is liver microsomes supplemented with a NADPH/NADPH regenerating system, as cofactor for CYP450-catalyzed reactions and a trapping agent [116,118]. Table 6.3 lists chemical traps that have been used in microsomal incubations for several reactive intermediates.

Methods of trapping RMs commonly include the use of GSH, GSH-ethyl ester, and *N*-acetyl cysteine (NAC), because of their nucleophilic nature they are able to conjugate spontaneously with electrophilic compounds. The most widely used trapping agent, GSH, contains a free sulfhydryl group, a soft nucleophile, that can react directly with soft electrophiles, including quinones, epoxides, nitrenium ions, alkyl halides, Michael acceptors, and so on to form stable GSH adducts [117,118]. GSH is present in a concentration of ~ 10 mM in the liver and can conjugate readily with metabolites with an electrophilic center, generated through the bioactivation by CYP450 enzymes. Hence, GSH serves as a natural trapping agent for chemically RMs and has been routinely used *in vitro* to screen and evaluate RM formation [119].

β -Mercaptoethanol has been successfully used to trap several RMs generated by microsomes and isolated enzymes. In most cases, where the structure of the intermediate can be deduced, the precursor of the β -mercaptoethanol adduct has been an α , β -unsaturated carbonyl [123], a quinone [124], or a quinone imine [124].

TABLE 6.3 Examples of Reactive Intermediates and Trapping Agents in Microsomal Incubation

Trapping Agents	Reactive Intermediate
GSH, GSH-ethyl ester, mercaptoethanol, cysteine, <i>N</i> -acetyl cysteine	Quinones, enones
GSH (ester), mercaptoethanol, cysteine	Thiophenes
Cyanide	Iminium ions
Semicarbazide, methoxylamine	Aldehydes, furans
Lysine	Imides, aryl halides
lysine + cysteine	Furan, epoxide
TEMPO	Free radical trap

GSH-ethyl ester has been used as a more lipophilic thiol [124] and was shown to increase the mass spectrometry (MS) sensitivity by ~10-fold for the detection of RMs [124]. Trapping of free radicals employs spin-trapped reagents, for example, electron paramagnetic resonance spectroscopy in the presence and absence of the spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide [125]. Iminium ions can be trapped with cyanide as well as detection of the trapped intermediate, and commonly the incorporation of radiolabeled cyanide is used as confirmation of iminium formation [122,126]. Hard electrophiles such as aldehydes are trapped with hard nucleophiles such as semicarbazide [127,128] and methoxylamine [129,130]. Semicarbazide has also been used to identify furan ring opening and thiophene bioactivation.

While the detection of adducts (GSH/NAC/cyano, etc.) early in the drug discovery process provides the information about RM formation, it is absolutely essential that the data need to be placed in proper context before discarding drug candidates associated with this liability. There are numerous examples of drugs that form GSH conjugates in these assays and also exhibit covalent protein binding, but which are not associated with a significant incidence of toxicity. Moreover, toxic drugs that form RMs through non-CYP-mediated bioactivation process will not be detected by the routinely used microsomal assays [131].

6.6.2 Mercapturate (NAC) Conjugate as *In Vivo* Indicators of Drug Bioactivation

A common mechanism for the detoxification of toxic electrophilic compounds occurs via GSH conjugation [16]. The GSH conjugates can be further processed into breakdown products such as mercapturate conjugates (Fig. 6.4) and are excreted in urine [132]. Therefore, mercapturate conjugates are potential biomarkers of bioactivation that are not dependent on cell death to be released into an accessible compartment (e.g., urine) [133]. GSH conjugates are unsuitable biomarkers of metabolic activation *in vivo* because they are only very rarely eliminated in urine and seemingly only at very low concentrations [134]. They have been found as *N*-acetylated derivatives [135].

Human urinary mercapturates have been recognized for many years as particularly useful biomarkers in assessments of human exposure to chemically diverse environmental and biogenic electrophiles [132,136,137]. Recently, through the employment of advanced MS techniques [138], there has been a revival of interest in the bioanalytical possibilities of mercapturates [139,140]. Mercapturate biosynthesis (Fig. 6.4) has been modeled as an interorgan pathway, with the liver serving as the major site of GSH conjugation and the kidney as the primary site for the conversion of GSH conjugates to cysteine conjugates [132,141]; however, in some cases, it can be an entirely intrahepatic process even in rats [142]. GSH adducts of bendamustine are metabolized completely in human liver, only the mercapturates and further metabolites appearing in bile [115]. The cysteine adduct is the sole thioether metabolite of APAP eliminated in human bile, with the APAP cysteine adduct and mercapturate being excreted in urine [143]. Any drug mercapturate produced extrahepatically might also be eliminated in bile: rat liver possesses an efficient mechanism for uptake and biliary excretion of circulating mercapturates [144]. Although the metabolism of GSH adducts can yield numerous S-linked products via modifications of the tripeptide moiety [115,135], as in the case of carbamazepine [145,146] mercapturates are not invariably found among

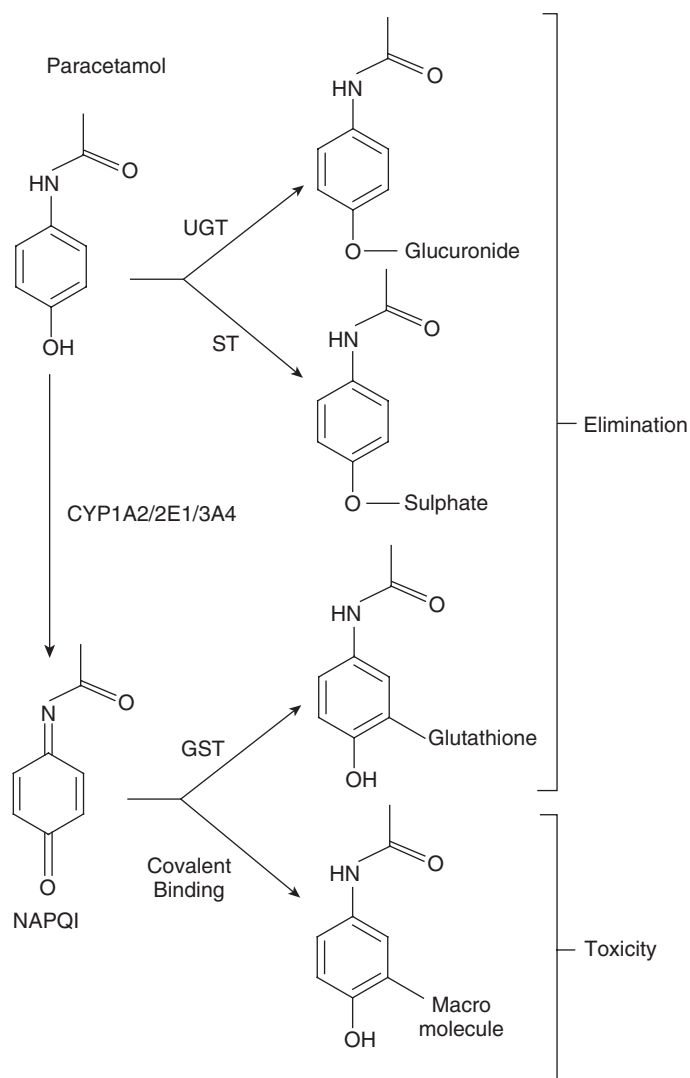


Figure 6.4 Metabolic activation of APAP. APAP is primarily detoxified by glucuronidation and sulfation in the liver. APAP can also undergo conversion to the chemically reactive species *N*-acetyl-*p*-benzoquinone imine (NAPQI) by cytochrome P450. NAPQI can undergo bioinactivation via GSH conjugation, but when GSH stores are depleted, free NAPQI can oxidize and covalently modify proteins resulting in hepatotoxicity. The toxicological and pharmacological properties of the molecule are a function of the redox potential of the molecule [151].

the excreted metabolites, and there is evidence for a species-selective absence of carbamazepine mercapturates from human urine [145,147]. When considered alongside the frequent observation that a compound can be metabolized to several GSH adducts [145,146,148–150], these findings indicate that a careful analysis of a drug's metabolites is necessary to identify a thioether derivative suitable for use as a biomarker of metabolic activation.

S-Phenyl mercapturic acid and *N*-methylcarbamoyl mercapturic acids have been proposed by American Conference of Governmental Industrial Hygienists (ACGIH) [152] as biomarkers for purposes of evaluating exposure to benzene and *N*, *N*-dimethylformamide, respectively. Among the human pharmaceuticals the mercapturate conjugates (Fig. 6.5) of APAP [143], phenacetin [153], felbamate [154], and valproic acid [155] have been used for the quantitative assessment of *in vivo* bioactivation.

6.7 CASE STUDIES

6.7.1 Acetaminophen

6.7.1.1 The Role of Metabolism in APAP Hepatotoxicity. APAP is a commonly used analgesic and antipyretic, which is safe at therapeutic doses (4 g/day). The pharmacological activity is thought to be attributed to the inhibition of cyclooxygenase activity and a reduction in prostaglandin synthesis [156–158]. However, in cases of overdose, acute liver failure develops that is owing to centrilobular hepatic necrosis. To date, APAP hepatotoxicity still remains a major clinical problem and is the single biggest cause of acute liver failure in the United Kingdom and United States [159]. APAP provides an important tool with clinical relevance to study toxicological consequences of drug metabolism across *in vitro*, *in vivo*, and clinical systems.

At therapeutic doses, about 55% and 30% of renally excreted metabolites are as the glucuronide and sulfate conjugates, respectively [160]. A small proportion of the

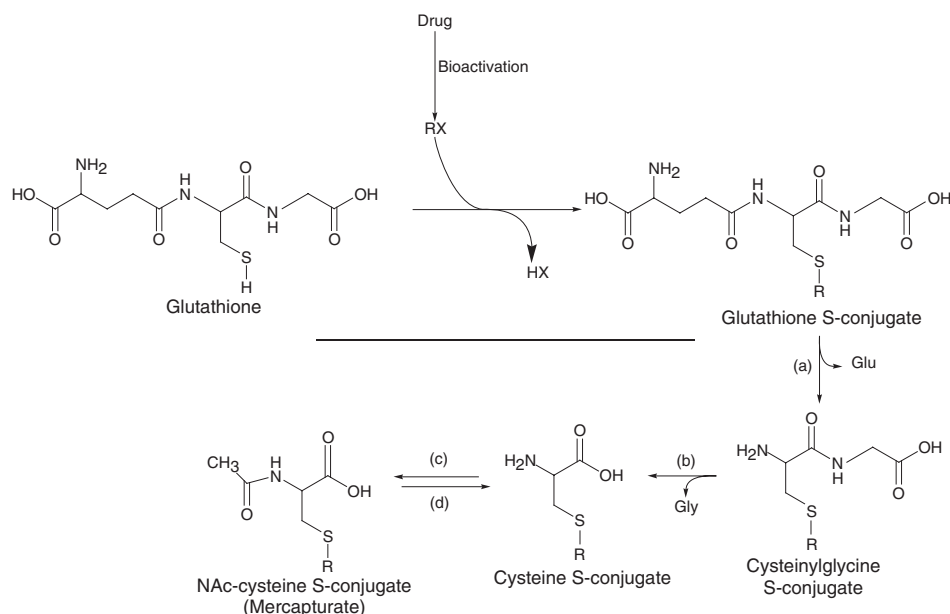


Figure 6.5 Mercapturate biosynthesis pathway. Steps are catalyzed by (a) γ -glutamyl-transpeptidase; (b) dipeptidase: cysteinylglycine dipeptidase, and aminopeptidase; (c) cysteine conjugate *N*-acetyltransferase; and (d) *N*-deacetylase [132]. RX, reactive species; Glu, glutamate; and Gly, glycine.

therapeutic dose (5%) is bioactivated primarily by CYP2E1 and also CYP3A4 and CYP1A2 oxidations to the electrophilic intermediate NAPQI [161]. NAPQI is readily detoxified by GSH conjugation and is excreted in the urine as a cysteine or mercapturate product [162].

In cases of overdose, low-capacity sulfation pathways become saturated and a greater fraction of the dose undergoes glucuronidation and oxidation. Under these conditions, NAPQI accumulates and cellular stores of GSH become depleted owing to the shift in NAPQI formation and GSH synthesis (Fig. 6.6) [162–166]. The standard treatment for APAP intoxication is NAC, which replaces hepatic GSH after depletion or conjugates to NAPQI and prevents toxicity, although this is most beneficial if given within 16 h of the overdose.

6.7.1.2 Cellular Consequences of APAP Bioactivation and Bioinactivation. The depletion of hepatic GSH has been shown to be an obligatory step to enable NAPQI to

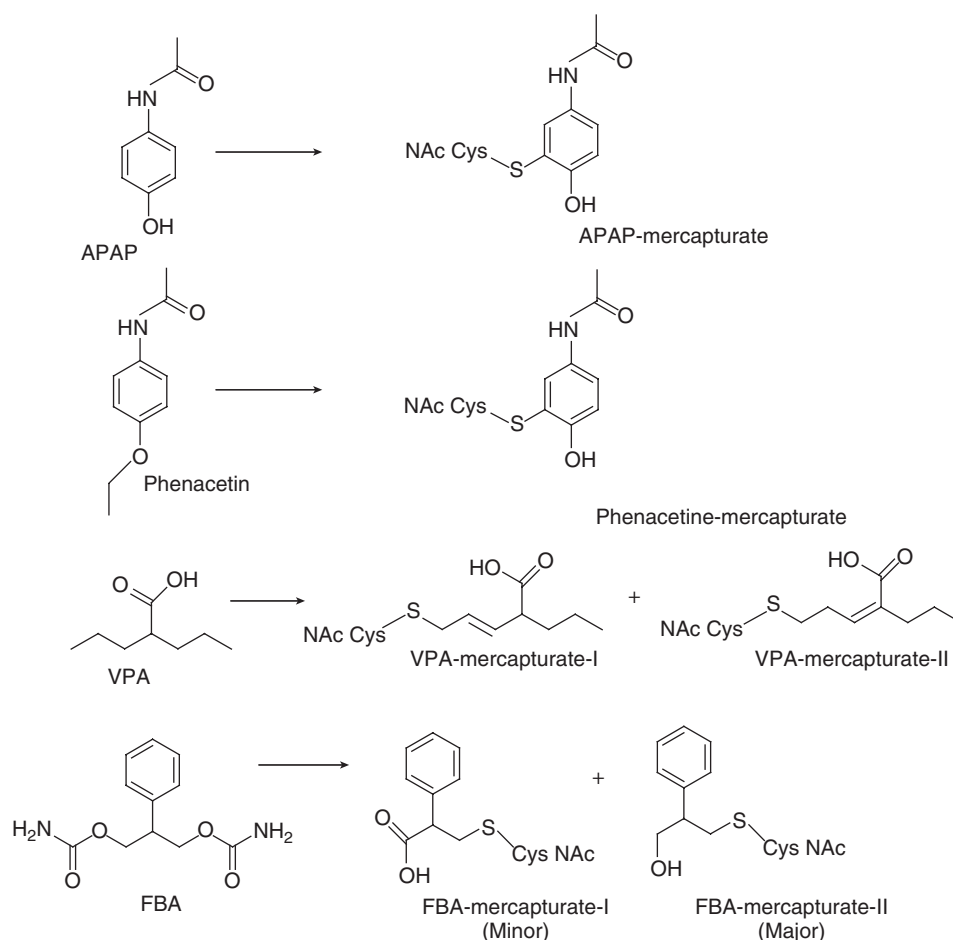


Figure 6.6 Structures of APAP, phenacetin, valproic acid, felbamate, and their mercapturate conjugates. APAP, acetaminophen; VPA, valproic acid; and FBA, felbamate.

covalently modify and potentially inhibit the function of a number of critical proteins within cells, leading to cell death and has been discussed previously [97]. Examples of critical proteins whose function is inhibited by NAPQI modification include γ -glutamylcysteine ligase catalytic subunit [167], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [168], aldehyde dehydrogenase [169], and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase [170]. As outlined earlier in the chapter, covalent binding has been hypothesized to contribute to mitochondrial dysfunction, and the resulting disruption in Ca^{2+} and ATP homeostasis is thought to be a critical step in the development of cell death associated with APAP overdose. Moreover, the importance of mitochondrial protein binding in the mechanism of APAP toxicity is highlighted by observations with the nontoxic regioisomer of APAP, AMAP. AMAP undergoes an overall similar level of covalent binding but significantly less covalent binding to mitochondrial protein than APAP [105]. Therefore, the extent of covalent binding and which proteins are modified, rather than covalent binding per se, represent a major factor underlying APAP hepatotoxicity.

The massive chemical stress mediated by an APAP overdose leads to an immediate adaptive defense response in the hepatocyte. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as Nrf2, which “sense” chemical danger and orchestrate cell defense. Thus, with respect to APAP, Nrf2-dependent genes of immediate significance are those involved in GSH synthesis such as γ -glutamate cysteine ligase, GSH transferases, glucuronyltransferases, and heme oxygenase. Importantly, it has been observed that nuclear translocation occurs at nontoxic doses of APAP and at time points before overt toxicity [171]. However, with increasing doses of APAP, there is progressive dislocation of nuclear translocation, transcription, translation, and protein activity [167] as the rate of drug bioactivation overwhelms cell defense through the oxidative modification of critical proteins. The cytoplasmic protein Kelch-like ECH-associated protein 1 (Keap1) directly binds to Nrf2 and represses transcription by promoting proteasome-dependent degradation of the protein. It has also been demonstrated that Keap1 is an adaptor molecule for the ubiquitin ligase complex and directs the rapid degradation of Nrf2. When cells are exposed to electrophiles, Nrf2 is liberated from the Keap1-mediated degradation process and accumulates in the nucleus to activate antioxidant response element (ARE)-mediated gene transcription [172]. Somatic disruption of the *Keap1* gene does not interfere with the development of the morphological and physiological integrity of the liver. However, specific knockout of the *Keap1* gene in mouse hepatocytes confers a strong resistance to drug-induced toxicity [172]. This would indicate that the constitutive activation of Nrf2 and concomitantly its target genes is advantageous for mice to overcome xenobiotic toxicity.

6.7.1.3 Inflammation in an Acetaminophen Model of Drug-Induced Liver Injury.

A widely studied model of DILI has been acetaminophen-induced liver injury (AILI) in mice (Table 6.4). Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of cellular contents known as *damage-associated molecular patterns*. These include High mobility group box protein 1 (HMGB1) and the heat shock proteins that act as signals that can activate cells of the innate immune system, which reside in the liver. This, in turn, leads to hepatic infiltration of inflammatory cells that may contribute to the progression of liver injury by producing proinflammatory mediators such as cytokines and chemokines as well as reactive oxygen and nitrogen

TABLE 6.4 Summary of Inflammatory Models Used in APAP-Induced Hepatotoxicity

References	Inflammatory Model(s)	Reported Effect
100,101	KC depletion	Protection against liver injury at early time points, more susceptible at later time points
114	Neutrophil depletion	Significantly protected against liver injury
102	NK/NKT cell depletion	Significantly protected against liver injury
107	Anti-TNF- α /IL-1- α /IL-1ra	Increased susceptibility to liver injury
106	Anti-TNF- α , TNF-p55 receptor 1 ^{-/-} mice	Reduced mortality and liver injury
122	TNFR1 ^{-/-} mice	Increased susceptibility to liver injury
109	IFN- γ ^{-/-} mice	Significantly protected against liver injury
119	IL-6 ^{-/-} mice	Increased susceptibility to liver injury
110	IL-10/4/6 ^{-/-} mice	Significantly protected against liver injury
	IL-10/4 ^{-/-} mice	Increased susceptibility to liver injury
120	IL-13 ^{-/-} mice	Increased susceptibility to liver injury
112	Anti-TLR9, TLR9 ^{-/-} , Nalp3 ^{-/-} mice	Reduced mortality and liver injury
113	Soluble RAGE	Reduced mortality and liver injury
117	LPS	Increased susceptibility to liver injury

species [173]. Neutrophils accumulating in necrotic areas of the liver after APAP overdose were first recognized by Mitchell *et al.*, but the relevance of this accumulation to the pathophysiology is still unclear [174]. A study in C3Heb/FeJ mice revealed that neutrophils accumulate in the liver shortly after hepatic injury, but there was no evidence of neutrophil activation. Additionally, antibodies targeted at these cells did not protect against AILI [175]. In a rat model of AILI, a significant reduction in neutrophil accumulation showed no protection of liver injury [176]. These findings are in contrast to other models in which neutrophils are involved in the course of injury where pretreatment with an antineutrophil antiserum attenuated AILI in rats [177].

Other studies have demonstrated that mice treated with gadolinium chloride to inactivate Kupffer cells (KCs) [178,179] and liposome/clodronate to deplete KCs [179,180] are protected against APAP toxicity. Nevertheless, reports suggest that KC depletion confers protection at early times after APAP treatment [180] but can lead to more severe injury at later time points [179]. The use of an anti-NK1.1 monoclonal antibody to deplete natural killer (NK) cells and natural killer T (NKT) cells significantly protected mice from APAP toxicity probably owing to the reduction in the levels of the proinflammatory cytokine IFN- γ [181]. However, as has been observed with KC depletion, there are also conflicting reports suggesting that NK/NKT cells play little

role in the overall outcome of liver injury in the mouse model of APAP hepatotoxicity [182,183]. This has been supported by a recent study that suggested NK and NKT cells only contributed to liver injury when dimethyl sulfoxide was used to solubilize APAP [183]. Given the contradictory data available on neutrophils and KCs, it is clear that more studies are required to determine their precise role in DILI.

The involvement of various proinflammatory mediators such as TNF- α [184–186], IL-1ra [187], and IFN- γ [181,188], in promoting tissue damage have been demonstrated, whereas other cytokines such as IL-10 and IL-4 [189] have been found to be hepatoprotective in models of APAP hepatotoxicity. Receptors involved in promoting an inflammatory response such as IL-1 receptor [186], p55 TNF- α receptor-1 [185], Toll-like receptors TLR4 and -9 [190,191], and receptor for advanced glycation end products (RAGEs) [192] have been found to be important in promoting an inflammatory response and subsequent pathogenesis.

6.7.1.4 Comparison of the Inflammatory Response in Animal Models of Acetaminophen-Induced Liver Injury. Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those on DILI. NK and NKT cells were found to play a critical role in the severity and progression of AILI (500 mg/kg, i.p.) by secretion of cytokines and recruitment of inflammatory cells (mainly neutrophils) into the liver [181,193]. An increase of hepatic neutrophil accumulation as early as 4–6 h with a significant threefold increase by 24 h was observed in APAP-treated mice with an associated increase in mRNA levels for cytokines TNF- α and IFN- γ [181]. Depletion of NK and NKT cells (using an anti-NK/NKT cell antibody or an antineutrophil antibody) inhibited liver injury with markedly reduced accumulation of neutrophils in the liver. Subsequent depletion of neutrophils also significantly protected mice against AILI. However, in a recently published fed CD-1 mouse model of AILI, no evidence of hepatic inflammatory cell infiltration was observed over a 24 h time course [27].

Nontoxic doses of APAP (100–400 mg/kg, i.p.) administered 2 h after a dose of Lipopolysaccharide LPS (44×10^6 EU/kg) to fasted C57BL6 mice have been shown to sensitize animals to AILI [194]. Application of LPS led to significantly greater TNF- α production than APAP alone. With APAP treatment alone there was a modest, sustained accumulation of neutrophils from 3 to 24 h after 300 mg/kg APAP. However, neutrophils were not apparent in livers of mice treated with a nontoxic dose of APAP (175 mg/kg), but significant accumulation occurred when this dose was coadministered with a noninjurious dose of LPS. Two hours after treatment with LPS (0 h after APAP), serum TNF- α levels were large and rapidly decreased thereafter, confirming that the inflammatory response observed in this model was due to LPS [194]. These observations support the hypothesis that inflammation induced by LPS and associated elevated levels of TNF- α increase sensitivity to AILI.

Fasted transgenic C57BL6 mice lacking anti-inflammatory mediators such as IL-10, IL-4, IL-6, migration inhibitory factor (MIF), and IL-13 dosed with APAP (200–300 mg/kg, i.p.) displayed increased susceptibility to AILI [189,195–197]. Neutrophils were found to accumulate to a greater extent in IL-13 knockout (KO) mice than wild type (WT) treated with APAP with the level of TNF- α , IL-6, and IFN- γ increased at 4 h in WT and IL-13 KO APAP-treated animals compared to saline-treated controls [197]. These data suggest a hepatoprotective role for anti-inflammatory mediators in these models and that the balance between the Th1 and Th2 response

is an important determinant for AILI. To test this hypothesis, susceptibility to AILI was compared between two mouse strains, C57BL/6 (more susceptible to AILI and that develop predominantly a Th1 response) and BALB/c (less susceptible to AILI and that develop a Th2 response). About 24 h after exposure to APAP C57BL/6 mice produce high levels of TNF- α (proinflammatory), whereas levels in BALB/c mice show no difference. On the other hand, IL-6 (anti-inflammatory) levels were higher in BALB/c than C57BL/6 24 h after administration. These data support the hypothesis that AILI is associated with a Th1-dominant response in Th1/Th2 cytokine balance and TNF- α may play a role in toxicity [196].

However, the role of TNF- α remains controversial with contradictory reports, suggesting that TNF- α is involved in pathogenesis of DILI [185,186]; it is not involved in toxicity [27,184,185,187,189–198]; or it has a role in defense against toxicity and repair [199,200]. Data suggest that as most cytokines the effects of TNF- α in liver toxicity are pleiotropic with other cytokine networks being involved and it is the interplay between these different cytokine cascades that determine the nature of the inflammatory response associated with toxicity. Furthermore, the understanding of the regulatory mechanisms in the context of DILI that control the potential for damage-associated molecular pattern molecules or cytokines to activate immune cells and initiate an inflammatory response require further investigation. For example, recent evidence suggests that the caspase-dependent oxidation of HMGB1 is critical for inhibiting its immune stimulatory potential following apoptosis [27].

As well as APAP, other model hepatotoxins have been used to investigate the role inflammation plays during DILI (Table 6.5). Given the complexity of interplay between chemically mediated toxicity and inflammation and the differences observed between strains of mice, species, and *in vivo* model pretreatment, further investigation is required to define the precise role a cytokine has in response to the chemical insult or as part of the inflammatory response. Once this has been defined, then the value of the cytokine as a biomarker can be assessed.

6.7.1.5 APAP Toxicity and Circadian Rhythm. Susceptibility to AILI has been reported to follow a circadian rhythm [201,202]. Rodents administered with APAP at the start of the circadian light phase (early subjective day) were more protected than those dosed at the start of the dark phase (early subjective night). This temporal difference in hepatotoxicity can be explained by concomitant changes in drug disposition. Hepatic GSH content fluctuates over a 24-h cycle linked closely with feeding patterns. In rodents, GSH abundance peaks at early morning and reaches a nadir at early night. This reduction in GSH lowers the potential for the liver to bioinactivate NAPQI and allows the chemically RM to cause extensive damage as defined by serum biomarkers

TABLE 6.5 Inflammation in Other Models of DILI

DILI Model	Inflammatory Factors Investigated	References
LPS	Neutrophils, TNF- α , Kupffer cells	124–126
EtOH	Kupffer cells, TNF- α	127–130
Halothane	Macrophage inhibitory factor	118
Cocaine + LPS	Kupffer cells, nitric oxide	131,132
Galactosamine + LPS	TNF- α , TNF-p55 receptor	133,134

of DILI. Moreover, it has also been shown that the bioactivation pathway, Cyp2e1-mediated biotransformation of APAP, does not remain constant over the course of the day. Cyp2e1 has been shown to exhibit a mild oscillation with an increased expression at early night. The molecular mechanism underlying Cyp2e1 oscillation has been suggested to be linked with hepatocyte nuclear factor 1 α (HNF1 α) and the biological clock [203], but other mechanisms have been suggested, for example, diurnal rhythms in liver energy status. This parallel of increased bioactivation and reduced bioinactivation over the light phase in the rodent model increases the exposure of the liver to the toxic metabolite and suggests the temporal variation in APAP-induced hepatotoxicity.

6.7.2 Nefazodone

Nefazodone is an antidepressant that was withdrawn from the US market in 2004, following withdrawal in Canada and Europe, owing to incidences of hepatotoxicity. A black-box warning for hepatotoxicity stated that there was one case of liver failure resulting in death or transplant per 250,000–300,000 patient years and more than 20 deaths were reported to the FDA [204]. Patients who presented with nefazodone-induced hepatotoxicity did so 1–8 months after commencement of treatment and had been taking a dose of 200–400 mg daily [204–206]. In those patients with acute liver failure, histology demonstrated bile duct proliferation, cholestasis, and centrilobular necrosis [205]. It is still unclear how nefazodone caused hepatotoxicity in man and the three mechanisms currently presented in the literature are discussed below.

6.7.2.1 Reactive Metabolite Formation and Covalent Binding. Nefazodone is predominantly metabolized by CYP3A4 [207]. During metabolism in human and rat liver microsomes, it is bioactivated to several quinone imine and iminium ions that can be trapped with GSH and cyanide, respectively (Fig. 6.7) [39,208]. The major quinone imine is formed from hydroxylation para to the piperazinyl nitrogen that can then undergo two electron addition to form the corresponding quinone imine [208]. The bioactivation of nefazodone *in vitro* has led to the investigation of binding to liver protein as a mechanism of toxicity. It has been shown to covalently bind to human liver protein in microsomes, S9 fraction, and hepatocytes [39,208,209]. In human liver microsomes (HLMs), it has been demonstrated to bind at 1364 pmol/mg protein [209], a level of binding over 20 times that of the 50 pmol/mg threshold, which Evans *et al.* suggested as an upper level of “safe” binding. This binding was significantly reduced to 306 pmol/mg with a mixture of Uridine 5'-diphospho-glucuronic acid (UDPGA) and GSH and 254 pmol/mg in the more complete metabolizing system of hepatocytes. Studies that took into the account the intrinsic clearance also showed that there was a high rate of binding in liver microsomes [210]. Although it has been established that nefazodone covalently binds to liver protein, there is currently no literature that indicates which proteins are the binding targets of nefazodone and whether this binding may contribute to cell death.

6.7.2.2 Role of Bile Salt Export Pump (BSEP) Inhibition in Nefazodone Toxicity. Nefazodone is predominantly excreted in the bile. Approximately 60% of radiolabeled nefazodone that was intravenously administered to dogs was excreted in the feces [211] and in humans 49% of nefazodone was cleared in the urine [212], suggesting that the other route of clearance was through bile. Bile salt export pump (BSEP) is a

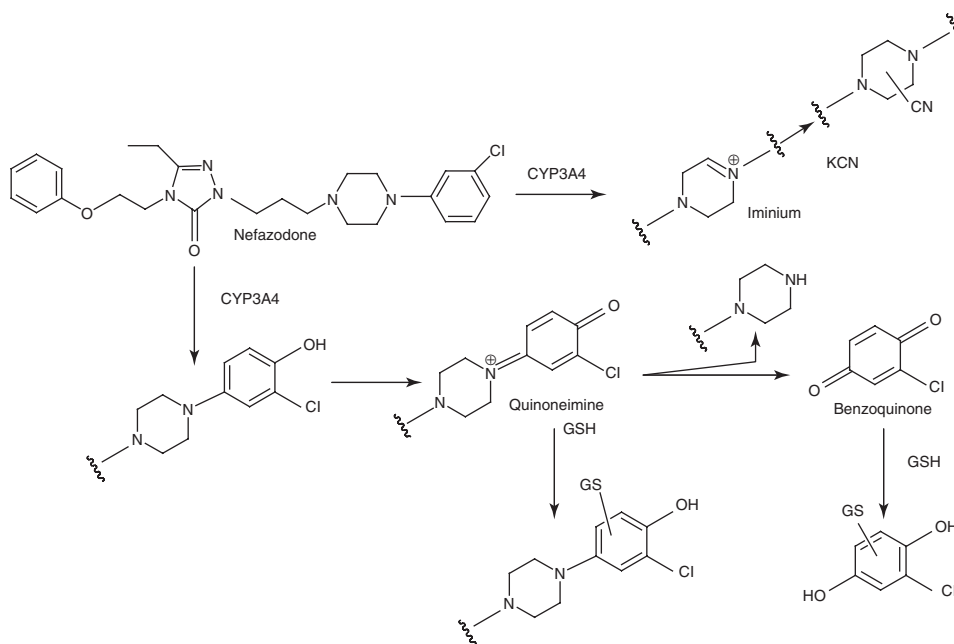


Figure 6.7 Characterized bioactivation pathway of nefazodone in human liver microsomes [122].

member of the ATP-binding cassette transporters and mainly responsible for the transport of bile salts and any other cholephilic compounds across hepatocytes from the blood plasma and into the bile canaliculi [213]. There have been investigations into the inhibition of BSEP as a cause of nefazodone hepatotoxicity [214]. Sandwich human hepatocytes, expressed BSEP in membrane vesicles and rats, were used to investigate the potential for nefazodone to be causing toxicity through inhibition of hepatobiliary transport [214]. Nefazodone caused a concentration-dependent inhibition of taurocholic acid in canaliculi with an $IC_{50} = 14 \mu M$ [214]. As the hepatocytes were fully capable of metabolism, it was not clear whether metabolites or parent compound was causing the inhibition. This led to the investigation of BSEP-mediated transport inhibition activity in membrane vesicles, which again resulted in concentration-dependent inhibition of taurocholic acid transport with $IC_{50} = 9 \mu M$ [214]. This suggested that the parent compound of nefazodone was causing the inhibition and not metabolites. The authors also explored the toxicity of nefazodone in hepatocytes using protein synthesis as an indicator of toxicity. Nefazodone produced a concentration-dependent decrease in protein synthesis. The effect of metabolism on toxicity was also considered, the authors used 1-aminobenzotriazole (ABT), a nonspecific inhibitor of CYP, to inhibit the metabolism of nefazodone in hepatocytes [214]. Incubation with nefazodone and ABT for 24 h resulted in a 45% decrease in total protein synthesis. Nefazodone alone resulted in almost full recovery after 24 h. This indicates that the bioactivation and covalent binding of nefazodone may not be the cause for hepatotoxicity.

6.7.2.3 Role of Mitochondrial Dysfunction in Nefazodone Toxicity. Increasingly, mitochondrial toxicity is being implicated in drug-induced organ toxicity. Patients with

underlying mitochondrial diseases may not have any symptoms until additional stress is placed on the mitochondria, such as drug-induced oxidative stress, that results in organ toxicity. As described earlier, the effect that nefazodone has on the mitochondria and the resulting hepatotoxicity has been investigated in isolated OXPHOS complexes, HepG2 cells, and sandwich human hepatocytes [89]. It has been demonstrated that in isolated OXPHOS complexes nefazodone inhibits complex I ($IC_{50} = 14 \mu M$) [89]. Using culture medium in which the primary energy source, glucose, has been replaced with galactose, HepG2 cells, which are more reliant on oxidative phosphorylation, exhibits 100% depletion of ATP when treated with $200 \mu M$ nefazodone [89]. A concentration curve in galactose grown HepG2 cells results in an $IC_{50} = 9 \mu M$ [89]. The HepG2 cells did not express CYP3A4, again suggesting that metabolism is not required for toxicity. Investigations of mitochondrial injury were also carried out in sandwich human hepatocytes. This resulted in significant cell loss, increased ROS, elimination of mitochondrial membrane potential, and depletion of intracellular GSH [89]. Maximum plasma concentrations of $2\text{--}4 \mu M$ has been reported in patients taking $200\text{--}400 \text{ mg/day}$, although there is no available data on plasma:liver ratio of nefazodone [215]. However, if nefazodone does inhibit its own excretion as discussed above, then accumulation in the liver could reach levels similar to that seen *in vitro*.

6.7.3 Acyl Glucuronides

Human populations are exposed to numerous chemical entities possessing a carboxylic acid moiety, including nonsteroidal anti-inflammatory drugs (NSAIDs), hypolipidemic drugs (clofibrate), anticonvulsants (valproic acid), and diuretics (furosemide). Several carboxylic acid drugs have been withdrawn from the market over the years as a consequence of rare but occasionally severe adverse effects. From a toxicological perspective, attention has been focused on the bioactivation of this functional group to form RMs, namely, coenzyme A thioesters and acyl glucuronides (AGs) [63], implicating drug bioactivation as an early step in the pathogenesis of ensuing adverse effects. Acyl glucuronidation represents one of the metabolic pathways in the elimination of many carboxylic-acid-containing drugs and metabolites [216], and therefore as a physiological clearance process is potentially important to the pharmacological effects of those compounds. This reaction is catalyzed by multiple human UDP-glucuronosyltransferases (UGTs) [217,218]. Generally, glucuronides are not only less biologically active than the parent aglycone [219] but also subject to more rapid biliary excretion because of their enhanced affinity for transporter proteins [220–225]. However, AGs represent an exception to this accepted principle, possessing intrinsic electrophilic reactivity. This reactivity is manifested through three main pathways: hydrolysis; acyl migration, and irreversible binding with cellular macromolecules. As a number of carboxylated drugs known to form AG metabolites are associated with ADRs in patients, AGs have come to be almost generically implicated in the possible causation of these toxicities. Although bioactivation liabilities connected with oxidative metabolism appear to have caused concern much more frequently than acyl glucuronidation [116] and initiate increasingly sophisticated structural optimization programs to minimize bioactivation [226], medicinal chemists have still derived reassurance from any evidence that an AG metabolite of a new drug is relatively stable [117,227,228]. As a further indication of an established perception of toxicological risk, attempts to connect drug metabolites with the toxicity that has forced the withdrawal

of a compound from development, as in the recent case of torcetrapib [229], still consider the possible involvement of AGs. Torcetrapib, a cholesteryl ester transfer protein inhibitor, was terminated in phase 3 trials because of an unexpected increase in cardiovascular events and death [230]. Administration of torcetrapib was shown to acutely increase blood pressure in both rodent and nonrodent species [230]. Glucuronide conjugates of two major carboxylic acid metabolites can be detected in different species [229]. What may be characterized as a lingering but ill-defined anxiety seems to surround drugs that are metabolized extensively to AGs in humans. Nevertheless, with the notable exception of the acute enteral toxicity of DCF in rats [231], the toxicological implications of AG formation have not been delineated clearly *in vivo*. Certainly, the use of probenecid [232] and the recent developments of bicifadine [233] and deferasirox [234] have continued, despite these drugs being metabolized by humans to unstable AGs.

6.7.3.1 Acyl Glucuronide Reactivity. The reactivity of an AG derives ultimately from the electrophilicity of its ester carbonyl group resulting in hydrolysis to the parent aglycone, intramolecular rearrangement, and intermolecular transacylation reactions. A structure effect on the degree of reactivity has been demonstrated with the rate order: acetic acid > propionic acid > benzoic acid derivatives. It was proposed that this order could be attributed to the inherent electronic and steric properties of each specific aglycone [235]. Consequently, as a number of drugs are associated with AG formation but not necessarily toxicity, the reactivity of the corresponding AGs has been under much investigation with the aim to delineate a relationship between AG reactivity and ensuing toxicity.

1- β -*O*-Acyl glucuronides may undergo intramolecular rearrangement through the migration of the acyl group to positions C-2, C-3, and C-4 of the carbohydrate moiety [236] (Fig. 6.8). The mechanism is thought to involve nucleophilic attack by the hydroxyl group on the adjacent carbon to generate an *o*-acid ester intermediate [237,238]. The intramolecular rearrangement of AGs is of particular importance because it leads to re-exposure of the hemiacetal function of the glucuronic acid moiety, thus allowing two chemical reactions: anomerization and nonenzymatic glycation [239]. Stability and, therefore, reactivity of the AG could potentially have significant implications on the likelihood of a drug causing toxicity.

In addition, 1- β -*O*-acyl glucuronides are particularly susceptible to hydrolysis, resulting in the release of the parent aglycone. A number of catalysts, including β -glucuronidases, nonspecific esterases, serum albumin, and hydroxide ion, can be involved in this reaction. The rate of hydrolysis is dependent on a range of parameters including pH, temperature, and aglycone structure [241]. It has been determined that the rate of hydrolysis, in parallel with acyl migration, can be a relevant parameter to investigate in connection to AG reactivity with cellular macromolecules [242]. An almost linear correlation ($r^2 = 0.995$) between the overall degradation rate (hydrolysis and intramolecular rearrangement) of AGs and the amount of drug bound to Human serum albumin (HSA) *in vitro* was reported for 9AGs. Subsequent investigations of AG reactivity also resulted in similar results with an improved correlation obtained when the fraction of bound drug (as a percentage of initial glucuronide present) was plotted against the degradation rate [235,243]. Exact degradation half-lives are variable across the AGs and is most likely dependent on the structure of the aglycone [244].

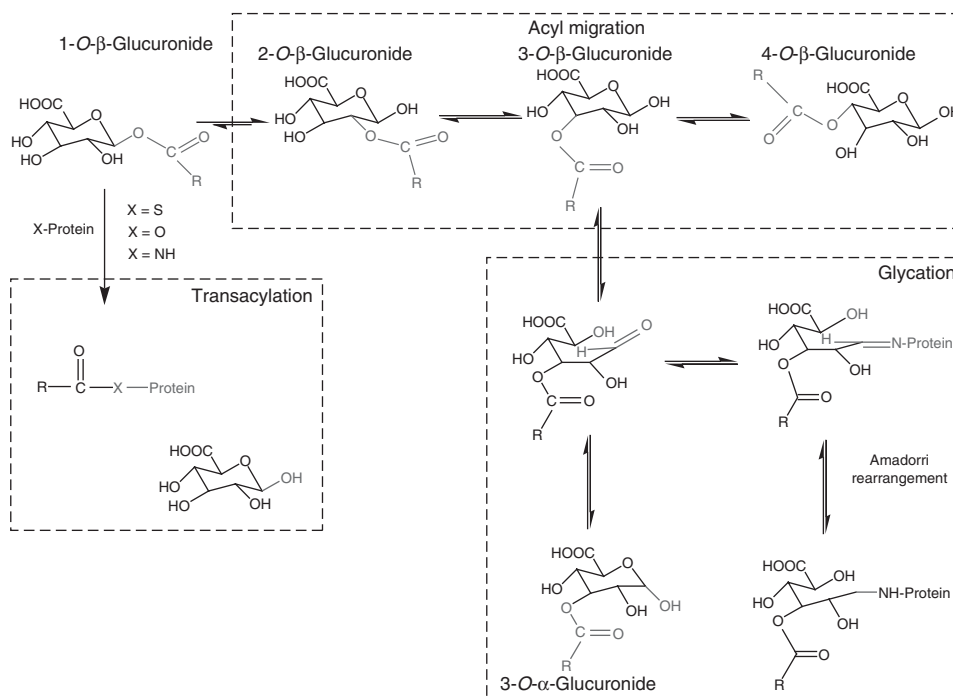


Figure 6.8 Mechanisms of AG rearrangement and covalent binding to protein. Rearrangement of the AG is shown, as the aglycone moves around the ring to the 2-, 3-, and 4-glucuronide positions. An example of rearrangement between the β and α positions is shown by the 3-isomer, with its corresponding covalent binding to protein via the glycation mechanism. This also occurs for the 2- and 4-isomers. Covalent modification of protein by the transacylation pathway is represented by the 1 β isomer. RCOO⁻ represents the aglycone. X represents nucleophilic groups on proteins susceptible to covalent adduct formation from the AG through transacylation. *Source:* Adapted from Bailey *et al.* [240]. (See color insert.)

The electrophilic properties of AGs also enable them to covalently bind to specific nucleophilic sites on biological macromolecules. While this reaction is considered to be a quantitatively a minor pathway of AGs, it has been implicated in the underlying mechanism of toxicity of certain carboxylic-acid-containing drugs such as hypersensitivity reactions [237,245–247]. Specifically, these immune-mediated reactions are governed by the generation of neoantigens, formed as a result of RMs binding to hepatic protein. These immunogenic adducts are then recognized by the immune system and elicit either antibody [107,173] or cytotoxic T-cell responses [173,245].

Several studies have been carried out to elucidate the mechanism for the formation of protein covalent adducts, with two pathways identified. The first is a transacylation mechanism involving nucleophilic displacement of the glucuronic acid from the ester group by macromolecular nucleophilic sites such as $-\text{NH}_2$ of lysine residues [248], $-\text{SH}$ of cysteine residues [249], or $-\text{OH}$ groups from tyrosine residues [250]. An alternative mechanism reported is the glycation pathway, where the aglycone moiety forms a protein adduct via the glucuronic acid moiety. Specifically, this requires acyl migration before binding, enabling the ring opening of the carbohydrate moiety and subsequent

anomerization. This results in the formation of open-chain aldehyde intermediates and the further condensation of the aldehyde group with the amine groups on lysines or DNA nucleosides to form imines (Schiff bases), which can further undergo rearrangement to a more stable 1-amino-keto-product (Amadori rearrangement) [251–254].

The formation of protein adducts is not only limited to plasma protein alone but can also involve other organs including the liver, kidney, skeletal muscle, and intestine, indicated by a number of animal studies [255,256]. Therefore, the formation of AGs could have implications in specific organ-directed toxicities and also systemic hypersensitivity reactions through both the binding to specific organs and plasma protein. However, the toxicological significance of covalent binding between drug and macromolecule remain speculative and still require definitive proof as protein adduct formation does not always lead to toxicity. This concept is best illustrated by APAP and its structural isomer AMAP, where AMAP at the same molar dose as APAP was found to covalently bind to cellular protein and yet did not result in toxicity. This introduced the concept of the critical protein hypothesis, delineating certain proteins as critical targets in hepatotoxicity [18,105]. Supporting this, the RM of APAP, NAPQI binds to a number of proteins. In mice, one of the major targets of NAPQI is urate oxidase, an enzyme lost during primate evolution. As a result, it is thought unlikely that irreversible binding to urate oxidase represents a role in APAP-induced hepatotoxicity in mice, as both humans and mice develop centrilobular necrosis with similar per kilogram doses [257]. This highlights the need to identify the critical protein adducts involved in the mechanism of toxicity.

Glucuronides of propionic acids have been reported to be more stable and less chemically reactive than those formed from acetic acid NSAIDs, generally possessing a much longer half-life *in vitro* at physiological pH. It is thought that this can most likely be attributed to the steric hindrance and electron donating effects of the α -methyl group [247]. Despite this, Benoxaprofen BNX-AG has been reported to react with HSA *in vitro*, forming protein adducts through both transacylation and glycation pathways [257]. The use of tandem mass spectrometry further enabled the identification of specific binding sites, which included Lys-159 and Lys-199 to a lesser extent. Ser-312, Ser-480, and Arg-222 were the other three major binding sites, which reacted only via nucleophilic displacement.

It is, therefore, well established that AGs are intrinsically reactive electrophiles, with the potential to bind to cellular macromolecules. However, the contribution of AG formation to underlying toxicological mechanisms remains unclear and is consequently under much investigation.

6.7.3.2 Toxicological Implications of Acyl Glucuronides. A number of carboxylate drugs have been found to undergo extensive metabolism to an AG, but with varying degrees of toxicity associated with administration. Specifically, the use of DCF has been associated with a number of adverse effects, the most prominent being induction of hepatic injury [242,258–261]. It has been reported that 15% develop abnormalities in aminotransferase plasma levels [258,262]. Cases of severe hepatic reactions are much rarer in frequency with estimates ranging from 1–2 cases per million prescriptions [263] to 6–18 cases per 100,000 person years [264]. Published cases of severe DCF hepatotoxicity equate to ~250 reports with a case fatality rate of 10% [265]. The high case numbers can be attributed to the large number of patients who

are prescribed DCF worldwide [76]. The injury associated with DCF-induced hepatotoxicity is consistently hepatocellular, resembling hepatitis, characterized by jaundice, and to varying degrees, fatigue anorexia, nausea, and vomiting [262]. The underlying pathogenesis associated with DCF hepatotoxicity is unknown, but drug bioactivation has been implicated. Consequently, investigations have been undertaken to delineate the importance of AG reactivity and to a lesser degree AG exposure.

The majority of investigations concerning AG adduct formation have focused on binding to HSA, which would explain systemic adverse effects associated with carboxylate drugs. However, certain drugs are associated with specific organ-related toxicity, such as DCF, which represents a paradigm for idiosyncratic hepatotoxicity. Consequently, the focus of investigations has centered on assessment of binding directly to liver macromolecules. In rodents, the majority of adducts were located on the canalicular plasma domain of the hepatocytes [266,267]. In addition, DCF adducts were also identified on the apical domain of enterocytes; however, it has not been conclusively proven that these are as a result of AG formation [268]. DCF adduct formation with enterocytes through acyl glucuronidation has been suggested based on a study by Seitz *et al.* [267] whereby administration of bile containing DCF-AG resulted in intestinal ulcer formation in Multidrug resistance-associated protein 2 MRP2-deficient rats, which did not occur when DCF alone was administered. The ability of DCF-AG to bind to serum albumin has yet to be determined in either *in vitro* or *in vivo*.

6.7.4 Nevirapine

Nevirapine (NVP) is a highly effective nonnucleoside reverse transcriptase inhibitor (nnRTI) used in the treatment of HIV-1 infection. NVP is used widely in developing countries as it is inexpensive and does not require refrigeration; however, it is associated with clinically restrictive side effects, skin reaction and hepatotoxicity that can occur either simultaneously or separately. Toxicity is thought to be mediated through RM formation either directly or through the induction of the immune system. Although NVP is highly effective, the incidence of severe or life-threatening hepatotoxicity has led to the FDA giving a black-box warning. The nature of NVP-induced liver injury is not clear; cases of hepatotoxicity vary in terms of severity, time to onset, and can occur with or without hypersensitivity reactions [269,270]. It is possible that the late-occurring reactions may be nonimmune in nature, while those occurring in the first three months may have an immune pathogenesis [271]. The time of onset and the nature of the NVP-induced skin rash suggest that the reaction is immune mediated and because of the low incidence it is likely that the reaction may be idiosyncratic [271,272].

6.7.4.1 Possible Pathways of Nevirapine Bioactivation. There appear to be several pathways for NVP bioactivation (Fig. 6.9). The cyclopropylamine group has the potential, via N-dealkylation, to become bioactivated to an aminium cation radical [120]. 12-OH NVP, a major metabolite in HLMs, is a substrate for sulfotransferase in rats [272], and it has been proposed that the sulfate ester dissociates to form a reactive quinone methide intermediate [272]. The quinone methide could also be generated, additionally or alternatively, by enzymic oxidation [117]. Hydroxyheteroaryl metabolites [273] are potential precursors of reactive quinone imines [117]. Finally, NVP might form one or more heteroarene epoxide intermediates in either of the pyridine

rings. Novel NVP GSH conjugates and mercapturates, potentially formed from multiple RMs, have been characterized [274]. Mercapturates were isolated from rat bile and characterized definitively by NMR as thioethers substituted at the C-3 and exocyclic C-12 positions of the methylpyridoring of NVP. It is proposed that NVP undergoes bioactivation to arene oxide and quinone methide intermediates [274]. Further evidence for metabolic activation of NVP includes NADPH-dependent irreversible binding of radiolabeled drug to rat liver microsomes (RLMs) [120], mechanism-based inhibition of microsomal CYP3A4 [117], and the metabolism of NVP by HLM and CYP3A4 to a single GSH adduct [117].

6.7.4.2 Animal Model of Nevirapine-Induced Skin Rash. Uetrecht and colleagues have characterized extensively a dose-dependent, NVP-induced skin rash in female Brown Norway (BN) rats that resembles the idiosyncratic cutaneous reaction seen in humans [275,276] and appears to be immune mediated [277]. The NVP-induced skin rash in BN rats is similar to that in human in terms of: the time of onset, sex dimorphism, the fact that incidence increases with dose, that an escalating dose of NVP can reduce incidence, and that rechallenge in rats and humans results in more severe rashes than on initial exposure [275]. Chen *et al.* [272] have proposed that the skin rash induced by NVP and 12-OH NVP in BN rats may be due to NVP quinone methide formed through dissociation of 12-OH NVP sulfonate in the skin. A mild hepatotoxicity, assessed by histopathological and plasma enzyme indicators, is induced in rats pretreated with NVP or dexamethasone [278]. This implies one or more NVP metabolites are causal agents of hepatotoxicity but no mechanistic explanation of the liver injury has emerged. Chen *et al.* [272] have suggested that the hepatotoxicity of NVP in humans is due to quinone methide formed *in situ* by CYP enzymes.

Evidence suggests that the 12-OH NVP pathway is responsible for NVP-induced skin rash, as administration of 12-OH NVP induces skin rash at 50 mg/kg/day as opposed to 150 mg/kg/day with NVP [272]. Cotreatment with ABT decreased urinary excretion of 2-OH NVP, 3-OH NVP, and 4-COOH NVP while increasing the urinary excretion of 12-OH NVP. The result was the formation of skin rash at 50 mg/kg/day in female BN rats and at 150 mg/kg/day in male BN rats, which normally unaffected [272]. However, on rechallenge, 12-OH NVP failed to elicit a skin rash, even if the initial rash was induced by 12-OH NVP [272]. It may be that NVP is needed to induce the CYP isoforms required for bioactivation to RM.

6.7.4.3 Potential Markers of Nevirapine Bioactivation. Novel NVP GSH conjugates and mercapturates, potentially formed from multiple RMs, have been characterized [274]. The formation of drug-GSH conjugates is often used as an indicator of bioactivation. GSH conjugates are generally excreted in the urine as mercapturates. Drug mercapturates have been used as biomarkers of exposure to an RM [154,155]. The identification of two novel NVP mercapturates in humans and rats and the development of a mass spectrometric assay to quantify them pose the opportunity to develop the mercapturates as biomarkers of NVP-induced skin rash [274]. To justify the use of a NVP mercapturate as a biomarker of exposure to an RM, a direct link between mercapturate formation and adverse event must be confirmed; the female BN rat model of NVP-induced skin rash can be utilized to do this. A direct link between mercapturate formation and skin rash incidence could be proved by using specific CYP450 inhibitors to modulate NVP metabolism.

6.8 SUMMARY POINTS

- There is overwhelming evidence to suggest bioactivation of drugs to chemically reactive metabolites (RMs) plays a major role in toxicity and ADRs.
- Chemically RMs can induce oxidative stress in the cell by introducing electrophilic species, stimulating production of ROS and disrupting thiol status through depletion of GSH.
- The cell can activate an immediate adaptive defense response via the upregulation of gene expression when the cell undergoes oxidative stress.
- If the oxidative insult overwhelms the cell's protective mechanisms cell death can ensue through a number of mechanisms, including mitochondrial dysfunction, DNA damage, protein binding, and induction of the immune response and inflammation.
- As a result of drug attrition occurring late in drug development—one of the main reasons for which is related to safety—we need to be able to detect the ability of a NCE to be bioactivated and whether the reactive intermediate is likely to cause hazards.
- Many pharmaceutical companies have used covalent binding to microsomal proteins as an indicator of a safety liability. However, the degree of covalent binding in microsomes is an indicator of bioactivation, but is not always indicative of toxicity and, hence, is poorly translated into safety outcomes in the clinic covalent binding. Work is currently ongoing to develop relevant human hepatic cell lines as an alternative to microsomes with the hope of generating a more physiologically relevant system and improving clinical translation of data.
- The development of clinically relevant biomarkers, which can successfully assess the propensity of a chemical to cause toxicity and will bridge the “disconnect” between preclinical *in vitro* and *in vivo* studies and clinical studies, in terms of successful prediction of safety liability, is crucial.

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7 Drug Metabolism in Drug Safety Evaluation

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7.1	Summary	1
7.2	Introduction	2
7.3	Stable drug metabolites	2
7.4	Reactive drug metabolites	7
7.5	Conclusions	17
	Abbreviations	18
	References	18

7.1 SUMMARY

Over the past two decades, drug metabolism has become an indispensable component of both research and development activities in the pharmaceutical industry, where it has played an increasingly important role in support of the safety assessment of new chemical entities (NCEs). In response to recent regulatory guidance on the safety evaluation of metabolites of candidate drugs in man, novel approaches have been adopted to define the identities and levels of circulating metabolites in the plasma of both human subjects and the animal species employed in safety assessment programs. Through these analyses, exposure margins are established for human drug metabolites that circulate above a certain threshold in order to provide assurance that the preclinical toxicology program takes into account metabolites of the drug candidate as well as the parent molecule. Metabolites judged to be “disproportionate” or “unique” by regulatory criteria may require more detailed evaluation. In considering the factors that need to be taken into account in assessing the safety of metabolites of NCEs, it is appropriate to classify biotransformation products into two categories based on their chemical reactivity, viz. “stable” and “chemically reactive.” The different experimental approaches employed to study these two groups are outlined and the implications of the findings for lead optimization efforts in drug discovery, and for later phase clinical development programs, are discussed in the context of establishing the human safety of the NCE.

7.2 INTRODUCTION

Traditionally, the primary focus of safety assessment paradigms in pharmaceutical development centered on measuring exposure to the drug candidate itself, first pre-clinically in animal models to support first-in-human studies and, subsequently, during clinical trials to establish appropriate safety margins for continued clinical development. However, with the advances that have occurred over the past two decades in the field of drug metabolism [1], notably in bioanalytical technologies for the detection, identification and quantitative analysis of drug metabolites, and in the tools of molecular biology that have enabled studies on the biological function and regulation of drug metabolizing enzymes and transporter proteins, increased attention has been paid to the pharmacological and toxicological importance of drug *metabolites* in the drug discovery and development process. Indeed, the level of scrutiny of biotransformation products has been heightened by the growing awareness that the adverse effects of many foreign compounds may be attributed, at least in part, to the actions of one or more metabolites of the administered agent, and it is in this context that drug metabolism as a discipline has become an integral component of the safety evaluation of NCEs [2]. Regulatory guidance documents now have been published on the safety assessment of drug metabolites [3,4], with an emphasis on those biotransformation products that circulate (or are anticipated to circulate) in human plasma, the overall goal of which is to provide assurance that preclinical safety findings are relevant to the human situation in terms of exposure to both parent drug *and* its circulating metabolites. For purposes of discussion, drug metabolites may be divided into two broad categories, namely, those that are sufficiently stable to be isolated from biological fluids and characterized by conventional analytical techniques [e.g., liquid chromatography–tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR)], and those that are chemically reactive in nature and rarely are detected as the native species, but whose existence may be inferred from stable downstream products of further biotransformation [e.g., conjugates with glutathione, γ -glutamylcysteinylglycine (GSH)]. In general, these two groups of metabolites act to cause toxicity by different mechanisms, distinct approaches are employed to study them, and the implications for drug development associated with stable versus reactive metabolites also differ. At the heart of the matter in both cases, however, is the question of predicting drug toxicity in humans from results obtained in animals, where species differences in drug metabolism (which normally are quantitative, rather than qualitative) may confound the interpretation of animal toxicology data in terms of human risk assessment [5].

7.3 STABLE DRUG METABOLITES

The issue of addressing, in a systematic manner, the involvement of stable drug metabolites as a key component of the safety assessment of new drug candidates was first highlighted in a position paper coauthored by representatives of member companies of the US Pharmaceutical Research and Manufacturers Association (PhRMA), which proposed a number of practical approaches to the problem [6]. This publication, which became known as the *MIST* (“Metabolites in Safety Testing”) document, proposed *inter alia* that those small molecule drug metabolites whose exposure in humans [as measured by area under the plasma concentration versus time curve (AUC_p) values]

exceeded 25% of that of the respective parent drug be viewed as “major metabolites” and that only such metabolites should be given further consideration in terms of verifying systemic exposure in one or more of the animal species employed in the preclinical safety assessment program. Recommendations also were made as to the specific, albeit very rare, case of a “unique” human metabolite, defined as a product of biotransformation that is detectable only in humans and not in any of the animal species investigated. In the years following publication of the MIST paper, numerous commentaries have appeared dealing with different aspects of the issue, such as whether absolute, as opposed to relative, metabolite exposure should trigger further safety evaluation of a given metabolite [7], the importance of the human radiolabeled absorption, distribution, metabolism, and excretion (ADME) study as the primary source of information on human metabolites for decision-making purposes [8], the impact of duration of drug administration on potential metabolite-mediated toxicity [9], and consideration of mechanisms of toxicity with regard to parameters such as dose, abundance, and duration of therapy [10]. Clearly, the challenge of addressing, in a comprehensive fashion, the safety of human drug metabolites is a complex and multifaceted issue.

In 2008, the US Food and Drug Administration (FDA) published a guidance document entitled “Safety Testing of Drug Metabolites” [3], which outlined the Agency’s views on best practices in this area. In some respects, this guidance paralleled the recommendations of the MIST paper [6], but differed significantly in other respects, notably in defining the threshold for human metabolites of interest as those whose AUC_p values exceeded 10% of that of the parent drug when measured under steady-state dosing conditions. Moreover, it was recommended that metabolites falling into this category should be demonstrated to be present in the plasma of at least one of the animal species selected for preclinical safety assessment at levels at least as great as those observed (or predicted) in human subjects at the highest clinical dose. Should this requirement for exposure margins (i.e., $\text{animal } AUC_p \geq \text{human } AUC_p$) not be met, then the metabolite in question is designated “disproportionate,” and additional safety studies may be required to assure its human safety. While in some cases, metabolite exposure in animals can be increased by administering progressively higher doses of the parent drug, this approach is not always successful because of a number of factors, for example, limited solubility in the dosing vehicle, saturation of drug absorption, and species differences in metabolic pathways. In these instances, the FDA guidance recommends that separate animal toxicology studies with the preformed (synthetic) metabolite should be considered. There appears to be general agreement that drug conjugates present in the systemic circulation do not, in general, pose a safety concern, although the FDA guidance singles out acyl glucuronides as being “toxic molecules,” a curiously broad generalization. Finally, the guidance emphasizes the need for information on human drug metabolism to be acquired as early as possible in the drug development process so that issues related to disproportionate human metabolites may be recognized and addressed before the initiation of large-scale (phase III) clinical trials.

The recommendations of the FDA guidance on MIST have a number of significant implications for the pharmaceutical industry, and many companies have revised (or are reviewing) their approaches to assessing the role of drug metabolites in the safety evaluation of NCEs. Central to many of the concerns over the MIST guidance are practical limitations that pertain to the conduct of drug metabolism studies in support

of drug development [11,12]. For example, it is widely accepted that the “gold standard” approach to quantitative metabolic profiling in human subjects is the radiolabeled ADME study [8,13], which typically is not performed until a relatively late stage of development (usually following the phase IIA proof-of-concept trials) and involves the administration of a ^{14}C -labeled variant of the drug candidate as a single dose; thus, definitive information on the presence of a disproportionate metabolite may not be available early in the development process, and the data would not be obtained under steady-state conditions which require a multiple-dose design. Moreover, any approach to assessing the toxicological properties of a drug metabolite that involves administration of the preformed compound is fraught with difficulties because of potential differences in the disposition of that metabolite when dosed as the synthetic material versus when the metabolite is formed *in vivo* from the parent drug [14,15]. Indeed, chemical synthesis of the metabolite of interest on a scale appropriate for *in vivo* studies may not be straightforward, particularly when issues of stereochemistry are taken into account, such that considerable time, effort, and financial resources may need to be expended for questionable return. Also, the 10% “cut-off” for a metabolite-of-interest seems unduly stringent, given that many situations are known in which the parent drug is a quantitatively minor contributor to the total drug-related material in human plasma due to extensive first-pass metabolism; in such cases, numerous circulating metabolites could exceed the 10% threshold and, if not present at exposure multiples greater than $1 \times$ in animals would require separate toxicology testing. Clearly, the resource implications associated with addressing these issues are nontrivial, and the scientific value of conducting such studies in many cases may be marginal at best and misleading at worst.

Interestingly, the International Conferences on Harmonization (ICH) counterpart of the FDA MIST guidance, termed *document M3* [4], also adopts a 10% threshold for circulating drug metabolites but references this value to “total drug-related exposure” and not to parent drug alone. This is a significant difference between the two regulatory guidances, and begs the question of how best to determine “total drug-related exposure” early in the drug development process, before the conduct of the radiolabeled human ADME study. Also, ICH M3 makes no mention of metabolite assessment under steady-state conditions and also suggests that the above 10% figure may be relaxed somewhat for therapeutic agents that are to be administered at a daily dose of <10 mg. How these differences between the FDA and ICH guidances will be resolved remains to be seen, although it is anticipated that the ICH guidance will take precedence. Regardless, it is apparent that in the future drug metabolites will be the focus of much greater regulatory scrutiny from a drug safety perspective than has been the case in the past.

In response to the guidances dealing with the issue of drug metabolites in drug safety evaluation, numerous publications have appeared dealing with topics such as new (nonradioactive) methodologies for detecting, identifying, and quantifying metabolites from both animal studies and early (first-in-human) clinical trials, based on NMR spectroscopy [16,17] and LC-MS/MS with high resolution mass analysis [18–22]. An alternative approach that retains the use of radioactivity for early human metabolism studies is the “microdose” technique, in which a very low dose of ^{14}C -labeled drug (typically $100\text{ }\mu\text{g}$ or less and $<1\text{ }\mu\text{Ci}$) is administered to volunteers in what may be regarded as a “pilot” human ADME study, and metabolite profiles in plasma determined by the combined use of (off-line) liquid chromatography (LC) and accelerator mass spectrometry (AMS) [23]. Since both the mass of drug candidate and the dose

of radioactivity are extremely low, such “Phase 0” human studies can be supported by a much-abbreviated preclinical safety package, thereby economizing on time and resources. However, this approach suffers from the limitation that, while radiochromatographic metabolite profiles can be reconstructed for the drug candidate of interest, no structural information is available from AMS, and therefore, additional work is required to identify any human metabolites that do not coelute on LC with previously characterized biotransformation products formed in animals or generated using *in vitro* systems. A related topic centers on our ability to predict circulating human metabolites from *in vitro* data, and an analysis of 17 molecules from the Lilly portfolio showed that in only 41% of cases were the plasma metabolite profiles adequately predicted from *in vitro* data [24]. A somewhat more favorable outcome was reported in a similar study by the Pfizer group [25], in which the success rate in predicting human excretory and circulating metabolite profiles from a panel of 48 compounds was judged to be >70% based on *in vitro* data obtained from pooled human liver microsomes, liver S-9 fraction, and/or human hepatocytes. However, it was noted that the predictability of secondary metabolites was less reliable than that with primary products of biotransformation, and the authors concluded that *in vitro* systems alone cannot mitigate fully the risk of disproportionate circulating metabolites in humans.

While the recommendations contained in the regulatory guidances on MIST likely will remain controversial for some time to come, it is noteworthy that many pharmaceutical companies, for example, Bristol–Myers Squibb [26,27], Lilly [28], and Pfizer [29], have published thoughtful analyses of the topic and, in some cases, have put in place formal strategies for the conduct of drug metabolism studies in support of drug safety evaluation that take into account the issues of disproportionate or unique human drug metabolites. There appears to be general agreement that a “flexible, tiered approach” should be adopted for the characterization and quantitative analysis of stable drug metabolites and that decisions on the need for safety studies on specific human metabolites be based on sound science and well-balanced judgment. Commentaries on MIST from the regulatory bodies themselves would appear to echo this sentiment [30–32].

Underlying many of the criticisms of the MIST guidances discussed above is the fact that relatively few examples exist where a stable drug metabolite has been clearly implicated as the causative agent in the adverse effects observed on dosing with the parent drug. This observation has been rationalized in terms of the normally modest structural changes that accompany mammalian biotransformation reactions, which suggests that most drug metabolites will exhibit off-target effects that are qualitatively similar to those of their respective parents, although quantitative differences can be expected because of changes in certain physicochemical properties, for example, polarity, which influences plasma protein binding and tissue penetration [12,26]. However, exceptions do exist, such as the neurotoxin 1-methyl-4-phenyl-pyridinium (MPP⁺), formed by monoamine oxidase-B (MAO-B)-catalyzed metabolism of the illicit drug impurity 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [33], the acyl glucuronide conjugate of gemfibrozil, which serves as a mechanism-based inhibitor of CYP2C8 and thereby causes a number of drug interactions [34], and the sulfate conjugate of troglitazone, which has been shown to inhibit the hepatic transport proteins BSEP and OATP and is believed to contribute to cholestatic liver injury in diabetic patients treated with troglitazone [35] (Fig. 7.1). In these examples, the toxicity observed following administration of the respective parent is believed to be due to the stable metabolite

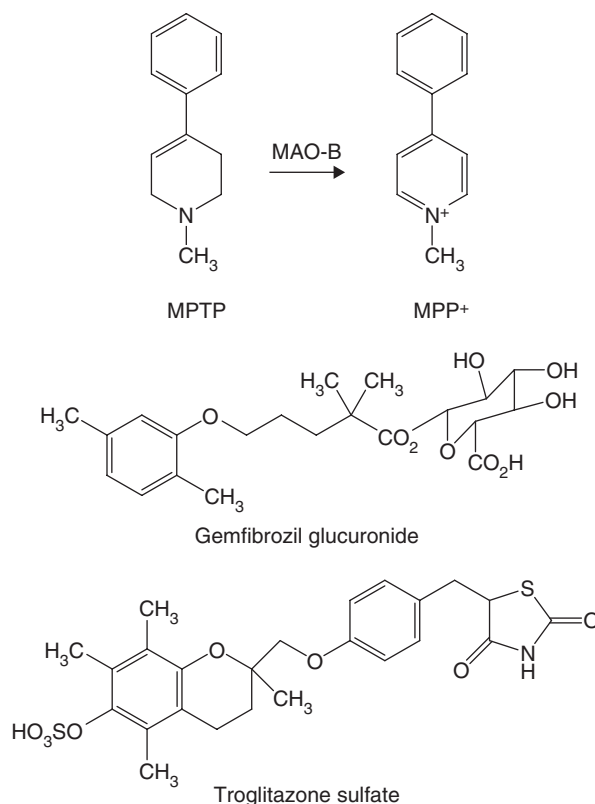


Figure 7.1 Structures of metabolites believed to be responsible for the adverse effects of their respective parent compounds. MPP⁺ is formed as a product of MAO-B-mediated oxidative metabolism, while the acyl glucuronide and sulfate conjugates of gemfibrozil and troglitazone result from the action of UDP glucuronosyltransferase and sulfotransferase enzymes, respectively.

in question. Of course, in certain instances, biotransformation of a drug molecule will result in the formation of a stable metabolite whose structure differs markedly from that of the parent, as may occur on an internal cleavage mediated by oxidative or hydrolytic processes, and the toxicological properties of that metabolite may well be quite distinct from those of the parent, for example, cyanide ion formed on hydrolysis of the naturally occurring glycoside amygdalin (Leatrole) [36]. The reverse situation also can apply, namely, that the toxicity resides in the parent drug but metabolism leads to a nontoxic product because of a significant change in structure–activity relationships (SARs) for the biological targets that mediate the therapeutic effects versus the toxicity. A classic example of the latter scenario is found with terfenadine (Seldane), a potent histamine H₁ antagonist that was marketed for the treatment of seasonal allergies. Although not appreciated at the time of launch in 1985, terfenadine binds avidly to human ether-à-go-go related gene (hERG) (the α -subunit of the cardiac I_{Kr} (potassium-dependent delayed rectifier current) potassium channel), blockade of which leads to delayed ventricular polarization, prolongation of the QTc interval and, in extreme cases, potentially fatal cardiac arrhythmias [37]. In contrast, fexofenadine, the major metabolite of terfenadine in humans and the most abundant drug-related material

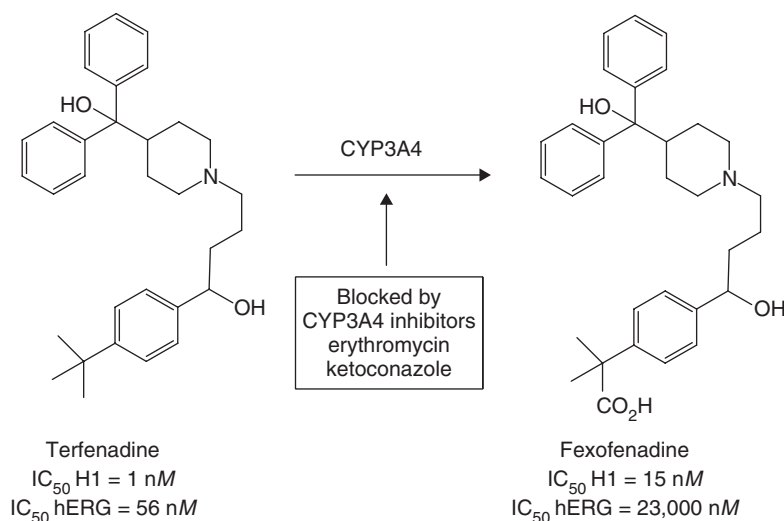


Figure 7.2 Metabolism of terfenadine to fexofenadine catalyzed by CYP3A4. This oxidation pathway results in a modest loss of activity at the histamine H1 receptor but in a large decrease in affinity for the hERG channel. Potent inhibitors of CYP3A4, for example, erythromycin and ketoconazole, effectively block the conversion of terfenadine to fexofenadine, and thereby increase exposure to terfenadine to levels that cause cardiotoxicity through prolongation of the QTc interval.

in the systemic circulation following an oral dose, has negligible binding affinity to hERG but retains good antihistamine activity. The conversion of terfenadine to fexofenadine in humans is catalyzed by CYP3A4 [38], and potent inhibitors of this enzyme, such as erythromycin and ketoconazole, block the metabolic “detoxification” process and lead to blood levels of terfenadine at which cardiotoxicity is manifest (Fig. 7.2). Recognition of the molecular basis of this serious drug–drug interaction led to the removal of terfenadine from the market in 1997 and its replacement by the metabolite fexofenadine (Allegra). While this drug interaction attracted widespread attention and had profound consequences for the pharmaceutical industry (introduction of high throughput screens for hERG binding in drug discovery programs), it also underscores the necessity to fully understand, at as early a stage as possible, the spectrum of off-target activities associated not only with the parent drug, but with its major circulating metabolites. This applies particularly to inhibition of the hERG channel (potential for cardiotoxicity) and to the inhibition of human CYP enzymes (potential for drug–drug interactions). While SARs have been relatively well established for hERG inhibition [39], much remains to be learned about those for CYP inhibition, and relatively few studies have examined the contribution of circulating human metabolites to drug–drug interactions that occur at the level of CYP inhibition [40].

7.4 REACTIVE DRUG METABOLITES

As pointed out above, there are relatively few cases where stable drug metabolites are believed to mediate the toxicity of their respective parent compounds. In contrast,

chemically reactive metabolites, most commonly generated via oxidative reactions catalyzed by the cytochrome P450 (CYP) family of enzymes [41], have been implicated in a variety of drug-induced toxicities [5,42,43]. The deleterious effects of these short-lived electrophilic species are thought to arise from their propensity to cause oxidative stress and/or to covalently modify biological macromolecules (primarily proteins and nucleic acids) and thereby alter normal cellular function. For example, covalent adduction of DNA by “hard” electrophiles can lead to mutagenesis, carcinogenesis, and teratogenesis [44], while “soft” electrophiles typically target nucleophilic centers on peptides and proteins and have been implicated in both target organ toxicities (notably those involving the liver [45–47]) and idiosyncratic reactions mediated by the immune system [48–51]. In the area of human pharmaceuticals, most reactive metabolites fall into the category of “soft” electrophiles, the formation of which can be inferred from their *S*-linked conjugates with the tripeptide GSH or through their covalent adducts with proteins which are most effectively detected with the aid of a radiolabeled analog of the drug [1,52]. It is of interest to note that, of the four broad categories of drug-induced toxicity proposed by Park *et al.* [53], reactive metabolites are proposed to play a central role in three of these mechanisms, when they have been associated mainly with hepatotoxicity, skin reactions, and blood dyscrasias [42]. However, it is equally important to point out that not all reactive metabolites are toxic, and the formation of adducts with GSH and proteins is not, *per se*, indicative of a hazardous compound [52]. Nevertheless, an analysis of drugs that have been removed from the market (or labeled with a “black box” warning) because of safety concerns has shown that, next to primary or secondary pharmacology, the major reason was direct organ toxicity, potentially associated with metabolic activation events. This suggests that reactive metabolite formation, while not an accurate predictor of drug toxicity in humans, represents a significant risk factor in drug development.

Growing concerns over the high attrition rates in drug development, and the recognition that toxicity is a major contributing factor, has led most pharmaceutical companies to implement strategies that aim to minimize potential metabolic activation liabilities in their development candidates. In 2004, the Merck group proposed a strategy based on an assessment of the covalent binding properties of lead compounds emerging from drug discovery programs, and outlined a set of decision-making criteria on which to advance lead candidates [54]. Metabolic profiling studies were performed in parallel to the measurements of covalent binding, focusing on the formation of GSH–drug conjugates whose structures provide a rationale for chemical modification to abrogate the formation of reactive metabolites [55]. Updates to the Merck strategy have been published as more practical experience has been gained [56–59], and a number of commentaries have appeared on approaches to minimize metabolic activation in drug discovery programs [60–63]. Much has been made of “toxicophores” or “structural alerts” (structural motifs that have been associated with metabolic activation to reactive electrophiles) [64], and Kalgutkar *et al.* [65] have published a comprehensive listing of functional groups that have been shown, in certain molecules, to undergo bioactivation to electrophilic intermediates. While helpful to the medicinal chemist engaged in drug design, it should be borne in mind that the presence of a toxicophore in a given drug structure is relevant only when that functional group is accessible to the appropriate activating enzyme in a biological milieu; in that context, it is important to assess experimentally whether metabolism of a promising drug candidate indeed occurs at the functionality of concern before rejecting the molecule from consideration. In some

examples, the toxicophore may be as simple as an electron-rich benzene ring, as was the case in the lead candidate in a cannabinoid-1 receptor (CB-1 R) inverse agonist program (compound **1**, Table 7.1), which underwent sequential metabolic oxidations to afford reactive arene oxide and *ortho*-quinone intermediates that were captured in the form of their GSH adducts [66,67]. Labeling of **1** with tritium demonstrated that this compound gave rise to very high levels of protein covalent binding (~3900 pmol-equiv/mg protein) in both rat and human liver microsomal preparations, and a series of analogs were prepared with the goal of reducing this bioactivation liability, while simultaneously retaining (or enhancing) the favorable pharmacological and pharmacokinetic characteristics of the lead structure. The modifications centered on reducing the electron density on the offending aromatic ring, through introduction of fluorine atoms (**2**), replacement of the phenoxy substituent by a 2-pyridyloxy (**3**) or 5-CF₃-2-pyridyloxy group (**4**), each of which led to progressively lower levels of covalent binding *in vitro*. The final compound in the series (**5**), which incorporated a nitrile substituent in the distal phenyl ring, exhibited a very low level of covalent binding (27 pmol-equiv/mg protein), had excellent Pharmacokinetic (PK) properties in rats and was almost an order of magnitude more potent at the CB-1 R than the initial lead. On the basis of these attributes, compound **5** was advanced into development, and became Taranabant, Merck's candidate CB-1 R inverse agonist for the treatment of metabolic syndrome [68].

In those situations, where electrophilic metabolites are highly reactive, alkylation of the apoprotein and/or prosthetic heme moiety of the responsible CYP enzyme may occur, resulting in mechanism-based inactivation of the enzyme. This phenomenon, which manifests as a time-dependent loss of enzyme activity *in vitro* (and often is the first indication that a new drug candidate is subject to metabolic activation), has potentially important safety implications for clinical drug–drug interactions with cosubstrates of the P450 isoform in question, and molecules with this undesirable property normally are “screened out” at the discovery stage [69–72]. Similarly, metabolism of certain functional groups, notably the methylenedioxyphenyl moiety and substituted aliphatic amines, affords electron-deficient intermediates that coordinate tightly to the Fe^{II} oxidation state of CYP heme and form inhibitory “metabolic intermediate” (MI) complexes that can be detected spectrophotometrically through their characteristic λ_{max} at 455 nm [71,72]. An example of a lead optimization program which focused on the removal of MI complex formation from an important lead molecule was reported by Tang *et al.* [73] and illustrates how an early understanding of potential metabolic liabilities in a given structural class can be key in guiding medicinal chemistry efforts. *In vitro* investigation of the melanocortin-4 receptor (MC4R) agonist **6** (Fig. 7.3), being explored for a weight-loss indication, showed that this compound inhibited CYP3A enzyme activity in nicotinamide adenine dinucleotide phosphate (NADPH)-fortified rat and human liver microsomal preparations and yielded a typical MI complex spectrum when incubated with recombinant CYP3A4 (Fig. 7.4). Since compound **6** contained an aliphatic amine moiety, it was hypothesized that sequential CYP-mediated N-oxidation afforded the corresponding hydroxylamine and nitroso derivatives and that the latter species coordinated with P450 heme iron to generate the observed MI complex. This hypothesis was tested by *in vitro* trapping experiments with cyanide ion [74], which resulted in the identification by LC-MS/MS of a cyano adduct whose formation could be rationalized by invoking capture, not of the nitroso species per se, but of the tautomeric oxime, followed by a further two-electron oxidation step of the adduct to give the final

TABLE 7.1 Covalent Binding and Rat Pharmacokinetics of Investigational CB-1 R Inverse Agonists

Compound	Ar	X	CB1R IC ₅₀ (nM)	Covalent Binding (pmol-equiv/mg)	Rat Pharmacokinetics ^a			
					CL _p [(mL/min)/kg]	<i>t</i> _{1/2} (h)	<i>C</i> _{max} (μM)	<i>F</i> (%)
1	Ph	H	2.03	3900	33	2.8	0.18	9
2	3,5-F ₂ -Ph	H	1.47	1700	35	2.4	0.30	19
3	2-pyr	H	1.80	910	25	2.2	0.44	29
4	5-CF ₃ -pyr	H	0.54	88	35	2.2	1.2	100
5	5-CF ₃ -pyr	CN	0.29	27	33	2.7	0.49	74

^aCL_p, rate of compound clearance from plasma; *t*_{1/2}, half-life of compound circulating in plasma; *C*_{max}, maximum concentration of compound achieved after an oral dose of 2 mg/kg; *F*, oral bioavailability.

Source: Adapted from Ref. 67.

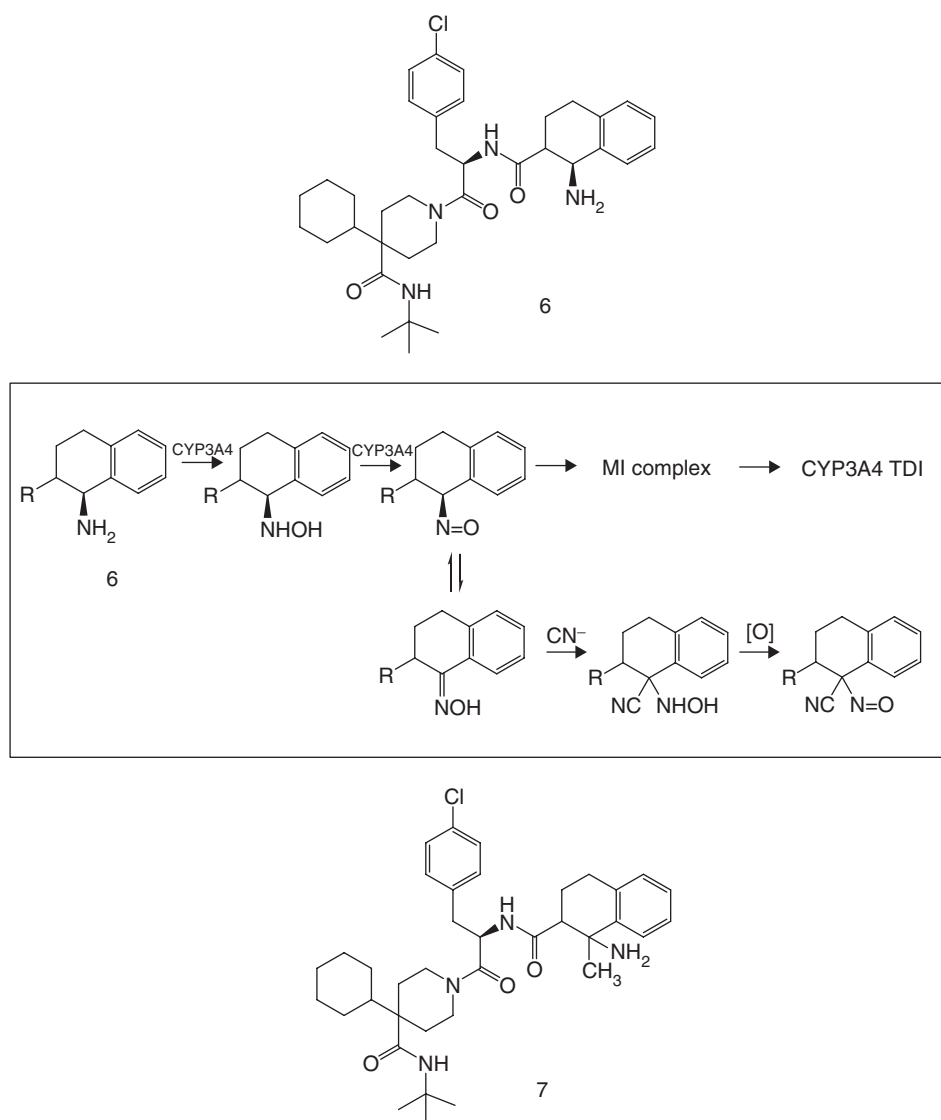


Figure 7.3 Structure of a lead MC4R agonist **6** and the proposed mechanism by which it caused time-dependent inhibition (TDI) of CYP3A4. Trapping experiments with CN^- , which afforded the indicated cyano-nitroso species, led to design of **7**, in which the methyl substituent α - to the primary amine introduced sufficient steric hindrance to suppress oxidation at this center to the inhibitory nitroso metabolite.

product (Fig. 7.3). These findings led to the design of compound **7**, in which a methyl group was introduced α - to the primary amine functionality in order to sterically hinder the offending N-oxidation reaction. This simple structural modification afforded a compound with greatly diminished MI complex formation and CYP3A inhibitory properties, thereby provided a drug candidate with an improved safety profile in terms of potential drug–drug interaction liabilities [73].

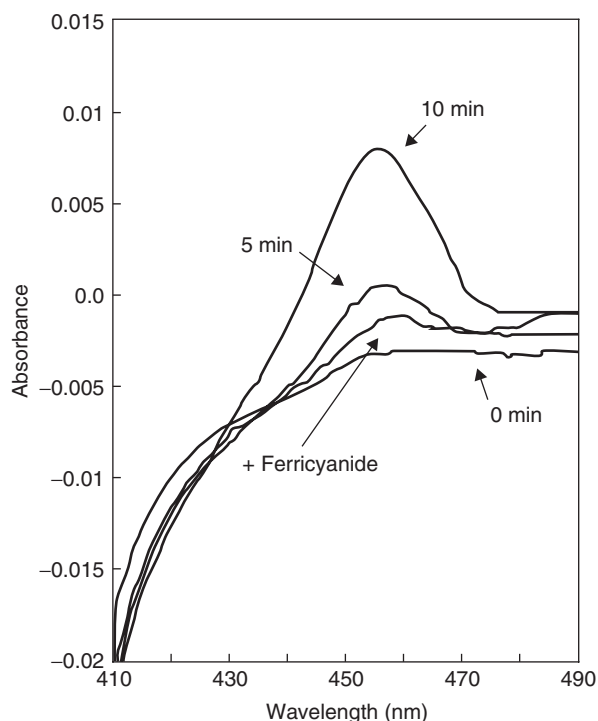


Figure 7.4 Spectrophotometric detection of the MI complex formed in incubations of **6** with recombinant CYP3A4. The absorbance increased with increasing incubation time (0, 5, and 10 min) and decreased on addition of potassium ferricyanide which converts heme iron to the Fe^{III} oxidation state and dissociates the MI complex. *Source:* Adapted from Ref. 73.

In the foregoing examples, it will be evident that a number of analytical techniques are employed in the detection of reactive metabolite formation, for example, UV spectroscopy for MI complexes, use of radiotracers for metabolic profiling and covalent binding studies, and *in vitro* nucleophilic trapping experiments with structural analysis by LC-MS/MS or NMR. By far, the most widely used nucleophile for the latter purpose is GSH, due both to its physiological role as an endogenous scavenger of potentially damaging electrophiles and to the favorable mass spectrometric characteristics of the resulting drug–GSH adducts under a variety of ionization conditions [75–78]. In recent years, a variety of LC-MS/MS-based methods have been reported for screening drug candidates for reactive metabolite formation *in vitro*, based on trapping experiments with GSH itself, or analogs such as GSH ethyl ester [79], quaternary ammonium derivatives of GSH [80], and stable isotope-labeled ($^{13}\text{C}_2$ and ^{15}N) GSH [81,82]. An interesting variation on this theme is the use of γ -glutamylcysteinyllysine (γ -GSK) as a bifunctional trapping agent, which has been shown to capture both “soft” and “hard” electrophiles [83]. The advent of LC-MS/MS systems with rapid scanning mass analyzers (e.g., time-of-flight) capable of operating under conditions of high mass resolution has provided an additional dimension in screening technology, based on elemental composition data and the technique of mass defect filtering [82,84–87]. Despite the power of mass spectrometry-based methods for the detection

and identification of GSH adducts, such approaches do not yield reliable information of a quantitative nature in the absence of authentic standards of the conjugates of interest because of differences (sometimes quite marked) in the ionization efficiency of individual adducts. Attempts have been made to address this problem by the use of ^3H - or ^{35}S -labeled GSH as trapping agents, with radiometric analysis of the products [88,89] or dansyl GSH followed by fluorometric analysis [90], both of which can provide quantitative information in the absence of synthetic reference materials. It is generally assumed that the reaction of GSH with reactive drug metabolites *in vitro* is predominantly a chemical process, although in certain cases, glutathione-S-transferase-mediated conjugation may contribute to product formation; analogs of GSH (or GSH surrogates, e.g., *N*-acetylcysteine) would not be appropriate for quantitative purposes in such situations since these nucleophiles do not serve as substrates for the transferase enzymes. While efforts have been made by some investigators (with limited success) to correlate thiol formation in liver preparations *in vitro* with measures of covalent binding *in vivo* and hepatotoxicity in animals or humans [91,92], it would seem that the greater value of GSH trapping experiments lies in the structural information that can be inferred on the identities of the reactive electrophiles being captured. When this insight is provided at a sufficiently early stage in drug discovery programs, rational structural changes can be made in lead compounds to block, or suppress, pathways of bioactivation, thereby minimizing the potential for reactive metabolite-mediated toxicities [93]. Although the foregoing discussion has focused on the detection of reactive metabolites using appropriate *in vitro* systems, extension of these studies to *in vivo* investigations typically rely on the collection of bile from animals dosed with the NCE of interest, and the identification of GSH adducts present in that fluid. Alternatively, the excretion of *N*-acetylcysteine (mercapturic acid) conjugates in urine, either of animals or human subjects, provides an insight into bioactivation processes occurring *in vivo* [77].

In assessing the potential risk to humans associated with a development candidate that has been demonstrated to undergo metabolism to reactive intermediates, a number of considerations need to be borne in mind, for example, the chemical tractability of the structural series from which the candidate was selected, the availability of existing treatments for the target disease, the severity of that disease (life-threatening or otherwise), the anticipated daily dose (and hence body burden of reactive metabolites), the expected duration of therapy (which will determine long-term exposure to reactive metabolites), the intended clinical population (adult vs children), and the balance between bioactivation and detoxification pathways *in vivo* [54,93]. It also should be recognized that chemically reactive intermediates will exhibit a large range of reactivities in a biological environment, which will influence their ability to migrate from sites of formation within the cell to other organelles, or even to distant organs. For example, the diquinonemethide metabolite of raloxifene, a selective estrogen receptor modulator, has been shown to have a half-life of <1 s in aqueous phosphate buffer at 5°C , while an *ortho*-quinone metabolite of the same drug exhibited a half-life of 69 min in the same medium at 37°C [94] (Fig. 7.5). Thus, while the majority of electrophilic drug metabolites have biological lifetimes that are too short to allow for their detection and characterization as the native species, a subset of these products will have relatively low chemical reactivities and therefore will be detectable in the circulation and excreta. A prominent example of the latter group is the class of acyl glucuronide conjugates, which are formed from many carboxylic acid-containing xenobiotics and frequently

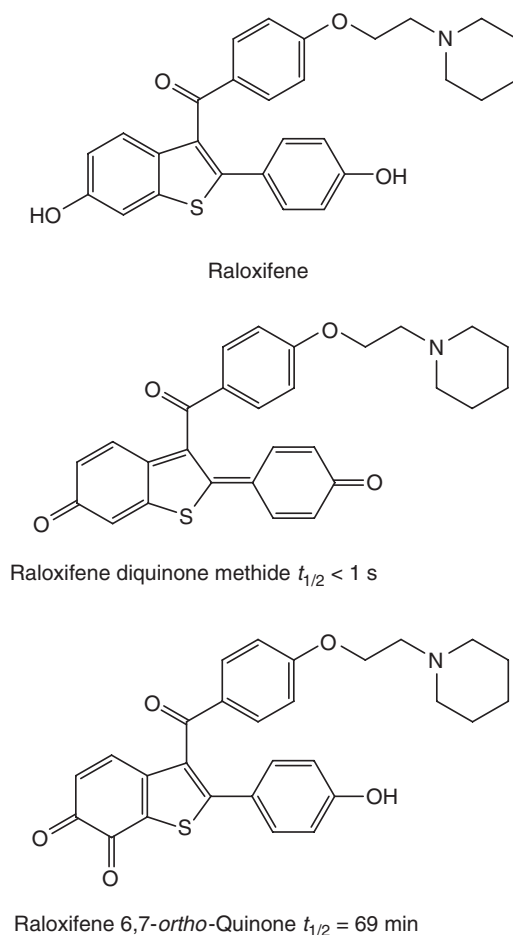


Figure 7.5 Structures of raloxifene and two quinoid metabolites with markedly different stabilities in aqueous phosphate buffer.

circulate in the bloodstream or are eliminated in bile or urine [95–98]. Owing to the propensity of these ester-linked conjugates to undergo intramolecular rearrangement reactions with resulting exposure of α -hydroxy-aldehydes from the sugar ring, they possess the ability to form stable covalent adducts to proteins. Direct acylation of proteins also can occur with acyl glucuronides (as is the case with the much more reactive acyl-coenzyme A thioesters, which usually do not escape the cell). In light of their ability to covalently modify proteins, concerns have been raised over the safety of acyl glucuronides as a structural class. Specifically, it has been noted that acyl glucuronide conjugates may be concentrated in hepatocytes and the biliary tree due to vectorial transport by export pumps, where they may bind selectively to canalicular membrane proteins [95]. Such concerns have been heightened by the fact that a relatively large proportion of drug withdrawals because of hepatotoxicity have involved carboxylic acid derivatives that yield acyl glucuronide conjugates [99]. Despite the fact that more recent investigations of some of these agents (e.g., zomepirac and tolmetin [100]) have

revealed pathways of bioactivation that involve metabolic *oxidations*, there has been a tendency to associate acyl glucuronide formation with a risk of adverse drug reactions, notably liver injury. Such a broad generalization seems inappropriate, since many carboxylic-acid-containing therapeutic agents that give rise to acyl glucuronides have a favorable safety profile, yet acyl glucuronide conjugates are referred to collectively in the FDA guidance as *toxic molecules* [3]. While it may be possible to assess the intrinsic reactivity of a particular acyl glucuronide *in vitro*, for example, by monitoring rates of intramolecular migration or direct acylation of model nucleophiles, there currently is a lack of consensus on the value of such information for risk assessment purposes. It is perhaps more appropriate, therefore, to treat this class of conjugates in the same fashion as stable drug metabolites, where exposure margins in animal species are taken as indices of safety in humans.

Unlike the situation with stable drug metabolites, there are no regulatory guidelines that deal specifically with chemically reactive metabolites, although the European Medicines Agency acknowledges that drug-induced hepatotoxicity may, in some cases, be mediated by reactive metabolites [101]. The lack of a direct correlation between the formation of electrophilic drug metabolites and drug-induced toxicity has confounded the interpretation of data on metabolic activation and covalent binding to proteins and underscores our general lack of understanding of the molecular mechanisms by which reactive intermediates damage cells [52]. While studies aimed at correlating the extent of covalent binding *in vitro* of hepatotoxic versus nonhepatotoxic drugs have yielded conflicting results [102,103], experimental protocols based on the use of human hepatocytes that incorporate estimates of dose (or body burden of reactive metabolites) have shown some promise as predictors of hepatotoxic potential [92,104–106]. It has been suggested that, for practical purposes, an estimated reactive metabolite body burden in humans of <10 mg/day may represent an acceptable risk [7,8], although this proposed upper limit probably will be highly dependent on the nature of the reactive metabolite in question, the identities of its macromolecular targets, and individual host susceptibility factors. Currently, very little is known about the protein targets of reactive drug metabolites and even less about those structural modifications induced by protein alkylation that have toxicological consequences [107]. Advances in the techniques of proteomics mass spectrometry have made it possible to address the structural issues [108], but analysis of the hundreds (or thousands) of proteins that are subject to chemical modification by reactive drug metabolites presents a formidable challenge. For these reasons, it seems prudent, in the context of drug discovery programs, to incorporate a formal assessment of metabolic activation of NCEs at an early stage (e.g., during lead optimization) with a view to minimizing, through appropriate structural modifications, the risk of downstream adverse drug reactions mediated by chemically reactive metabolites. As noted above, most major pharmaceutical companies now subscribe to this general approach, although individual corporate strategies differ in terms of the timing of such studies and their experimental design.

In considering the role of reactive intermediates in drug toxicity, it should be pointed out that some important therapeutic agents actually elicit their pharmacological effects through chemically reactive metabolites or products of chemical rearrangement that covalently modify their biological targets. Thus, the thieno-tetrahydropyridine antiplatelet agents (e.g., ticlopidine and clopidogrel [109]) and the proton pump inhibitors (e.g., omeprazole, esomeprazole, and lansoprazole [110]) undergo conversion *in vivo* to reactive sulfenic acid species which, in turn, form disulfide

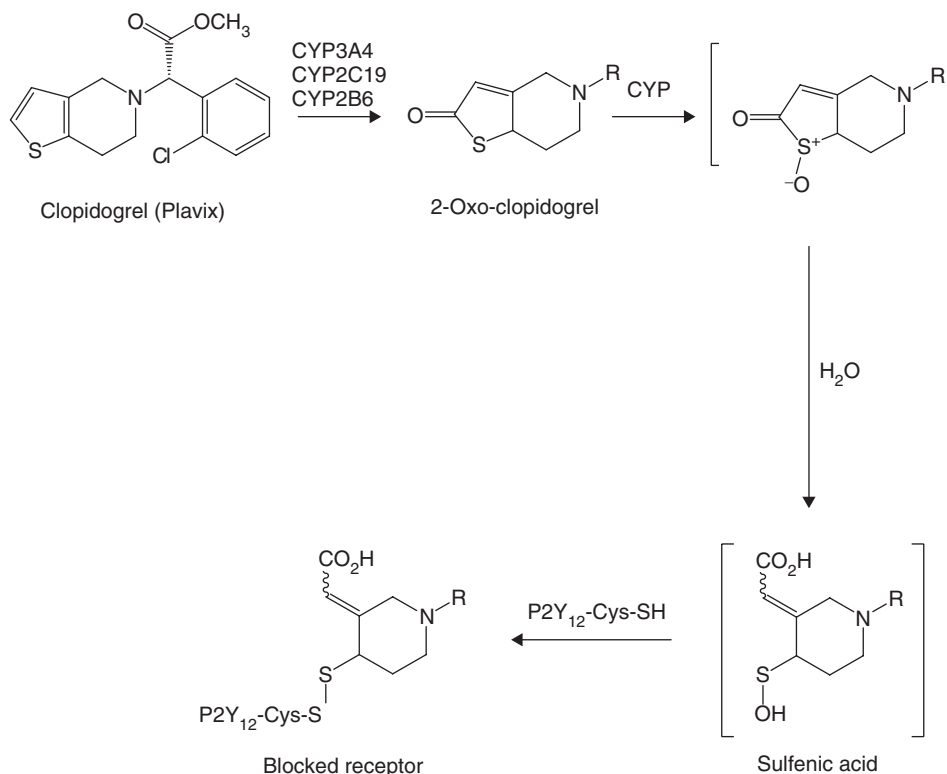


Figure 7.6 Cytochrome P450-mediated metabolism of clopidogrel to an acyclic sulfenic acid that binds covalently to an active site cysteine -SH group on the platelet P2Y₁₂ receptor.

linkages with active site -SH groups of their respective targets (P2Y₁₂ receptor on the surface of blood platelets and H⁺/K⁺ adenosine triphosphatase (ATPase) at the secretory surface of the gastric parietal cell). As an example, the mechanism by which clopidogrel (Plavix) inactivates its target receptor is depicted in Fig. 7.6. The relative safety of these widely used drugs probably is related to the high degree of specificity with which their active moieties interact with their target proteins, and the fact that the covalent linkage in each case is a disulfide bond, which can be reduced over time to regenerate the native protein. Interestingly, this concept of specific, reversible covalent modification of proteins has been advanced as a discrete strategy in the design of a new generation of potent, relatively long-acting therapeutic agents with low potential for toxicity [111].

Finally, reactive metabolites need to be taken into consideration when assessing the genetic safety of new pharmaceuticals, given the well-established role of drug metabolism in the genotoxicity and carcinogenicity of chemicals [44]. Traditionally, the potential of a new drug candidate and its mammalian metabolites to cause genetic toxicity is evaluated during the early stages of drug development through a combination of *in vitro* studies (e.g., using liver microsomal preparations from Aroclor-pretreated rats to enhance the production of metabolites) and *in vivo* assays (e.g., micronucleus assay in rat or mouse) [2]. Only in those cases where metabolism studies have identified a “unique” human metabolite, or where the structure of a metabolite of the

drug candidate of interest contains a genotoxicity “alert,” are follow-up studies normally performed with the metabolite per se. For example, when a human metabolite is not generated in rats or mice, assessment of its genotoxic potential could include the use of an alternative or optimized *in vitro* metabolic activation system or direct testing of the isolated or synthesized metabolite [112]. In recent years, a number of *in silico* approaches, involving expert systems or statistical modeling techniques, have been applied to predict human genotoxicity risk [113], and these have been adopted by both pharmaceutical companies and regulatory agencies as a screen for potentially problematic drug candidates, their known metabolites, and synthetic impurities in the active pharmaceutical ingredient. In situations where a drug candidate yields a human metabolite that tests positive in one or more assay in a genotoxicity panel, due consideration needs to be given to issues such as the intended therapeutic indication of the parent compound, the projected dose and duration of treatment, and the likely body burden of the genotoxic intermediate, in developing an overall strategy for risk assessment [114].

7.5 CONCLUSIONS

On the basis of the above discussion and the examples presented in this chapter, it will be evident that drug metabolism as a discipline plays a unique role within the pharmaceutical industry, in that it serves to bridge drug discovery projects to both preclinical and clinical drug development. In particular, a sound knowledge of the pharmacokinetic, metabolic, and dispositional characteristics of NCEs in animals, coupled with information on known or suspected human metabolites, represents a critical body of data for validating the choice of the animal species selected for the preclinical toxicology program. Evidence for the formation of chemically reactive metabolites has clear implications for the medicinal chemist with regard to logical directions for lead optimization, while at the clinical level, information on exposure margins for circulating stable metabolites dictates the need, if any, for further safety evaluation of specific biotransformation products. For all these reasons, drug metabolism studies represent an integral component of today’s drug safety assessment programs.

Looking to the future, one can anticipate that current advances in the fields of genomics, proteomics, and metabolomics will combine to provide a detailed insight into the numerous mechanisms by which foreign compounds elicit their specific adverse effects, and thereby provide a link between chemical structure and molecular toxicology. New tools will emerge at the drug metabolism/safety assessment interface, for example, the use of genetically engineered mouse models that express human P450 enzymes to address issues related to species differences in metabolism and toxicity [115,116]. An improved understanding of the role of redox-sensitive signaling pathways, for example, Kelch-like ECH-associated protein 1 (Keap1)-Nrf2-ARE [117], in protecting against the potentially deleterious effects of reactive drug metabolites, could lead to the development of *in vitro* screens for chemical stress and idiosyncratic drug toxicity, and improved biomarkers of cellular damage due to exposure to electrophilic drug metabolites likely will emerge, for example, induction of heme oxygenase-1 in human hepatocytes [118]. The intriguing question of what makes one human subject more susceptible to the toxic effects of a drug/drug metabolite than hundreds (or thousands) of other patients may be answered, at least in part, by genetic determinants

in the immune system, as has been shown to be the case with abacavir hypersensitivity which is strongly correlated with the presence of the human leukocyte antigen (HLA)-B*5701 [119]. It seems inevitable that the Holy Grail of predictive toxicology will emerge from close partnerships between drug metabolism and industrial safety assessment—an exciting prospect indeed!

ABBREVIATIONS

ADME	absorption, distribution, metabolism, and excretion
AMS	accelerator mass spectrometry
ARE	antioxidant response element
ATPase	adenosine triphosphatase
AUC _p	area under the plasma concentration versus time curve
CB-1 R	cannabinoid-1 receptor
CYP	cytochrome P450
FDA	the US Food and Drug Administration
GSH	glutathione, γ -glutamylcysteinylglycine
hERG	human ether-à-go-go related gene
ICH	International Conferences on Harmonization
IK _r	potassium-dependent delayed rectifier current
Keap1	Kelch-like ECH-associated protein 1
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MAO-B	monoamine oxidase-B
MC4R	melanocortin-4 receptor
MI	complex metabolic intermediate complex
MIST	metabolites in safety testing
NADPH	nicotinamide adenine dinucleotide phosphate
NCE	new chemical entity
NMR	nuclear magnetic resonance spectroscopy
SAR	structure–activity relationship
TDI	time-dependent inhibition
Nrf-2	nuclear factor-erythroid 2-related factor 2

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8 *In Silico* Models of Drug Metabolism and Drug Interactions

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8.1	Summary	1
8.2	Introduction	2
8.3	Tools	6
8.4	Applications	8
8.5	Incorporating <i>in silico</i> methods in drug discovery	22
8.6	Conclusions	29
	References	31

8.1 SUMMARY

Determination of the nature and the rates of any active processes that are involved in the absorption, distribution, metabolism, and elimination (ADME) of drug candidates is a primary goal of drug discovery. These processes include metabolism and active transport. Metabolism entails the (usually) irreversible enzyme-mediated conversion of the compound to a different form. Active transport entails the movement of an unchanged compound, mediated by a transporter protein, across a biological membrane (frequently the plasma membrane). Within drug discovery, *in vitro*, *in situ*, and *in vivo* methods for estimating human drug metabolism and transport are becoming increasingly supplemented by a wide range of *in silico* methods aimed at reducing costs, reducing animal usage, and increasing throughput of compound screening.

These active processes are potentially subject to drug–drug interactions, whereby one drug (or, in general, any xenobiotic) alters the rate of metabolism or transport of a second xenobiotic *in vivo*, leading to changes in plasma and target-site concentrations of the affected drugs. Several mechanisms for such interactions exist, including reversible or irreversible inhibition of transporters and enzymes and increases in their expression mediated by activation of nuclear receptor proteins. *In silico* methods are being developed to predict these effects, to reduce the cost and time burden of experimental screening and to mitigate the likelihood that undetected drug–drug interactions with undesirable consequences could compromise otherwise promising drug candidates.

Following a brief review of drug metabolism and drug–drug interactions (Section 8.2), we present a high level view of some of the areas in which *in silico* modeling can contribute to the quantitative or qualitative understanding and prediction of drug metabolism and drug–drug interactions (Sections 8.2.1 and 8.2.2). We then discuss some of the available *in silico* approaches to these problems (Section 8.3). We start our discussion with methods that just depend on descriptors calculated from compound structure (Section 8.3.1), go on to discuss more complex models that use compound structure more explicitly (Section 8.3.2), then discuss approaches that try to model in detail the interaction between compound and protein (Section 8.3.3). Following this, we discuss in some detail a number of specific application areas in each of which one, or more, of the previously described methods have been used (Sections 8.4.1–8.4.3). These application areas include prediction of the rate of metabolism of a compound; determination of the substrate and/or inhibitor specificity of enzyme and transporter isoforms; and activation of the pregnane X receptor (PXR), a nuclear receptor which is known to be involved in the xenobiotic-mediated induction of a number of drug-metabolizing enzymes and transporters.

The approaches discussed up to this point do not permit the understanding of the interactions between different active processes *in vivo*; we, therefore, then (Section 8.4.5) discuss physiologically based modeling, which, in principle, allows the creation of an integrated model of compound pharmacokinetics that allows the modeling and understanding of all relevant physiological processes involved in drug metabolism and interactions. We finish with a discussion of some practical aspects of applying *in silico* methods in drug discovery (Section 8.5) and our conclusions on the topic (Section 8.6). Having such a large and diverse subject matter to cover, we realize that we cannot be comprehensive and have therefore cited several relevant recent papers for readers to follow-up.

8.2 INTRODUCTION

Drugs and other xenobiotics are potentially subject to metabolic transformation by enzymes and enzyme systems located in the liver, intestine, and other organs. Metabolism of drugs usually leads to reduction, or total loss, of their therapeutic effect, in which case the rate of metabolism can be a prime determinant of their *in vivo* therapeutic utility. Metabolism can sometimes generate toxic metabolites [1], thus contributing to the toxicity of a drug, or to active metabolites, contributing to its therapeutic effect [2]. The latter effect can be deliberately harnessed in the production of prodrugs that have, for example, favorable absorption properties but require metabolism to generate the therapeutically active compound [3,4]. The bulk of xenobiotic metabolism is performed by the cytochrome P450 (CYP) superfamily of enzymes [5], which tend to transform compounds into more polar compounds. These metabolites can be either eliminated directly or become subject to further metabolism [6].

Until relatively recently, the role of active transport in the disposition of drugs had been poorly studied in comparison to the effort put into investigating and understanding drug transport. The effects of phosphoglycoprotein (PGP) (MDR1/ABCB1) and MRP1 (ABCC1) on drug disposition had originally been observed because of their roles in the development of multidrug resistance in cancer cells [7], and there was an appreciation

of their potential in inhibiting drug uptake from the gastrointestinal (GI) tract [8] and from the blood across the blood–brain barrier [9]. Some compounds must be transported into the interior of the hepatocyte to reach the metabolizing enzymes and some compounds can be transported out of the hepatocyte into the bile [10]; the rate of transport can have an effect on the overall rate of removal from the bloodstream [11]. Transporter proteins also affect the absorption of a compound from the gut—some help compound uptake and some pump compound back into the gut lumen [12]—and hence also have an effect on the overall extent of metabolism. Transporter polymorphism can have a significant effect on pharmacokinetics [13–15].

The activity of the metabolizing enzymes and transporter proteins can be induced by a range of drugs or environmental factors and can be inhibited by a range of drugs or foodstuffs [16]. Xenobiotic concentration in plasma or tissues can significantly be affected by interactions with other compounds and can vary by an order of magnitude or more [17]. This can lead to unwanted behavior: either a lack of efficacy at a standard dose caused by an increase in overall clearance or toxic effects caused by a decrease in overall clearance. For a compound with a narrow therapeutic window—when the effective dose is close to the toxic dose—this is particularly significant. Assessment of the potential of a clinical candidate to participate in drug–drug interactions is an integral part of review by regulatory bodies [17]. Compounds that are found to participate in drug–drug interactions must undergo *in vivo* tests to determine the extent of any *in vivo* drug–drug interactions [18].

Inhibition of CYP3A4 has been a particular concern in drug development, and pharmacokinetic changes following CYP3A4 inhibition have led to the withdrawal from market of a number of compounds. Terfenadine, cisapride, and astemizole are CYP3A4 substrates that, in excess, can cause cardiac arrhythmias. If their concentrations rise—due to the inhibition of their metabolism—adverse, potentially fatal, effects can ensue. These side effects on coadministration with CYP3A4 inhibitors led to their withdrawal. The significance of CYP3A4 inhibition relates to two factors. First is the preponderance of this isoform in the metabolism of many drugs and drug-like compounds. Second is that its substrates are often lipophilic (as discussed further below), which increases the likelihood of unwanted off-target interactions *in vivo*, such as those with the human ether-a-go-go-related gene (hERG) protein [19,20] that caused the arrhythmias noted above. The postmarketing discovery of enzyme inhibition has also led to the withdrawal of mibefradil, an inhibitor of CYP3A4 and PGP [21].

Induction of drug-metabolizing enzymes is less dangerous, insofar as the consequence is reduced concentrations of the substrate(s) of the induced enzyme(s), but this does raise the possibility of therapeutic failure of such substrates, with potentially serious consequences. A common mechanism for CYP induction is binding of ligand to nuclear receptors that regulate transcription, which alters the expression of the CYP genes [22]. This means that potential CYP inducers can be identified as potential binders to the relevant nuclear receptors. As examples, plasma levels of CYP3A4 substrates—for instance, oral contraceptives, triazolam, and midazolam—can be reduced by coadministration of CYP3A4 inducers [22–24], leading to a lack of efficacy at standard doses.

There are a number of transporter-based interactions documented in the literature: for instance, an increase in repaglinide plasma concentration following atorvastatin dosing due to an inhibition of OATP1B1 [25], an increase in plasma concentration of rosuvastatin following cyclosporin dosing due to the same effect [26], and an increase

in atorvastatin plasma concentration following rifampin dosing [27,28]. Transporter inhibition does not yet seem to have been as serious as metabolic inhibition, but the capacity for clinically significant changes in pharmacokinetics exists.

Just in this brief discussion of the biochemistry and physiology that affects xenobiotic metabolism, we find some questions that must be answered—qualitatively or quantitatively—during drug discovery, to produce high quality development candidates:

1. The overall rate of metabolism, which affects the tissue concentrations reached after administration, and consequent duration of action of the drug;
2. The identity of major metabolites along with knowledge of whether, or not, they are toxic and/or therapeutically active
3. The enzyme/enzymes that is/are responsible for the majority of metabolism and, where more than one enzyme is involved, the relative proportions of metabolism via each enzyme. This determines the potential for
 - a. Differences in metabolism between different individuals of the target population
 - b. Effects of polymorphisms in drug-metabolizing enzymes
 - c. Inducibility of drug metabolism, and the identities of potential inducers
 - d. Inhibitability of drug metabolism, and the identities of potential inhibitors
4. Potential effects on metabolism of other drugs that could be coadministered, whether by induction or inhibition of drug-metabolizing enzymes.

Furthermore, with the increasing recognition of the importance of active transport in affecting the disposition and elimination of drugs, there is a corresponding increase in emphasis being given to determining the analogous properties (excluding 2) relevant to drug transport in the liver, kidneys, intestines, and other tissues.

8.2.1 Why Is *In Silico* Modeling Necessary?

All the above properties can be determined, in principle, by appropriate *in vitro* assay. But a liability in any one property could cause costly clinical failure of a candidate, so there is a consequent drive to determine these properties as early as possible within drug discovery. This leads to increasing costs and compound requirements in lead generation and optimization, as additional assays are developed and used to inform the discovery process. This generates competition for resources in lead identification and lead optimization that requires careful prioritization for successful outcomes at affordable costs. Improvements in assay miniaturization and throughput [29] have helped offset these costs, but there is still a potentially crucial role for *in silico* modeling to inform prioritization of assays, and hence to ease the competition for resources throughout drug discovery. First, predictive models for *in vitro* assay endpoints can be used to reduce the number of assays to be performed and/or to increase the number of compounds—whether real or virtual—that can be screened. Second, models can be used to extrapolate from *in vitro* assay endpoints (or predictions thereof) to predict *in vivo* pharmacokinetics. A variety of *in silico* techniques are used across the entire range of ADME, toxicity, pharmacology, and physicochemical properties [30–40], of which applications relating specifically to drug metabolism and drug–drug interactions [17,41–47] are a subset.

8.2.2 Problems for *In Silico* Modeling

This leaves us with a number of problems that could be addressed by *in silico* modeling.

1. Predict the total rate of metabolism of a compound.
2. Predict whether a compound is metabolized by a given enzyme, or enzyme isoform, and the rate of such metabolism. One difficulty is that the enzymes, such as CYPs and uridine 5'-diphosphoglucuronic acid (UDPGA) glucuronosyl transferases (UGT), that control a significant fraction of drug metabolism are promiscuous, metabolizing a wide range of substrates. If interactions with a given metabolizing enzyme are predicted, predict the kinetics of transformation, and also the metabolite or metabolites formed.
3. Predict whether a compound is transported by a given transporter and the rate of such transport. Just like the metabolizing enzymes, the transporters are promiscuous proteins that interact with a variety of drugs, and any interaction with a transporter is a potential source for drug–drug interactions with compounds that affect that transporter. If interactions with a given transporter are predicted, this can give some idea of the population variability that may be expected.
4. Predict whether a compound inhibits metabolism or transport of itself or other compounds. Is the compound likely to prevent the metabolism or transport of other compounds? Does it affect important enzymes, risking interactions with many other drugs, or does it just affect a relatively insignificant enzyme?
5. Predict whether a compound induces metabolizing enzymes or transporters. Does the compound bind to a nuclear receptor, causing increased expression of CYP or transporter protein? Which enzymes does it induce; are they important, or relatively insignificant in the metabolism of the drug, or of coadministered drugs?
6. Predict or model, quantitatively, total *in vivo* clearance or concentration time profiles. What effects do metabolism and transport have on the compound's pharmacokinetics, and how do they interact with absorption and distribution. If one can model *in vivo* metabolism and transport to the extent of individual enzymes, one could begin to understand the compound's susceptibility to population variability and enzyme induction or inhibition. A model of this class is a useful tool: one can ask the questions “by how much do I have to inhibit metabolism to increase plasma concentration of my drug or a probe substrate by x -fold?” or “by how much do I have to induce metabolism to decrease plasma concentration of my drug or a probe substrate x -fold?,” where the value of x can be dependent on the drug's therapeutic window. It is possible to begin to understand the true *in vivo* significance of an interaction discovered *in vitro*.
7. Predict or model, quantitatively, inhibition or induction effects due to the dosed compound or caused by a coadministered drug. With knowledge of effective and toxic concentrations, a model of this class directly addresses changes in the therapeutic window of the compound or of a coadministered drug. Such a model also aids in the design of *in vivo* clinical studies that examine an interaction in detail. One may, perhaps, attempt full prediction from *in vitro* data, or one may take the single-compound pharmacokinetics as given and just attempt to model the compound–compound interaction.

8.3 TOOLS

In this section, we discuss in some detail the different *in silico* approaches to drug metabolism and drug–drug interaction. Generally, we must consider compound interactions with varying promiscuous proteins, whether transporters, metabolizing enzymes, or nuclear receptors. Given this similarity among the different problems, we look at each modeling approach in turn and discuss some advantages and disadvantages of the methods.

8.3.1 Compound Descriptor Methods

A compound can be characterized by a set of molecular descriptors that are classed according to the dimension of the chemical formula used to derive them. One-dimensional descriptors are calculated from the empirical formula and include quantities such as the molecular weight and atom counts. Two-dimensional descriptors come from the planar projection of the chemical structure and include, for example, the number of hydrogen bond donors and acceptors. Finally, the three-dimensional structure of the compound gives rise to three-dimensional descriptors such as charge distribution parameters like the dipole and quadrupole moments [48]. These molecular descriptors form the basis of a quantitative structure–activity relation (QSAR), which relates these molecular descriptors with a specific activity, such as metabolic rate or protein binding affinity, for a training set of compounds [39,49,50]. A huge number of molecular descriptors can be calculated for each compound [48]. However, sensitivity analysis can identify those descriptors having greatest influence over the structure–activity relationship, and restricting the descriptors to this select group helps prevent over-fitting to the compound training set. The major advantage of using compound descriptor methods to predict enzyme metabolism/inhibition is that structural knowledge of the active site is not required so this method can be used to predict enzyme activity when the enzyme structure is unknown.

8.3.2 Compound Structure Methods

The following methods utilize the compound geometry: either the three-dimensional structure or its two-dimensional projection onto a plane. The geometry provides a structural understanding of binding into an active site and we therefore expect compound structure methods to be superior to methods based purely on compound descriptors (Section 8.3.1). Furthermore, as with compound descriptor methods, compound structure-based methods do not utilize the protein structure and can therefore be used when the protein structure is unknown [51–55].

8.3.2.1 Pharmacophore Methods. A pharmacophore is the collection of the most important structural features relevant for a given biological activity from a series of molecules with a similar mechanism of action [56]. Pharmacophore features are, for example, hydrogen bond donors, hydrogen bond acceptors, acidic centers (centers with a negative charge at neutral pH), hydrophobic regions, and aromatic rings [57]. The set of features with their corresponding positions within the molecule then define the pharmacophore. By comparing a set of known enzyme substrates to a set of known

nonsubstrates, the common set of pharmacophore features required for binding to the enzyme active site can be identified. This method can therefore classify other compounds as being substrates or nonsubstrates, depending on whether they contain the same pharmacophore features. However, it will not predict the strength of binding to the enzyme active site and so cannot differentiate between compounds of the same class.

8.3.2.2 Comparative Molecular Field Analysis. Comparative molecular field analysis (CoMFA) is a particular three-dimensional QSAR technique. It requires that a training set of compounds are all aligned, according to some rule, within a three-dimensional grid. The steric and electrostatic fields are calculated for each compound at each point on the grid, which are then correlated with the compound activity. CoMFA is a widely used method in drug discovery, but its success is sensitive to the molecular alignment step. By aligning the compounds, the assumption is made that all compounds have the same orientation in the active site. In reality, however, different compounds will bind to the active site in different orientations, perhaps even several orientations for a given compound [58].

8.3.2.3 Comparative Molecular Similarity Indices Analysis. Comparative molecular similarity indices analysis (CoMSIA) is another three-dimensional QSAR technique where molecules are aligned on a grid and the steric and electrostatic fields calculated [59]. However, CoMSIA is considered superior to CoMFA as the hydrogen bond donor, hydrogen bond acceptor, and hydrophobic fields are also taken into account. At each grid point, these interaction fields are converted to a similarity index [60], which is the property that is correlated with the compound activity. The steric and electrostatic fields, given by the Lennard–Jones and Coulombic potential, respectively, can be excessively large for grid points close to atoms of the compound. The Gaussian function of the similarity index can be designed to moderate this dominant behavior, ultimately resulting in greater predictive accuracy [61].

8.3.3 Protein Structure Methods

The following methods require the three-dimensional structure of the protein, which restricts these methods to systems where this is known, or at least predicted using protein homology models [62]. Since the proteins typically contain several thousands of atoms, these methods are computationally expensive and are not high throughput. However, this disadvantage is somewhat offset since these methods can reveal the exact nature of binding into the active site of an enzyme.

8.3.3.1 Molecular Dynamics. A molecular dynamics simulation provides an atomistic view of enzyme–substrate binding over time. It can capture the different orientations and conformations of the compound in the active site, and the role of solvent molecules if they are included in the simulation too [63]. The accuracy of the simulations depend on the classical force field used to model the interactions between compound and enzyme, which may require parameterizing to higher level quantum calculations. Add this to the already computationally intensive molecular dynamics simulation and it is clear that this method cannot currently be used as a general screening tool in drug discovery.

8.3.3.2 Docking. As the name suggests the compound is docked into the binding site of the enzyme. Each atom pair between the ligand and the enzyme is considered, the separation between the two atoms calculated, and the result converted into an atom-pair potential according to a scoring function. The total binding potential is the sum of the potential energies across all atom pairs. Where entropic and solvation effects have been incorporated into the scoring function, the free energy of binding is obtained [64,65]. However, knowledge-based atom-pair potentials are parameterized on selected sets of ligand–protein structures and may not predict well outside this domain [64].

8.4 APPLICATIONS

Here, we present a variety of examples where *in silico* methods have been used to characterize substrates of CYP, UDP-glucuronosyltransferase, and the PXR according to the different problems that are outlined in Section 8.2.2.

8.4.1 Prediction of the Rate of Metabolism

We start with prediction of overall rate of metabolism, not because it is one of the easiest to address by *in silico* modeling—far from it—but because it is a fundamental property that can significantly affect the likelihood of its progression in drug discovery. Consequently, the *in vitro* determination of metabolic rate (using isolated hepatocytes, or subcellular fractions such as microsomes or S9) is a routine screen utilized in lead optimization, or even lead identification, during drug discovery. Even modest drug discovery programs can result in large numbers of compounds being screened for this property [66]. Consequently, any contribution that *in silico* modeling can make to reduce the need for *in vitro* screening is potentially valuable.

The problem has been studied since the early days of QSAR modeling. For example, Hansch *et al.* [67] developed linear regression models for oxidative deamination of aliphatic amines and benzylamines by a variety of *in vitro* systems (purified amine oxidases, liver extracts, and beef liver mitochondria), for glucuronide formation of benzoic acids and aliphatic alcohols in the rabbit *in vivo* and for hippuric acid formation, also in the rabbit *in vivo*. The models they developed were for small series (7–30 compounds) of closely related, relatively simple compounds, having calculated $\log P$ s in the range 0.7–3.5. They considered hydrophilic/hydrophobic nature, hydrogen bonding capability, polarization, dipole moment, molar volume, pK_a , and electronic and steric properties. In essence, they found that the logarithm of the rate of metabolism (MR) was a parabolic function of calculated $\log P$, of the general form:

$$\log(\text{MR}) = -a \cdot \log P^2 + b \cdot \log P + c$$

with, for a small number of cases, a contributing term from the Taft intramolecular steric substituent coefficient, E_s . These relationships had correlation coefficients between 0.8 and 0.95 on the training set, thus explaining between 64% and 90% of the variance of the training sets. Now, these relationships are unlikely to generalize beyond the training sets (because of the relatively large ratio of fitted parameters to compounds), and are not necessarily applicable to larger, more complex drugs and drug leads, but they did provide valuable insight into important characteristics of drug metabolism.

First, the parabolic fits imply the existence of a $\log P$ value ($\log P_0$) for which metabolic rate is predicted to be maximal. The authors interpreted this as a balance between increasing lipophilicity enhancing the ability of compounds to diffuse through the lipoidal membranes (*in vitro* or *in vivo*) to reach the active site(s) of the metabolizing enzyme(s) and the tendency, when lipophilicity is particularly high, to strongly adhere to lipids or lipophilic proteins, so slowing this necessary diffusive step. They observed that, across the range of series and systems they investigated, the mean $\log P_0$ was ~ 2.0 . Second, particular determinants of metabolic rate were observed for certain compound series. Thus, predictability of the *in vivo* rate of glucuronidation of primary aliphatic aldehydes was improved by incorporating E_s with a negative coefficient, indicating that as the site of glucuronidation becomes more hindered, the rate of glucuronidation increases, probably because the competing oxidative metabolism is more sterically demanding and becomes more strongly inhibited. This was not observed for secondary aliphatic alcohols, in which oxidation is already hindered.

Recent developments in *in vitro* assays for drug metabolism in drug discovery have tended toward the deployment of high throughput, standardized, assays within pharmaceutical and biotech sectors using isolated cells or subcellular fractions such as microsomes or S9 [29]. Routine use of these assays both provides the capability to generate large data sets of standardized data for model building and defines the framework within which predictions from models are judged. Chang *et al.* [66] generated models for intrinsic clearance (CL_{int}) in a rat hepatic microsomal incubation using an isocitrate dehydrogenase nicotinamide adenine dinucleotide phosphate (NADPH)-generating system. The model was developed using $\sim 28,000$ drug leads across a broad chemical space with a calculated $\log P$ normally distributed between -3.7 and 10 , molecular weights normally distributed between 140 and 839 , and intrinsic clearance strongly right-skewed between 1.57 and $989 \mu\text{L}/\text{min}/\text{mg}$ protein. The model was a two-way classifier to distinguish between those compounds with $CL_{\text{int}} \leq 87 \mu\text{L}/\text{min}/\text{mg}$ and those with $CL_{\text{int}} > 87 \mu\text{L}/\text{min}/\text{mg}$.

The model was a random forest and had an overall predictive accuracy of 83% on an internal test set and 77% on an external test set that was in a distinct area of property space from the training set compounds. Descriptors important in the model were a double-bond E-state descriptor, $\log P$, and fractional hydrophobic and fractional hydrophilic van der Waal's surface areas (which are completely correlated). The result is a potentially useful model, but the study illustrates the challenge in modeling xenobiotic metabolism to obtain useful results, even when using very large training sets.

The difficulty in predicting CL_{int} is unsurprising. Not only are xenobiotic-metabolizing enzymes promiscuous with respect to substrate structure [68,69], but CL_{int} is itself a composite property. For an enzyme displaying Michaelis–Menten kinetics, it is defined as the ratio of the maximal rate, V_{max} , and the Michaelis constant, K_m :

$$CL_{\text{int}} = \frac{V_{\text{max}}}{K_m} \quad (8.1)$$

Many compounds can be metabolized by a number of P450 isoforms present in the microsomal fraction so that the total rate of metabolism, v , is the sum of metabolic rates of the individual isoforms. If each of n isoforms displays Michaelis–Menten

kinetics toward a compound,

$$v = \frac{V_{\max,1} \cdot S}{K_{m,1} + S} + \frac{V_{\max,2} \cdot S}{K_{m,2} + S} + \cdots + \frac{V_{\max,n} \cdot S}{K_{m,n} + S} = \sum_i^n \frac{V_{\max,i} \cdot S}{K_{m,i} + S}$$

where S is the drug concentration at the active site of the enzyme.

In this case, the total intrinsic clearance is given by

$$CL_{\text{int}} = \sum_i^n \frac{V_{\max,i}}{K_{m,i}}$$

assuming that $S < K_{m,i}$ for all isoforms. Thus, for a compound that is metabolized by n isoforms, CL_{int} is a combination of $n V_{\max}$ and $n K_{m,i}$. Even for a compound whose rate of metabolism is quantitatively dominated by a single isoform, CL_{int} is a conflation of two composite parameters. Using the Briggs–Haldane definition of $V_{\max}$ and K_m for a single enzyme/isoform,

$$V_{\max} = E_0 \cdot k_2$$

and

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

where E_0 is the total concentration of the isoform, k_2 the rate constant for the irreversible catalytic step converting the enzyme–substrate complex to generate the product, k_1 the association rate constant of the substrate and enzyme, and k_{-1} the corresponding dissociation rate constant of the enzyme–substrate complex. Thus, the two composite parameters of the Michaelis–Menten equation correspond to four different fundamental properties. E_0 is, in principle, measurable and, for the specific case where the rate of catalytic conversion is much less than the rate of dissociation of the enzyme–substrate complex ($k_2 \ll k_{-1}$), then

$$K_m \approx \frac{k_{-1}}{k_1}$$

which is the dissociation constant for the enzyme–substrate complex. Thus, in the simplest case, prediction of CL_{int} requires the implicit prediction of an underlying association step, which could be expected to be dominated by bulk properties relating to hydrophobicity/hydrophilicity, polarity, and hydrogen bonding, and of a transformative step, which could perhaps be expected to depend more on electronic and steric properties. The significant descriptors identified by the Chang model reflect the importance of hydrophobicity/hydrophilicity, hydrogen bonding, and electronic but not steric factors.

In contrast to the complexity inherent in attempting to predict CL_{int} in microsomes, predicting the properties of individual enzymes/isoforms offers a more tractable proposition. The difficulties should not be underestimated, because of the inherent promiscuity of drug-metabolizing enzymes, but the individual enzyme provides a far simpler system to understand than the complex microsomal fraction. In addition, the full

range of tools bringing in structural data about the isoforms concerned (Section 8.3) is available for studying these simpler systems, whereas the prediction of CL_{int} in multi-isoform systems, such as microsomes, is essentially limited to statistical methods, such as QSAR.

Ultimately, prediction of drug metabolism via the properties of individual isoforms should prove more valuable than prediction of, say, microsomal CL_{int} . This latter property can be measured in relatively high throughput screens, requiring a relatively small amount of compound [29]. Data on the effects of individual drug-metabolizing enzymes on a potential drug (such as the relative rates of metabolism, inhibibility by other drugs, and nonlinearity of metabolism), while being increasingly required by regulatory agencies and/or to predict likely population pharmacokinetics of clinical candidates compounds, are more expensive, time consuming, and/or require more compound to obtain. This combination of potentially providing greater cost savings, at the same time as offering problems that are more soluble indicates that prediction of the properties of individual drug-metabolizing enzymes is the route that offers greatest potential in drug discovery. That said, the advantage of developing models for microsomal CL_{int} is the very availability of data for model generation. The work of Chang *et al.* implies that a large “global” model for CL_{int} using data generated across many areas of chemistry has potential benefit in screening new chemistry and could thus be used to virtually screen libraries or hits for lead identification.

8.4.2 Drug Interaction with Specific Enzyme Isoforms

Changing the focus of study from composite systems (such as isolated cells, microsomes, or S9 subcellular fractions) to simpler systems, such as individual enzyme isoforms, increases both the toolset available for model development and the number of properties that can potentially be predicted. The additional tools include (Section 8.3) docking and molecular dynamics methods (where the crystal structures are available), field alignment methods, such as CoMFA and COMSIA, and pharmacophore methods. The additional properties that can be determined, and hence potentially predicted, with *in silico* models include various measures of the propensity of a particular enzyme isoform to metabolize a given compound or, alternatively, the propensity of a given compound to inhibit the metabolism of other compounds by a particular isoform. These are two different problems, though with common underlying bases that relate to the complementarity—in ways that need to be defined and determined—between a compound’s structure and the structure of active/allosteric sites on the enzyme form in question. Each of these problems must be tackled during drug discovery, and we address each in turn in the following sections.

8.4.2.1 Isoform-Specific Metabolism. The identities of the enzyme isoforms responsible for metabolizing a given drug, and the distribution of metabolism between them, determine the pharmacokinetics of a drug in a heterogeneous population, the potential for nonlinear pharmacokinetics as a consequence of enzyme saturation, and the potential for enzyme inhibition and induction to affect the drug’s pharmacokinetics, toxicity, and therapeutic efficacy. Experimental methods are available for identifying the enzymes, such as measuring metabolism in expressed enzyme systems or inhibiting metabolism in microsomes by isoform-specific inhibitors. Since metabolism plays

such an important role in determining the efficacy and toxicity of a compound, it is necessary to understand the metabolic fate of potential drug entities early on in the drug discovery process. A large number of compounds presented at this stage would render a full metabolic screen of them all unfeasible and therefore there is a clear need to develop reliable predictive models of metabolism instead.

First, we focus our attention to simply predicting whether a compound is a substrate for metabolism. This problem has been tackled using a wide variety of molecular descriptors and machine learning methods and we outline a few studies [70–73] in Table 8.1. The predictive accuracies for classifying whether a compound is a substrate of an enzyme isoform span the range 65–97%, but estimated accuracies depend on the chemical domain spanned by the training and validation sets, so it not a straightforward process to rank and compare models from different studies. However, given a compound is predicted to be a substrate, the conditional probability it is actually a

TABLE 8.1 Summary of Selected Studies that Predict Whether a Compound Is a Substrate/Nonsubstrate for Metabolism

References	Enzyme System	Method	Accuracy	Notes
Sorich <i>et al.</i> [70]	Human UGT	Support vector machine	76%	Averaged accuracies across all isoforms
		Partial least squares determinant analysis	66%	
		Bayesian regularized artificial neural network	71%	
Sorich <i>et al.</i> [71]	Human UGT	Partial least squares determinant analysis	69% and 71%	Averaged accuracies across all isoforms
		K-means clustering plus genetic algorithm	72%	
Balakin <i>et al.</i> [72]	CYP450	Kohonen self-organizing maps	CYP3A4 91% (high K_m predictions) 97% (low K_m predictions) All CYP isoforms 82% (high K_m predictions) 65% (low K_m predictions)	Accuracies calculated from compound training set
Yap and Chen [73]	CYP450	Support vector machine	CYP3A4 95% CYP2D6 95% CYP2CP 97%	

The predictive accuracy of each of the methods used is given.

substrate can be shown to be

$$P(\text{sub}|\text{pred}) = \frac{1}{1 + \frac{1-\text{Sp}}{\text{Se}} \times \frac{1-f}{f}}$$

The specificity (Sp) is the probability a nonsubstrate is correctly identified as being a nonsubstrate by the model. Likewise, the sensitivity (Se) is the probability a substrate is correctly identified to be a substrate by the model. Finally, f is the fraction of actual substrates in our total set of compounds. Taking the worst case scenario that the model accuracy is only 65%, and assuming this represents both the specificity and sensitivity,

$$P(\text{sub}|\text{pred}) = \frac{1}{1 + 0.538 \times \frac{1-f}{f}}$$

Therefore, if overall only 10% of the compounds are actually enzyme substrates ($f = 0.1$), then the model will predict a subset of these compounds to be substrates, 17% of which will actually be substrates. Thus, the model will have increased the proportion of substrates by almost a factor of 2, from 10% of the total set to 17% of the predicted subset. Using the model accuracy in this way can help judge whether applying a model for screening purposes would result in worthwhile savings in time and expense.

In silico docking is a computationally expensive method of predicting drug metabolism and therefore cannot be routinely used to screen large numbers of compounds. It is particularly valuable, however, for providing an atom-level understanding of the drug–enzyme binding complex and has been used to study drug binding to CYP2D6 [74]. This study revealed that metoclopramide can adopt two positions in the enzyme active site, but only one of these binding conformations had been observed experimentally. Analysis of the second binding conformation implied the production of a second metabolite that had not previously been identified, but which was later verified by experiment.

Molecular dynamics simulations, like *in silico* docking methods, are computationally demanding but can provide insight into the mode of binding for a small number of drugs. Seifert *et al.* [75] analyzed the dynamics of warfarin in the active site of CYP2C9. The crystal structure of this warfarin-bound complex cannot by itself predict the subsequent metabolites, as the warfarin sits too far away from the catalytic heme group. Molecular dynamics simulations, on the other hand, revealed two ways of binding to this catalytic center, resulting in the formation of the two experimentally observed metabolites. In a more recent study [76], the role of water within the active site of CYP2D6 was investigated using molecular dynamics simulations. Hydration sites were identified and these water molecules, when included in an *in silico* docking protocol, improved the reliability in predicting the sites of metabolism for methylenedioxy-*N*-methylanphetamine.

Both docking and molecular dynamics methods predict the site of metabolism on a compound by identifying the energetically favorable binding conformations in the active site of an enzyme. The compound atom that predominantly interacts with the catalytic group of the active site is identified as being the site of metabolism. In the absence of an enzyme structure, an alternative way of estimating possible sites of metabolism is to consider the electronic environment surrounding the hydrogen

atoms of the compound [77,78]. If it is assumed that removal of the hydrogen is a rate-limiting step, then hydrogen atoms requiring least energy to dissociate from the molecule are most likely to be at the sites of metabolism. Calculating dissociation energies requires expensive quantum mechanical calculations, but semiempirical methods have been developed, which reduce the computation time with minimal compromise on accuracy [79]. Although these methods are far too costly to be used in early stage drug discovery, it has been proposed that there is potential value in employing these methods to assess metabolic transformations of lead compounds in the preclinical phase [78].

8.4.2.2 Inhibition of Metabolism. While some drugs are metabolized by CYP450 or UGT enzymes, other drugs will inhibit this metabolism, potentially resulting in drug–drug interactions that may compromise the success of clinical trials. Prediction of enzyme inhibition in early drug discovery is becoming increasingly important and we summarize several studies of this kind in Table 8.2 [43,73,80–82].

The studies summarized in Table 8.2 show that compounds can be predicted to be strong or weak enzyme inhibitors with an accuracy ranging from 66% to 96%, with the support vector machine method developed by Yap and Chen [73] achieving accuracies at the higher end of this range.

Kriegel *et al.* [80] applied partial least squares methods to a combination of 2D, 3D, and quantum mechanical descriptors resulting in an overall accuracy of 66%. In addition, a model based purely on quantum descriptors (accuracy of 54%) did not outperform a model composed purely of 2D descriptors (accuracy = 58.3%). This is surprising as unlike 2D descriptors, quantum mechanical descriptors are derived from the 3D chemical structure, and a molecule's electronic structure offers great insight into its binding potential. However, on this evidence, it seems the considerable computational overhead required to calculate quantum mechanical descriptors does not translate to increased predictive ability.

Hudelson *et al.* [81] built four models to predict compounds that inhibit CYP2C9, the predictive ability ranging from 74% to 90%. To increase reliability, it was proposed that a prediction should only stand when all four models agreed whether a compound was an enzyme inhibitor. Surprisingly, there was agreement across all four models for only two thirds of the compounds tested, and so in this case, one-third of compounds could not be classified. This stringent rule was then relaxed so that a result would stand if at least three of the models agreed, increasing coverage to the majority of compounds and resulted in a predictive accuracy of 91%.

Vasanthanathan *et al.* [82] tested a variety of models on a large test set of 7000 compounds, resulting in predictive accuracies in the range 67–76%, and since the test set is so large, these values can be taken to be a reliable measure of the predictive accuracy of enzyme inhibition by machine learning methods. The nonlinear support vector machine and random forest methods gave slightly superior accuracies, 75% and 76%, respectively. At the other end of the scale, a decision tree based on Lipinski's rule-of-five descriptors managed to classify 67% of test compounds correctly. However, this decision tree is easy to interpret and provides structural insight into CYP1A2 inhibitors—hydrophobicity and hydrogen bond donors/acceptors being important features for classifying CYP1A2 inhibitors. This structural interpretation is not as transparent from the machine learning methods. On balance, one must decide

TABLE 8.2 Summary of Selected Studies that Predict Whether a Compound Is an Enzyme Inhibitor

References	Enzyme System	Method	Accuracy	Notes
Kriegel <i>et al.</i> [80]	CYP3A4	Partial Least Squares:	66%	—
Yap and Chen [73]	CYP450	Support vector machine	CYP3A4 92–96% CYP2D6 92–94% CYP2C9 95%	—
Marechal <i>et al.</i> [43]	CYP3A4	Docking	Strong ($k_i < 10 \mu\text{M}$): 5/7 or 7/7 Weak ($k_i < 100 \mu\text{M}$): 5/5	Depends on the crystal structure used
Hudelson <i>et al.</i> [81]	CYP2C9	Line walking recursive partitioning	88%	—
		Normal equation recursive partitioning	90%	
		Gravity	86%	
		Substructure discovery using examples	74%	
Vasanthanathan <i>et al.</i> [82]	CYP1A2	Support vector machine (linear)	73% (77%)	Accuracies calculated from compound training set in brackets
		Support vector machine (nonlinear)	75% (82%)	
		Random forest	76% (100%)	
		k-nearest neighbors	74% (83%)	
		Decision tree	71% (97%)	
		Lipinski-decision tree model	67%	

The predictive accuracy of each of the methods used is given.

whether the additional accuracy obtained from machine learning methods offsets the ease of use and straightforward interpretation of the Lipinski's rule of five.

Finally, as mentioned in Section 8.4.2.1, the computational expense of the docking method restricts its application to a small number of compounds. That said, Marechal *et al.* [43] were able to discriminate well between strong ($k_i < 10 \mu\text{M}$) and weak ($k_i < 100 \mu\text{M}$) inhibitors using this technique, but its main selling point is in the detailed structural information it reveals regarding the mode of binding in the active site of the enzyme.

8.4.3 Transporters

8.4.3.1 Identifying Substrates for Transporters. To fully understand drug metabolism and drug–drug interactions, one must consider active transport into and out of cells as it may be significant compared with passive diffusion for certain drugs, and there has been corresponding interest in developing *in silico* prediction models to predict transporter activity [83].

In Table 8.3, we summarize results from selected studies that have developed QSAR models to predict the affinities for a range of transport proteins: P-glycoprotein, organic cation transporters, and nucleotide transporters [84–91]. Osterberg and Norinder [84] conclude that variables associated with hydrogen bonding and high polarizability correlate with high P-glycoprotein ATPase activity. Other studies also highlight the importance of hydrophobic features [90–92], which may represent aliphatic or aromatic hydrophobes [92].

TABLE 8.3 Summary of Selected Studies that Have Developed *In Silico* Models to Predict the Affinity of Drugs to Transporter Proteins

References	Transporter Protein	Method	Predictive Accuracy	Correlation Coefficient Between Experimental and Observed Values
Osterberg and Norinder [84]	P-Glycoprotein	Partial least squares	—	0.718
Bednarczyk <i>et al.</i> [85]	Human organic cation transporter	Forward stepwise regression	—	Pharmacophore based model: 0.86 Molecular descriptor based model: 0.95
Chang <i>et al.</i> [86]	Human nucleotide transporters	CoMFA	—	
	hCNT1			0.98
	hCNT2			0.83
	hENT1			1.00
Gombar <i>et al.</i> [87]	P-Glycoprotein	Stepwise discriminant analysis	86.2%	—
Xue <i>et al.</i> [88]	P-Glycoprotein	k-nearest neighbors	70.8%	—
		Probabilistic neural network	74.4%	
		C4.5 decision Tree	71.5%	
		Support vector machine	79.4%	
Suhre <i>et al.</i> [89]	Organic ion transporters	Forward stepwise regression	—	2D-QSAR: 0.81 CoMFA: 0.97
Chang <i>et al.</i> [90]	P-Glycoprotein	Pharmacophore model	—	0.87
Zalloum and Taha [91]	P-Glycoprotein	Genetic partial least squares	—	Training set: 0.8 Validation set: 0.6

8.4.4 Induction of Metabolizing Enzymes or Transport Proteins

The PXR is a nuclear receptor that regulates the expression of metabolizing enzymes and P-glycoprotein efflux transporters. A drug activating PXR can therefore influence the metabolism and transport of itself, or a second drug dosed simultaneously, and may lead to drug–drug interactions. A pharmacophore for PXR ligands has been identified by Ekins and Erickson [93] and consists of hydrogen bond acceptor and hydrophobic features, similar to the pharmacophore for CYP3A4 substrates and inhibitors, but in different positions. The pharmacophore alone was able to differentiate very potent activators from weak activators. More involved methods are required to build more reliable predictive models for PXR activation, some of which are summarized in Table 8.4 [94–96].

A comparison of the predictive accuracies obtained by Ung *et al.* [94] and Khandelwal *et al.* [95] reveals that the support vector machine method is superior to other methods investigated. The predictive accuracies obtained by Ung *et al.* were roughly 10% greater than those achieved by Khandelwal *et al.*, even when they both used the support vector machine technique. The most likely explanation for this discrepancy is perhaps in the calculation of the accuracy statistics, rather than the way in which the methods were applied in the two studies. Khandelwal *et al.* used a 145-compound test set to measure the predictive accuracy, whereas Ung *et al.* adopted a 10-fold cross-validation study in which the test sets each contained only 20 compounds. The larger test set of Khandelwal *et al.* could conceivably cover a greater area of chemical space

TABLE 8.4 Prediction of PXR Activators and Nonactivators

References	Transporter	Method	Accuracy	Notes
Ung <i>et al.</i> [94]	Human PXR	k-nearest neighbors	75.0%	Recursive frequency elimination (Guyon <i>et al.</i> 2002) used to select relevant molecular descriptor
		Probabilistic neural network	77.7%	
		Support vector machine	79.6%	
Khandelwal <i>et al.</i> [95]	Human PXR	Recursive partitioning	63.45% (87.50%)	Accuracies calculate from compound training set in brackets
		Random forest	65.52% (73.45%)	
		Support vector machine	66.90% (94.35%)	
Ekins <i>et al.</i> [96]	Human PXR	Docking	47.06–57.98%	The range of predictive accuracies obtained from docking into six different crystal structures

and would therefore be a greater test of the model's ability to predict human PXR activators.

In contrast to the machine learning methods, *in silico* docking as employed by Ekins *et al.* [96] resulted in a much worse predictive accuracy, barely greater than a 50–50 guess. However, the authors also investigated the performance of 3D-, 4D- and 5D-QSAR. Surprisingly, 3D-QSAR methods, such as CoMFA and CoMSIA, also performed poorly, a reason being that the PXR active site is flexible, allowing small molecules to bind in several orientations. In CoMFA and CoMSIA, the compounds must be aligned and so these two methods would not capture the multiple orientations, which contribute to the overall observed enzyme activation. 4D-QSAR, on the other hand, does consider multiple conformations, orientations, and stereoisomers of each compound and improved the predictions for the training set compounds, although the prediction of the test set compounds was still poor. 5D-QSAR is an extension of 4D-QSAR, also considering the induced fit of the compound in the active site of the protein, and was the only QSAR method capable of making test set predictions of steroidal PXR activation with a reasonable level of success.

8.4.5 Physiologically Based Models

The modeling approaches discussed hitherto only provide information about the existence or extent of the interaction between a compound and a protein. These data may be useful in early stage drug development, providing an indication of the likelihood of such an interaction, but do not provide any indication of the clinical significance of this interaction: crudely, if significant interactions occur only when the test compound concentration is greater than some lower bound, we also need to know the time for which the compound concentration is greater than this lower bound after the compound is dosed. To obtain information about the clinical significance of a compound–protein interaction, it is necessary to integrate interaction data with pharmacokinetic data. Indeed, interactions between a compound and metabolizing enzymes or transport proteins determine the compound's pharmacokinetics.

8.4.5.1 Single-Compound Pharmacokinetics. The use of physiologically based pharmacokinetic (PBPK) models to predict or model pharmacokinetics has been widely discussed [97–100] and the prediction or modeling of single-compound pharmacokinetics is a prerequisite of the prediction or modeling of interactions between compounds. A number of commercial products exist or it is possible to implement basic PBPK models in-house.

The gross structure of a whole-body PBPK model is shown in Fig. 8.1. Dosed compound flows with blood from the arteries through the organs into which it can distribute or from which it may be cleared from the body. After passing through the organs, compound arrives in the venous blood, from which it enters the pulmonary circulation and then flows back into the arteries. While this gross structure is generally agreed, the variations that are observed in the details of PBPK models mean that there is as yet no well-defined and agreed generic structure for a PBPK model that can be relied on in all situations. Different groups model different organs, and sometimes organs, particularly the liver, are supposed to be segmented [101]. Other models include the dynamics of plasma protein binding and transport effects in the

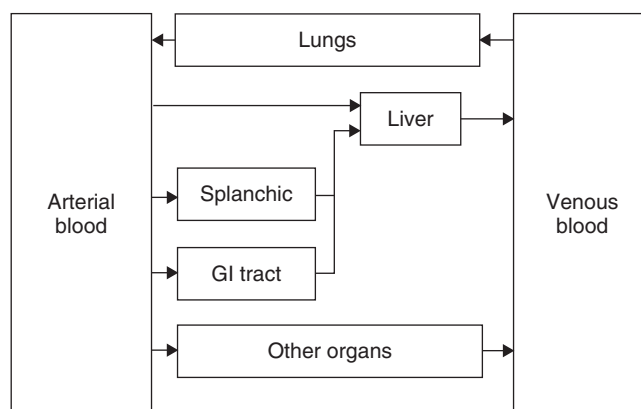


Figure 8.1 The structure of a generic PBPK model. Boxes (“compartments”) represent blood pools (arterial and venous), organs, and tissues. Arrows represent blood flows between compartments. Different models differ primarily in the number of organs and tissues that are explicitly represented.

liver [102,103]. Sometimes organs are lumped into “central” and “peripheral” compartments [104,105]; this makes a model less physiological, but may make it easier to parameterize from *in vivo* data. Organs are commonly modeled as being well-stirred, with rapid equilibration of free compound between plasma and tissue; but taking typical values for compound permeability, it seems that this rapid equilibration may be an unrealistic assumption [105–107]. In any case, to model transporter effects, it is necessary to include permeability barriers in the organ model; organs are sometimes subdivided into blood, interstitial, and intracellular compartments [108]. The inclusion of transporter effects is a relatively recent development [109–115].

The modeling of absorption from and transport along the GI tract is an art in itself. Physiological models of the GI tract consist of a sequence of segments through which flow dissolved and undissolved compound (Fig. 8.2), with some modeling of dissolution, and with absorption of the dissolved compound [12,107,116,117]. Metabolism in the walls of the GI tract is also sometimes included [118,119].

The extent of distribution in PBPK models is predicted using a competition between partitioning into tissue lipids and binding to plasma proteins. A few variants on the idea are reported in the literature [120–122]; as yet there is no definitive method. The octanol–water partition coefficient is commonly used as a measure of lipophilicity; some groups use, instead, affinity to phospholipid membranes [123,124], and there does not seem to be agreement on the best measure of lipophilicity to use in general. It should be noted that these models rely on the partition coefficient directly rather than on its logarithm: an error of half a log unit in the logarithm translates to a threefold error in the partition coefficient and—for lipophilic compounds—a threefold error in the volume of distribution even before difficulties in the measurement of plasma protein binding are taken into account.

It is also necessary to parameterize the rate of metabolism in a PBPK model. If *in vivo* data are available, one can just optimize the model to fit the measured data, but if they are unavailable, one must predict the rate of metabolism using *in vitro* or *in silico* data. The prediction of metabolic clearance from *in vitro* data has recently

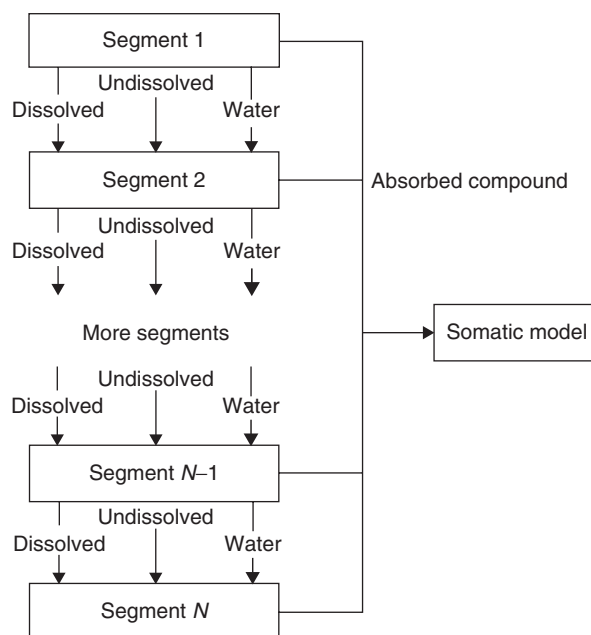


Figure 8.2 The generic structure of gastrointestinal tract absorption modeling in PBPK. The GI tract is treated as a series of segments (compartments) in sequence. Dissolved and undissolved compound plus water (more precisely the gastric or intestinal fluid) flows from a compartment to the next in the series. Dissolved compound is available for absorption across the GI tract epithelium into the body, which can be represented by a PBPK model (as in Fig. 8.1). Different models differ in the number of segments that are used to represent each part of the tract.

been reviewed [125]. Large uncertainties and variabilities in the *in vitro* data mean that, generally, predictions of *in vivo* clearance based on *in vitro* data alone can be statistical at best.

Some success is reported in using generic PBPK models for statistical predictions of pharmacokinetic parameters such as clearance [108,126] and, until the details of mechanistic extrapolation from *in vitro* to *in vivo* are resolved, this is perhaps the most that can be expected from predictive PBPK modeling in early drug discovery. Later in drug discovery and in drug development more effort can be expended on developing a customized and optimized model for a particular compound, when *in vivo* data are available for the calibration of such models. These optimized models can be used for explanatory purposes [127] and to design detailed *in vivo* trials that assess specific aspects of pharmacokinetics [128]. The detailed analysis of such models allows the determination of the controlling parameters and can give good mechanistic understanding of *in vivo* pharmacokinetics [127,129].

8.4.5.2 Interactions Between Compounds. The interaction most commonly modeled mechanistically is the inhibition of CYP-mediated metabolism. PBPK models are separately developed for each interacting compound using *in vitro* or *in vivo* data. These models are used to predict compound concentration at the critical sites for drug–drug interaction such as the liver or intestinal mucosa. *In vitro* interaction data

can then be inserted to link the two models. A relatively detailed model of metabolism is necessary: in general, compounds may be metabolized by multiple enzymes, so to model correctly the effect of the inhibition of a given enzyme, one must account for the relative contributions of all enzymes that significantly metabolize the compounds in question. For compounds that can be modeled with single-enzyme metabolism, this step is unnecessary, but this will not in general be the case. Interactions between more than two compounds can be handled in a similar way. Irreversible inhibition, in which a metabolizing enzyme is permanently inactivated, demands a different type of model to reversible inhibition. To predict irreversible inhibition, some idea is needed about protein turnover [130]; the actual protein concentration must be evolved as part of the simulation.

Successes for this approach have been reported in the literature (Table 8.5) [101,104,105,119,123,130–137]. Some use mechanistic PBPK modeling to predict single-compound pharmacokinetics; others use a more empirical approach in which physiological or semiphysiological models of single-compound pharmacokinetics are fit to *in vivo* data. The overwhelming majority study CYP3A, presumably because CYP3A-family enzymes are the most common drug metabolizers.

In principle, this general approach could also be used for interactions controlled by the inhibition of active transport; all that is needed is a mechanistic model of transporter activity *in vivo* [109–112,114] coupled to some *in vitro* transporter inhibition data.

TABLE 8.5 Physiological and Semiphysiological Metabolism Inhibition Models Found in the Literature

Model Type	Compounds	Inhibited Enzyme(s)	References
Whole body	Midazolam/itraconazole	CYP3A	Vossen <i>et al.</i> [123]
Whole body	Midazolam/clarithromycin	CYP3A	Quinney <i>et al.</i> [104]
Whole body	Midazolam/diltiazem	CYP3A	Zhang <i>et al.</i> [105]
Whole body	Midazolam (cimetidine/itraconazole/ erythromycin)	CYP3A	Yamano <i>et al.</i> [131]
Whole body	Various CYP3A4 substrates/various CYP3A4 inhibitors	CYP3A4	Fenneteau <i>et al.</i> [119]
Whole body	Midazolam/phase 1 compound	CYP3A4	Chenel <i>et al.</i> [132,133]
Whole body	Tesofensine/itraconazole	CYP3A4	Lehr <i>et al.</i> [134]
Whole body	CYP3A4 substrates/ketoconazole, verapamil	CYP3A4	Perdaems <i>et al.</i> [134]
Whole body	Diltiazem/triazolam/ <i>N</i> - desmethyldiltiazem	CYP3A	Rowland Yeo <i>et al.</i> [130]
Whole body	Midazolam/ketoconazole	CYP3A	Chien <i>et al.</i> [136]
Whole body	Midazolam/macrolide antibiotics	CYP3A	Ito <i>et al.</i> [137]
Isolated perfused liver	<i>R</i> -bupuralol/ bunitrol/debrisoquine	CYP2D	Haddad <i>et al.</i> [101]

TABLE 8.6 Physiological and Semiphysiological Mechanistic Induction Models Found in the Literature

Model Type	Compound	Enzyme Induced	References
Whole body	2,3,7,8-Tetrachloro-dibenzo- <i>p</i> -dioxin	CYP1A1, CYP1B1	Kohn <i>et al.</i> [144]
Whole body	2,3,7,8-Tetrachloro-dibenzo- <i>p</i> -dioxin	CYP1A2	Emond <i>et al.</i> [140] Wang <i>et al.</i> [141] Santostefano <i>et al.</i> [142] Leung <i>et al.</i> [142]
Semiphysiological	Isoniazid	CYP2E1	Chien <i>et al.</i> [145]
Semiphysiological	Phenobarbital	Various	Magnusson <i>et al.</i> [146]
Semiphysiological	Artemisinin	Unspecified	Gordi <i>et al.</i> [147]
Semiphysiological	Cyclophosphamide	Unspecified	Hassan <i>et al.</i> [148]

But while these are all promising successes, it must of course be noted that, as discussed above, the quantitative prediction of even single-compound pharmacokinetics is challenging. It would be surprising if prediction of drug–drug interactions was simpler than the prediction of plasma clearance: a mechanistic model of metabolic drug–drug interaction requires, to some extent, an understanding of metabolic clearance. Given the challenges in the quantitative prediction of metabolic clearance from *in vitro* data [125], it is easy to see how difficult it is to accurately predict *in vivo* inhibition of metabolism.

Heuristic models of CYP induction relate the extent of *in vitro* induction to the rate of *in vivo* metabolism [138]. This essentially applies sensitivity analysis to the single-compound pharmacokinetics: develop a working model for the victim compound in base conditions and just rerun the model using various hypothetical rates of metabolism following induction.

Mechanistic models of enzyme induction require some concept of protein turnover and gene expression [139] and require the enzyme concentrations to be evolved as part of the simulation. There are two mechanisms for CYP induction that have been modeled. Both result from a change to the balance between protein synthesis and degradation: the more common mechanism is an increase in protein synthesis following nuclear receptor binding, the less common is a decrease in protein degradation due to stabilization caused by xenobiotic binding to the protein. Some of the mechanistic induction models found in the literature are given in Table 8.6 for induction by dioxin [140–144], isoniazid [145], phenobarbital [146], artemisinin [147], and cyclophosphamide [148]; these are all customized models for a particular interaction and are not *a priori* predictive.

8.5 INCORPORATING IN SILICO METHODS IN DRUG DISCOVERY

In previous sections, we have described a number of *in silico* methods and discussed some specific applications to predict drug metabolism and drug–drug interactions. These methods perform an important function by helping increase our understanding

of the properties of compounds that determine their fates and interactions *in vivo*. To have maximum impact, however, in a drug discovery program, the methods must support and inform the selection of (virtual) compounds to be synthesized or (real) compounds to be selected for screening assays. In this section, we consider ways whereby the methods can be incorporated into the drug discovery process, with a view to inform and help direct medicinal chemistry efforts toward designing compounds with required characteristics. In this respect, the methods so far described do not stand alone. The capability to beneficially employ them in drug discovery depends on

- the availability of necessary supporting software;
- appropriately skilled and experienced staff;
- effective communication between modelers, drug metabolism/pharmacokinetics (DMPK) scientists, and medicinal chemists.

This can be illustrated with a simple example in which systemic clearance is to be predicted in order, for instance, to estimate plasma half-life. In this case, the effect on predicted *in vivo* plasma clearance of uncertainty in a prediction of hepatic clearance diminishes in relative importance as the predicted hepatic clearance increases toward hepatic blood flow. This is intuitively apparent from the effect of blood flow on limiting clearance, but sensitivity and error analyses of PBPK models can quantify the effect, can process large numbers of compounds in a short time, and can do so for those less obvious relationships that can exist between model inputs and outputs. Use of sensitivity and error analysis can thus indicate the acceptable bounds in uncertainty and bias on any given prediction or set of predictions.

As a simple, illustrative, example, we can first consider the case of predicting lipophilicity, in the form of the octanol/water partition coefficient, P . The partition process can be viewed as the creation of cavities in the two solvents (octanol and water)—an endoergic process—and the insertion of a solute into the solvents, which gives rise to various solute/solvent interactions (hydrogen bonding, electrostatic, van der Waal's, etc.), which may be endo- or exoergic. The balance of these processes will determine the overall energy change, and hence equilibrium position, of the partitioning (Fig. 8.3). Many *in silico* methods exist for this property (reviewed in Refs 149 and 150), but the method of Abraham and coworkers [151] has made the above relationships explicit:

$$\log P_{\text{oct}} = a + b \cdot E - c \cdot Z + d \cdot A - e \cdot B + f \cdot V$$

where $\log P_{\text{oct}}$ is the logarithm (base 10) of P . E , S , A , B , and V are properties of the particular solute:

- E is the excess molar refraction.
- Z is the polarizability/dipolarity.
- A is the hydrogen bond acidity (donor capability).
- B is the hydrogen bond basicity (acceptor capability).
- V is the McGowan's characteristic volume.

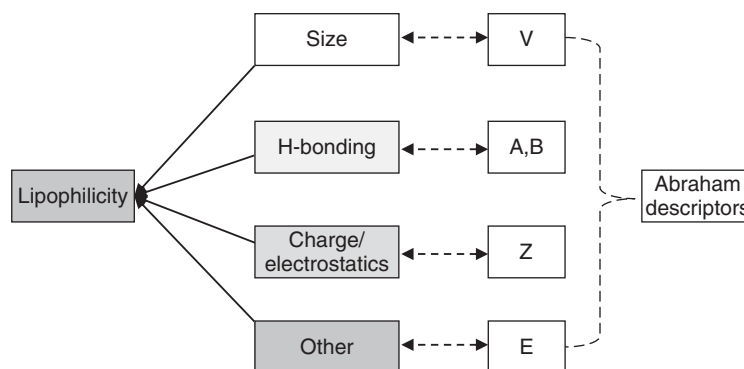


Figure 8.3 Relationships between Abraham descriptors, compound characteristics, and lipophilicity. V , A , B , Z , and E are the Abraham descriptors.

$a-f$ are constants with specific values for any partitioning system. For octanol/water, the partitioning is dominated by fV (i.e., molecular size, which favors octanol) and eB (hydrogen bond basicity, which favors water). E and V can be calculated for a compound directly from atom and bond contributions, whereas S , A , and B require manual summation from specified molecular fragments. While more accurate $\log P$ predictors currently exist [149,150], in terms of implementation in a drug discovery process, the Abraham method has the following points in its favor:

- The model involves a limited number of (six) unknown parameters, reducing the risk of being overtrained on reasonably sized training sets.
- The descriptors are readily interpretable in terms of fundamental properties of compounds.
- The small number, and the nature, of the descriptors facilitates their understanding by nonspecialists.
- The model is linear with respect to its descriptors, so that the way in which changes in any descriptor's value affects the predicted $\log P$ is clear.

The problems of interpreting and utilizing metabolism and drug interaction are harder than the analogous problem for $\log P$. Involving, as they do, interaction between a small molecule (or, in the case of drug interactions, two small molecules) and an enzyme or transporter protein active site, simple explanations are less frequent and correlations are weaker than for $\log P$. Additional properties, relating to molecular shape are more important and such properties are, of necessity, less amenable to simple interpretation. Lewis [152] has collated information from a number of sources to specify characteristics of individual human P450 isoforms, in a qualitative/semiquantitative manner in terms of planarity, acid/base characteristics, volume, and lipophilicity. Lewis [153] has also defined QSARs for substrates (and inhibitors) of the human P450 CYP2 family (2A6, 2B6, 2C8, 2C19, 2D6, and 2E1) in terms of a small number of readily definable descriptors, including lipophilicity, molecular weight, and hydrogen bonding capability. These QSARs have, however, been generated using small data sets so, while they can provide some useful insight into potentially important features that define

substrate specificity, the ability of the QSARs to extrapolate to other compounds is doubtful. Methods for predicting CYP450 substrate specificity have been reviewed by Ekins *et al.* [154] and de Groot [155].

Turning in more detail to the identification of CYP3A4 substrates, the key CYP450 isoform for drug metabolism in humans, a number of approaches, including pharmacophore [156], QSAR [72,73], and docking [157], have been attempted.

Balakin *et al.* [72] developed a model for the Michaelis constant, K_m , of CYP450 substrates. As we have discussed in Section 8.4.1, the Michaelis constant does not, by itself, predict the rate of metabolism of a compound. For this, the V_{max} is also required. The Michaelis constant does, however, provide information about binding to the active site, potential for nonlinearity in metabolism, and potential for drug–drug interactions mediated by inhibition of metabolism. For CYP3A4, Balakin *et al.* had experimental K_m values for 126 compounds, categorizing the values as low ($< 10 \mu M$), medium ($10\text{--}100 \mu M$), or high ($> 100 \mu M$). For differentiating between low and high (ignoring medium) K_m values, six descriptors were found to be important. Listed in order of decreasing importance, these are as follows:

1. Zagreb (a measure of topological complexity).
2. Number of H-bond acceptors.
3. Number of rotatable bonds (a measure of flexibility and also correlated to size).
4. A measure of partial negative surface area (PNSA-1).
5. Number of H-bond donors.
6. $\log P_{oct}$.

For all except the two hydrogen bonding descriptors, the differences were statistically significant by a two-tailed t test (Table 8.7). It can thus be seen that the most important determinant between low and high K_m is a complex descriptor (Zagreb), whose interpretation is not obvious—and therefore whose utilization in guiding medicinal chemistry is not trivial. Consequently, to use the results of this model to guide chemistry, medicinal chemists would require appropriate computational tools and/or databases, either to screen libraries for compounds or to design compounds having appropriate properties [158–160]. Three of the properties (numbers of hydrogen bond acceptors and donors and number of rotatable bonds) are more amenable to quick assessment, as is $\log P_{oct}$ (on the assumption that $\log P_{oct}$ predictive software is almost ubiquitous and/or chemists would rapidly gain experience in estimating the lipophilicity

TABLE 8.7 Important Descriptors for Differentiating Between Strong and Weak Binders to Human CYP3A4

Property	Mean Value for Strong Binders ^a	Mean Value for Weak Binders ^a
Zagreb index	~ 210	~ 120
Number of rotatable bonds	9	4
PNSA-1	~ 260	~ 175
$\log P_{oct}$	~ 4.8	~ 2.6

^aStrong and weak binders are distinguished by low ($< 10 \mu M$) and high ($> 100 \mu M$) K_m , respectively.

within a particular series). Furthermore, by inspection of Table 8.7, it can be seen that the model predicts marked differences in $\log P_{\text{oct}}$ and the number of rotatable bonds for strong and weak binders, so there are some, at least, clear, readily identifiable differences between strong and weak binders.

Yap and Chen [73] adopted a different approach, identifying compounds as CYP3A4 substrates or nonsubstrates (368 and 334, respectively) on the basis of reports—or lack of such—in the literature of a compound being metabolized by a particular P450. The groups of descriptors that were selected as important during the modeling process were mostly composite, and therefore difficult to interpret, namely

- 3D-MorSE—encode 3D features such as van der Waal's volume, electronegativities, and polarizabilities.
- Radial distribution function (RDF)—encode information about bond lengths, ring types, planar and nonplanar systems, and atom types.
- Randic molecular profiles—encode interactions between atoms in a molecule and information on molecular shape.

The simpler, noncomposite, descriptors ($\log P$, hydrogen bond acceptors and donors, etc.) provided less information about the substrate/nonsubstrate (and, analogously, the inhibitor/noninhibitor) classification of substrates with respect to CYP3A4. Thus, substrates had mean Moriguchi $\log P$ of 2.2 ± 1.9 compared with 1.6 ± 2.06 for nonsubstrates. This contrasts with the findings of Balakin [72], where low and high K_m compounds were differentiated by statistically significant mean $\log P$ of ~ 4.8 and ~ 2.6 , respectively (Table 8.7). The difference arises both from the nature of the problem being addressed and the amount of information being sought. Identifying whether a compound is a substrate for metabolism of CYP3A4 is more difficult than identifying whether it binds tightly to the protein's active site, in which bulk-phase hydrophobic interactions can exert a significant effect. Yap and Chen were, furthermore, seeking a classification for all possible compounds into substrate/nonsubstrate, whereas Balakin *et al.* had restricted themselves to the widely separated classes of low and high K_m compounds and were not attempting to address the intermediate category of medium K_m (between 10 and 100 μM).

The study of Ung *et al.* on the activation of PXR also provides interesting insight into the interpretation of molecular descriptors and their potential for informing medicinal chemistry [94]. They point out the tremendous range of compounds that are known to be activators, and the range of structural features they exhibit, including aromatic and saturated multiring structures, halogen- and oxygen-rich compounds, and compounds with chain-like structures, such as discodermolide and β -carotene. This is consistent with PXR's flexible, predominantly hydrophobic, binding site that permits binding of a wide range of substrate sizes in multiple conformations [161]. Despite the flexibility of the site, which might lead to the expectation that complex descriptors would be preferred in the model, certain molecular descriptors were selected that are related to identifiable structural features in compounds. Table 8.8 lists some of these descriptors and their values for activators and nonactivators. These descriptors relate to size, polar surface area, flexibility, and the number/electronic and topological state of certain types of heteroatoms (nitrogen and halogens). The number of rotatable bonds and Kier molecular flexibility index indicate that, on average, activators are slightly smaller and less flexible than nonactivators. Polar surface area is slightly lower for activators

TABLE 8.8 Selected Descriptors for Differentiating Between Activators and Nonactivators of Human Pregnane X Receptor

Property	Activators ^a	Nonactivators ^a
Number of rotatable bonds	4.45	5.99
Kier molecular flexibility index	5.84	6.24
Polar surface area	61.9	69.5
Number of halogens	1.16	0.8
Number of chlorines	1.02	0.27
Number of nitrogen atoms	0.8	1.79
Atom-type E-state sum for chlorine	6.33	1.63
Atom-type E-state sum for >NH	0	0.45
Atom-type E-state sum for =N-	0.31	1.15
Atom-type E-state sum for >N-	0.15	0.94

^aFigures are the mean values for compounds predicted by the model to be activators or nonactivators.

(although it might not be so when normalized for molecular size). Activators tend to have more halogen and less nitrogen atoms than nonactivators. However, for simple descriptors (numbers of rotatable bonds, halogens, chlorines, and nitrogens), the differences are small, particularly when taking into account that the values are limited to integers; these descriptors may not be particularly informative. Thus, the presence of four or five rotatable bonds is more likely to indicate an activator than nonactivator, while six is more likely to indicate an activator. One chlorine is more likely to indicate an activator than a nonactivator, but a significant proportion of nonactivators must have at least one chlorine. For halogens, in general, there is very little difference between the mean values for activators and nonactivators, both of which are close to 1. Two nitrogen atoms in a molecule are more likely to indicate a nonactivator, while one is more likely to indicate an activator.

While being less amenable to immediate interpretation, the E-state indices [162], which encode information about the electronic and topological environment of particular atoms, offer the potential for greater discrimination on a continuous scale. Thus, the E-state values for chlorine and for different bonding configurations of nitrogen (see the last five rows of Table 8.8) provide such information for discriminating between activators and nonactivators of human PXR, in which the nonintegral mean values and values between them can have a true meaning. Thus, it can be immediately inferred from the mean number of chlorine atoms in predicted nonactivators (0.27) that a significant proportion of them must have at least one chlorine atom. In contrast, the nonintegral values of the E-state sums of chlorines in predicted activators and nonactivators (6.33 and 1.63, respectively) provide information about the number of chlorines, and the modulating electronic field effects of the other atoms in the molecule, weighted by their topological distance (i.e., the number of bonds) from the chlorine(s). Similarly, the mean numbers of nitrogen atoms in predicted activators (0.8) and nonactivators (1.8) indicate some uncertainty around the status of compounds having one nitrogen atom. Again, the relative values of the E-state indices for the different configurations of nitrogen are well separated, helping distinguish between the two classes of compounds. The drawback in using such descriptors has already been addressed in reference to the model of Balakin *et al.* for CYP3A4 binding.

A further means of exploiting predictions of drug metabolism and drug interactions is to use them as inputs to appropriate PBPK models, linking the prediction of a drug's properties to its expected behavior *in vivo*. Sensitivity/error analysis of a PBPK model (i.e., the quantification of the dependence of its outputs on the value of any of its inputs) will provide information about how uncertainty and/or bias in the predictions of a drug's property will affect its expected behavior *in vivo*. This type of analysis makes use of the ease of rerunning simulation models with appropriate variation of the input and can be used in one of two related ways:

1. By scanning an input value through a (potentially hypothetical) range and recording the predictions of the PBPK model for each input value, the sensitivity of the predictions to the value of the input can be ascertained. This is known as *sensitivity analysis*.
2. By sampling the predicted distribution of the input (which can be generated from the error statistics of the model that generates the input), the corresponding distributions in the PBPK model's predictions can be determined. This is an aspect of *error analysis*.

Because PBPK models require quantitative inputs on the whole, the most effective use of this approach requires that the models for the input properties generate quantitative values or classifications with classes with well-defined upper and lower limits. As we have discussed, many models for predicting drug metabolism and drug–drug interactions are binary classification models (i.e., for substrate/non–substrate and inhibitor/noninhibitor), so there is still significant progress to be made in model development in this area to realize the prediction of *in vivo* behavior from *in silico* metabolism and drug interaction models.

We complete this section by emphasizing that DMPK represents just one part of a drug's property set and that the significance of its DMPK properties—whether determined by experiment or by *in silico* modeling—has to be viewed in the context of its other properties, particularly its on-target and off-target pharmacology. Of the properties we have discussed in this chapter, it is particularly important to view those that can cause drug–drug interaction (i.e., inhibition of metabolism and induction of metabolism/transport) in this context. The propensity of any potential new drug to actually cause such interactions *in vivo* is dependent on the concentrations it is required to achieve *in vivo*. These are, in turn, governed primarily by its activity against its target(s). Low activities against targets will require high concentrations *in vivo*, increasing the likelihood of interactions with other drugs. In a similar manner, the most effective use of PBPK models that can predict plasma concentrations is dependent on knowledge of the expected therapeutic window of the compound, which is in turn determined by its primary pharmacology and its dose-limiting toxicity. The most efficient screening strategies will be those that bring together useful data in pharmacology, toxicity, and DMPK, each having similar costs of information relevant to the particular stage of the discovery process. This could entail a combination of *in vitro* and *in silico* data, the balance between the two depending on the cost of information. High throughput *in vitro* screens for drug metabolism properties [66,163] are able to generate the large amounts of data required for producing reliable models and also reduce the cost of generating primary assay data, potentially reducing the need for predictive models. Set against this, the use of systems for automated QSAR model development [164] can

drastically reduce the time and cost of model development and their regeneration from evolving data sets. If target activity can be predicted *in silico*, so permitting hits to be identified virtually, then the ability to predict metabolic and *in vivo* drug–drug interaction properties can result in a significant recovered opportunity cost by permitting deferral of the synthesis of potentially unsuitable compounds [66].

8.6 CONCLUSIONS

In silico methods are making significant contributions to our growing knowledge of the factors that determine how drugs and other xenobiotics are processed *in vivo*. Numerous methods have been developed and employed over the past 30 years. In this chapter, we have endeavored to do justice to a number of these methods, to their contributions to the furthering of knowledge and their practical applications in the drug discovery process. We have described two major areas of application. Each area is extremely complex in its own right; this must be borne in mind when assessing current capabilities and future progress.

The first is concerned with the prediction of interactions between drugs and individual proteins (drug-metabolizing enzymes, transporters, and nuclear receptors), including the simultaneous interactions of multiple drugs with an enzyme or transporter that can give rise to mutual or unilateral inhibition of metabolism or transport. The complexity in this area arises from the well-documented promiscuity of the proteins involved. A given enzyme/transporter/receptor can metabolize/transport (or be inhibited by)/be activated by multiple xenobiotics. A given xenobiotic can be metabolized by/transported by (or inhibit)/activate multiple proteins. The challenge of *in silico* modeling is to identify those interactions that will/will not occur, and the strength of those interactions, as measured by rates of metabolism or transport, binding constants, inhibition constants, and so on. For many of the applications we have described, a large part of the challenge entails the collation of a reliable data set of a large enough size for model development. For any given problem, it is not guaranteed that a suitable data set can be generated, because of the huge number of combinations of xenobiotic/protein interactions that must be considered. For example, development of a QSAR model to discriminate between the metabolism of compounds by (say) four isoforms of CYP450 requires reliable data for each compound and for each isoform. It frequently arises that the absence of reported metabolism of a compound by a particular isoform is taken to indicate that the compound is not metabolized by that isoform but this is not, of course, the same thing. Without definitive data, such assumptions can compromise the modeling process from the start. Extend this to the myriad other interactions of interest—with UGT enzymes, ABC transporters, nuclear receptors, and so on, and the whole model development and application structure can be fatally undermined. Improvements in reliable, high throughput, low volume assay methodologies will significantly increase the availability of reliable data for model development in the future.

In assessing the progress and utility of *in silico* model development in this area, the differences between practice in the drug discovery industry and the majority of reports in the scientific literature must be borne in mind. Many the papers we have discussed in Section 8.4 have shown modest predictive ability for a range of properties related

to drug metabolism and drug–drug interactions. We should not be surprised with these results, given the data available, the relatively small data sets, and the diversity of the compounds that can be processed by, or bind to, the proteins in question. These types of models are generally termed *global* models, in that they are generated using data for compounds scattered across a wide range of chemistry—almost invariably using data on marketed drugs. This is not necessarily because of the desire to generate global models, but because of the limitations of data availability in the public domain. The practicalities of drug discovery are different. Discovery projects focus on a small number of specific areas of chemistry, so chemical diversity (and the problems introduced by this) is much reduced. The need for a model to extrapolate or interpolate into an area of chemical space that is poorly represented in the training set is consequently reduced. This matter has been discussed by Weaver and Gleeson [49] in relation to the development of QSPR models for predicting plasma protein binding and inhibition of CYP3A4-mediated metabolism. It seems reasonable to regard the prediction statistics of global models as a lower bound on what should be achievable with models on more focused areas of chemistry.

The second application area we have considered is that of PBPK modeling, by means of which data (whether from *in vitro* or *in silico* sources) on drug metabolism and drug–drug interactions can be integrated to predict *in vivo* outcomes. The key modeling challenges for the prediction of drug–drug interactions are (i) to predict the concentrations of drugs at the active sites of the relevant enzymes/transporters and (ii) to be able to appropriately scale, if necessary, kinetic parameters (e.g., $V_{\max}$, K_m , and K_i) to the *in vivo* case. These two are dependent, as the ability to predict concentrations depends, in part, on the scaling of kinetic parameters of drug transport and metabolism. The complexity in this case arises from the multiple potential *in vivo* processes (absorption, distribution, metabolism and elimination, each potentially via multiple competing routes) to which a xenobiotic can be subject and which affect prediction of drug concentration at the active site(s). In most cases, in drug discovery and development, appropriate data required to model some—maybe most—of these processes will be lacking. Therefore, predictions must be interpreted in the light of what is, and what is not, known about the processes affecting the fate(s) of a particular compound *in vivo*. The evolution of predictive PBPK models is absolutely dependent on the development of appropriate assays for these processes and the knowledge to enable data to be successfully scaled to the *in vivo* case. This knowledge frequently lags significantly behind assay development and is frequently a significant impediment to new applications of PBPK modeling. Having said that, we have described a number of applications where the technique has already been used to provide valuable information and interpretation concerning *in vivo* drug metabolism and drug–drug interactions.

It can be seen that *in silico* techniques are able to provide valuable insight and predictivity regarding drug metabolism and interactions *in vitro* and *in vivo*. Significant work remains to be done on improving predictability for known routes of metabolism and transport, and the challenge is continually growing as further routes are discovered, and hence opportunities for drug–drug interactions to arise are identified. In fact, this very increase in the known complexities involved in drug metabolism and interactions requires that the contribution of *in silico* methods in this area increases to help keep drug discovery costs under control and to inform and guide the discovery selection process.

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9 Methods for Determination of Enzyme Kinetics and Metabolic Rates

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9.1	Summary	1
9.2	Background	2
9.3	<i>In vitro</i> systems to study enzyme kinetics	2
9.4	Basics of enzyme kinetics	8
9.5	Atypical enzyme kinetics	12
9.6	<i>In vitro</i> – <i>in vivo</i> correlations	18
9.7	Concluding remarks	21
	Abbreviations	21
	References	22

9.1 SUMMARY

Determination of the *in vitro* kinetics of metabolism of drugs is critical to the drug discovery and development process. This typically involves using some type of enzyme system (e.g., human liver microsomes, expressed enzymes, and hepatocytes) to measure metabolite formation rates and enzyme involvement (reaction phenotyping). For most compounds, this process usually follows the Michaelis–Menten type of enzyme kinetics resulting in a hyperbolic rate of metabolite formation. However, for some compounds, an atypical kinetic profile may be observed, for example, sigmoidal, biphasic, or substrate inhibition kinetics. Regardless, the proper kinetic equation must be used to describe the kinetics and obtain the most accurate kinetic parameters. These estimated parameters are then used for *in vitro*–*in vivo* correlation (IVIVC) analyses to predict the human *in vivo* pharmacokinetics and the doses to be used in phase I clinical trials.

9.2 BACKGROUND

The introduction of a new chemical entity to the market is a long and expensive process with many potential pitfalls [1]. An integral component of drug discovery and development is determination of the enzymes catalyzing metabolism of the drug, the kinetics involved therein, and the resulting metabolic products. Previously, most of these efforts were focused on the cytochrome P450 (P450 or CYP) enzymes, the primary enzymes involved in oxidative metabolism. However, as drug discovery efforts were focused on reducing P450-mediated metabolism, other enzyme families such as the glucuronosyl transferases (UGTs), the sulfotransferases (SULTs), the flavin monooxygenases (FMOs), gained importance, resulting in efforts to determine the kinetics of these enzymatic reactions, as well. The kinetics of metabolizing enzyme reactions contributes to the half-life of a drug and its elimination rate, thus affecting the dosing regimen and potentially the route of administration. In addition, the resulting metabolic products are not always inactive or innocuous, potentially resulting in toxicity or other adverse effects. Thus, the proper identification of the enzymes involved in metabolism of a drug, the resulting metabolic products, and the kinetics of these processes is paramount to successful drug development.

This chapter summarizes the current state of knowledge in enzyme kinetics, including both typical and atypical enzyme kinetics. Also included is a discussion of the various *in vitro* systems employed to determine enzyme kinetic rates and the resulting products, as well as the strengths and weaknesses of each system. Finally, methods of determining kinetics with the intent of estimating *in vivo* clearances are discussed, as are the basics of enzyme inhibition.

9.3 IN VITRO SYSTEMS TO STUDY ENZYME KINETICS

There are three well-established systems to study P450 kinetics (microsomes, hepatocytes, and expressed enzymes), as well as a newly developed cell line, HepaRG, that is being tested for suitability to study drug-metabolizing enzyme kinetics. Each of these systems offers unique advantages and disadvantages for conducting *in vitro* determinations of enzyme involvement and kinetics of reactions. In fact, one usually employs a complement of the various systems to maximize the information gained, while optimizing the use of resources and reducing overall time to derive the desired information.

9.3.1 Human Liver Microsomes (HLMs)

The most commonly used system for determining drug-metabolizing enzyme kinetics are human liver microsomes (HLMs), prepared from human livers. In this case, the endoplasmic reticulum fraction is isolated from human liver tissue, typically obtained from tissue that was deemed nontransplantable. This system retains all the P450s as well as other microsomal enzymes such as the UGTs and FMOs [2]. Obviously, microsomes may be prepared from any animal species, permitting study of metabolism in that species. HLMs are relatively easy to prepare, convenient to use, can be stored frozen (-80°C) for long periods of time with little relative loss of activity, and are comparatively less expensive than other systems. Because they can be prepared in

relatively large quantities and then stored frozen, the same “batch” can be used for an extended period of time, allowing direct comparison of results conducted over time. It is relatively common for investigators to create a “master mix” of HLMs, pooled from multiple donors. Frequently, proportions of HLMs from various donor livers, wherein the relative activity of various P450 enzymes has been characterized are mixed to create a “master mix” with known isoform activity levels assumed to be representative of the population at large (in contrast to representing only a single individual). These pooled HLMs also avoid the potential use of a prep from an individual liver that might be deficient in metabolism by a particular isoform due to genetic polymorphisms that might provide misleading results.

HLMs offer additional advantages, including the fact that the enzymes are also anchored in the lipid membranes as they would be anchored in the liver *in vivo*. With respect to the P450s, HLMs also contain the redox partners cytochrome P450 reductase (CPR) and cytochrome *b*₅ (*b*₅) at physiologically relevant concentrations. Finally, all the drug-metabolizing enzymes that are present in HLMs are in physiologically relevant ratios, which is important in that *in vitro* data suggest that protein–protein interactions may occur that can alter enzymatic activity, and are dependent on ratios of the enzymes involved. To use HLMs, one simply adds a physiological pH (7.4) maintaining buffer, the enzyme cofactor nicotinamide adenine dinucleotide phosphate (NADPH), and substrate [3,4]. Since multiple enzyme isoforms are present in the preparation, determining the individual isoform(s) involved may be somewhat less straightforward but can be accomplished by coincubation with isoform-specific inhibitors or antibodies [5].

As with any system, there are also disadvantages to the use of HLMs for determining enzyme activity and isoform involvement. Depending on the time between tissue harvest, microsome preparation, and freezing, enzyme activity can be lost to varying degrees depending on the time duration. Freezing whole liver tissue first, then thawing and preparing HLMs can also reduce enzyme activity. In addition, once thawed for use, HLM preparations usually only maintain consistent activity levels for up to 1 h at 37°C, the usual temperature for conducting *in vitro* HLM incubations. It should also be realized that HLMs will not contain either cytosolic enzymes (e.g., SULTs) or transporter proteins [e.g., P-glycoprotein (PgP)]. This lack of the total complement of enzymes precludes evaluation of “total” metabolism of a compound if multiple enzyme families are involved. However, since both UGT and P450 enzymes are present in HLMs, one can add the necessary cofactors for both to permit simultaneous activity by both enzyme systems and thus, to evaluate the overall metabolism by both systems, though this requires care in determining the incubation conditions. More typically, each route of metabolism is evaluated separately. Finally, it should be realized that HLM preps do not contain the necessary machinery for enzyme synthesis and thus, cannot be used for enzyme induction experiments. In spite of these limitations, HLMs remain the primary “workhorse” of drug metabolism studies, given their relatively low cost, ease of use, and availability.

While study on CYPs in HLMs essentially only requires NADPH as a cofactor and an appropriate buffer, the study on UGTs require more involved conditions. Like with the CYPs, a cofactor, uridine-5′-diphosphoglucuronic acid (UDPGA), that provides the glucuronic acid for transfer also needs to be included [6]. However, in contrast to the CYPs, since UGT proteins exist with their active side facing the luminal part of the endoplasmic reticulum, access to substrates and cofactors requires them to pass

through the membrane to access the active enzyme site. Hence, membrane disruptors such as alamethicin (pore-forming agent), sonication, or use of a surfactant (e.g., Brij 58) are required to obtain activities that are readily measurable. Disruption of the membrane is usually conducted on ice for a period of 15–30 min before beginning incubation with substrate. Saccharolactone, a β -glucuronidase inhibitor is sometimes also added to prevent an artificial lowering of UGT activity [7]. Finally, recent research has suggested that a number of K_m values generated earlier may have been artificially elevated due to the presence of inhibitory long-chain unsaturated fatty acids in the incubations that can compete with substrates for the UGT active site. However, it appears that the inclusion of albumin can restore K_m to values that result in more accurate IVIVC predictions. Thus, some researchers advocate for the inclusion of albumin in all incubations evaluating UGTs; however, this opinion is not shared by all.

FMOs are a microsomal enzyme that can metabolize soft nucleophiles, such as nitrogen- and sulfur-containing compounds and, in particular, heterocycles with these atoms. Since FMOs can catalyze analogous reactions to the CYPs, such as N- and S-oxidation, it is important to distinguish between the two enzyme families to assure the correct implications are made. The incubation conditions for FMOs are the same as CYPs, that is, it requires substrate, NADPH, and buffer but at a slightly alkaline pH of 8–9 for optimal activity. To distinguish between CYP and FMO metabolism, the microsomal mixture can be incubated at 45°C for 5 min in the absence of NADPH, causing degradation of the FMOs but leaving the CYPs unaffected. The reaction is then carried out under normal conditions and if the activity goes away, the implication is that FMOs were involved since they would have been degraded during the preincubation. Nonionic detergents that inhibit CYPs but not FMOs can also be used to delineate between the two enzyme families.

9.3.2 Expressed Enzymes

With current technology, almost any individual P450 isoform human or animal cDNA can be expressed in cell systems, such as *Escherichia coli* bacteria, yeast, or insect cells (e.g., *Trichoplusia*), and the resulting enzymes can subsequently either be fully purified or membrane preps can be generated [8,9]. In addition, CPR or b5 can be coexpressed in these cells (with membrane preps as the final form) [10,11]. The enzymes thus obtained are suitable to determine kinetics of a substrate from a single P450, assuming that the cDNA from only a single enzyme was expressed. Expressed enzymes are the simplest system and can provide unequivocal evidence of the ability (or lack of ability) of an enzyme to metabolize a substrate. Expressed enzymes also allow for discernment of the reaction kinetics of a single enzyme, which may be confounded by multiple enzyme involvement in systems with the full enzyme complement. Expressed enzymes offer additional advantages that make them an attractive system in certain circumstances. For example, because only one enzyme is present, they can be used to identify isoforms that make a minor contribution to the metabolism of a substrate that might become more important when the primary isoform is inhibited [12]. This situation would be difficult to discern in a system with the full complement of enzymes that might overshadow this contribution by a more minor enzyme. Hence, expressed enzymes can provide comprehensive information on all the isoforms metabolizing a substrate. In addition, atypical kinetics (see below) are also most accurately elucidated using single enzyme systems, since confounding may occur in multiple enzyme systems

[3]. Finally, expressed systems allow the most convenient study of the kinetics of polymorphic enzymes, since allelic variants are easily expressed in sufficient quantities in these systems. Locating and genotyping sufficient quantities of, for example, HLMs, expressing an allelic variant, is currently difficult.

Incubations using expressed enzymes are, as with microsomes, a straightforward affair with only the addition of an appropriate cofactor [such as NADPH for P450s, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) for the SULTs, and UDPGA for UGTs], buffer at pH 7.4 and the substrate being required. These systems are generally robust enough to provide activity for up to 45 min with a subsequent drop in activity and also hardy enough to withstand a few freeze thaw cycles. Expressed enzyme preparations of CYPs, UGTs, *N*-acetyltransferases (NATs), FMOs, aldehyde oxidases (AOs), SULTs, monoamine oxidases, and esterases are now commercially available and are valuable tools in reaction phenotyping and metabolic pathway elucidation.

It should be realized that expressed enzymes also possess certain disadvantages. For example, since they do not contain the full complement of enzymes, overall clearance is more difficult to estimate and the relative importance of individual enzymes may be overestimated. The fact that a substrate is metabolized by an enzyme in a single-enzyme system does not indicate that this will be observed *in vivo* since a number of variables such as enzyme ratios, ratios of redox transfer proteins (see below), and unbound substrate concentrations at the enzyme active site can influence the metabolism. Also, the concentrations of CPR and b5 used in the expressed enzyme systems are designed for maximal activity and are not reflective of physiological levels. In addition, for fully purified enzymes, lipid vesicles (typically prepared from dilauroylphosphatidylcholine or didecanoylphosphatidylcholine) need to be added to the system as an artificial membrane system to provide a milieu for the hydrophobic portions of the enzyme to anchor. This again is an artificial system and may not be fully representative of complex *in vivo* architecture and interactions. These kinds of systems are termed *reconstituted expressed enzymes*, as opposed to expressed systems, wherein the enzyme is maintained in the membrane environment of the cell system.

A reconstituted recombinant enzyme incubation requires the expressed CYP enzyme, lipid vesicles, appropriate level of CPR (2:1 or greater CPR:P450), b5 (1:1 or greater b5:P450), reaction buffer at pH 7.4, and NADPH, all maintained at 37°C. Since this incubation typically requires a greater number of constituents to be mixed as compared with HLMs, there is typically greater variability in kinetic parameters depending on ratios, order of addition, type of lipids used, and so on. In addition, some P450s such as CYP3A4 are more challenging to reconstitute into lipids and elicit activity, as opposed to others such as CYP2C9 and CYP2D6. Clearance predictions can be made from expressed enzymes, but this usually requires incorporation of a relative activity factor (RAF) established using probe substrates of the enzyme to standardize the activity of the P450 isoform to microsomal activity and an accounting for the relative ratios of each enzyme in a typical liver system [13].

Recently, the issue of CYP enzyme protein-protein interactions has been discussed [14,15] adding an additional layer of potential complexity. In these cases, the presence of a second CYP isoform can modulate (either increase or decrease) the activity of the second isoform. Since expressed enzyme systems typically involve the incubation of a single CYP isoform, one would not expect these interactions to occur in this type of system. However, if these do occur in HLMs, conjectured but not conclusively

proven, then the *in vitro*–*in vivo* extrapolations made from expressed enzyme systems involving a single isoforms may be less accurate than anticipated. A second potential complication can occur when expressed enzymes are mixed in a cocktail fashion for studying enzyme involvement and inhibition interactions, since these protein–protein interactions may then occur in these expressed enzyme incubations containing more than one CYP isoform.

9.3.3 Human Hepatocytes (Fresh and Cryopreserved)

Human hepatocytes contain the entire complement of drug-metabolizing enzymes, induction machinery, and most transporters [16,17]. They also contain all the cofactors and other redox proteins in appropriate levels [18]. Thus, they provide a system very similar to an actual human liver (with the exceptions of blood flow and intercellular interactions). They can be used for longer (e.g., 8–10 h) experiments and hence, may be more beneficial in the study of slowly cleared substrates, though it should be realized that the activity of hepatocytes does decline with time [19,20]. A significant advantage of human hepatocytes (either fresh or cryopreserved) is their ability to synthesize new enzyme protein and thus, they are ideally suited for enzyme induction experiments. The second primary advantage of human hepatocytes is that they contain the full complement of enzymes and transporters and thus, are ideally suited to evaluate total clearance by metabolism and transport. However, this may also be a disadvantage if one wishes to identify the specific isoform(s) involved in metabolism or the kinetics of an individual process.

Fresh hepatocytes, isolated from donor livers, can be maintained in appropriate media and be utilized to determine enzyme kinetics. However, fresh hepatocytes are less readily available, more difficult to maintain than, for example, HLMS. They are also expensive and need to be used relatively soon after donor harvest and preparation [21,22]. Because this timeframe from harvest to preparation and final use can be variable in length, it is not uncommon to observe substantial intersubject variability. Additionally, it should be realized that since hepatocytes are obtained from recently deceased donors, liver function may have been compromised and multiple drugs may have been present at harvest. This could lead to induction or inhibition of the metabolizing enzymes. The extensive handling procedures and expensive materials cost offset some of the advantages of using fresh hepatocytes. Cryopreserved hepatocytes were developed to overcome some of the disadvantages of freshly isolated hepatocytes. These hepatocytes are cryogenically frozen and thus, can be stored for long periods before use, making them more attractive than fresh hepatocytes that typically need to be used within a few days of extraction [23,24]. Cryopreserved hepatocytes can also be pooled, as is done with microsomes, to reduce the impact of interindividual variation. Though originally less active, recent advances in preparation and preservation techniques have resulted in cryopreserved hepatocyte preparations with activity similar to freshly isolated hepatocytes [23].

Incubations with cryopreserved hepatocytes have been standardized by suppliers of these tissues. Thawing and reconstitution of these cells, followed by incubation with buffer and substrate is the typical protocol. Fresh hepatocytes do not require the thawing and reconstitution steps that are required of cryopreserved hepatocytes, but may require additional Percoll filtration steps to improve viability. Since hepatocytes

contain all the necessary cofactors and additional proteins, no additional components need to be added to the media, although these incubations are typically done in 5% CO₂ at 37°C. Recent reports also suggest that cryopreserved hepatocytes, due to the process of instant freezing, are better suited for the study of AO activities that tend to be lost in fresh hepatocytes within 24 h post isolation [25].

9.3.4 Liver Slices

Liver slices are prepared from liver tissue using a microtome to create ultrathin slices. They contain all the drug-metabolizing enzymes and transporters, allow for intercellular communication, and in addition the role of passive diffusion can be elucidated [26,27]. However, liver slices exhibit a rapid decrease in activity (and typically activity lower than hepatocytes), are subject to interslice variation, cannot be pooled to overcome interindividual variation, are expensive to produce, and require special equipment to produce [28]. For these reasons, liver slices are not frequently used in kinetics determination.

9.3.5 S9 Fraction

The S9 fraction, such as microsomes, is prepared by homogenization of the tissue followed by ultracentrifugation to isolate the desired fraction. In this case, the S9 fraction is the supernate typically resulting from the spin before the 100,000g spin that is used to isolate the microsomes. Thus, this fraction of tissue will also contain the cytosolic and mitochondrial enzymes, though the activity can be lower than that of the microsomal fraction [29]. The presence of cytosolic conjugative enzymes such as SULTs, NATs, AO, and xanthine oxidases make the S9 fraction a more versatile system than microsomes. Though at one time used to measure overall metabolism, the requirement to add necessary cofactors and the availability of human hepatocytes has reduced the use of S9 fractions in determining enzyme kinetics and drug metabolism reactions. Incubation conditions for S9 fractions are similar to those with microsomes, that is, substrate, buffer, and appropriate cofactor need to be added. Typically, higher concentrations of protein, 2–4 mg/mL final, are used due to the inherent diluted nature of this system.

AOs are being increasingly recognized for their role in drug disposition, are a cytosolic enzyme, and hence found in S9 and cytosolic fractions and hepatocytes [30]. Their use does not require any special incubation conditions apart from enzyme, substrate, buffer, and recognition. Esterases are present ubiquitously, and hence, any tissue fraction, be it microsomes, cytosol, or a tissue homogenate, can be used to determine the esterase activity and substrate specificity. No unique cofactors are required to be added for esterase activity.

In contrast to the above cytosolic enzymes, the SULTs, another cytosolic drug-metabolizing enzyme, require addition of the cofactor PAPS to supply the sulfate group that is transferred onto the phenolic oxygen during an SN₂ substitution reaction. Similarly, NATs can be assayed for activity in cytosolic protein or S9 at protein concentrations of 0.2–1 mg/mL, 1 mM ethylenediaminetetraacetic acid (EDTA), dithiothreitol (1 mM; optional), phosphate buffer, pH 7.4, and acetyl CoA (2 mM). Acetyl CoA is the cofactor supplying the acetyl group that is added onto a nitrogen atom.

9.3.6 HepaRG Cells

The HepaRG cell line was created following immortalization of liver cells from a donor with hepatocellular carcinoma [31]. The cells were subsequently cultured and passaged with the cells then differentiated by the addition of dimethyl sulfoxide (DMSO). Once DMSO is added to the medium, the cells differentiate into hepatocyte-like granular cells with one or two nuclei. In addition, during this process, bile cannicular-like structures are formed [32]. As compared with isolated human hepatocytes, HepaRG cells exhibit comparable or greater levels of many drug-metabolizing enzymes (CYP1A2, CYP2B6, CYP2C9, CYP2E1, and CYP3A4, SULTs, UGTs, and glutathione-S-transferases), transporters, and induction elements (constitutive androstane receptor, peroxisome proliferator-activated receptors, aryl hydrocarbon receptor, and pregnane X receptor) [33,34]. In addition, the bile cannicular-like structures provide another level of detail and physiology to these cells [35,36]. Endogenous proteins such as albumin, haptoglobin, and aldolase B are also present in these cells [34]. Together, these factors suggest that HepaRG cells are very similar in morphology and physiology to liver cells, making them an appropriate system to study enzyme kinetics, inhibition, induction, and long-term toxicity. In addition, the HepaRG cells are stable for up to two weeks after differentiation and hence are useful to study slowly metabolized substrates, as well as conduct long-term toxicity and mutagenicity studies.

However, there are some identified disadvantages of HepaRG cell lines. For example, at the present time, they do not contain all P450s at physiologically appropriate ratios—for example, CYP2D6 is not present since the donor may have been a CYP2D6 poor metabolizer [34]. In addition, since it is a cell culture system, constant maintenance is required and sufficient time must be allowed (over 30 days) for the cell line to be cultured and differentiated. Despite these disadvantages, the HepaRG cell line appears to have promise toward providing qualitative and quantitative information on drug metabolism, although further characterization of activity and enzyme levels is required.

9.4 BASICS OF ENZYME KINETICS

9.4.1 Michaelis–Menten Kinetics

Enzyme kinetics are assumed to be governed by processes that are saturable and can be described mathematically by the Michaelis–Menten (Michaelis–Menten, sometimes called *Henri–Michaelis–Menten equation*) equation (Eq. 9.1), which states that at low concentrations of substrate, a first-order linear relationship exists between the rate and substrate concentration, and at high saturating concentrations of substrate, the rate is independent of substrate concentration and reaches a maximal value [37]. This maximal value is termed $V_{\max}$ or the maximum velocity, while half the concentration required to achieve this maximum velocity is termed K_m . With respect to the single-substrate reaction (Scheme 9.1), it should be realized that K_m is equal to $(k_{-1} + k_2)/k_1$ and thus is the amalgamation of several rate constants. With respect to affinity, unfortunately, K_m is frequently (and incorrectly) used interchangeably with K_S ($K_S = k_{-1}/k_1$), which is the substrate dissociation constant. Though K_m may sometimes approximate K_S when k_2 is very small relative to k_1 and k_{-1} , the two do not have to be equal, and numerous examples exist where these parameter values vary dramatically. $V_{\max}$ is

**Scheme 9.1**

indicative of the capacity of the enzyme to turnover that particular substrate, a higher $V_{\max}$ indicates faster turnover of substrate. The kinetic scheme and equation governing Michaelis–Menten equation are given as follows:

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]} \quad (9.1)$$

where $V_{\max} = k_2 \cdot [ES]$, which is a measure of the rate of product formation, $K_m = (k_{-1} + k_2)/k_1$ is the Michaelis constant, and $K_S = k_{-1}/k_1$ is the dissociation constant for the ES complex.

Figure 9.1a depicts the classic hyperbolic enzyme kinetics plot. Several assumptions have been made in the generation of the Michaelis–Menten equation. It is assumed that the $[ES]$ complex is in a quasi-steady state, that is, its rate of concentration change

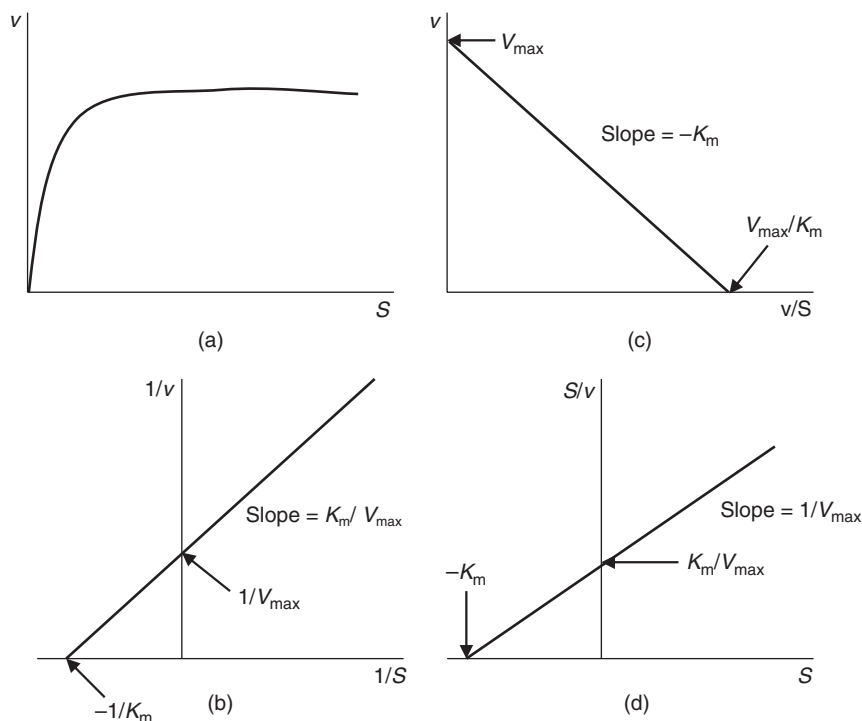


Figure 9.1 Kinetics of enzymes following Michaelis–Menten (MM) kinetics: (a) the classic hyperbolic plot, (b) the Lineweaver–Burk (LB) double reciprocal plot, (c) the Eadie–Hofstee (EH) plot, and (d) the Hanes–Woolf plot. EH, LB, and EH plots can be used for determining whether the data follows MM kinetics, while all plots can be used for determination of the kinetic parameters— $V_{\max}$ and K_m .

is zero since each molecule of [ES] converted to product is replenished instantly. The other key assumption is that total enzyme concentration is constant and is the sum of free enzyme, [E], and the [ES] complex. Additionally, the concentration of the substrate is much higher than the enzyme, and this combined with the total enzyme concentration being constant implies that the rate has a pseudo-first-order dependence on substrate concentrations (at low nonsaturating substrate concentrations).

The Michaelis–Menten equation can be rewritten into a number of forms to allow for easier visual analysis of the data and calculation of kinetic parameters [37] (Fig. 9.1).

9.4.2 Lineweaver–Burk (LWB) Double Reciprocal Plot

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (9.2)$$

The Lineweaver–Burk (LWB) plot depicts the reciprocal of velocity versus substrate concentration and results in a straight line with a slope equal to $K_m/V_{\max}$, a y -intercept representative of $1/V_{\max}$, and an x -intercept equal to $-1/K_m$ (Fig. 9.1b).

9.4.3 Eadie–Hofstee (EadieHofstee) Plot

$$v = -K_m \cdot \frac{v}{[S]} + V_{\max} \quad (9.3)$$

The Eadie–Hofstee (EadieHofstee) plot is graphically represented as v versus $v/[S]$ and results in a straight line with slope equal to $-K_m$, with the y -intercept representing $V_{\max}$ and the x -intercept equal to $V_{\max}/K_m$ (Fig. 9.1c).

9.4.4 Hanes–Woelf Plot

$$\frac{[S]}{v} = \frac{1}{V_{\max}}[S] + \frac{K_m}{V_{\max}} \quad (9.4)$$

In the Hanes–Woelf plot, where it is plotted as $[S]/v$ versus $[S]$, giving a straight line with a slope representative of $1/V_{\max}$, where the y -intercept is equal to $K_m/V_{\max}$ and the x -intercept is equal to $-K_m$ (Fig. 9.1d).

Though each of the above graphical representations can give quick estimations of the kinetic parameters K_m and $V_{\max}$, in general, nonlinear regression fitting of the data to the Michaelis–Menten model is preferred for more accurate estimations. These types of nonlinear regression fits have been made easier with the advent of inexpensive, readily available, and easy to use kinetic modeling programs.

The first step in determination of enzyme kinetics, viz $V_{\max}$ and K_m values, or depletion rate constants, is the verification of time and protein (enzyme concentration) linearity. One must assure that the proposed length of the incubations is not so long that metabolite formation rates are affected by enzyme degradation and that the conditions do not consume all cofactors, such as NADPH or too much substrate that could also affect metabolite formation rates. To accomplish assurance of linearity of time, one usually conducts incubations at 4–5 time points, typically ranging from 5

to 45 min. In the case of enzyme concentration, again, 4–5 protein concentrations are employed. With regard to substrate concentration, a concentration near the expected $V_{\max}$ or at least equal to the expected K_m should be employed. Finally, determination of enzyme kinetics is also based on the assumptions of rapid equilibrium and steady-state conditions. To this end, the investigator should assure that <10% substrate consumption occurs throughout the experiment to maintain steady-state conditions. Obviously, the above methodologies are dependent on the ability to measure the formation of metabolite, as the measurement of the amount of “product” formed.

9.4.5 Use of Substrate Depletion Methods to Determine Enzyme Kinetics

Early in the drug discovery process, it is desirable to obtain estimates of clearance rates for the molecule to assess its viability for further development, before metabolic pathways of drug removal have been determined, and before expending significant resources and time to synthesize metabolite(s). While the traditional methods of assuring time and protein linearity as well as steady-state kinetics for the determination of enzyme kinetics is preferred, many less labor-intensive methods also exist. Since K_m values are in general of greater interest in the drug development process than $V_{\max}$ values, determining K_m from a substrate disappearance $t_{1/2}$ assay has been described and the theoretical validation of the technique explained [38,39]. The first-order rate of degradation of substrate over a range of substrate concentrations is determined, and the inflection point of the plot of first-order rate versus substrate concentration can give a very reasonable approximation of K_m . It should be noted that determination of the substrate disappearance $t_{1/2}$ for the prediction of *in vivo* clearances should typically be done at substrate concentrations well below K_m to assure linearity. However, K_m values are generally not known for new chemical entities, and hence, in an attempt to assure substrate concentrations below K_m , typically $1\ \mu M$ substrate concentration is utilized.

P450 kinetics has been extensively studied and K_m values from submicromolar to millimolar range have been obtained. Compounds with submicromolar K_m values typically are good substrates and can sometimes display saturable metabolism *in vivo*. The observed range of K_m values for UGTs has been reviewed extensively by Kiang *et al.* [40]. While UGTs are in general considered to be high K_m reactions, a closer analysis reveals that a wide range of K_m values starting from single digit micromolar ($5\ \mu M$) up to millimolar levels have been observed. However, the bulk of K_m values observed for the UGTs are in the double digit micromolar range and above, suggesting that saturation kinetics will not be observed *in vivo* since drug concentrations seldom reach this range. Even with K_m values in the range of $5\ \mu M$, the risk of nonlinear kinetics (due to saturation of clearance) for the UGTs is less likely when only unbound concentrations of the drug is considered.

In the case of the FMOs, the reported K_m values are in a much tighter range, from around $2.5\ \mu M$ to approximately $60\ \mu M$ [41–49]. For AOs, a molybdoflavoprotein that prefers to oxidize carbons with low electron density (nitrogenous heterocycles) [50,51], the reported K_m values have ranged from $3.4\ \mu M$ to upward of $400\ \mu M$ [52–55]. As suggested from these reports, K_m values for the FMOs and AOs are rarely in the low single digit micromolar concentrations, and hence, a $1\ \mu M$ starting substrate concentration for $t_{1/2}$ determinations is appropriate for these enzymes. However, for

the sulfonyltransferases, the K_m values span a broader range ($0.5\ \mu M$ to $\sim 1\ mM$) [56–63], but generally are in the low micromolar range.

One should use the lowest possible protein concentration to minimize nonspecific protein binding and to be cognizant of incubation time as microsomes are generally stable for only 45–60 min [64]. Also, it should be realized that a minimum of 15–20% of the drug needs to be cleared to provide sufficient analytical resolution. In the case of slowly cleared substrates, large errors may be introduced due to difficulty in dissecting the rate of depletion of substrate from analytical error. Substrate depletion methods can be used to determine half-lives that are used in estimating intrinsic clearances [65] (see Section 9.6).

9.4.6 Caveats to be Considered in the Determination of Enzyme Kinetics

The effects of solvents on the activity of enzymes also require special consideration, since most drug candidates are nonpolar and require some solvent component for dissolution. Numerous studies have demonstrated that various organic solvents can either activate or inhibit different isoforms, depending on the substrate and enzyme source. For example, at concentration of 0.3%, methanol and acetonitrile had no effect on CYP1A1, CYP2D6, CYP2B6, and CYP2C9. However, at a concentration of 1%, methanol inhibited these same enzymes by 12–26% and acetonitrile inhibited CYP1A1, CYP2B6, CYP2A6, CYP3A4, CYP2C19, and CYP2D6 by 10–60%, depending on the isoform. In contrast, even at concentrations as low as 0.1%, DMSO inhibited CYP3A4, CYP2C19, and CYP2D6 by 15–25% [66]. Ideally, one should try to use <0.3% of methanol or acetonitrile to dissolve substrates, though when not feasible, one should use as little organic solvent and preferably <1%.

9.5 ATYPICAL ENZYME KINETICS

While Michaelis–Menten kinetics are the most commonly observed kinetics among the drug-metabolizing enzymes, there is a growing database of substrate–enzyme combinations that display atypical kinetics. These non-Michaelis–Menten kinetics have been observed most frequently in CYP3A4 and CYP2C9, and they have also been observed in CYP2D6, CYP1A2, and CYP1A1. In addition to P450s, SULTs, UGTs, and PgP, transporter proteins have also been observed to demonstrate atypical kinetics. The application of the Michaelis–Menten equation to substrate metabolism displaying atypical kinetic behavior may result in errors in the estimation of *in vitro* intrinsic clearance, which is subsequently used in the estimation of *in vivo* clearance. Atypical kinetics is generally believed to occur when two substrate molecules (either two molecules of the same substrate or two different substrates) are present in the active site simultaneously [67]. It should, however, be recognized that multiple conformers of the enzyme or enzyme dimers [68] have also been suggested to contribute to the occurrence of atypical kinetics.

The following sections describe the various kinds of atypical kinetics, the equations governing them, and the diagnostic plots to determine the nature of the kinetics [69–72]. While this chapter focuses predominantly on CYPs, it should be noted that SULTs, UGTs, and transporters also display atypical kinetics. These have been reviewed previously and hence are not dealt with here [73].

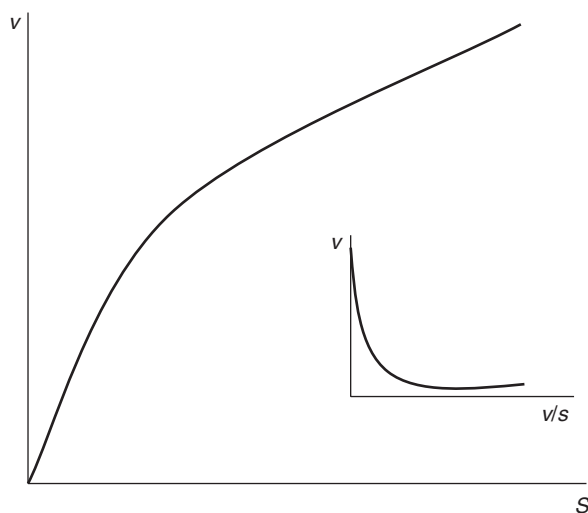


Figure 9.2 Biphasic kinetics. The inset shows the diagnostic EH plot for the same.

9.5.1 Biphasic Kinetics

Biphasic kinetics (Fig. 9.2) is observed when the metabolite formation rate appears to initially behave as if it would begin to saturate but instead continues to increase in a linear fashion, such as that occurring with the CYP2C9 substrate naproxen [67]. In this case, two molecules of the same substrate are present in the enzyme active site. One of the catalytic sites behaves as a low K_m and low V_{max} (high “affinity,” low capacity) site, while the other catalytic site behaves as a high K_m and a high V_{max} site (low “affinity,” high capacity). Hence, at low substrate concentrations, the first catalytic site is more represented in the velocity–substrate plot, while at higher substrate concentrations closer to K_{m2} , the second catalytic site is more prominent. As seen in Fig. 9.2, saturation is not observed, but a linear increase in velocity is observed at high substrate concentrations. The Eadie-Hofstee plot for this kind of kinetics is shown in Fig. 9.2 (inset). This type of plot will also be visible if two enzymes are present and both catalyze a single-substrate reaction, one enzyme with a low V_{max} and K_m and the other enzyme with a high V_{max} and K_m . When a substrate exhibits biphasic kinetics, but the Michaelis–Menten equation is incorrectly applied, an underprediction of clearance (V_{max}/K_m) typically occurs. When substrate depletion experiments are used to determine intrinsic clearance, one must be careful to use an appropriate substrate concentration (typically as low as possible, e.g., $1\ \mu M$ or lower) so as to best reflect metabolism at the low K_m site (which is typically nearer actual physiological concentrations of the drug), rather than a higher concentration that might reflect the high K_m site and typically not physiological.

The equation used to describe biphasic kinetics (Eq. 9.5) is as follows:

$$v = \frac{(V_{max1} \cdot [S]) + Cl_{int}^* [S]^2}{K_{m1} + [S]} \quad (9.5)$$

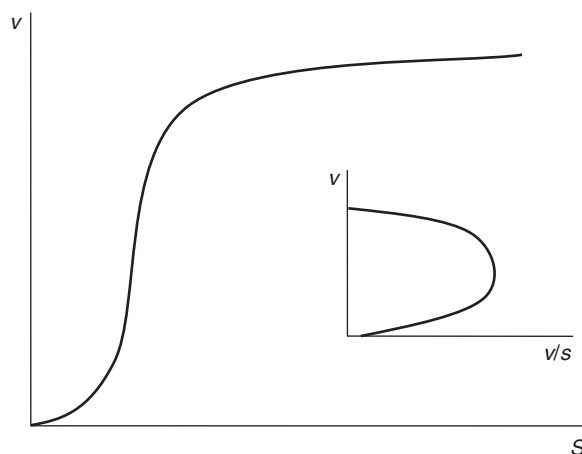


Figure 9.3 Autoactivation kinetics. The inset shows the diagnostic EH plot for the same.

where $V_{\max 1}$ and K_{m1} are the parameters governing the first catalytic site and Cl_{int2} is $(V_{\max 2}/K_{m2})$ for the second catalytic site, that is, Cl_{int2} is the slope of the linear portion of the plot that occurs at high substrate concentrations.

9.5.2 Autoactivation (Sigmoidal Kinetics)

Autoactivation (Fig. 9.3) occurs when a substrate activates its own metabolism, again presumably when two substrate molecules occupy the active site at the same time resulting in a sigmoidal kinetic profile [67]. Detecting sigmoidal kinetics can be challenging since the changes in the substrate–velocity plot at low substrate concentrations are often subtle, sometimes making it difficult visually to distinguish from Michaelis–Menten kinetics. Thus, it is suggested that the data be plotted in an Eadie-Hofstee plot (Fig. 9.3, inset) to better assess the type of kinetics being exhibited. It should be recognized that sufficient analytical sensitivity and precision are also paramount in establishing autoactivation kinetics. When autoactivation (sigmoidal) kinetics are observed in a two binding site model, $V_{\max 2} > V_{\max 1}$ (when $K_{m1} = K_{m2}$) or $V_{\max 1} = V_{\max 2}$ (when $K_{m2} < K_{m1}$) [67]. Hence, two independent solutions exist to the sigmoidal equation. The two binding sites can have naturally differing $V_{\max}$ and K_m values, or the binding of the first substrate molecule could cause a conformation change in the enzyme active site, thereby altering the $V_{\max}$ and K_m values of the second binding site. If the $V_{\max}$ and K_m values of the two binding sites are similar in values, then a hyperbolic profile will be visible. Use of the Michaelis–Menten equation to predict kinetic parameters for a substrate exhibiting autoactivation kinetics will result in an estimation of $V_{\max}$ and K_m more reflective of the high $V_{\max}$ site, most likely leading to an underestimation of intrinsic clearance at the more physiologically relevant concentrations that usually occur near K_m of the lower $V_{\max}$ site. In substrate depletion experiments, as with biphasic kinetics, the starting substrate concentration will determine whether the intrinsic clearance of the first or second catalytic site is estimated.

The Hill equation (given below) can be used for a simplistic fit of autoactivation data:

$$v = \frac{V_{\max} \cdot [S]^n}{K' + [S]^n} \quad (9.6)$$

where n is the degree of cooperativity; a value of n equal to unity represents no autoactivation, whereas a value of $n > 1$ suggests cooperativity and thus, sigmoidicity. Note that this equation gives the parameter K' , which is analogous to, but not the same as, K_m . If a sufficient number of data points are employed, one can use Equation 9.7 to estimate the $V_{\max}$ and K_m values for each of the two binding sites:

$$v = \frac{\frac{V_{\max 1} \cdot [S]}{K_{m1}} + \frac{V_{\max 2} \cdot [S]^2}{K_{m1} \cdot K_{m2}}}{1 + \frac{[S]}{K_{m1}} + \frac{[S]^2}{K_{m1} \cdot K_{m2}}} \quad (9.7)$$

where $V_{\max 1}$ and K_{m1} are the kinetic parameters for the first binding site and $V_{\max 2}$ and K_{m2} for the second binding site.

9.5.3 Heteroactivation

Heteroactivation occurs when coincubation with another molecule (effector) results in an increase in the metabolism of the primary substrate [67,74]. The effector molecule is frequently also a substrate for the same enzyme, but this is not an absolute requirement. Importantly, in the case of heteroactivation coincubation of substrate, the effector does not result in inhibition of the primary substrate, as might be expected. It is generally believed that as with the other atypical kinetics phenomena discussed herein, the effector and substrate are believed to be present in the active site simultaneously, despite the fact that they are different molecules. It should also be noted that effector molecules binding to an allosteric site outside the active site have been reported [75], possibly causing conformational changes within the active site that might result in cooperativity. It has also been observed that the effector molecule is capable of undergoing metabolism simultaneously with the substrate [10]. This suggests that both the effector and substrate are present simultaneously in the catalytic region of the enzyme [67,76]. The following equation has been used to describe data exhibiting heteroactivation kinetics:

$$v = \frac{V_{\max} \cdot [S]}{K_m \frac{\left(1 + \frac{[B]}{K_B}\right)}{\left(1 + \frac{\beta[B]}{\alpha K_B}\right)} + [S] \frac{\left(1 + \frac{[B]}{\alpha K_B}\right)}{\left(1 + \frac{\beta[B]}{\alpha K_B}\right)}} \quad (9.8)$$

where the parameter α is related to the change in K_m due to the presence of the effector ($\alpha < 1$ is reflective of a decrease in K_m), β represents change in $V_{\max}$ due to the presence of the effector ($\beta > 1$ is reflective of an increase in $V_{\max}$), $[B]$ is the effector concentration, and K_B is the dissociation constant of the effector.

Obviously, when determining the clearance of substrates *in vivo*, one does not include a second substrate molecule and so though $V_{\max}$ and K_m are affected by heteroactivation, it would not be operable in typical enzyme kinetics incubations. However, during *in vitro* drug–drug interaction exploration, heteroactivation can be problematic as it will be difficult to predict the degree of interaction that will occur *in vivo*. Studies have been conducted to determine whether heteroactivation occurs *in vivo*. *In vitro* studies demonstrated that diclofenac metabolism by CYP3A4 is stimulated in the presence of quinidine [77]. To assess whether this phenomenon also occurs *in vivo*, the metabolism of diclofenac in monkeys in the presence and absence of quinidine was studied and it was observed that an $\sim 57\%$ decrease in the plasma concentration of diclofenac was observed when quinidine was coadministered as compared with diclofenac alone [78]. In humans, demonstration of heteroactivation has been less clear. Hutzler *et al.* [79] were only able to demonstrate an $\sim 10\%$ activation of flurbiprofen metabolism by dapsone in humans, despite substantial heteroactivation occurring *in vitro*. Though not directly studied, Egnell *et al.* [80], using a meta-analysis from multiple studies, predicted based on modeling that the coadministration of felbamate resulted in a 20–47% increase in the ratio of carbamazepine-epoxide to carbamazepine plasma concentrations due to heteroactivation of CYP3A4-mediated carbamazepine metabolism. A recent study examined the heteroactivation of CYP3A4-mediated metabolism of the dye Nile Red with α -naphthoflavone as an activator [81]. α -Naphthoflavone affected the sequential kinetics of Nile Red and increased the catalytic efficiency of the secondary metabolite production at the expense of the primary metabolite. Another recent paper provides the closest biophysical evidence for a two-site model based on longitudinal T_1 NMR relaxation and docking studies on carbamazepine-mediated heteroactivation of midazolam metabolism by CYP3A4. Midazolam metabolism typically results in greater quantities of 1'-OH-midazolam compared with 4-OH-midazolam when incubated with CYP3A4. However, this ratio declines in the face of high midazolam concentrations or the presence of carbamazepine. The distances of protons at the site of metabolism as determined from the NMR studies were used in docking simulations to demonstrate that when a single molecule of midazolam is present in the active site of CYP3A4, the 1'-CH₃ position of the molecule is closer to the catalytic heme, hence leading to the production of more 1'-OH-midazolam as compared with 4-OH-midazolam. However, at higher midazolam concentrations and when midazolam and carbamazepine are coincubated, two midazolam molecules or one midazolam molecule and one carbamazepine molecule assume a stacked position within the active site. This is predicted to result in the 4'-position of midazolam being oriented closer to the active heme than the 1'-CH₃ position. This observation could explain the shift in ratios of 1'-OH-midazolam to 4-OH-midazolam on homo- or heteroactivation.

9.5.4 Substrate Inhibition

Substrate inhibition (Fig. 9.4) occurs when, at high concentrations of the substrate, the velocity of metabolite formation decreases [67]. Figure 9.4 (inset) depicts the Eadie-Hofstee plot for substrate inhibition. Substrate inhibition is also thought to occur due to the presence of two molecules of the same substrate in the active site simultaneously. In the case of non-overlapping binding sites, substrate inhibition is observed when $V_{\max 2} < V_{\max 1}$ (and $K_{m1} = K_{m2}$ or $K_{m1} > K_{m2}$). When K_m and $V_{\max}$ of both sites are similar, substrate inhibition can still occur if a substrate molecule binding to

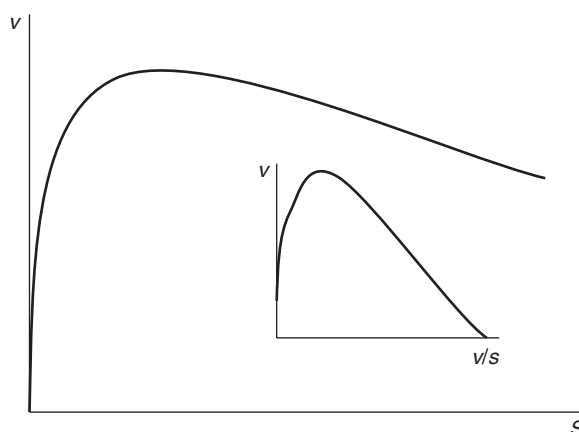


Figure 9.4 Substrate inhibition kinetics. The inset shows the diagnostic EH plot for the same.

one site physically hinders binding to the other site. Substrate inhibition can also occur when the substrate molecule binding to the second binding site causes a change in conformation or efficiency of metabolism at the first binding site. In this scenario, the second binding site is unproductive. One must be careful in interpretation of substrate inhibition in that conditions forced on the system by the inclusion of high substrate concentrations can also cause conformational changes in the enzyme, affect electron transfer, stimulate ionic interactions, or shield ionic interactions, leading to the changes in substrate metabolism rate that mimic true substrate inhibition kinetics. It is unlikely that substrate inhibition will be observed *in vivo* due to the inability to reach the typically high substrate concentrations needed to observe the effect.

The following equation is used to describe data exhibiting substrate inhibition:

$$v = \frac{V_{\max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} \quad (9.9)$$

where K_i is the inhibition constant of the binding of the second molecule causing the inhibition. One should be careful to not confusion substrate inhibition with product inhibition, which can result in a similar kinetic profile. To do so, one must consider the amount of product formed and the inhibition constant for the product toward the reaction. In general, substrate inhibition is much more common than product inhibition.

9.5.5 Partial Inhibition

Partial inhibition occurs when saturating concentrations of an inhibitor are unable to completely inhibit metabolism of a substrate [70]. This phenomenon is usually indicative of mixed inhibition, wherein a productive enzyme-substrate-inhibitor complex is formed. This complex can continue to form product, hence accounting for the partial inhibition. The inhibitor can either bind to the protein in an allosteric site or in the catalytic pocket of the enzyme, resulting in either conformational changes or steric hindrance, thus affecting the binding and metabolism of the substrate.

9.5.6 Substrate-Dependent Inhibition

Because of the presence of multiple binding sites within an enzyme active site, the potential exists for two molecules that are substrates for the same enzyme to bind to the enzyme but not result in either inhibition or activation. This can result in substrate-dependent inhibition, wherein an inhibitor will inhibit the metabolism of one substrate for the enzyme (i.e., both inhibitor and substrate bind to the same site) but not inhibit the metabolism of another substrate for the same enzyme (i.e., the substrate and inhibitor bind to different sites within the enzyme active site). This is particularly problematic during *in vitro* assessment of drug interactions using a probe substrate. One may miss additional potential interactions with an enzyme when only a single probe substrate is used. In these cases (especially CYP3A4), one should employ at least two probe substrates that are thought to bind to different sites within the active site to best assure assessment of full interaction potential. Substrate-dependent inhibition has been observed for CYP2C9, CYP3A4, and CYP2D6, where K_i values have been found to differ by orders of magnitude depending on the substrate [82–85]. Recently, substrate-dependent inhibition of CYP2D6 was demonstrated, while also suggesting the occurrence of two-site inhibition, such as noncompetitive and uncompetitive inhibition. The observation of both noncompetitive and uncompetitive inhibition further substantiates the possibility that CYP active sites are large enough to accommodate multiple substrates simultaneously.

At least one instance of substrate-dependent inhibition *in vivo* has been reported [86]. Mibefradil (CYP3A4 substrate) coadministration increased triazolam area under the curve (AUC) ninefold, but isradipine (another CYP3A4 substrate) coadministration had a minimal effect on triazolam AUC. Hence, although all mibefradil, isradipine, and triazolam are substrates of CYP3A4, only mibefradil had an effect on triazolam disposition, demonstrating the occurrence of substrate-dependent inhibition in humans.

9.6 IN VITRO–IN VIVO CORRELATIONS

An important objective of drug discovery research is the determination of rate of removal of drug *in vivo* and the residence time of the drug in the body. The process of scaling up from *in vitro* systems to *in vivo* systems, commonly referred to as *IVIVCs*, was first developed by Rane *et al.* [87] and further refined by Houston [88] and later Obach *et al.* [65]. The clearance rate of a drug determines its rate of removal from the body. Clearance is defined as the volume of blood cleared of drug per unit time per unit body weight and includes drug removal mechanisms from the gut, liver, kidney, and other organs. To allow direct comparisons between the *in vitro* and *in vivo* situations, one typically estimates the intrinsic clearance (Cl'_{int}), which is the capacity to clear drug from blood in the absence of protein binding and blood flow restrictions. Intrinsic clearance (Eq. 9.10) has the units of the rate of removal of drug per unit concentration:

$$Cl'_{int} = \frac{v}{[S]} \quad (9.10)$$

As described previously, the Michaelis–Menten equation (Eq. 9.1) can be used to fit data that conform to a hyperbolic profile.

In vivo, the concentrations of substrate in the blood rarely approach K_m , such that the $[S]$ in the denominator can be eliminated and Equation 9.1 simplifies to the following:

$$v = \frac{V_{\max} \cdot [S]}{K_m} \quad (9.11)$$

Rearranging Equation 9.11 to place $[S]$ on the left-hand side of the equation makes it clear that the following equalities exist:

$$Cl'_{\text{int}} = \frac{v}{[S]} = \frac{V_{\max}}{K_m} \quad (9.12)$$

Such that the $V_{\max}/K_m$ determined from *in vitro* work serves as an approximation of Cl'_{int} , which can be related to the *in vivo* Cl'_{int} . As discussed above, this is predicated on the assumptions that there are no blood flow restrictions, rate is proportional to concentration, that is, the substrate concentrations are in the linear portion of the Michaelis–Menten equation, and there is no protein binding.

If multiple metabolites are formed, the velocities for the formation of all metabolites can be subsequently summed up to determine total intrinsic clearance:

$$Cl'_{\text{int}} = \sum \frac{V_{\max}}{K_m} \quad (9.13)$$

Early in drug discovery, the metabolic pathway(s) of drug removal has typically not been defined. In this case, the method of substrate depletion (see above) to determine half-life can also be applied to determine intrinsic clearances [65]. Since clearance is also defined as the rate of drug removal per unit time, it is analogous to the first-order rate constant ($k_{\text{elimination}}$). Assuming first-order linear kinetics, clearance is equal to the slope obtained from a velocity–substrate plot and can be related to half-life using the following equation:

$$Cl'_{\text{int}} = k_{\text{elimination}} = \left(\frac{0.693}{\text{in vitro } t_{1/2}} \right) \left(\frac{\text{mL incubation}}{\text{mg microsomes}} \right) \quad (9.14)$$

The clearance thus obtained is in units of mL/min/mg of microsomal protein. The substrate depletion method has also been validated to predict K_m [38]. This methodology has the particular advantage that the determination of K_m in this manner does not require knowledge of metabolites and pathway.

The intrinsic clearance, as obtained from above calculations, is then scaled up from microsomal quantities to a whole liver. This requires the use of a scaling factor that considers the typical amount of microsomal protein per gram of liver. Multiple values of microsomal protein per gram of liver have been used, ranging from 21 to 77 mg protein/g liver, although a value of 40 mg/g liver is the most widely used scaling factor [65,89]. A more recent review has advocated for the use of a scaling factor of 32 mg/g liver (29–34.4, 95% confidence interval) [90]. This intrinsic clearance can then be scaled from per gram of liver to an average liver weight per person (Eq. 9.15).

$$Cl'_{\text{int}} = \left(\frac{0.693}{\text{in vitro } t_{1/2}} \right) \left(\frac{\text{mL incubation}}{\text{mg microsomes}} \right) \left(\frac{45\text{-mg microsomes}}{\text{g liver}} \right) \left(\frac{20\text{-g liver}}{\text{kg body weight}} \right) \quad (9.15)$$

where the unit of intrinsic clearance is mL/min/kg.

In vitro incubations can also be performed with hepatocytes and as with microsomes, appropriate scaling factors have been developed. The number of hepatocytes per gram of liver has been reported to be between 99×10^6 cells/g of liver ($74\text{--}131 \times 10^6$ cells/g, 95% CI) [90] and 120×10^6 cells/g of liver [65] for use in scaling up to the whole liver. Expressed enzymes can also be used to determine intrinsic clearance, although as previously mentioned, there are disadvantages in using this approach since it might overestimate the contribution of the enzyme. A RAF is necessary to standardize the activity of each P450 isoform to its corresponding microsomal activity, which can then be scaled up [13]:

$$\text{RAF} = \frac{\text{Cl}'_{\text{int-HLM}}}{\text{Cl}'_{\text{int-recP450}}} \quad (9.16)$$

This factor needs to be determined for each isoform in the individual expressed enzyme preps using a specific probe substrate, for example, midazolam for CYP3A4, bufuranol for CYP2D6, phenacetin for CYP1A2, and diclofenac for CYP2C9 [13].

Since drugs can bind nonspecifically to proteins during *in vitro* incubations, it is necessary to account for protein binding in the determination of intrinsic clearances:

$$\text{Cl}'_{\text{int,u,inc}} = \left(\frac{0.693}{f_{\text{u,inc}} \cdot t_{1/2}} \right) \left(\frac{\text{mL incubation}}{\text{mg microsomes}} \right) \left(\frac{45\text{-mg microsomes}}{\text{g liver}} \right) \left(\frac{20\text{-g liver}}{\text{kg body weight}} \right) \quad (9.17)$$

where $f_{\text{u,inc}}$ is the fraction unbound drug in microsomes. When $V_{\text{max}}/K_{\text{m}}$ values are used to calculate intrinsic clearance, only the values of K_{m} need to be adjusted ($f_{\text{u,inc}}^* K_{\text{m}}$) since V_{max} values will be unaffected. Hepatic clearance (Cl_{H}) can then be estimated from intrinsic clearance using a hepatic model, such as the well-stirred model [91].

The equation for hepatic clearance using the well-stirred model is as follows:

$$\text{Cl}_{\text{H}} = \frac{Q \cdot \text{Cl}_{\text{int}}}{Q + \text{Cl}_{\text{int}}} \quad (9.18)$$

where Q is the blood flow to the liver estimated at 20 mL/min/kg for humans.

Incorporating both blood binding and protein binding of the drug, the equation takes the following form [13]:

$$\text{Cl}_{\text{H}} = \frac{Q \cdot f_{\text{u}} \frac{\text{Cl}'_{\text{int}}}{f_{\text{u(microsome)}}}}{Q + f_{\text{u}} \frac{\text{Cl}'_{\text{int}}}{f_{\text{u(microsome)}}}} \quad (9.19)$$

where f_{u} is the free fraction of the drug in blood and $f_{\text{u(microsome)}}$ is the free fraction in microsomes. Initial studies suggested that blood and microsomal binding may cancel each other out, but several subsequent studies have shown that the free concentration of the drug needs to be utilized in all clearance estimates [92]. A suite of 29 acidic, basic, and neutral compounds were tested for protein binding, and basic compounds exhibited

the greatest microsomal binding as compared with acidic and neutral compounds. In addition, for all compounds, inclusion of both plasma and microsomal binding resulted in the most accurate scale-up estimates [38]. Two reports have provided methodologies to empirically estimate protein binding of drugs in either microsomes or hepatocytes, providing an alternative to experimentally determining protein binding for each drug candidate [93,94].

These estimates of hepatic clearance are made using the assumption that the concentration of drug in the blood is equal to the concentration in the plasma. However, if the blood to plasma concentration ratio of the drug is different from unity, a correction needs to be included [65]:

$$Cl_{bl} = \frac{Cl_p}{B/P} \quad (9.20)$$

where Cl_{bl} is the blood clearance and B/P the blood to plasma concentration ratio of the drug.

It should be realized that the liver may not be the only organ responsible for clearing the drug. In this case, to determine the total clearance of the drug, one must sum (Eq. 9.21) the clearances from all organs involved in the clearance of the drug from the body, for example, excretory clearance from the kidneys and gut metabolism, though estimation of these individual clearances may not be straightforward:

$$Cl = Cl_{hepatic} + Cl_{renal} + Cl_{intestine} + Cl_{pulmonary} + \dots \quad (9.21)$$

Multiple studies have attempted to use the above IVIVC approach to evaluate the accuracy of predicting the hepatic clearance of a variety of drugs [64,65,92, 95–99]. In general, both microsomes and hepatocytes (fresh and cryopreserved) perform reasonably well in estimating *in vivo* clearance, although all systems tend to underpredict the clearance. Additionally, fresh and cryopreserved hepatocytes were equally proficient in generating clearance estimates for use in predicting *in vivo* clearance when both protein and plasma binding were considered.

9.7 CONCLUDING REMARKS

Determination of *in vitro* enzyme kinetics is critical to drug discovery and drug development. Utilization of the appropriate system and appropriate equations are important to avoid the generation of inaccurate estimates. Several *in vitro* systems (e.g., microsomes, hepatocytes, and expressed enzymes) can be used for this purpose. One must be cognizant of the potential for atypical kinetics and use appropriate kinetic models. When conducted properly, accurate estimates of *in vitro* kinetics can be used to successfully predict the pharmacokinetic properties of drugs, *in vivo*.

ABBREVIATIONS

AUC	Area under the Curve
b5	Cytochrome <i>b</i> ₅

CPR	Cytochrome P450 Reductase
DMSO	Dimethyl Sulfoxide
FMOs	Flavin Monooxygenases
HLC	Human Liver Cytosol
HLMs	Human Liver Microsomes
IVIVC	<i>In vitro</i> – <i>In Vivo</i> Correlations
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
P450s	Cytochrome P450
PgP	P-Glycoprotein
RAF	Relative Activity Factor
SULTs	Sulfotransferases
UGTs	Glucuronosyltransferase

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10 Utility of Molecular-Based *In Vitro* Assays in Metabolic Liability of Drug Candidates

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10.1	Introduction	1
10.2	Protein microarrays	2
10.3	DNA microarrays	4
10.4	Summary	14
	Acknowledgments	15
	References	15

10.1 INTRODUCTION

Advances in molecular-based technologies and increased knowledge of genomics and proteomics have resulted in a greater understanding of the metabolic pathways and in particular, the polymorphisms that may cause less than optimal drug pharmacology, unexpected pharmacokinetics, and potential toxicities. The success of the Human Genome Project and improved *in vitro*–*in vivo* extrapolations, helping in the early attrition of potentially problematic drug candidates. These evaluations aid in the planning and design of clinical trials and selection of appropriate subjects for clinical therapies, particularly in oncology, and for drugs with a narrow therapeutic index. Cellular and subcellular fractions from specific organs including the liver, kidneys, and intestines are routinely utilized to assess metabolic and transporter influences on the bioavailability and pharmacokinetics of drug candidates. This chapter briefly describes systems used to understand the metabolic liability of drug candidates at the molecular level and reviews microarray analysis.

The nucleic acid arrays described in this chapter have a common origin in the DNA blotting methods pioneered by Southern in the early 1970s [1]. Majority of the current methodologies continue to use immobilized or tethered DNA or RNA that is hybridized with a second nucleic acid, and the unknown targets are identified by decoding hundreds of thousands of sequences in parallel analyses on a single sample and in a single assay. Direct comparisons of parallel tests on multiple samples (different tissues, individuals, or treatment groups) provide relevant data that can be used to assess the safety and efficacy of drug candidates and drugs.

In drug development, microarrays that can identify from tens to thousands of unique proteins or nucleic acid sequences are used to assess changes in levels of multiple proteins or transcripts after *in vitro* treatment over two or more days or *in vivo* treatments upward of one week. Arrays are typically printed on multiwell plates or glass slides with specific protein or DNA binding sequences that serve to capture target proteins or DNA. Samples from control and treatment cohorts can be compared to determine their gene profile parameters and potential differences due to treatment.

Data mined from the Human Genome Project for targets of therapeutic agents in combination with combinatorial chemistry improved structure–activity relationships and from high throughput screening have lowered later stage attrition of drug candidates and yielded candidates with a higher safety profile and improved efficacy. Owing to this, microarray technology has been incorporated into preclinical assessment programs to prioritize and further understand the potential liabilities of drug candidates early in the drug discovery and development [2]. Microarrays are also used to understand mechanisms of toxicity, identify biomarkers, and accurately diagnose tumor status based on gene expression alone. While these assays have many additional uses such as understanding coordination of biological pathways, this chapter focuses on measuring changes in gene expression to provide critical information regarding the pharmacokinetics, dynamics, metabolism, and safety profiles of compounds.

10.2 PROTEIN MICROARRAYS

10.2.1 Functional Analysis

Over the past decade, a variety of functional proteomics methods have been used to understand protein–protein interactions. Many of these assays are now conducted in high throughput, for example, the yeast two-hybrid assay [3,4], the protein complementation assay [5], affinity purification coupled with mass spectrometry [6,7], peptide phage display [8], oriented peptide library screening [9], and protein microarrays [10,11].

The yeast two-hybrid and protein complementation assays entail co-expressing a “bait” and “prey” protein in the same cell. If the two proteins interact, a reporter gene is expressed or a reporter protein becomes active. Major advantages of these assays are that they are conducted within living cells and no biochemical purification steps are required. The methods allow for efficient assessment of large numbers of potential interactions and have been used to conduct numerous genome-wide investigations [12–18]. Drawbacks of these techniques are that there is limited control over the assay conditions, integrity of the proteins cannot be assessed, and concentrations and posttranslational modification are difficult to determine and manipulate.

Methods based on affinity purification/mass spectrometry are powerful and compatible with genome-wide investigations [6,7,19]. The limitation is the restricted ability to control the environment or state of the interacting proteins. For these experiments, an epitope-tagged target protein is expressed in a cell of interest, where it interacts with endogenous proteins or complexes. Following affinity purification of the bait protein, interaction partners are identified by tandem mass spectrometry. This technique is well suited to identifying physiologically relevant complexes, making the choice of the type and state of the cells used important in defining the outcome. This assay works well when the interaction partners are highly expressed in the cells used but has the potential to provide false negatives for proteins that are not expressed or are present at low concentrations.

The methods described above provide primarily binary data, that is, proteins are reported either to “interact” or “not interact.” Genomic and proteomic investigations coupled with computational analyses would provide more predictive and reliable extrapolations if the data were quantitative and could be incorporated into predictive models of cellular behavior. Protein microarray technology can complement these approaches and provide a way to study protein–ligand interactions *in vitro* in a noncompetitive format.

In a typical protein microarray experiment, the target proteins are spotted in a regular pattern at high spatial density on a solid support, usually a chemically derivatized glass substrate or a glass-supported nitrocellulose membrane (Fig. 10.1). The spotted proteins become immobilized on the surface and are incubated with a labeled probe (e.g., a protein, peptide, nucleic acid, or small molecule). Then, protein–ligand interactions are identified using the label on the probe. For example, if the probe has been labeled with a fluorophore, the array is simply scanned for fluorescence. To date, this approach has been used primarily to investigate Src-homology 2 (SH2), phosphotyrosine binding (PTB), and PDZ-domain-containing proteins [20–22], many of which are critical to signaling in the liver. Going forward, investigations of other domain families (e.g., BH3-domain-containing protein, which are critical for mitochondrial apoptotic signaling, or RING-domain-containing proteins essential for ubiquitin-mediated protein degradation) will greatly enhance our understanding of fundamental liver biology.

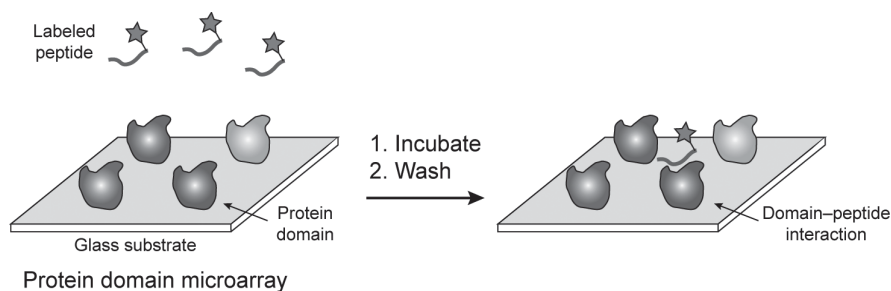


Figure 10.1 Protein domains are immobilized on a solid support (glass substrate) and probed with solution-phase, fluorescently labeled peptides. After an incubation step, the arrays are washed and scanned for fluorescence. Spots comprising domains that recognize the peptide become fluorescent. Figure and related text are adapted from Kaushansky *et al.* Ref 11. (See color insert.)

10.2.2 Analysis of Changes in Cellular Signaling

Although protein domain microarrays provide valuable data with regard to molecular affinities and interactions that occur in a cell, a broader view of cellular pathways impacted by drug treatment, for example, is necessary. A number of new technologies have made analyses that were previously only possible by traditional Western blotting, possible on a smaller, more efficient scale. This is particularly useful in the context of analyses that require the testing of hepatocyte-derived samples since primary human hepatocytes are difficult and costly to obtain in large numbers.

Antibody microarray technology uses a very small amount of total cell lysate per measurement. In an antibody array, a wide variety of antibodies are attached to a glass slide and lysates, which have been modified to be labeled on either the N-terminus or the C-terminus with a fluorescent tag, are incubated with the slide. These arrays are commercially available and able to monitor differences among a wide range of signaling proteins. Alternatively, arrays can be fabricated from lysates. In a lysate microarray, each sample is printed on a glass slide coated with nitrocellulose. The slide is then treated similar to the membranes used in Western blots. Preliminary assessments of antibody microarrays are time and resource consuming, as it is critical to validate each antibody used for a particular cell line by comparing data obtained on control arrays to that obtained by Western immunoblotting. Although antibody validation is a challenge, the ability to obtain Western-blot-like information from a very small number of cells (thousands, instead of the millions used in Western blots) is a significant advantage and provides researchers with the capacity to monitor a large number of cellular signals at the protein level while using a small number of cells, such as primary hepatocytes.

10.2.3 Advantages of Protein Lysate Array Technologies in Absorption, Distribution, Metabolism, and Excretion (ADME)

Protein lysate and antibody array technology are viable options for assessing functionality of proteins expressed in tissues, such as the liver or kidneys, and molecular changes that take place on a protein level after perturbations to liver or renal biology (e.g., drug treatment). New technologies that allow for the monitoring of binding events and/or signaling changes with very small sample size allow for unparalleled insights into organ biology.

10.3 DNA MICROARRAYS

10.3.1 Background

The microarray was forged from the convergence of disparate technologies. Advances in the semiconductor industry have yielded “on-chip synthesis,” allowing for high density, *in situ* production of short oligonucleotide sequences that encompass individual probes from the genome. These oligonucleotide expression chip platforms may be produced with tens to tens of thousands of oligonucleotides, depending on the experimental design [23]. Another high throughput platform consists of spotting prefabricated polymerase chain reaction (PCR) products on a matrix [24]. Both platforms provide a stage by which sample RNA may be hybridized to the associated probe sequence and quantified to assess the functioning genome, also known as a *microarray expression analysis* [25].

In general, DNA-based microarrays are analysis tools that use nucleic acid fragments for the simultaneous and parallel examination of the changes in the levels of gene expression for individual genes and/or groups of related genes in response to environmental or biological factors. In addition, this array-based technology can assess individual genetic variations derived from polymorphisms, mutations, and genomic abnormalities associated with disease states [26–29].

Traditional solid-phase arrays use matrices made of materials such as glass, silicon, or membranes to which the nucleic acid probes are affixed. The hybridizing targets are RNA transcripts that are extracted from biological samples and converted to cDNA. In the dual color strategy, these are then converted to cDNA in conjunction with differing fluorescent labels of green (control sample) or red (test sample). The amount of labeled cDNA binding to a specific stabilized sequence is directly related to the intensity of the fluorescent signal. If the intensity of the red target is greater than the green target for a specific probe, then this indicates that the associated gene has been upregulated in the test sample as compared to the control sample. Yellow represents equal binding of the control and the sample, that is, labeled cDNA, and no change in gene expression [30–32]. Therefore, incorporation of dissimilar fluorescent dyes in the reference and experimental cDNA pools allows for differential gene expression analysis. Single color schemes provide absolute quantification of gene expression on a single matrix and may be more amenable to comparative studies that are temporally distinct [33]. In both scenarios, mRNA representing the steady-state balance between active transcriptional and degradation events within the biological source are embodied in the cDNA population [34], allowing for an expression pattern comparison of different genes among samples. Various detection methods, computational and integration software, and databases are available for subsequent data analysis and storage [35–39] and are detailed later in the chapter.

Limitations of gene expression microarrays include “noise” in the signal, leading to a higher than optimal background signal from the system. In some cases, regions of sequence homology can lead to target cross-hybridization, requiring additional confirmation using techniques such as Northern blots or S1 nuclease protection assays. Other causes of variation are the same as in all other systems, for example, human errors in pipetting, RNA extraction and labeling efficiency, microarray production, and differences in response to treatment among individual samples. Furthermore, posttranscriptional and posttranslational events necessary for appropriate gene expression are not captured.

Because of these potential complications, the microarray experimental setup should be guided by predetermined parameters and the actual design should be dictated by the questions that need to be answered. Ideally, biological and technical replicates should be included to account for variability and allow for distinction between background noise and actual signal, and data analysis and interpretation should be straightforward [40,41]. Technical replicates are useful in accounting for variations in sample preparation and labeling; biological replicates are necessary to address issues such as inherent basal variation between the reference samples. The distinction between biological and technical replicates can be somewhat vague and is determined to a great extent by sample size and availability. However, as the process progresses from starting sample to labeled sample to scanned image, background noise accumulates as a consequence of biological, biochemical, and physical effects. Standardized microarray methods can minimize technical variation, making biological variation the most relevant parameter

to be addressed [42]. In addition, these allow comparison of data from different platforms. Both cDNA- and oligonucleotide-based platforms have been used concurrently for mutual multiplexed verification and data integration using standardized methods [43,44].

10.3.2 Microarrays in ADME

Traditionally, microarrays have been used in drug discovery predominantly for therapeutic target identification and validation [45,46]. The data are used to follow the dynamic progression of disease states within a complex biological network and measure changes with drug exposure [47,48]. Literature is replete with examples of microarray gene expression studies that demonstrate the utility of microarrays in gene discovery programs. For example, much investigation has delineated gene expression signatures for tumor diagnosis, prognosis, or classification [49–52] and additionally, has explored drug response and drug resistance in tumors [53]. In fact, responses to a variety of drugs have been reported using microarrays, for example, 15 genes were found to be similarly involved and affected in cell growth, signaling, and trafficking in the cerebral cortex of mice chronically treated with lovastatin, pravastatin, and simvastatin [54]. Simvastatin modified expression of 23 genes in addition to those changed by all three drugs, including some involved in apoptosis. In another study, gene chip analysis of human duodenal biopsies and blood samples in subjects treated with valacyclovir was used to correlate drug pharmacokinetics with multiple intestinal genes [55]. The data from this study helped identify 4F2hc, an activator of the human oligopeptide transporter (HPT1), as being highly relevant to valacyclovir transport. Furthermore, microarray analysis was used to assess the changes in drug-metabolizing enzymes and transporters by the endothelin receptor-type A antagonist CI-1034 and the atherosclerosis drug avasimibe. Data revealed up- and down-regulation of several ADME genes, indicating the potential for multiple drug–drug interactions in the clinic [56,57]. More recently, the technique has been applied to determine individual variability in baseline expression of clinically relevant drug-metabolizing genes among a large population of human liver samples [58]. Establishing this foundation is crucial for the subsequent analysis of putative inductive and inhibitive roles of a potential drug compound.

Microarray analysis can be used to determine drug-mediated changes at the level of genes associated with absorption, distribution, metabolism, and excretion. Monitoring these global alterations in gene expression can help to predict potential drug interactions and cytotoxicity. Formats based on tissue or cell platforms are beneficial, but they are not necessarily amenable to an array design. Tissue arrays deal primarily with formalin-fixed, paraffin-embedded tissue blocks for testing antibodies against various normal tissue and tumor panels to screen antibodies, identify disease biomarkers, and assess antibody or biomarker specificity. These arrays are not used to assess activity. Cell arrays involve the creation of microfluidic devices or platforms of various cell types, such as bacterial, mammalian, and either transiently or stably transfected cells, for a range of cell-specific, gene-specific studies. The technology is not very applicable to or validated for ADME assays and does not offer a distinct advantage over the existing methods. Enzyme arrays can be performed in plates or slides and may use hydrogels or other biological matrices. For example, in oncological research, a protein array containing 170 breast cancers was profiled for 21 cytochrome P450 (CYP) enzymes to assess the expression profile of these proteins in breast cancer in order to

identify potential biomarkers and aid decisions regarding optimal adjuvant hormonal therapy [59]. For the most part, however, these protein arrays are not comprehensive or widely available for all enzymes and suffer from attenuation of enzyme activity when immobilized in gels or on a surface. In contrast, use of DNA microarrays is well established, and products are commercially available [35,37].

These assays can be an initial or adjunct screen to indicate which *in vitro* cell-based, gene-specific, or enzyme-specific assay should be performed to define the potential effects of drug treatment. The microarray results may be the first-line indicator of potential drug–drug interactions, particularly if the drug induces gene expression of multiple drug-metabolizing enzymes, nuclear xenobiotic receptors (NXRs), or transporters. These gene expression analyses can also be performed with quantitative polymerase chain reaction (qPCR) or reverse transcriptase polymerase chain reaction (RT-PCR). Microarrays simply permit the concurrent, convenient screening of one or more compounds against many targets. These arrays decrease assay time and generate a more complete assessment of possible off-target effects and potential for drug–drug interactions or adverse drug effects.

10.3.3 Biological Systems in ADME

Microarray advances are permitting high throughput and global transcriptome analysis of ADME-associated proteins and subsequent effects engendered by pharmaceutical agents. *In vivo* studies entail dosing animals with exogenous compounds and RNA extraction directly from the tissue to determine changes in transcriptional profiling. Well-characterized xenobiotics have been used to determine ADME gene expression patterns in rodents [60], and these correlate with existing data [34,61]. In addition, the effects of a compound may be compared to existing reference databases to define its ADME gene expression signature [62].

Laboratory rodent models are more genetically and environmentally uniform than human systems and do not include the intrinsic heterogeneity of the human population. In a study comparing transporter expression in 75 human donors with expression in 27 rhesus monkey donors [63], human transporter expression showed comparatively small variations when evaluated against human CYP3A4 transcripts. However, transporter variability was consistently lower in the monkey livers examined. A parallel study examining basal expression levels of other ADME genes demonstrated similar results, with higher variability in humans relative to the rhesus population [58]. Human gene expression data did not exhibit normal distribution patterns, indicating that the mean data could not illustrate the wide range of potential expression profiles.

Human hepatic cell lines have been used as the biological paradigm for measuring metabolic liabilities, as they have relatively limited variability [64–67]. However, these cell lines have functional disadvantages that include potentially aberrant nuclear receptor pathways, atypical metabolic enzyme expression, and inability to maintain other appropriate hepatocyte phenotypes [68,69]. The cell lines deviate phenotypically among laboratories and across passages [70], perhaps as an effect of differing culturing and subculturing conditions. Wilkening and Bader quantified gene expression of various drug-metabolizing enzymes during 10 consecutive intralaboratory passages of HepG2 cells and observed significant fluctuations in expression profiles with succeeding passage number [71]. The culturing environment was consistent at each passage. Therefore, the alterations were most likely due to inherent instability of the cell line.

Differences in drug-metabolizing activities have also been noted while monitoring within a single passage of HegG2 cells [71]. Similar modifications in nuclear receptor and transporter pathways are likely [72].

There is much greater variability among human individuals, and the quantitatively more limited variations in the cell lines do not represent the natural diversity of the human population. Primary human hepatocytes from different donor livers are the most acceptable model for determining the ADME properties of a drug candidate, and the resultant data have been shown to correlate with the *in vivo* human response. Use of multiple donors addresses potentially divergent individual responses to xenobiotics. Historically, catalytic enzyme activity has been the principal end point to detect induction instigated by a xenobiotic although mRNA has been shown to be a more sensitive assay that is not affected by potentially concomitant inhibition [73]. The U.S. food and drug administration (USFDA) has recently recognized this fact, and the 2012 drug interaction guidance recommends using mRNA analysis with three or more preparations of human hepatocytes as the end point for predicting interactions of drug-metabolizing enzymes and transporters. Extending customary PCR methods to microarray assessment of drug interactions in cultured hepatocytes creates a more complete profile for a drug candidate.

10.3.4 Strategy

The vast amount of data derived from a microarray analysis can be a bottleneck since commercial arrays can have thousands of genes. Investigation of the specific question of induction benefits from a simplified system that focuses only the relevant drug-metabolizing networks. Gene expression alterations in response to archetypal inducers have been examined reliably in cultured primary human hepatocytes using a platform with a condensed number of drug-metabolizing proteins, including some transporters [74]. Low density DNA microarrays have also been used effectively to validate the response to classic inducers in cultured cynomolgus monkey hepatocytes [75]. In addition, custom microarray slide chambers have been used to assess success of transporter inhibition mediated by small interfering RNA (siRNA) [76]. In this study, generated data were normalized to a control gene that expressed regularly across all samples, and fold change was determined by comparing the normalized values from treated and reference samples.

These types of custom microarrays in which all probe sequences are involved in drug metabolism, conjugation, or transport permit interrogation for both reported and unreported drug effects on gene expression and the investigation of coordinate regulation of gene expression following drug treatment. Since all genes on these microarrays are members of integrated regulatory gene expression pathways, these can serve as a marker or internal control for induction or suppression of gene expression as well as for drug selectivity or specificity in the modulation of gene expression (Fig. 10.2).

If the response is specific in this simplified system, then all members of the pathway should be affected. It can offer a measurement of potential off-target effects if multiple genes are affected or genes common to multiple pathways are affected. Furthermore, it permits the interrogation of expression levels for genes and pathways for which no *in vitro* or cell-based assay exists. The microarray format also lends itself quite easily to the analysis of multiple cell lines or hepatocyte lots versus a single drug or the analysis of multiple drugs versus a single cell line or hepatocyte lot, or tissues harvested from

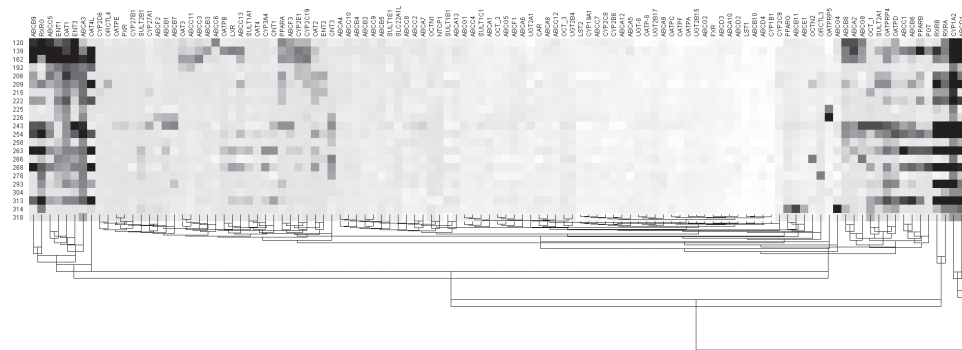


Figure 10.2 Basal level ADME-associated gene expression in 22 untreated human hepatocyte lots. Total RNA isolated from 22 untreated cryopreserved human hepatocyte samples was labeled and hybridized to individual DTETM microarrays (NoAb BioDiscoveries, Mississauga, ON). Gene expression values represent the normalized, mean value (relative to a constitutively expressed control gene, GAPDH) from three individual microarray hybridizations ($n = 12$ for each gene). Fold change data was generated by comparing normalized gene expression levels between treated and untreated samples. *White squares* indicate the lowest level of fold change in gene expression ($<1\times$). *Black squares* indicate the highest level of fold change in gene expression ($>10\times$). Basal variations were noted among individuals. (See color insert.)

in vivo studies. A complete gene expression profile can be established for any combination of drug/cell line/hepatocyte lot and gene/regulatory pathway (Fig. 10.3).

10.3.5 Microarrays in Toxicogenomics

Toxicity is a complex interaction of a toxin and a biological system. When a toxin is delivered to the site of action, it interacts with a target molecule or alters the biological environment. This can cause cellular dysfunction and disrepair, resulting in toxicological response such as necrosis, cancer, fibrosis, or disease. The number of mechanisms by which a drug elicits a toxicological response is proportional to the number of mechanisms maintaining homeostasis, such as mitochondrial function, cellular activity, signal transduction, and gene expression. Therefore, monitoring a single marker to assess the toxicological response will neither accurately nor adequately predict a drug's toxicological mechanism. A holistic approach must be employed to capture the specifics of a complex process. To do so, Systems Toxicology integrates technologies and information from several “-omics” disciplines to profile a toxicological event [77]. One aspect is the monitoring of gene regulation in response to exposure to toxicants, known as *toxicogenomics*.

Toxicogenomics developed in the 1990s from the availability of genome-wide DNA sequencing and the converging technology of microarrays or “chips.” This allowed for measuring the expression levels of thousands of genes from the exposure to a toxicant. The sequencing of the genome powers microarray expression analysis with candidate oligonucleotide sequences covering multiple mechanisms, signal pathways, and polymorphic deviations that may influence a toxicant's response. The mRNA is quantified to form a transcript profile for a toxic insult. This in turn may be compared to similar profiles with known outcomes to better predict the toxicity potential of a test article and potential mechanism of action [78,79]. This is an oversimplification of the complexity in determining toxicity. Concentration, time course, species differences, target tissue, and target cells are a few of the variables that add to the layers of complexities in toxicogenomics. In addition, toxicogenomics cannot be viewed alone but needs to be integrated with other facets of toxicology, such as metabolomics, proteomics, pathology, biochemistry, and other disciplines, depending on the mechanism of action. This section will solely focus on the aspects associated with toxicogenomics.

10.3.6 Biological Systems in Toxicogenomics

In vivo studies have traditionally dominated toxicology. Preclinical evaluations on a rodent (typically rat) and a nonrodent species (in most cases, dog or monkey), followed by escalating dose studies in phase I clinical trials, are used to evaluate the safety profile of a drug candidate. The *in vivo* interactions among organs, tissues, and cells allow for a thorough investigation of the drug candidate in the preclinical species as tissues are harvested for biochemical, pathological, and mRNA analyses. A typical animal study consists of several dosing concentrations, including a vehicle control, to assess acute versus chronic toxicity over time [77], and observations are made on parameters such as weight, behavior, and organ function.

For toxicogenomic evaluations, biological samples are harvested and gene profile analysis is performed, comparing the dose time variables to the control treatment. The data are combined, and significant outcomes are recorded [80]. For example, brown

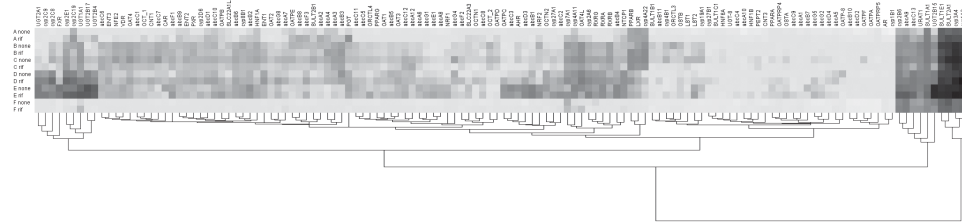


Figure 10.3 Induction of ADME-associated gene expression in six human hepatocyte lots treated with rifampicin for 24 h. Total RNA isolated from six human hepatocyte lots treated with either rifampicin (*rif*) or vehicle (*none*) were labeled and hybridized to individual DTE_xTM microarrays. Gene expression values represent the normalized, mean value (relative to a constitutively expressed control gene, GAPDH) from three individual microarray hybridizations ($n = 12$ for each gene). *White squares* indicate the lowest level of ADME-associated gene expression ($<1\times$) relative to GAPDH. *Black squares* indicate the highest level of ADME-associated gene expression ($>10\times$) relative to GAPDH. (See color insert.)

Norway rats were exposed to oral dosing of hexachlorobenzene, and microarray samples were prepared from blood, thymus, liver, spleen, and mesenteric lymph nodes [81]. Results indicated that the spleen and mesenteric lymph nodes exhibited significant changes for genes associated with granulocytes, chemokines, cytokines, and immunoglobulins [82]. A limitation of the preclinical evaluations is identifying the appropriate animal model, as these in many cases do not reflect the human response, particularly with regard to metabolites formed and toxicological responses. A large number of drugs fail in the clinic because of unexpected toxicities that were not observed in the preclinical species. In many cases, human *in vitro* systems better reflect, at least qualitatively, the clinical response. Furthermore, efforts have been put forth to limit animal testing and bridge the information gap using *in vitro* evaluations [83].

In vitro testing utilizes immortalized or transformed cell lines or primary cells as the biological source. Generally, homogeneous cell culture allows for a specific view into the interaction of a compound and a single cell type, such as using hepatocytes to study the potential for hepatotoxicity [84] or isolated CD4 T cells and B cells to assess immunotoxicity [31]. *In vitro* systems are amenable to efficient testing of complex regimens of concentration and time. Interspecies comparisons can be made under similar conditions in order to reduce experimental bias. In a paper presented by Kienhuis *et al.*, acetaminophen-induced hepatotoxicity in rat and human primary hepatocyte cultures was compared by using toxicogenomic data, as well as comparison of *in vitro* and *in vivo* rat data, from which relevant common pathways were identified [85]. However, *in vitro* systems have caveats, such as appropriate cell model and conditions and lack of support cells to retain *in vivo* function. Relying on a single cell type or even a mixed culture from a single organ may not provide the appropriate conditions in order to capture a toxic event. In the case of terfenadine, cardiotoxicity occurs when cardiomyocytes are exposed to high concentrations of the parental drug. Clinically, this may occur from an overdose of terfenadine or from inhibition of its intestinal CYP3A4-mediated metabolism, allowing accumulation in the blood to overdose levels. *In vitro* studies employing myocytes confirmed terfenadine effects on cardiotoxicity, while its metabolite terfenadine oxalate had no effect. A screen solely utilizing cardiomyocytes would implicate terfenadine as a cardiotoxin, as is the case; however, this would not account for the common clinical outcome of being safe because of metabolic clearance [86].

Conversely, cells lacking metabolic capacity may underestimate potential cytotoxicities when the metabolite is cytotoxic compared to its parental form. Therefore, cells retaining metabolic pathways may be required to accurately predict metabolically dependent cytotoxicity, as in the case of aflatoxin B1. When metabolically competent cells, such as primary hepatocytes or HepaRG, were employed, microarray analysis provided evidence supporting previously reported involvement of p53, as well as several novel genes FHIT, BCAS3, and SMYD3 in association with aflatoxin B1 genotoxicity [87].

Variations among cell systems and in the case of primary hepatocytes, among individual donors must be defined. Harris *et al.* [88] investigated three genotoxic hepatocarcinogens and one nongenotoxic hepatotoxin in primary hepatocytes from multiple donors and the HepG2 cell line. The results showed that both primary hepatocytes and HepG2 provided similar gene expression profiles; however, there were significant differences among the individual human donors. Although 2172 genes were modulated from all donors, individual donors expressed a portion of these. One donor expressed

only 29% of the affected genes, and 40% of the commonly identified genes were measured in all the donors. This illustrates the variation in idiosyncratic responses among humans and the difficulty extrapolating a single mechanism of action given the inherent variation. *In vitro* systems with appropriate constitutive activities must be vetted with *in vivo* responses within and between species to ensure clinical relevance and relevant extrapolations.

10.3.7 Bioinformatics

Toxicogenomic evaluations generate vast amounts of data since a DNA chip may have tens of thousands of individual genes, and multiple samples (time and dose-dependence) from different species add to the complexity. Bioinformatics has been very useful in melding statistics, computer science, database management, and analytical approaches in order to organize, visualize, and prioritize biological information [89]. The first line of analysis is to normalize the data to remove systematic bias and artifacts due to experimental conditions [31]. The resulting values are then processed for comparison among conditions or among experiments.

The standard approach for toxicogenomics studies is to determine which genes are differentially expressed, that is, which genes are induced or suppressed compared to the control. A common output is a tree-based visualization or heat map that is a graphic representation of a data matrix with a variable hierarchy of gene expression and associated sample. This tree is a simple representation of data for a single gene. It may be organized to bin up- or down-regulated genes in rank order by clustering the row vectors or relationships between samples by clustering the column vectors [32,90,91].

A statistical comparison of classes attempts to separate the gene expression into two or more groups according to specific exposure conditions. This is an effort to connect genes to an event. A *volcano plot* is a type of scatter plot made by graphing significance versus fold change [25]. The regions of interest are the points toward the top at the far right or far left of the graph. To visualize more complex relationships among multiple classes, Venn diagram may be employed to integrate the size of the information with cardinality of the sets and the intersection of their relationships [92,93].

In order to attempt mechanistic analysis, the abstract relationships among the data may be annotated by linking the genes with biological databases in order to include biological information such as proteomics, metabolomics, and phylogenetics. Together, the information may give insight into a gene's function, tissue expression, localization, and clinical ramifications from which signal transduction pathways may be identified or developmental-dependent phenotype is implicated [31].

Toxicogenomic databases provide a repository for gene expression as pathway and mechanistic information from both *in vitro* and *in vivo* testing. Toxicogenomic databases are available both commercially and as open access. The Chemical Effects in Biological Systems (CEBS) database maintained by the National Institute of Environmental Health Services (NIEHS) is "designed to display in the context of biology and study design, and to permit data integration across studies for novel meta-analysis" [94]. The EDGE database developed at the McArdle Laboratory for Cancer Research, University of Wisconsin (Madison, WI) is organized to address the differences in study design and platforms [90], while the Comparative Toxicogenomics database focuses

on cross-species comparative studies of gene expression [95]. From the “Minimum Information About Microarray Experiment (MIAME)” guidelines [31], a database was produced by the Functional Genomics Data Society (FGED) to advocate “for open access to genomic data sets” [96]. The Human Toxicogenomics Initiative (HTGI) consists of both public and private entities is working toward organizing this vast amount of data and publishing it in a public database [97].

10.4 SUMMARY

DNA microarray technology can be used efficiently to measure gene expression alterations with exposure to a compound, such as the xenobiotic induction of transporter and drug metabolism genes. Results obtained from these platforms correspond well to responses measured with activity and RT-PCR. Yet, the capability of simultaneous quantitation of many transcripts provides high throughput analysis of multiple end points. Custom cDNA microarrays can be used to examine the modulation of gene expression levels of a defined set of ADME-associated genes, focusing on the relevant proteins. Furthermore, the emphasis on transcript analysis for induction in the recent regulatory guidance creates higher relevance for these models and underscores the importance of microarrays in future drug discovery programs. Protein microarrays based on either purified proteins or cellular lysates allow for a more thorough and fundamental understanding of the signaling events that are at play during drug treatment. As these platforms are further developed, monitoring transcriptional as well as translational and posttranslational changes in response to drug treatment will become standard.

The widespread use of various microarray-based methodologies has also bolstered new disciplines, such as computational biology, bioinformatics, and toxicogenomics. More work on standardization is necessary to fulfill the expectations of toxicogenomics to adequately assess safety risks of drugs and chemicals. Standards for exposure assessment and hazard screening will need to be identified and implemented. This will require that the scientific community and policy regulators agree on acceptable screening methods, integrated databases, and reasonable decision trees. To move toward this end, variability in susceptibility of distinct populations must be determined as well as mechanistic information with clinical relevance. Given the historical reliance on animal studies, cross-species extrapolation will need to be refined and redefined in the context of gene expression and mechanism of action. Dose–response relationships may increase our understanding of low level exposures, as well as exposure to multiple therapeutics, chemicals, and behavioral influences, but will require validation with clinical outcomes. Little is known about the health impact of fetal and neonatal exposures and their effects, and this area still requires extensive work.

The microarray field has been moving toward standardization in assay design and data analysis. This will allow interpretation of results that can be understood in the context of preexisting data. As microarray tools become more sophisticated, the ability to incorporate and measure concurrent effects on multiple biological pathways will permit integration of entire networks in order to understand the global responses to a xenobiotic.

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11 Applications Using Caco-2 and TC7 Cells for Drug Metabolism Studies

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11.1 Summary	1
11.2 Introduction	2
11.3 Intestinal Metabolism	3
11.4 Comparison Between Intestin/Caco-2/TC7	4
11.5 Using Cell Models	7
11.6 Example of Protocol: Using Caco-2 Cells for Passage Studies	10
11.7 Conclusion	13
References	14

11.1 SUMMARY

Gastrointestinal tract, particularly the small intestine, is the site of absorption of orally ingested xenobiotics including nutrients and drugs. The objective of this absorption is the passage of these molecules from the intestinal lumen to the blood and/or to the lymphatic circulation. Moreover, the oral route is the major route used for administration of drugs since dosing is convenient and noninvasive and many drugs are well absorbed by gastrointestinal tract. It is therefore easy to understand that knowledge and the prediction of the passage of these molecules through the intestinal barrier are essential for the development of these compounds. The complexity of this barrier justifies the existence of many predictive models and explains the numerous studies done to develop and improve these patterns. This chapter provides an overview of key issues regarding the intestinal barrier, commonly used cell models and their weaknesses. Also proposed are an example of protocol for the use of model Caco-2 cells and a set of controls for validating the model.

11.2 INTRODUCTION

The intestinal barrier, in a general point of view, can be defined as the boundary between the intestinal contents and the internal medium of the human body, represented by the enteric circulation [1,2]. To be more precise, the intestinal barrier can be defined histologically, chemically, or metabolically.

11.2.1 Histological Point of View

The intestinal mucosa consists of a simple columnar epithelium, a lamina propria (collagen matrix containing blood and lymphatic vessels), and a muscularis mucosa [3]. The intestinal mucosa possesses villi, whose function is to increase the surface of absorption. The epithelium constitutes the essential element of the intestinal barrier and is composed of four major cell types: enterocytes (possessing microvilli, and implicated in the absorption), goblet cells (producing mucus), enteroendocrine cells, and Paneth cells [4]. Intestinal epithelium is a dynamic system continuously renewed from stem cells located in invaginations called *crypts of Lieberkuhn* [5]. The epithelial cells are connected by junctional complexes including adherens junctions, desmosome and especially tight junctions at the origin of the intestinal barrier impermeability.

11.2.2 Chemical Point of View

To reach the bloodstream, a molecule present in the intestinal lumen must cross several layers possessing specific physicochemical characteristics. Thus, the molecule will pass through the mucus layer (aqueous solution of mucin), the unstirred water layer (aqueous interface between the intestinal lumen and the plasma membrane), the lipid bilayer of the enterocytes plasma membrane, and the basement membrane associated with intestinal epithelium and reach the capillaries, then cross the basement membrane associated with the endothelium and the endothelium itself. All of these steps can limit the passage of a molecule.

11.2.3 Metabolical Point of View

If the liver is the organ of reference concerning the metabolism, the intestinal metabolism should not be underestimated. Indeed, the metabolic activity of the intestinal mucosa is the second largest of the human body after the liver. The metabolizing enzymes, such as aminopeptidases, dipeptidyl peptidase IV, esterase, sulfotransferase (SULT), UDP-glucuronyltransferase (UGT), glutathione-S-transferase (GST), cytochrome P450 (CYP) superfamily, and membrane transport systems, including P-glycoprotein (ABCB1), expressed by enterocytes, serve as a biochemical barrier [6]. Some works even seem to demonstrate that after normalization for the relative abundance of P450 enzymes in the organ, the metabolic activities of the intestine and liver are comparable. This ability to transform and/or to efflux molecules into the lumen of intestine is also a crucial element of the barrier intestinal.

The intestinal barrier is extremely complex and difficult to reproduce *in vitro*. There are many systems that attempt to mimic: organotypic models (everted gut sac, Ussing chamber), cellular models (cell lines: Caco-2, TC7, etc.), which all have advantages and limitations. The choice of one of them will depend mainly on the problematic of the molecules studied and resources available.

11.3 INTESTINAL METABOLISM

11.3.1 Longitudinal and Transverse Heterogeneities

The intestinal metabolism, in animals or humans, has been the subject of a very extensive work. Many difficulties have been encountered, leading sometimes to discordant results. Thus, one can be cited: (i) the low expression levels of intestinal metabolic enzymes relatively the liver; (ii) intra- or interspecies variability of expression of biotransformation enzymes; (iii) ethical and technical limitation for studies in humans; (iv) variability of sample preparation techniques; and (v) structural and functional heterogeneity of the intestine.

The human intestine is divided into two parts: the small intestine, subdivided into duodenum, jejunum, and ileum, and the colon. They differ in their histological structure and by their metabolism. Thus, when considering axially mouth rectum (longitudinal axis) of the gastrointestinal tract, the metabolic activity of the intestine is particularly highest in the upper part of the small intestine, with a maximum observed at the proximal jejunum [7]. It has been shown that the total P450 content increased slightly between the duodenum and the jejunum, then decreased markedly at the ileum. If this heterogeneous distribution concern phase I enzymes (i.e., CYP3A4, 2C9, or 2C19), phase II enzyme (i.e., GST, UDPGT) distribution is relatively homogeneous in small intestine but with a lower level of expression in the colon. These observations were consistent with the fact that absorption of most of the xenobiotics occurs in duodenum and proximal jejunum and with the fact that relative abundance of goblets cells increases from small intestine to colon. In the same way, the peptide transporter PepT1 (SLC15A1) appears to have higher expression in the duodenum than in the ileum [8]. However, the decrease of expression and/or activity, from duodenum to the colon, seems not to be associated with all membrane transport systems. Indeed, some reports has been demonstrated, by analyzing the rate of mRNA levels along the gastrointestinal tract, that expression of the drug efflux pump P-glycoprotein increases from the stomach to the colon [9], even if other studies have shown that the expression of P-glycoprotein is correlated with CYP3A4 distribution [10,11].

Another level of heterogeneity corresponds to crypt-villus axis (transverse axis) [7]. Three cellular phenomena are observed along this axis: proliferation, differentiation, and apoptosis. Proliferative cells (stem cells) reside in the crypts, while apoptotic cell are observed in the top of villi. Approximately three days after the terminal differentiation, the cells reach the villus apex, undergo apoptosis and are exfoliated in the intestinal lumen. In the crypts, stem cells generate four major differentiated cell types: enterocytes, goblet cells, and enteroendocrine cells, which migrate to the top of the villus, and Paneth cells that remain at the crypt [12,13]. The junction between the crypts and the villi is the limit from which the cells develop their specific characteristics. This organization is observed not only in the small intestine but also in the colon, except for a colon that has no villi or Paneth cells [14]. The differentiation gradient of enterocytes along the crypt-villus axis results in the existence of a gradient of expression and/or activity of metabolic enzymes. It was thus shown that microsomes prepared from crypts cells contained much less CYPs than microsomes prepared from the mature villus cells. The maximum activity was observed at the middle third of the villus. A similar gradient exists for phase II enzymes, especially for GST and UDPGT

[7,15]; P-glycoprotein is also highly expressed in villus cells but is barely detectable in crypt epithelium [16].

11.3.2 First-Pass Intestinal

The first-pass effect is defined as *partial* or *complete extraction* of the dose of a xenobiotic that takes place between administration site and the systemic circulation. Metabolism is a key element of this process. To reach the systemic circulation, a molecule, administered orally, must pass three potential sites of metabolism: the intestine, liver, and lung. To cross the intestinal barrier, a molecule can use the transcellular or the paracellular route [16]. Only molecules using the transcellular mode may be submitted to first-pass effect. During the cellular transfer, these compounds can undergo the action of phase I and phase II metabolic enzymes and can be expelled out of cells by membrane transport systems [17,18]. The first-pass effect is also facilitated by amplification systems of the intestinal luminal surface (i.e., valvulae conniventes, villi, microvilli) that allow a surface of absorption, and thus of potential metabolization, considerable. Despite the great variability of expression in humans, CYP3A enzymes, especially CYP3A4, are a key element of the intestinal first-pass effect [18–20]. These enzymes represent 80% of CYPs in the small intestine and about 50% of drugs are substrates for these enzymes. P-glycoprotein is another key element of the intestinal first-pass effect. This transporter belongs to the family of ATP-binding cassette (ABC) transporters and is present at the brush border of enterocytes. It is interesting to note that CYP3A4 and P-glycoprotein have many substrates in common and that their intestinal distributions may be correlated [10,11]. All these observations led to the hypothesis of coordinated actions between these two detoxifying proteins [21].

11.4 COMPARISON BETWEEN INTESTIN/Caco-2/TC7

11.4.1 Cellular Intestinal Model

Much attention is paid to the use of epithelial cell cultures for studies of drug transport mechanisms, especially during drug development in pharmaceutical companies. As the use of primary intestinal epithelial cells is limited by the rapid loss of their differentiated characteristics during culture, the attention has turned to the use of human established lines of tumor origin, which display a number of properties characteristic of differentiated intestinal cells with variable degrees of differentiation. Some of them, such as Caco-2, HT-29, and T84 cells have been shown to exhibit most of morphological and functional characteristics of enterocytes. Other cell lines, such as HCT8, HRT18, or SW1116, express only a partially intestinal differentiated phenotype [22]. The limitations of cell models must not be overlooked, but they offer the advantage of relative simplicity and are suitable for automated procedures. Caco-2 cells are the most used cellular model in studies on passage and transport. They were derived from a human colorectal adenocarcinoma. In culture, Caco-2 cells, despite their colonic origin, express the majority of the morphological and functional characteristics of small intestinal absorptive cells, including phase I and phase II enzymes and drug transporters; they differentiate spontaneously into polarized intestinal cells possessing an apical brush border and tight junctions between adjacent cells, and they express hydrolases and typical microvillar transporters.

Moreover, these cells, particularly Caco-2 cells, can be cultivated as monolayers of differentiated cells on a semipermeable membrane. Thus, separating the apical compartment from the basolateral compartment, which correspond to the intestinal lumen side and the serosal side, respectively, is possible. These cell lines, originated from tumors, are, however, out of the *in vivo* physiological environment; therefore, extrapolation of the data to the *in vivo* situation may be difficult; a similar conclusion can be otherwise drawn for most of *in vitro* culture systems.

11.4.2 Phase I Enzymes

Among the enzymes of phase I, CYPs have been the object of the largest number of studies [23–25]. There may be differences between these works, especially for those using human intestine as a model. These differences are related to techniques used (sample preparation, analytical techniques) and may also reflect human variability. Using enterocytes isolated from human small intestine, Zhang *et al.* have analyzed the expression of CYPs. The used technique allows the analysis of enterocytes from the villi without contamination by crypts cells. Several CYPs were detected at mRNA level: CYP1A1, 1B1, 2C, 2D6, 2E1, 3A4, and 3A5. In contrast, at the protein level, only CYP1A1, 2C, and 3A4 were detected. Other studies have demonstrated that CYP2C isoforms present in the intestine were 2C9 and 2C19 [23]. Regarding CYP3A5, it has been highlighted, at the protein level, in a small proportion of the caucasian population [26].

Despite the differences that may exist between the works, they agree that the main CYPs are those of the CYP3A family, especially the isoform 3A4. However, these CYPs are not or slightly (according to studies) expressed in Caco-2 cells [26–28]. Thus, Sun *et al.* [29], comparing the expression profile of many genes, including CYP3A4, have shown that Caco-2 cells expressed about 150 times less CYP3A4 the human duodenum. Treatment with 1 α ,25-dihydroxyvitamin D₃, an inducer of CYP3A4 at the mRNA level, and transfection of CYP3A4 cDNA are two ways for increasing CYP3A4 expression levels in Caco-2 cells [30,31]. However, expression levels do not reach the levels observed *in vivo*. Other CYPs have been highlighted in this cell line, at least at mRNA level, such as CYP1A1, 2E1, and 2B6. Caco-2 cells are widely used to study the passage of the intestinal barrier and is described in the literature as the reference model to study intestinal drug absorption. However, on the intestinal first-pass effect, due to the low expression of CYP3A4, it seems that these cells do not constitute a model of choice.

To overcome absence expression of CYP3A, the Caco-2 clone TC7 has been developed. This cell line, generated by passing Caco-2 cells 198 times, expresses CYP3A4 and 3A5 (at least at mRNA level) at a higher level than their parental counterparts. In addition, CYP3A4 is inducible by 1 α ,25-dihydroxyvitamin D₃ in this cell line. Moreover, several studies have demonstrated that TC7 cells are a good alternative to the Caco-2 parental line for drug transport studies [27,28,32].

11.4.3 Phase II Enzymes

The main phases II enzymes are present in the intestine, including UGT, SULT, acetyl-transferase (ACT), and GST [29,33–35] (Table 11.1). These enzymes are characterized by their large interindividual variations and their most important expressions in the

TABLE 11.1 Example of Phase II Metabolizing Enzyme Expressed in Human Small Intestine, Caco-2 Cells and TC7 Cells^a

	Small Intestine	Caco-2	TC7
GST	Alpha, mu, pi	A1, A2, A3, A4, O1, P1, T2, Z1	
UGT	1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A10, 2B4 2B7, 2B15, 2B28, 2B17	1A1, 1A6, 1A9, 2B9	1A1, 1A3, 1A6, 2B7
SULT	1A1, 1A2, 1A3, 1B1, 1E1, 2A1	1A1, 1A2, 1A3, 1B1, 1C1, 1C2, 2A1	1A1, 1A2, 1A3, 1B1, 1C1, 1C2, 2A1, 1E1

^aRefs 29, 33–35, and 37–43.

small intestine compared to colon [36]. In the Caco-2 cells, at least at mRNA level, the GST class μ , π , and especially α (A1, A2, A3, and A4) has been highlighted [34,37]; UGT (especially after induction) such as the UGT 1A1, 1A6, 1A9, and 2B9; and SULT such as SULT 1A1, 1A2, 1A3, 1B1, 1C1, 1C2, or 2A1. From a general point of view, the activity of phase II enzymes is lower in the Caco-2 cell line than in the small intestine. However, some enzymes have higher expression in this cell line than in intestine. This is the case, for example, of the SULT 1A2, 1A3, 1C1, or 2A1 [29,37–40].

Several studies have shown that phase II metabolism is more important in TC7 cell line than in Caco-2 cells, especially with respect to glucuronidation and sulfoconjugation levels [41] (Table 11.1). In these two cell lines, as in the enterocytes, the expression of phase II enzymes is in fact strongly linked to the differentiation state.

11.4.4 Drug Transporters

Drug transporters, belonging either to the solute carrier (SLC) family or to the ABC family, play a major role in drug absorption or secretion in the intestine. Indeed, the ABC pumps P-glycoprotein, multidrug resistance protein (MRP) 2 (ABCC2), and breast cancer resistance protein (BCRP, also known as ABCG2), located to the apical pole of enterocytes, are important in preventing the absorption of some chemicals into the systemic circulation, through expelling them in the lumen of the intestine. Other transporters located at the brush border pole of intestinal cells, such as the peptide transporter, PEPT1, or the organic anion transporters, OATP-A (SLCO1A2) and OATP-B (SLCO2B1), are by contrast involved in drug uptake into enterocytes. At the basolateral pole, organic solute transporters (OST α /OST β) and MRP3 (ABCC3) may facilitate the removal of the intracellular solutes into the basolateral side, thereby enhancing the absorption of drugs.

Most of the transporters found in human intestine are also detected in Caco-2 cells, including P-glycoprotein, BCRP, MRP2, PEPT1, OATP-B, and the monocarboxylate transporter (MCT)-1 (SLC16A1) [42]. The expression of transporters in Caco-2 cells such as OATP-B, BCRP, and MRP2 differ, however, more than fivefold than that found in the ileum, indicating that Caco-2 cells do not strictly reflect the pattern of transporter expression displayed by small intestine. In addition, it is noteworthy that the source of the Caco-2 cells, the culture conditions, and the culturing time are likely to affect

transport proteins; P-glycoprotein, MRP2, and PEPT1 mRNA levels have thus been shown to differ by at least a twofold factor in two independent Caco-2 cell clones [43]. Such differences may directly contribute to heterogeneity in transport activity between Caco-2 cells cultured in different laboratories [44].

With respect to transporter expression in TC7 cells, slightly higher mRNA levels of SLC transporters were found in these cells when compared to Caco-2 cells; these differences between the two cell lines were, however, considered as marginal [45].

Functional assays with Caco-2 cells, that is, permeability studies (see the following sections), may be performed with referent substrates for transporters: digoxin, vinblastine or paclitaxel (for P-glycoprotein), sulfobromophthalein (for MRPs and OATPs), methotrexate, estrone-3-sulfate, and pheophorbide A (for BCRP) and glycylsarcosine (for PEPT1). In addition, referent inhibitors of transporters may be used: verapamil or cyclosporin A (inhibiting P-glycoprotein), probenecid (inhibiting MRPs and OATPs), and fumitremorgin A (inhibiting BCRP). A recent paper from the International Transporter Consortium has proposed guidelines for investigating whether a compound may be a substrate for P-glycoprotein or BCRP using Caco-2 cells; an efflux ratio, basal to apical versus apical to basal, >2 , is in favor of a potential transport by P-glycoprotein or BCRP, which has to be confirmed by the use of transporter inhibitors [46]. Moreover, decision trees for P-glycoprotein and BCRP inhibitor interaction studies using bidirectional transport assays with Caco-2 cells are also provided in this chapter.

11.5 USING CELL MODELS

As Caco-2 cells are the most popular cellular model in studies on passage and transport, this and the following sections will only concern these cells.

11.5.1 Conditions of Culture

The complete morphological and functional differentiation of Caco-2 cells requires three weeks in culture under conditions described in Table 11.2. Many modifications of these conditions have been reported. Thus, the support of culture can be coated with, for example, type 1 collagen or matrigel, and the inactivated serum concentration decreased.

TABLE 11.2 Culture Conditions of Caco-2 Cells

	Caco-2 Cells
Culture medium	Dulbecco's modified Eagle's medium (classically)
Inactivated fetal calf serum (heat-inactivated at 56°C for 30 min)	Usually 20% (range, 5–20%)
glucose	25 mM (final concentration)
Nonessential amino acids	1%
Glutamine	2 mM (final concentration, range: 2–4 mM)
Cell density at seeding	$1 \times 10^5 - 4 \times 10^5$ cells/cm ²
pH	Usually 7.4
Medium change	Every 24–48 h

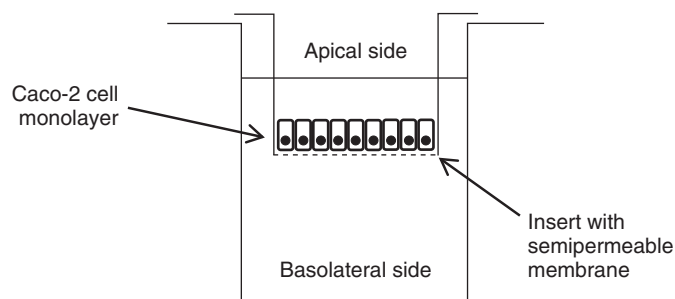


Figure 11.1 Schematic description of cells culture in nacelle system.

The enterocyte-like phenotype of Caco-2 cells is enhanced when cells are grown on semipermeable membrane (Fig. 11.1). In this case, Caco-2 cells can proliferate and differentiate in monolayer, reproducing the intestinal epithelium organization, where the apical compartment corresponds to the intestinal lumen and the basolateral compartment to the serous site. This method of culture allows the creation of an accessible apical and basolateral pole, with a possible asymmetry in the composition of culture medium. This asymmetry may allow, for example, studies of the passage of molecules from apical to basolateral side or from basolateral to apical side.

11.5.2 Essential Elements

The variation between laboratories can be associated to the heterogeneity of the Caco-2 cell line as well as to many factors influencing the morphological and functional characteristics of these cells. Among these factors, we can cite: the origin of cells, the culture conditions, the number of passage, the culture time, and the study conditions (concentration of drugs, materials used, temperature, methods of analysis, etc.) [6,47]. Thus, the transepithelial electrical resistance (TEER, a marker of the integrity of the cell monolayer) [48], or the expression of P-glycoprotein, [49–51] are influenced by cell differentiation level or by passage numbers of cells. Moreover, many studies show the influence of fetal calf concentration in the culture medium. Indeed, the serum provides hormones, growth factors, attachment factors, and other compounds necessary for growth and differentiation of Caco-2 cells. However, the exact composition of serum is often not fully characterized and varies from batch. This variation, for some authors, may favor the selection of a predominant clone over the course of passages, contributing to interlaboratory variability [52,53]. It is obvious that the removal of this variability is illusory. However, some rules can be observed to reduce it, such as to establish a master bank and a working bank to be sure to be in ranges of moderate numbers of passage, to try to always use the same serum; to strictly follow the protocols used for changing the culture medium or for reseeding cells, and to establish control of differentiation before the use of Caco-2 cells such as the analysis of the tight junction formation. These controls allow to reduce the variability of results and also to value the model.

11.5.3 Validation of Models

The use of Caco-2 model requires different controls [6,54] (Table 11.3). The first controls concerns the identification of the differentiated state of Caco-2 by measuring

TABLE 11.3 Example of Control and Methods of Control Used during Validation of the Caco-2 Cell Model^a

	Method Proposed for the Control	
Cell monolayer integrity	Measuring the TEER	TEER measurement can reveal toxicity or the tight junctions opening. TEER range of Caco-2 cells: 150–1600 ohm cm ²
	Measuring permeability of test compounds: • mannitol • PEG 400 • Lucifer yellow	These molecules do not usually spend the intestinal barrier
Cell differentiation	By measuring expression of differentiation markers: • sucrase isomaltase • dipeptidyl peptidase IV • alkaline phosphatase aminopeptidase	The expression of these markers is correlated with cell differentiation that depends on confluence of cells
Model validation (for passage studies)	By measuring the permeability of reference molecules	Proposed molecules with their apparent permeability (Papp): • Testosterone: 73×10^{-6} cm/s • Propranolol hydrochloride: 28×10^{-6} cm/s • Methothrexate: 1.2×10^{-6} cm/s The United States pharmacopeial (USP) convention proposes these molecules. If the Papp values obtained with Caco-2 cells are the same as the values given by USP ($\pm 20\%$), the model can be considered to be valid

Abbreviation: TEER, transepithelial electrical resistance.

^aRefs 6, 56, and 57.

the expression of differentiation markers such as sucrase-isomaltase (SI), dipeptidyl peptidase IV, alkaline phosphatase, or aminopeptidase. These enzymes are present at the brush border of enterocytes and some of them, such as SI, correspond to specific differentiation markers of the intestine. These checks can be performed on Caco-2 cells grown on plastic supports or on semipermeable membrane.

The second concerns the formation of cell layer and tight junctions (if cells are cultivated on semipermeable membrane). The integrity of the cell layer can be monitored by measuring TEER, which may be associated with the study of the passage of molecules such as lucifer yellow, an hydrophilic dye that does not pass the intestinal barrier.

Finally, the third control concerns the passage of the intestinal epithelium, represented by the monolayer of differentiated Caco-2 cells, by the study of passage of

reference molecules. The choice of reference molecules is difficult. The following compounds have been proposed by the US Pharmacopeia: testosterone, propranolol hydrochloride, and methotrexate; permeability values obtained should not be different $\pm 20\%$ of the proposed values [55].

If this part is related to the use of Caco-2 cells, it should be noted that most of the concepts described are also to be associated with the use of TC7 cells. However, keep in mind that TC7 cells possess some different characteristics, compared to Caco-2 cells, that is, a lower expression of P-glycoprotein (but remains higher than in the human small intestine); a doubling time shorter; a lower TEER than Caco-2 cells, and expression of CYP3A4. Thus, if controls and validation methods are the same for these two cell models, the experimental results obtained are not necessarily directly comparable.

11.6 EXAMPLE OF PROTOCOL: USING Caco-2 CELLS FOR PASSAGE STUDIES

This section provides a sample protocol for using Caco-2 cells in order to study the passage of various molecules through the cell monolayer formed by Caco-2 cells.

11.6.1 Cell Culture

The Caco-2 cells are used between passages 25 and 35. The culture medium (DMEM, 4.5 g glucose/L) is supplemented with 20% decomplexed fetal calf serum, 1% nonessential amino acids, 1% L-glutamine and antibiotics (100 μ g/mL penicillin and 100 mU/mL streptomycin). It is renewed every 48 h. The cells are seeded at 100,000 cells/cm² on a semipermeable membrane (Transwell, 0.4 μ m, COSTAR) and maintained in culture for 21 days after confluence. With these culture conditions, the cell confluence is usually obtained in four days.

11.6.2 Expression of Sucrase-Isomaltase (SI)

To determine whether our culture conditions lead to differentiated Caco-2 at the time of their use, we have studied expression of SI. The study of mRNA level of SI and β -actin was assessed by RT-PCR. The primers used for the study of mRNA of SI and β -actin of Caco-2 are: SI forward, 5'-gcccgcttccaagtgatta-3'; SI reverse, 5'-aaattgcagggtccaga-3'; β -actin forward, 5'-ggccatctcttgctc-3'; β -actin reverse, 5'-gccagagcaagagag-3'. The results show that, in our conditions of cell culture, enterocytic differentiation appears between the 5th and the 10th day of culture (Fig. 11.2).

11.6.3 Measurement of the TEER and Passage of Lucifer Yellow

Measurement of TEER of cell monolayer and the low passage of lucifer yellow, a dye not passing the epithelial barrier, are markers for the development of tight junctions. The TEER measure can be achieved through a volt-ohm meter (Evoma, World Precision Instruments Sarasota, FL). It is realized from the day after seeding (day 0) and after 3, 7, 14, and 22 days of culture. The intrinsic resistance of the semipermeable membrane

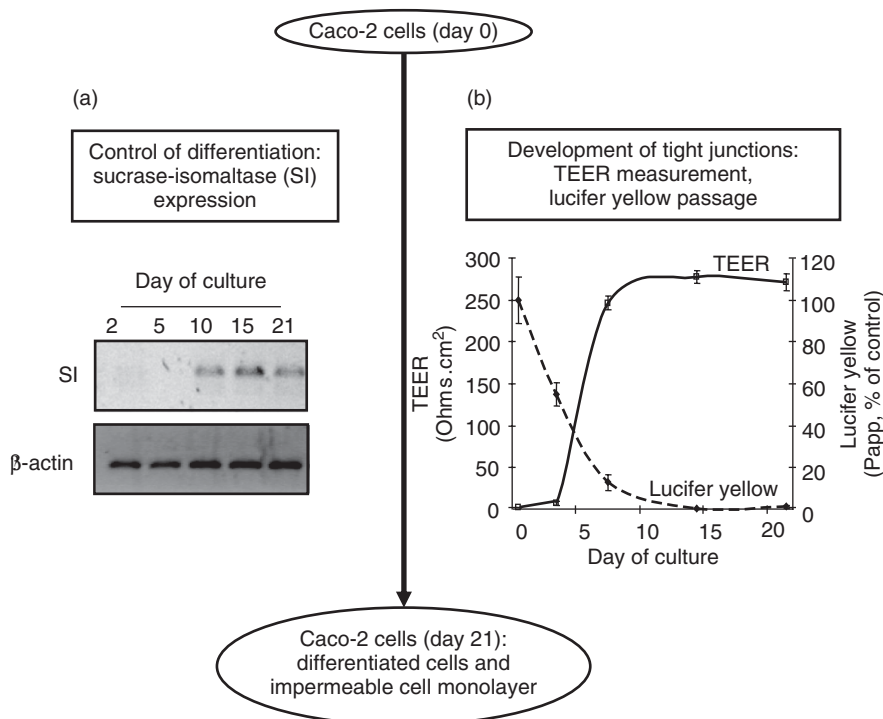


Figure 11.2 (a) Kinetic expression analysis, by RT-PCR, of sucrase-isomaltase (SI) and of β -actin in Caco-2 cells. (b) Measurement of TEER and the passage of lucifer yellow. These measurements were made from the day of seeding on semipermeable support (day 0) and after 3, 7, 14, and 22 day of culture. The results are expressed in Ohm- cm^2 for TEER and percentage of control (amount of cell-free passage) for lucifer yellow.

is subtracted from the total resistance (cell + membrane). After correcting the value obtained by the culture surface, the results are expressed in Ohm cm^2 ($\Omega \text{ cm}^2$). The permeation rate of lucifer yellow is determined by Papp calculation (see below). The sharp increase of TEER associated with the decrease in Papp of Lucifer yellow (Fig. 11.2) shows that our experimental conditions allow the formation of an impermeable cell monolayer.

11.6.4 Calculation of Apparent Permeability (Papp)

The current mode of implementation of the experience of passage is described in Fig. 11.3 [31]. There is a passage from the cell support (Fig. 11.1), which corresponds to the donor compartment or apical compartment in different acceptor compartments, or basolateral compartment, at regular time intervals. Using calculations as described in the Table 11.4, the curve representing the variations, in the acceptor compartment, of the quantity (Q) of interest molecule in function of time (T), can be built. The Papp were determined by linear regression of changes in concentrations in the acceptor compartment in function of time and by measuring the slope obtained (represented by dQ/dT , Table 11.4). The coefficient of apparent permeability (Papp) is expressed

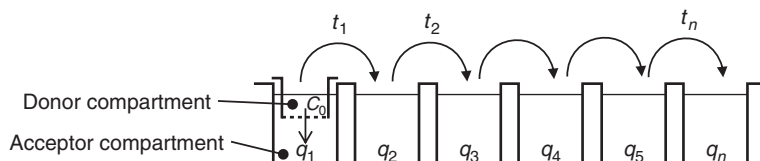


Figure 11.3 A schematic representation of the method used for measurement of passage (donor compartment to acceptor compartment) of drug across monolayer of Caco-2 cells. C_0 , initial concentration; q , quantity of drug passing in acceptor compartment; t , interval time.

TABLE 11.4 Steps for Apparent Permeability (Papp) Determination

(1) Using disposition described in Figure 11.3, construction of the curve corresponding to the variations, in the acceptor compartment, of the quantity (Q) of interest molecule, in function of time (T).	Where $T_0 = 0$ and $Q_0 = 0$; $T_1 = t_1$ and $Q_1 = q_1$; $T_2 = t_1 + t_2$ and $Q_2 = q_1 + q_2$; $T_n = t_1 + t_2 + \dots + t_n$ and $Q_n = q_1 + q_2 + \dots + q_n$ (generally $t_1, t_2, \dots$ and t_n are identical)
(2) Determination of the slope dQ/dT by linear regression of the curve obtained in (1).	
(3) Calculate the coefficient of apparent permeability (Papp) by: Papp (cm/s) = $(dQ/dT) \times [1/(A \times C_0)]$	Where C_0 = initial concentration in donor compartment; A = surface-exposed (cm^2); dQ/dT = slope determined in (2).
(4) Construction of the curve corresponding to the slope (dQ/dT) in function of the initial concentration (C_0) in the donor compartment.	If the curve obtained is saturable, this indicates that the mode of passage probably involves a transport system. If the relationship between the slope (dQ/dT) and C_0 is linear, this indicates that the mode of passage is probably passive.

in centimeter per second. Measuring the slope (dQ/dT) as a function of the initial concentration (C_0) may be also used to determine if the passage uses a passive or active mode (Table 11.4).

The permeation rate of testosterone, propranolol hydrochloride, methotrexate, and the lucifer yellow are used as reference compounds. The US Pharmacopeia specifies the concentration tested ($C_0 = 0.3$ mM) and the Papp values to obtained (1.2×10^{-6} cm/s for methotrexate, 28×10^{-6} cm/s for propranolol, 73×10^{-6} cm/s for testosterone) [55].

Figure 11.4 shows the variations in quantity of different compounds in the acceptor compartment (basolateral) as a function of time. The results are expressed as a percentage of the initial amount at time zero in the donor compartment (apical). The slopes of linear regression curves obtained allow the calculation of various Papp (Fig. 11.4a). No significant difference was observed by comparing our values and those proposed by the US Pharmacopeia (Fig. 11.4b).

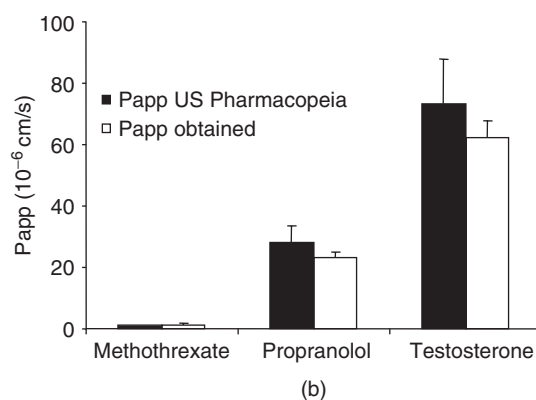
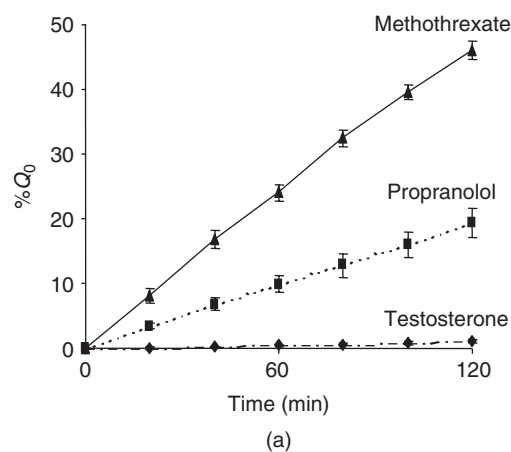


Figure 11.4 Passage study, across Caco-2 cell monolayer, of the reference molecules. (a) Evolution of methothrexate, propranolol hydrochloride, and testosterone concentrations in the acceptor compartment in function of time. Results are expressed as a percentage of the initial quantity (Q_0) in the donor compartment. (b) Comparison of apparent permeability (Papp) obtained with those proposed by the US Pharmacopeia. The results are expressed in 10^{-6} cm/s.

In summary, these results show that our culture conditions allow obtaining a fully differentiated cell monolayer.

11.7 CONCLUSION

Among the different models used to study the passage of the intestinal barrier and/or intestinal metabolism, the intestinal cell lines are the most used. Despite their faults, they are popular because of their ease of use, maneuverability, and ability to conduct studies that can last more than a few hours. However, the major problem that remains unresolved is the variability inter- and/or intralaboratory. In this section, some solutions are proposed, but they cannot solve the problem. Thus, the reference drugs proposed by the US Pharmacopeia are considered by some users as too rigid limits

and not accepted by all users. Another approach is the use of a collection of laboratory values, with internal specifications and criteria of acceptance for each assay system. The development of methods for isolation of enterocytes and goblet cells from human biopsies and culture techniques for maintaining these cells at an acceptable level of differentiation over several days is a goal that has likely to deserve further studies.

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12 *In Vitro* Liver Systems to Study Induction/Inhibition: Prediction of *In Vivo* Metabolism and Drug–Drug Interactions

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12.1	Summary	1
12.2	Introduction	2
12.3	Metabolic stability	3
12.4	CYP enzyme inhibition	9
12.5	CYP enzyme induction	21
12.6	Future perspective	30
	References	30

12.1 SUMMARY

Cytochrome P450 (CYP) enzymes expressed in liver microsomes are mainly involved in the biotransformation of drugs. CYP enzyme-related metabolic activity is a major determining factor in the pharmacokinetic profile, including bioavailability. A change in the metabolic activity derived from CYP inhibition and induction by comedication often results in adverse events or failure of drug therapy. The metabolic stability, CYP inhibition, and induction are key parameters in the optimization of lead structures and the selection of new molecular entities (NMEs) in drug discovery programs. Regarding these screenings, the advent of various evolutionary technologies has facilitated a paradigm shift from the traditional system to a robust moderate to high throughput system that meets the demands of modern drug discovery. Furthermore, the prediction of clearance of NMEs and alteration of the plasma concentration profiles

associated with drug–drug interactions (DDIs) caused by comedication has become an important task in pharmacokinetic studies. This chapter reviews advanced assay systems for metabolic stability, CYP inhibition, and induction, as well as predictive methods for pharmacokinetic profiles and DDI-associated changes in their profiles in humans.

12.2 INTRODUCTION

Pharmacokinetic profiles and DDIs such as drug-metabolizing enzyme inhibition and induction of NMEs in humans have increasingly become recognized as critical parameters of the developability of drugs [1]. Drug metabolism is a major factor governing pharmacokinetics. CYP enzymes are major drug-metabolizing enzymes and can catalyze the biotransformation of two thirds of commonly used drugs [2] and many other compounds such as steroid hormones, toxics, and carcinogens [3–5]. CYP enzymes consist of a superfamily of heme-thiolate proteins, and CYP enzymes responsible for drug metabolism are classified into three families, namely, CYP1, CYP2, and CYP3. The liver is the principal organ of drug metabolism and one or more of the various CYP enzymes expressed in the microsomal fraction are mainly involved in the metabolism. Shimada *et al.* [6] demonstrated that the most abundant CYP enzymes in the human liver are CYP3A subfamilies at 30% of the total spectrally determined CYP content, followed by the CYP2C subfamilies (20%), CYP1A2 (13%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 (<2%), and CYP2B6 (<1%). Accordingly, metabolic screening has been performed using human liver microsomes and fresh or cryopreserved human hepatocytes. Furthermore, those materials have been utilized to evaluate CYP inhibition, and hepatocytes in primary culture have been an indispensable tool for the assessment of CYP induction.

There is a growing awareness of the importance of predicting the pharmacokinetic profile in humans based on preclinical *in vitro* and/or *in vivo* data. The estimated human clearance (CL) allows predicting an effective dose by taking the efficacious plasma concentration into consideration, and the estimated plasma concentration profile, including maximum plasma concentration ($C_{\max}$) and half-life ($t_{1/2}$) in humans, are incorporated into the design of the dose regimen of the clinical study. Similarly, with regard to the inhibition and induction of CYP enzymes, the prediction of a change in CL by comedication provides an important position when selecting development candidates or creating a strategy for clinical DDI studies.

This chapter focuses on *in vitro* liver systems used to study metabolic stability, and CYP enzyme inhibition and induction both to optimize pharmacokinetics during early drug discovery and to characterize NMEs during late drug discovery or drug development. The predictive models to evaluate CL and/or plasma concentration profile are also reviewed, together with the evaluation of the influence of DDI on the pharmacokinetic profile in humans. Recently, it has been highlighted that first-pass metabolism in the small intestine has a great impact on bioavailability, and *in vitro* and *in vivo* studies have been directed toward the assessment of metabolism of CYP3A substrates in the small intestine [7]. However, this chapter focuses on such metabolic events in the human liver.

12.3 METABOLIC STABILITY

12.3.1 *In Vitro* System for Evaluating Metabolic Stability

In vitro systems of microsomes or hepatocytes are routinely used in drug metabolism studies to obtain an estimate of metabolic stability, usually expressed as intrinsic clearance (CL_{int}). In general, the CL_{int} can be estimated from the metabolite formation assay using the following equation:

$$CL_{int} = \frac{v}{S} = \frac{V_{max}}{K_m + S} \quad (12.1)$$

where S is the substrate concentration, v the metabolite formation rate, K_m the Michaelis constant, and V_{max} the maximum metabolite formation rate. In the drug discovery phase, identification of the main metabolites using *in vitro* or *in vivo* samples is difficult due to the labor-intensive and time-consuming processes. Hence, substrate depletion assay is widely used to estimate CL_{int} from the depletion or degradation rate constant (k_{dep}) of the substrate in microsome or hepatocyte incubation [8–10]. CL_{int} is estimated at a low substrate concentration such as 0.5–1 μ M [11–15], assuming that K_m values are at least higher than 5–10 μ M.

Drug metabolism is a major determinant factor to pharmacokinetics of NMEs. In prediction of the pharmacokinetic behavior, hepatic clearance (CL_H) can be estimated from the CL_{int} using physiological models: well-stirred model, parallel tube model, and dispersion model [16,17]. It is highly likely that the CL is calculated mainly based on the well-stirred model, simplified method among them, because the CL values estimated from the well-stirred model were reported to be within an acceptable range when compared with the other two models [16]. Assuming that drugs are cleared via hepatic metabolism from the body, the CL can be expressed by hepatic clearance (CL_H), which is calculated by the following equation based on the well-stirred model:

$$CL = CL_H = \frac{Q_H \times f_p \times CL_{int, in vitro}}{Q_H + f_p \times CL_{int, in vitro}} \quad (12.2)$$

where Q_H is hepatic blood flow, $CL_{int, in vitro}$ the intrinsic clearance (CL_{int}) scaled up to whole body using a scaling factor (microsomal recovery from the entire liver), and f_p the unbound fraction of protein binding in blood. The intrinsic clearance ($CL_{int}/f_{u,inc}$), which is corrected by unbound fraction ($f_{u,inc}$) of microsomal or hepatocyte incubation, is utilized on a case-by-case basis. Both unbound fractions are determined by equilibrium dialysis and ultracentrifugation [18]. An equilibrium dialysis method using 96-well apparatus has been developed for efficiently determining plasma protein binding [19].

Although knowledge of the binding parameters would be an essential part in precisely predicting CL, the metabolic stability has been a major indicator to guide the direction of lead optimization. Increased throughput of the metabolic screening has been achieved by developing an advanced robotic system in miniaturized format, such as 96-well plates [20–24]. Currently, Xu *et al.* [25] proposed a streamlined and intelligent workflow for metabolic stability that allows for high throughput analyses based on a novel postincubation pooling strategy coupled with ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS). Establishment of a semiquantitative

relationship between CL_{int} and *in vivo* CL is a prerequisite for metabolic screening. However, some investigators pointed out a poor correlation between CL_{int} and CL in a relatively large data set, including various compounds [12,24,26]. Noticeably, Komura *et al.* [24] demonstrated that there was a certain relationship among derivatives with the same core structure, and even if a derivative showed a poor correlation, considering the f_p and/or $f_{u,inc}$ into the estimation of CL_{int} dramatically improved the relationship of $f_p \times CL_{int}$ versus CL or $f_p \times CL_{int}/f_{u,inc}$ versus CL. Moreover, Obach [27] and Riley *et al.* [28] demonstrated that the $CL_{int,in vitro}$ was correlated with *in vivo* intrinsic clearance ($CL_{int,in vivo}$) estimated from CL using physiological models incorporating both unbound fractions.

In the pharmacokinetic profiling for NMEs, linear pharmacokinetics would be qualitatively guaranteed on comparing the K_m value with the effective plasma concentration predicted in humans [8,29]. Obach and Reed-Hagen [8] applied the substrate depletion assay where the K_m value can be estimated from a concentration-dependent change in k_{dep} of unchanged compounds. For substrates metabolized possibly via a predominant pathway by one CYP enzyme, the estimated K_m values were comparable to those from metabolite formation assays using recombinant CYP enzyme. A similar result for K_m values was reported by Komura *et al.* [30]. Recently, a newly developed multiple depletion curve method has been reported to provide accurate estimates of K_m , in addition to CL_{int} [31].

Like liver microsomes, hepatocytes with integrated drug-metabolizing enzymes, including CYP enzymes, are an indispensable tool for metabolic studies. Cryopreserved human hepatocytes, which are preliminarily well characterized by metabolic activity of each CYP enzyme, are commercially available, and pooled or individual cryopreserved hepatocytes have been utilized. However, it is a question whether cryopreserved hepatocytes retain drug-metabolizing activities during storage. Griffin and Houston [32] comparatively evaluated CL_{int} for 20 biotransformations using fresh and cryopreserved rat hepatocytes. There was a tendency for both hepatocytes to provide a similar CL_{int} except for some highly cleared compounds that showed lower activity in cryopreserved hepatocytes. Additionally, comparable CL_{int} values in fresh and cryopreserved human hepatocytes were proven by Lau *et al.* [33] and Naritomi *et al.* [14].

In general, plasma protein binding, as well as metabolic stability, affect the *in vivo* pharmacokinetic behavior, and the dose-dependent CL of some drugs arises from concentration-dependent protein binding, particularly for drugs that are governed by drug metabolism [34,35]. A challenged approach, whereby test compounds were incubated with hepatocytes in the presence of serum, has been developed by Shibata *et al.* [13]. The approach, based on substrate depletion assay and termed the serum (or plasma) incubation method, provided CL_{int} values that included the influence of plasma protein binding, and the obtained value was regarded as the relevant parameter for predicting *in vivo* CL. Further investigation using the plasma incubation method with rat and human hepatocytes was performed to assess species differences in nonlinear pharmacokinetics of propafenone [10]. The nonlinear disposition in humans would be ascribed to the saturation of hepatic metabolism because of the low K_m values, which was in contrast to the linear pharmacokinetics in rats, which was associated with the offset of saturable metabolism by nonlinear plasma binding in rats. The plasma incubation approach was applied to investigate chiral-conversion-associated pharmacokinetics of ibuprofen in adjuvant-induced arthritis rats [36]. The developed substrate depletion

assay combined with plasma incubation can produce *in vivo* relevant parameter, including two different factors of metabolic stability and plasma protein binding, and must be a valuable tool in the drug discovery phase [17].

12.3.2 *In Vitro* System for Evaluating the Contribution of CYP Enzymes to Total Metabolism

The estimation of fraction (f_m) of drug metabolized by the target enzyme is required to precisely predict the effect of CYP inhibition and induction. Not only the identification of CYP enzyme involved in the major metabolic pathway of an NME but also the evaluation of contribution of the CYP enzyme to total metabolism has been conducted by investigating the effects of chemical inhibitors or an antibody against CYP enzymes on the metabolite formation rate in human liver microsomes after elucidation of the major metabolic pathways [37,38]. Further investigations have then been performed by comparing the metabolite formation rate with either recombinant human cytochrome P450 (rhCYP) enzymes or separate samples of human liver microsomes with a wide range of CYP activity [22,39]. The metabolic activity in each rhCYP enzyme needs to be extrapolated to the relevant data in human liver microsomes. Nakajima *et al.* [40] proposed the concept of relative activity factor (RAF) for the extrapolation by which ratios of marker activity specific to a certain CYP enzyme between rhCYP enzyme and human liver microsomes was used. The RAF method has been widely utilized to study the involvement of individual CYP enzyme in the formation of each metabolite [41–43].

There are practical difficulties involved in identifying the detailed chemical structures of the major metabolites during drug discovery and early drug development. The substrate depletion assays, which are a useful tool to estimate total metabolic intrinsic clearance [8,44], are also applied to evaluate the contributions of CYP enzymes to the metabolic elimination in human liver microsomes using chemical inhibitors specific to each CYP enzyme [9,45]. Furthermore, Emoto and Iwasaki [46] demonstrated the usefulness of a combination method of the RAF method and substrate depletion assay in early drug discovery.

Some of the drugs and NMEs are eliminated via multiple pathways, not only catalyzed by CYP enzymes but also conjugative enzymes. The usefulness of hepatocytes for evaluating the contribution of CYP enzymes in propafenone and propranolol metabolism, which are mediated by phase II conjugation alongside CYP2D6 [47], was demonstrated. The contribution of CYP2D6 to the metabolism of both drugs in human hepatocytes was consistent with corresponding *in vivo* data and was different from human liver microsomes that exhibited the overestimation of CYP2D6 involvement.

12.3.3 Prediction of *In Vivo* Pharmacokinetics in Humans

12.3.3.1 Physiological Approach (*In Vitro*–*In Vivo* Extrapolation). One of the major purposes of preclinical pharmacokinetic studies is to select NMEs and/or support a design of clinical studies including DDI, by the prediction of CL and plasma concentration profiles in humans [48]. The prediction of human CL is based on *in vitro*–*in vivo* extrapolation using the physiological models in which the $CL_{\text{int}, \text{in vitro}}$ obtained in *in vitro* systems is incorporated [16,49].

The first successful prediction of *in vivo* hepatic CL of YM796 from *in vitro* data with human liver microsomes and rhCYP enzymes was demonstrated by Iwatsubo *et al.* [50]. Moreover, a pronounced species difference in *in vivo* CL was kinetically explained by the K_m and V_{max} values in rat, dog, and human liver microsomes [51]. Retrospective prediction using marketed drugs and/or development candidates has been performed by some investigators. Obach *et al.* [52] evaluated the effect of plasma protein binding on CL prediction based on the well-stirred or parallel tube model. Impressively, not incorporating the f_p value in the physiological models yielded better predictability than taking the f_p value into consideration. On the other hand, investigation using a relatively large data set by Ito and Houston [53] demonstrated that a prediction model incorporating rather than ignoring the f_p value provided a better correlation between the predicted $CL_{int, in vitro}$ and the observed $CL_{int, in vivo}$. Furthermore, when both the f_p and $f_{u, inc}$ values were canceled out under the assumption that both binding parameters are equivalent, there was reportedly a poor relationship between the predicted $CL_{int, in vitro}$ and the observed $CL_{int, in vivo}$ values, particularly for acidic compounds [28]. This relied on the fact that the f_p values were lower than the $f_{u, inc}$ values, for acidic compounds unlike basic compounds.

The importance of nonspecific binding in *in vitro* system with microsomes and hepatocytes, on the CL prediction, was demonstrated by Riley *et al.* [28]. Compared with considering only plasma protein binding, incorporating both binding parameters in the well-stirred model produced a relatively better relationship regardless of the classification of acidic, neutral, and basic properties. Similar findings were reported in the prediction study of 29 drugs conducted by Obach [27], who concluded that the inclusion of both binding values gave the best agreement between the observed CL and the CL predicted from *in vitro* data. It should be noted that the unbound fractions in microsome and hepatocyte systems can be calculated using lipophilicity- or chemical structure-based *in silico* approaches [54–57].

It has been realized that there is a common issue of underestimation in various reported prediction studies. Some attempts have been made to clarify the underlying mechanism for the underestimation. Using benzodiazepine derivatives, which are subject to CYP-mediated elimination, kinetic profiles were compared between human liver microsomes and cryopreserved hepatocytes that possess the full spectrum of drug-metabolizing enzymes and transporters [58]. Although almost similar CL_{int} values were obtained with both evaluation systems, the retrospective analysis exhibited 5.6-fold underprediction in human hepatocytes as evidenced in liver microsomes.

A scaling factor has been regarded as a determining parameter of predictability. When scaling up the CL_{int} from microsome or hepatocyte studies to whole body basis, generally, a physiological scaling factor, which is obtained from average recovery of microsomal protein/number of cells per gram of liver multiplied by the average liver weight in humans, is utilized. However, the employed microsomal content and number of cells range widely from 29 to 77 mg protein/g liver and from 86 to 135×10^6 cells/g liver, respectively [59], and the most widely utilized values seem to be 45 mg protein/g liver and 120×10^6 cells/g liver, respectively. Ito and Houston [53] reported that the use of an empirical scaling factor, instead of the physiological scaling factor, dramatically improved predictability. In their analysis, the empirical factor was determined by regression analysis to obtain the best fit of $CL_{int, in vitro}$ and $CL_{int, in vivo}$ values for various drugs. Recently, Sohlenius-Sternebeck *et al.* [60] proposed a prediction method based on a regression line that adjusts for systematic underprediction. Namely,

the regression lines were drawn from the correlation between $CL_{\text{int}, \text{in vitro}}$ from rat liver microsome and hepatocyte studies and $CL_{\text{int}, \text{in vivo}}$ from intravenous studies with 52 drugs. The use of hepatocyte regression, in particular, yielded high predictability that CLs for 82% of the compounds were predicted within twofold error. An approach using the animal scaling factor was established by Naritomi *et al.* [61]. The animal scaling factor was determined by dividing $CL_{\text{int}, \text{in vivo}}$ by $CL_{\text{int}, \text{in vitro}}$ obtained from *in vitro* and *in vivo* studies of the individual compound in rats or dogs and was a drug-dependent intrinsic factor that possibly included unknown or unidentified mechanisms governing drug disposition. Correction of the scaling factor gave better prediction within twofold of the observed value. An impressive finding for the underprediction was reported by Huang *et al.* [62]. Their group highlighted that permeability and efflux status in P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP)-expressing cells are a key factor affecting predictability of CL from CL_{int} and would be associated with the underprediction. For compounds that displayed high passive permeability and that are not good substrates toward the efflux transporters, the CLs would be predicted reasonably from the CL_{int} . In contrast, for compounds that showed either poor permeability or good substrates toward the transporters, the CL_{int} was unlikely to be predictive of CLs possibly due to a significant role of the transporters on their eliminations from the body.

It is increasingly realized that transporter-mediated liver uptake is involved in pharmacokinetic behavior with evidence that plasma concentrations of cerivastatin were increased with the inhibition of uptake transporter by the coadministration of cyclosporine A [63,64]. On the basis of the difference in concentration-dependent depletion profile between rat liver microsomes and hepatocytes, Parker and Houston [65] revealed that the liver uptake was a rate-limiting step for hepatic metabolism of saquinavir, nelfinavir, and ritonavir. A media loss assay was developed with rat hepatocytes to evaluate the impact of hepatic uptake on CL_{int} [66]. In contrast to the conventional assay in which the initial depletion rate constant is estimated from whole incubate (medium plus cells), the new concept was based on estimation of the disappearance rate constant only from the medium, which significantly improved predictability for a data set of 36 AstraZeneca compounds. Hence, the media loss assay can evaluate the hepatic active uptake process that confounded the CL prediction. Furthermore, the disposition of atorvastatin, cerivastatin, and indomethacin, known as substrates of uptake transporters, was investigated based on the measurement of cell and media concentration–time data in rat hepatocytes [67]. The developed physiological pharmacokinetic model that describes the ratio of the intracellular to extracellular concentration successfully predicted the effect of active uptake on the hepatic CL of atorvastatin and cerivastatin. Such a model would be a useful tool to predict *in vivo* CL of compounds that are subject to active liver uptake. In fact, using the *in vitro* kinetic data derived from human hepatocytes, analysis with a complicated physiologically based pharmacokinetic (PBPK) model incorporating a hepatic uptake process can successfully predict the *in vivo* pharmacokinetic profile of pravastatin, which is a typical substrate to solute organic anion-transporting peptide (OATP)1B1/1B3 [68].

12.3.3.2 Empirical Approach (Allometric Scaling). Alongside the *in vitro*–*in vivo* scaling described above, one of the best described techniques for predicting CL is allometric scaling, in which many physiological processes (blood flow, creatinine CL, heart rate, glomerular filtration, etc.) and organ size are defined by a power–law relationship

with the body weight of the species [69,70]. Owing to its simplicity, allometric scaling has been widely used to obtain human CL by extrapolating CL in animal species. Several methods have been developed; that is, simple allometry (SA), maximum life-span potential (MLP), brain weight (BrW), and rule of exponent (ROE) approaches associated with the choice of SA, MLP, or BrW based on the exponent of SA. Ward and Smith [71] developed a liver blood flow (LBF) method in which CL in humans is predicted based on the ratio of human and animal LBF rate. Monkey LBF method provided the best accuracy among the examined approaches, including allometric scaling [71,72]. Sinha *et al.* [73] reported that the ROE approach based on unbound oral clearance (unbound CL/F) gave the highest predictability among the examined allometric methods. Unbound fraction corrected intercept method (FCIM), by which both ratios of unbound fraction between rat and human plasma and a fixed power coefficient of 0.77 are considered, can be utilized for compounds that are not applicable to the ROE approach [73,74].

Interestingly, the concept that each property in the empirical and the physiological approach is integrated has been proposed by Lavé *et al.* [75,76]. The integrated approach was conducted using CLs in animal species that were normalized by a ratio of *in vitro* metabolic activities between hepatocytes of human and animal species and yielded 80% prediction within an error of twofold the observed CL.

Although the major disadvantage of allometric scaling is its empirical nature, this can, in some cases, be recognized as an advantage. Since it is different from *in vitro*–*in vivo* scaling, the application of allometric scaling does not need to clarify physiological mechanisms underlying elimination from the body and to understand species differences in the elimination mechanism [77]. In the early phase of drug development, characterizing other elimination mechanisms than the typical hepatic metabolism process, such as oxidation, is in many cases impossible. With regard to compounds that are eliminated from the body via unknown or multiple mechanisms, allometric scaling must be an indispensable predictor to human CLs.

12.3.3.3 Comparison of Empirical and Physiological Approaches. Comparative assessment between empirical and physiological approaches has been conducted by several investigators. Ito and Houston [53] demonstrated that the best method would be prediction from *in vitro* data using an empirical scaling factor. However, the use of a physiological scaling factor instead of the empirical scaling factor had similar accuracy as SA analysis. Obach *et al.* [52] evaluated the accuracy of human CLs predicted by an *in vitro* microsomal study without plasma protein binding (f_p) and by allometric scaling. The average error expressed by predicted/actual values was within a similar range (1.95 vs 1.91). Similar predictability between the empirical and the physiological approaches was also reported by Shiran *et al.* [78] and Tamaki *et al.* [79]. The latter group evaluated the comparative predictability of empirical and physiological approaches using data sets that were similarly characterized by a wide range of lipophilicity and CL. In contrast, using 16 marketed drugs, the ROE method was found to be more accurate than the *in vitro*–*in vivo* scaling method with *in vitro* microsomal data [80]. One issue of this kind of study is that each conclusion is derived from a different data set. To identify the best predictive method, predictability should be compared based on the same data set with a wide range of physicochemical properties.

12.4 CYP ENZYME INHIBITION

Comedication with two or more drugs is quite common in the treatment of diseases. One drug is often found to inhibit the drug-metabolizing enzymes of the second drug, mainly leading to an increased plasma concentration. As a consequence, such DDIs cause a risk particularly in patients given drugs with a narrow therapeutic index. In 1998, adverse drug reactions were reported as one of the most common causes of death of hospitalized patients in the United States, and most adverse effects involved drug metabolism [81]. Terfenadine [82], mibefradil [83], astemizole [84], cisapride [85], cerivastatin [86], and nefazodone have been withdrawn from the market due to toxicity or QT prolongation attributable to increased exposures by DDI [87]. Furthermore, DDIs have a huge impact on sales, as can be seen with cimetidine, a drug for the treatment of acid reflux, which lost sales because of DDIs that emerged in clinical and postmarketing approval studies of competing products [88]. To avoid costly failures of drug development, the assessment of DDIs has become an increasingly important task of the pharmacokinetic department.

The assessment of CYP inhibition is conducted both during the drug discovery and drug development phases. During drug discovery, the data of CYP inhibition are utilized to optimize the chemical structures and to identify NMEs with no or weak inhibitory activity by simplified moderate to high throughput methods. During drug development, detailed evaluation of CYP inhibition causes inhibitory kinetic data to both predict a change in plasma concentration profiles based on the mathematical models and to clarify the need for clinical DDI studies; for example, according to the FDA draft guidance [89] (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf>), it was noted that CYP inhibition can be classified as either reversible or time-dependent inhibition (TDI) [90]. For reversible inhibition and TDI, our review focuses separately on assays that meet the requirements for the drug discovery and the development phases.

12.4.1 *In Vitro* Systems for Evaluating CYP Inhibition

12.4.1.1 Reversible Inhibition. Reversible inhibition involves rapid association and dissociation of drugs and enzymes and can be classified into competitive, noncompetitive, and uncompetitive modes based on the inhibitory mechanisms. The assay conditions of reversible inhibition have been described, together with the prediction criteria of the clinical relevance of competitive CYP inhibition, in the FDA's draft guidance and wave site [89]. Probe substrates and specific inhibitors of CYP enzymes are summarized in Table 12.1, together with time-dependent inhibitors. The guidance-defined method mainly using marketed drugs as practical probe substrates seems to be a gold standard for the assessment of CYP inhibition, in which the IC_{50} (inhibitor concentration causing a reduction of enzyme activity by 50%) or the K_i (inhibition constant) obtained from the dose response curve is estimated as a clinical relevant parameter to predict the possibility of a DDI in humans. However, the lead optimization process requires moderate to high throughput CYP inhibition screening to reduce the cost and shorten the time frame. The advantages and disadvantages of the various developed methods are shown in Table 12.2.

TABLE 12.1 Probe Substrates, Inhibitors, and/or Inducers in *In Vitro* CYP Inhibition or Induction Assessment

CYP Enzyme	Perspective of PhRMA (MBI) ^a		FDA Draft Guidance ^b		
	Substrate	Positive Control	Substrates	Inhibitors	Inducers
CYP1A2	Phenacetin Ethoxyresorufin	Furafylline	Caffeine Ethoxyresorufin Phenacetin ^c Tacrine Theophylline	Furafylline ^c α -Naphthoflavone	Lansoprazole Omeprazole
CYP2B6	Bupropion	Ticlopidine Thiotepa	Bupropion ^c Efavirenz ^c S-Mephenytoin Propofol	Clopidogrel Phencyclidine Ticlopidine Thiotepa Sertraline	Phenobarbital
CYP2C8	Paclitaxel Amodiaquine	Gemfibrozil-glucuronide Phenelzine	Amodiaquine Rosiglitazone Taxol ^c	Gemfibrozil Montelukast ^c Pioglitazone Quercetin ^c Rosiglitazone Trimethoprim	Rifampicin
CYP2C9	Diclofenac Tolbutamide S-Warfarin	Tienilic acid	Diclofenac ^c Flurbiprofen Phenytoin Tolbutamide ^c S-Warfarin ^c	Fluconazole Fluvoxamine Fluoxetine Sulfaphenazole ^c	Rifampicin

CYP2C19	S-Mephenytoin Omeprazole	Ticlopidine	Fluoxetine S-Mephenytoin ^c Omeprazole	Nootkatone Ticlopidine	Rifampicin
CYP2D6	Dextromethorphan Bufuralol	Paroxetine	Bufuralol ^c Debrisoquine Dextromethorphan ^c	Quinidine ^c	None identified
CYP3A4	Testosterone Midazolam	Mifepristone Verapamil Troleandomycin Erythromycin	Erythromycin Dextromethorphan Midazolam ^c Nifedipine Terfenadine Testosterone ^c Triazolam	Azamulin Ketoconazole ^c Itraconazole ^c Troleandomycin Verapamil	Rifampicin

^aGrimm *et al.* [91]; the list of substrates and inhibitors for time-dependent inhibition is based on perspective of Pharmaceutical Research and Manufacturers of America.

^bFDA's wave site, <http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm#inVitro> [89].

^cPreferred substrates and inhibitors listed in the FDA's wave site [89].

TABLE 12.2 Advantages and Disadvantages on Various Methods for Assessment of CYP Inhibition

Methods	Advantages	Disadvantages
Fluorescence	• Very high throughput	• Low selectivity and only use of recombinant CYP enzymes • K_i or IC_{50} that shows relatively low correlation with the corresponding data derived from marketed drugs
Radioactivity	• High throughput • Use of marketed drug with high selectivity as probe substrate • Use of recombinant CYP enzymes, liver microsomes, and hepatocytes	• Limited availability of probe substrates • High possibility of nonspecific binding when microsome concentration is high
LC/MS/MS	• Moderate–high throughput • Use of marketed drug with high selectivity as probe substrate • Use of recombinant CYP enzymes, liver microsomes, and hepatocytes • Inhibition on well-defined metabolic pathway • Cassette dosing (cocktailed approach) • Results applicable to new drug application (NDA)	• Ion suppression • High possibility of nonspecific binding when microsome concentration is high

Fluometric-based assays are regarded as a high throughput screening method by which substrates converted to fluorescent metabolites by CYP enzymes are utilized. Since the substrates exhibit low specificity toward each CYP enzyme, recombinant CYP enzymes but not human liver microsomes need to be used. Crespsi *et al.* [92] developed high throughput screening using 3-cyano-7-ethoxycoumarin as the substrate of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 and resorufin benzyl ether as the substrate of CYP3A4. The obtained IC_{50} values showed high correlation to those of the traditional method using phenacetin, diclofenac, *S*-mephenytoin, bufuralol, and testosterone as the respective substrates. A similar microtiter plate assay with recombinant CYP enzymes and fluorescent probes was reported by Yamamoto *et al.* [93], who demonstrated comparable K_i values for nine CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) to the reported values of the conventional assay using human liver microsomes. Such a good relationship between the two assays was also reported by Bapiro *et al.* [94]. On the other hand, different results were found due to low sensitivity when VIVID®Red as the fluorescent substrate was utilized for CYP3A4 inhibition [95]. Furthermore, relatively lower IC_{50} values of CYP2C9, CYP2D6, and CYP3A4 in the fluometric assay than the conventional assay were found and were possibly associated with nonspecific binding

due to high microsome concentrations in the latter assay [96]. The reduction of microsomal concentration from 0.5 to 0.1 mg/mL led to an increase in the relationship. To increase the throughput, as well as to reduce the used amount of test compound, the fluometric assay was miniaturized to a 384-well plate format [97], and an integrated robotic system that consisted of liquid handlers, a plate hotel, and an incubator was applied to the fluometric assay [98].

Importantly, Kenworthy *et al.* [99] have recommended that multiple CYP3A4 probes, representing individual substrate groups, are used for *in vitro* assessment of CYP3A4-mediated DDI. Their group evaluated the inhibitory effects of 34 CYP3A4 substrates in the metabolism of 10 CYP3A4 probes in rhCYP3A4. As a consequence, erythromycin, cyclosporine, and testosterone were categorized as the large molecular group and dextromethorphan, diazepam, and benzodiazepine derivatives as the second group. Dihydropyridine derivatives such as felodipine and nifedipine showed a different inhibition profile from the other two groups. It was thus recommended that different CYP3A substrates (two or more substrates) chosen from each group are applied to the CYP3A inhibition assay. The sensitivity of various fluorescent substrates to CYP3A4 such as benzoxyresorufin (BzRes), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-benzyloxyquinoline (BQ), and dibenzylfluorescein (DBF) was comparatively evaluated by Stresser *et al.* [100]. BFC and BQ were the most and the least sensitive substrates, respectively, for evaluation of CYP3A4 inhibition. Additionally, IC₅₀ values from DBF correlated to those from testosterone 6 β -hydroxylation. Chauret *et al.* [101] also showed evidence for a similar correlation between DFB (3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-furan-2-one and testosterone turnover. On the basis of the aspects of high sensitivity and/or clinical relevant substrate, BFC, DBF, and DFB are regarded to be suitable fluorescent CYP3A4 substrates for the high throughput assay.

Amelioration of conventional assay with marketed drug substrates was advanced with a short running time per sample analysis by developing the technologies of analytical equipment such as LC/MS/MS. Establishing simultaneous determination methods for metabolic activities of seven or nine CYP enzymes using LC/MS/MS allowed increasing the throughput in the assay [102,103]. Given that the use of two different CYP3A substrates was required, further development of simultaneous analysis, including two CYP3A activities (midazolam and dextromethorphan), was conducted by Li *et al.* [104]. Interestingly, as a cassette probe dosing/cocktail approach, a simultaneous incubation method using several probe substrates was developed [97,105–107]. In a validation study of the cocktail approach by Zientek *et al.* [97], the estimated IC₅₀ values of furafylline to CYP1A2, sulfaphenazole to CYP2C9, ticlopidine to CYP2C19, quinidine to CYP2D6, and ketoconazole to CYP3A4 were almost consistent between the cassette dosing and single dosing approaches.

In general, the IC₅₀ value is estimated based on the remaining CYP activity at multiple inhibitor concentrations such as 5–10 points. To increase the efficiency, various methodologies for the estimation of IC₅₀ have been developed. Moody *et al.* [108] developed two-point IC₅₀ determination in automated radiometric inhibition assay. The IC₅₀ estimated from 5 and 50 μ M inhibitor concentrations showed a good relationship with full IC₅₀ determination regardless of CYP enzymes. Moreover, a great challenge by Lin *et al.* [109] employed one-point IC₅₀ determination to maintain screening throughput instead of cassette incubation and pooled sample analysis. On the basis of the experiences of their group, there were high possibilities that the cassette dosing

generated additional interactions and that pooled sample analysis could potentially compromise MS/MS detection in case of strong inhibitors. The IC_{50} values calculated based on the one-point assay was in good agreement to those obtained by the eight-point assay, with slope of 0.95–1.02 for CYP enzyme activities. Additionally, Nakamura *et al.* [110] pointed out the possibility of ion suppression in LC/MS/MS analysis, which probably misleads the inhibitory effect of test compounds; that is, stable isotopes as internal standards of the quantification of metabolites of probe substrates are, in many cases, not commercially available. Use of [$^{13}C_4$ -, ^{15}N]-midazolam and 1'-hydroxymidazolam as the substrate and internal standard, respectively, successfully improved the effect of ion suppression.

Pharmacokinetics of some drugs involves various mechanisms such as uptake transporters, intracellular protein binding, and lysosomal uptake in addition to CYP-mediated metabolism. Hepatocytes are considered to retain those functions, and the usefulness of cryopreserved human hepatocytes in the assessment of DDI, alongside metabolic stability, was demonstrated by Li *et al.* [111]. *In vitro* DDI study for four hepatic uptake substrates, including atorvastatin and pitavastatin, was implemented using rat and human hepatocyte systems [112]. Those substrates were concentrated up to 1000-fold in the intracellular space of rat hepatocytes; however, only a fivefold difference in the IC_{50} values was pronounced. This was due to intracellular binding rather than extensive hepatic uptake. The importance of intracellular binding was also revealed by Brown *et al.* [113], who employed miconazole, fluconazole, ketoconazole, quinine, fluoxetine, and fluvoxamine with a high cell-to-medium ratio ranging from 4.2 to 6000. The cell-to-medium ratio was not correlated with the ratio of the K_i values between rat hepatocytes and liver microsomes; instead, there was a relationship between the unbound K_i values of the two assays. Accordingly, the high cell-to-medium ratio was not ascribed to hepatic uptake but to intracellular nonspecific binding. Intracellular accumulation associated with the binding must be considered as a key factor in *in vitro*–*in vivo* extrapolation. Importantly, McGinnity *et al.* [114] reported the usefulness of inhibition data of hepatocytes rather than recombinant CYP enzymes in the assessment of inhibition of quinidine and propafenone, CYP2D6 inhibitors. A significant difference in unbound IC_{50} ($IC_{50,u}$) toward the metabolism of bufuralol values between rhCYP2D6 and human hepatocytes was found with $IC_{50,u}$ of 0.02 versus 0.5 μM for quinidine and 0.02 versus 4.1 μM for propafenone, which was in contrast to similar $IC_{50,u}$ values for other examined CYP2D6 inhibitors.

As reported in the metabolic stability study, the plasma incubation method with human hepatocytes was applied to evaluate CYP inhibition by ketoconazole and fluconazole, a strong and a moderate CYP3A inhibitor, respectively [115–117]. On the basis of the concept that the examined inhibitor concentration in the plasma incubation is clinically relevant, *in vivo* inhibition potential was successfully derived from a relationship between the inhibitor concentrations and the remaining metabolic activity of the probe substrates in the plasma incubation, taking the f_m value into consideration. Moreover, the plasma incubation approach with human hepatocytes was applied to develop a single preincubation time method, which provided reliable prediction of DDI, regardless of CYP3A inhibitory mechanism [118].

For a drug that is biotransformed via multiple pathways mediated by CYP enzymes, in the general approach, the K_i value obtained in the individual metabolite formation need to be converted to the K_i value of whole metabolism by the weighted method with

the contribution fraction (f_m) of the inhibited CYP enzyme to CL for each pathway [114]. The substrate depletion assay has also been used to evaluate *in vitro* DDI [119] and can easily provide the K_i value of the inhibitor to whole metabolism. The estimated K_i values were similar to those of the metabolite formation assay in case of inhibition of omeprazole to diazepam metabolism. Hence, substrate depletion assay would enable to easily generate a K_i value for drugs that are eliminated via unidentified metabolic pathways.

12.4.1.2 Time-Dependent Inhibition. TDI, in general, results from irreversible covalent binding or quasi-irreversible noncovalent tight binding of a chemically reactive intermediate to enzymes that catalyze its formation. Grimm *et al.* [91] has distinguishably defined TDI and mechanism-based inactivation (MBI). TDI only indicates a kinetic definition and can be defined as a compound that time dependently increases an inhibitory effect during incubation with enzymes before the addition of substrates. MBI can be referred to as a *subset of TDI* demonstrating that enzymes act on the substrate to form a chemically reactive metabolite that subsequently inactivates the enzyme [120]. Accordingly, TDI, in some cases, includes reversible inhibition of the product (metabolite) formed during the incubation with enzymes. On the basis of the results of biochemical experiments, MBI can be classified into two groups: quasi-irreversible and irreversible inhibition.

Assessment of TDI has generally been performed by a shift in IC_{50} after preincubation with human liver microsomes or recombinant CYP enzymes in the presence of NADPH before incubation with CYP enzyme-selective probe substrates (Table 12.1). The shift in the IC_{50} value depending on the preincubation time cannot distinguish MBI and inhibition of the formed metabolite. In a general approach, after preincubation with human liver microsomes at high concentrations of 1–2 mg/mL, at least 10- or 20-fold dilution is necessary to quench further inhibition of probe substrate metabolism by the test compound or its metabolite. Use of a probe substrate concentration under saturation conditions ($>four\text{- or fivefold } K_m$) is recommended to minimize further inhibition of probe substrate metabolism by the test compound and its formed metabolite.

The rate of CYP enzyme inactivation is proportionally dependent on the inhibition concentration as described by the following equation [121]:

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]} \quad (12.3)$$

where k_{obs} is the pseudo first-order rate constant of inhibition at the inactivator concentration $[I]$, k_{inact} the maximum inactivation rate, and K_I the inactivator concentration when the rate of inactivation reaches half that of k_{inact} . The k_{obs} can be determined from percentage remaining activity in multiple time- and concentration-dependent TDI assay. The logarithm of percentage remaining activity is plotted against the preincubation time for each concentration of test compounds, and the k_{obs} can be obtained by a slope from the linear regression analysis, and the k_{obs} and the inactivator concentration are fitted into Equation 12.3. Hence, to determine K_I and k_{inact} , five to six preincubation time points and multiple inactivator concentrations are required, and the TDI study is a time-consuming and labor-intensive work. Tables 12.1 and 12.3 indicate probe substrates and inactivators, and K_I and k_{inact} for CYP3A-mediated TDI using human liver microsomes, rhCYP3A4 or human hepatocytes, respectively.

TABLE 12.3 *In Vitro* Parameters for Time-Dependent Inactivators in Humans

	Enzyme Sources	Substrates	K_I (μM)	k_{inact} (min^{-1})	References
Amprenavir	HLM	Testosterone	0.37	0.59	[122]
Azamulin	HLM	Midazolam	0.17	0.68	[123]
Clarithromycin	HLM	Midazolam	15.5	0.0192	[121]
	HLM	Nifedipine	15.9	0.0244	[121]
	HLM	Testosterone	12.9	0.0324	[121]
	HLM	Midazolam	5.49	0.072	[124]
Diltiazem	HLM	Midazolam	8.7	0.0050	[123]
	HLM	Testosterone	4.7	0.0230	[123]
	rhCYP3A4	Midazolam	0.49	0.010	[125]
Erthinylestradiol	rhCYP3A4	Midazolam	23	0.09	[125]
Erythromycin	HLM	Midazolam	12.1	0.0215	[121]
	HLM	Nifedipine	11.3	0.0295	[121]
	HLM	Testosterone	10.9	0.0352	[121]
	rhCYP3A4	Midazolam	8.82	0.12	[126]
	rhCYP3A5	Midazolam	No TDI	—	[126]
	Hepatocyte	Midazolam	11	0.07	[127]
Fluoxetine	HLM	Midazolam	5.26	0.017	[124]
	Hepatocyte	Midazolam	1	0.01	[127]
Lopinavir	HLM	Testosterone	1.0	0.11	[122]
Mifepristone	rhCYP3A4	Midazolam	0.61	0.080	[126]
	rhCYP3A4	Midazolam	No TDI	—	[126]
Nelfinavir	HLM	Testosterone	1.0	0.22	[122]
Ritonavir	HLM	Testosterone	0.17	0.40	[122]
Saquinavir	HLM	Testosterone	0.65	0.26	[122]
Troleandomycin	HLM	Midazolam	2	0.03	[127]
	rhCYP3A4	Midazolam	0.26	0.12	[126]
	rhCYP3A5	Midazolam	No TDI	—	[126]
	Hepatocyte	Midazolam	0.4	0.05	[127]
Verapamil	HLM	Midazolam	2.55	0.0277	[121]
	HLM	Nifedipine	3.12	0.0486	[121]
	HLM	Testosterone	5.79	0.0591	[121]
	HLM	Testosterone	2.6	0.038	[123]
	rhCYP3A4	Midazolam	0.74	0.041	[125]

Abbreviation: HLM, human liver microsomes.

As a simplified screening system for TDI with moderate to high throughput, IC_{50} shift assay using fluometric substrates (3-cyano-7-ethoxycoumarin for CYP1A2, CYP2C9, CYP2C19, and CYP2D6; BzRes and BFC for CYP3A4) has been developed [128]. In this assay, IC_{50} was continuously measured without terminating the enzyme reactions. Time-dependent reduction in IC_{50} values was found for quasi-irreversible inhibitors (isosafrole, erythromycin, troleandomycin, and diltiazem) and irreversible inhibitors (furafylline, propranolol, and mifepristone), which was in contrast to the constant IC_{50} for typical reversible inhibitors such as sulfaphenazole, tranilcypromine, quinidine, and ketoconazole. Similarly, Sekiguchi *et al.* [129] also conducted TDI assay of CYP3A4 using fluorescent substrate (BFC). In contrast, a disadvantage of

those fluometric assays is that metabolite inhibition and MBI cannot be distinguishably evaluated.

Single time- and concentration-dependent assay has been proposed as the first screening for TDI [121,125]. Watanabe *et al.* [121] reported automated single time- and concentration-dependent inhibition assay using midazolam as the CYP3A probe substrate, combined with a 10-fold dilution process of preincubation to avoid the effect of product inhibition. Using 171 marketed drugs, a diagram to evaluate the possibility of MBI-associated *in vivo* DDI was successfully described from the remaining activity of midazolam 1'-hydroxylation in the MBI assay and *in vivo* DDI information that included therapeutic blood or plasma concentration. Their group also developed a screening method to distinguish quasi-irreversible and irreversible inhibitors by the addition of ferricyanide into the preincubation solution. Noticeably, the assessment of MBI does not depend on the substrates (midazolam, nifedipine, and testosterone), while reversible inhibition requires the use of different types of substrates. A two-time point shift assay for TDI with a 5- or 10-fold dilution of preincubation solution was applied to the assessment of five major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) [123]. Moreover, to increase the throughput in the TDI assay, a cassette dosing approach was applied to the estimation of K_I and k_{inact} of prototypic inhibitors in cultured human hepatocytes [127].

A different strategy to evaluate TDI of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 was proposed by Lim *et al.* [130]. In their strategy, test compounds are first evaluated in apparent partition ratio (APR) screening, followed by detailed study with time- and concentration-dependent inhibition, and differentiation of quasi-irreversible inhibition and irreversible inactivation by oxidation of potassium ferricyanide and/or dialysis. APR was estimated by plotting percentage remaining activity as the function of the molar ratio of the test compound to CYP enzyme. Among 19 mechanism-based inhibitors and 4 quasi-irreversible inhibitors, the assay identified ritonavir, mibefradil, and azamulin as highly effective mechanism-based inhibitors of CYP3A, with an APR of 1.4–2.1, and isoniazid as a less effective inhibitor of CYP2C19 with an APR of 13,491. Such a widely dynamic range is regarded as an advantage in APR screening.

CYP3A5, along with CYP3A4, one of the major CYP enzymes, is expressed in adult human liver and small intestine [131], although the expression is genetically polymorphic [132]. The time-dependent inhibitors such as verapamil and its metabolites reportedly showed different inhibitory kinetics for CYP3A4 and CYP3A5 [133]. In addition, some of the compounds investigated by Soars *et al.* [126] did not show TDI to rhCYP3A5 but rhCYP3A4. Therefore, there is a possibility that a variable expression ratio of CYP3A4/3A5 in human liver microsomes contributes to interindividual variations in CYP3A-mediated DDI [134].

12.4.2 Prediction of *In Vivo* CYP Inhibition

12.4.2.1 Static Prediction Model. Assessment of the possibility of DDI and/or risk based on the predicted data has been included in the framework of pharmacokinetic studies. The prediction methods can be divided into two groups; namely, the static model describing the fraction of remaining CYP activity at a fixed inhibitor concentration and a PBPK model describing the time-dependent fraction of the remaining CYP activity associated with the plasma concentration–time curve of the inhibitor after administration.

In the static model, the most widely utilized method is the FDA-recommended approach based on $[I]/K_i$ ratio, in which $[I]$ indicates the mean steady-state $C_{\max}$ value for total drug (bound plus unbound) after administration of the highest proposed clinical dose [89]. An estimated $[I]/K_i$ ratio exceeding 0.1 is considered positive and follow-up *in vivo* assessment and/or detailed evaluation using mechanistic models are recommended.

The critical point of the static is which inhibitor concentration to use preferentially as the surrogate of the inhibitor concentration in the surroundings of the target enzymes in the liver because direct measurement of the inhibitor concentration is impossible. Some surrogate inhibitor concentrations have been investigated comparatively to evaluate the possibility of *in vivo* DDI. Komatsu *et al.* [135] challenged DDI prediction based on reversible inhibition between tolbutamide and various sulfonamides using K_i values estimated from an *in vitro* experiment in human liver microsomes. Irrespective of competitive or noncompetitive inhibition, the ratio of CL_{int} in the presence ($CL_{\text{int},+I}$) and absence of inhibitor ($CL_{\text{int},-I}$) can be described as the following equation when the substrate concentration is much lower than the K_m value:

$$CL_{\text{int},+I} = \frac{CL_{\text{int},-I}}{1 + \left(\frac{C_{\text{hep,inlet,u}}}{K_i} \right)} \quad (12.4)$$

where $C_{\text{hep,inlet,u}}$ is the unbound concentration of inhibitor. To avoid the false negative predictions, $C_{\text{hep,inlet,u}}$, which presents unbound maximum hepatic inlet concentration, was calculated as the sum of maximum unbound plasma concentration in the systemic vein ($C_{\max} \times f_p$) and the portal vein ($I_{\max} \times f_p$) from gastrointestinal absorption after oral administration, under the assumption that unbound inhibitor concentration in the liver equals that in the plasma. The blood concentration in the portal vein can be estimated as follows:

$$I_{\max} = \frac{D \times k_a \times F_a}{Q_h} \quad (12.5)$$

where k_a is the absorption rate constant, F_a the fraction of absorption, D the dose of administered inhibitor, and Q_h the hepatic blood flow. Approximately 4.8- and 1.8-fold increases in area under the plasma concentration curve (AUC) of tolbutamide, which were predicted by the coadministration of sulfaphenazole and sulfamethizole, were within a similar range as the reported *in vivo* data.

A comprehensive study on the prediction methods for reversible inhibition using Monte Carlo simulation [136] indicated that the use of maximum unbound hepatic inlet concentration did not provide false negative prediction, often accompanied with a produced overestimation, whereas an attempt using maximum unbound blood concentration in the systemic vein produced a false negative prediction. Furthermore, Ito and Houston [137] intensively investigated the effect of various estimates of inhibitor concentrations on the predictability of DDI based on the results of 193 DDI studies involving CYP2C9, CYP2D6, and CYP3A4. Excluding mechanism-based inhibitors, the use of total hepatic inlet concentration did not result in false negative prediction. This method may be a preferential approach in the selection of NMEs, which need to avoid false negative predictions, although the incidence of false positive predictions is controversial.

Importance of f_m and k_a values was highlighted by several investigators. The use of f_m values and the free hepatic inlet $C_{\max}$ successfully identified drugs that showed at least twofold increase in the AUC of the CYP-probe substrates, excluding known mechanism-based inhibitors from among 40 examined drugs [138]. In addition, Brown *et al.* [139] focused on the impact of f_m and k_a values on the prediction accuracy based on 115 clinical DDI studies involving CYP2C9, CYP2D6, and CYP3A4. The incorporation of f_m values into *in vivo* predictions using hepatic input plasma concentration resulted in 84% of studies within twofold of the *in vivo* data. In addition, the refined k_a value calculated from pharmacokinetic analysis reduced the number of overpredictions of CYP2D6 and CYP3A4. Ohno *et al.* [140] developed a robust and practical model, in which the time-averaged apparent inhibition ratio of CYP3A4 (IR_{CYP3A4}) calculated primarily based on AUC increases in DDI studies with 18 CYP3A inhibitors and midazolam was incorporated. The proposed method with f_m and IR_{CYP3A4} values is a versatile tool that enables the easy prediction of AUC increase by CYP3A4-mediated unknown interaction.

Regarding TDI, retrospective investigation by Ito *et al.* [141] focused on what inhibitor concentration was appropriate to predict midazolam and macrolide interaction. Application of maximum unbound hepatic inlet concentration gave a more accurate prediction, whereas underprediction tended to be observed in case of maximum unbound systemic concentration. Galetin *et al.* [142] evaluated 37 *in vivo* cases of irreversible inhibition of macrolides and diltiazem on the metabolism of CYP3A4 substrates, considering the inhibitory effect on the intestinal first-pass metabolism, using the following equation:

$$\frac{AUC_I}{AUC} = \frac{F'_G}{F_G} \times \frac{1}{\frac{f_{m,CYP3A4}}{1 + \sum_{i=1}^n \frac{k_{inact,i} \times I_{u,i}}{k_{deg,CYP3A4} \times (K_{I,u} + I_{u,i})}} + (1 - f_{m,CYP3A4})} \quad (12.6)$$

where $f_{m,CYP3A4}$ is the fraction of probe substrate metabolized by CYP3A4, $k_{deg,CYP3A4}$ is the *in vivo* degradation rate constant of CYP3A4 in the liver, I_u is either the average unbound systemic plasma concentration after repeated administration or the maximum unbound hepatic inlet concentration, and F'_G and F_G are the intestinal availability in the presence and absence of inhibitor, respectively. The use of unbound systemic plasma concentration of the inhibitor and f_m value resulted in 89% of studies predicted within twofold of the *in vivo* data.

The prediction accuracy of TDI is very sensitive to the CYP enzyme degradation rate. However, regarding CYP3A4 enzyme, the reported turnover ranged widely from 26 to 79 or 36 to 140 h, estimated from the *in vitro* and *in vivo* data, respectively [91]. Obach *et al.* [143] reported a prediction method for CYP3A-related MBI incorporating an *in vivo* CYP enzyme degradation rate constant estimated from clinical pharmacokinetic data, which was a similar model as to that by Galetin *et al.* [142], additionally with intestinal inhibitory kinetics. When using unbound systemic $C_{\max}$ as the *in vivo* inhibitor concentration, the most accurate predictions of DDI was obtained with a mean error of 1.64-fold. Additionally, using IC_{50} , IC_{50} fold-shift, K_I , and K_{inact} obtained in irreversible inhibition analysis, the relationship between each parameter was investigated. Surprisingly, fold-shift of IC_{50} before and after preincubation, which is often used as an indicator in the first screening, showed no correlation with K_{inact}/K_I , one of the indicators for potential irreversible inhibition. A good relationship was noted

between the shifted IC_{50} determined after 30-min preincubation and the K_{inact}/K_I or K_I value. Burt *et al.* [144] also reported that K_{inact}/K_I was correlated with $1/IC_{50}$ value after either a 10- and 30-min preincubation.

12.4.2.2 Physiologically Based Pharmacokinetic Model. In addition to K_i or K_I generally used as an index of hepatic enzyme inhibition, a key factor to the predictability is which inhibitor concentration is used. Those retrospective assessments using fixed inhibitor concentration would have given an important indication that the use of unbound hepatic inlet inhibitor concentration and unbound systemic inhibitor concentration appears to yield better prediction for reversible and irreversible inhibition, respectively. Not only to increase the predictability of DDI but also to describe the plasma concentration profile of substrates altered by a concomitantly given drug, the use of a plasma concentration–time curve of the inhibitor simulated from a PBPK model is essential instead of the fixed inhibitor concentrations.

With regard to reversible inhibition, estimation of *in vivo* relevant K_i value allowed quantitative prediction of CYP-mediated DDI [145]. The *in vivo* K_i values were determined by fitting the plasma concentration–time profiles of a substrate and an inhibitor to a simple PBPK model. Increased ratios of AUCs predicted using the *in vivo* K_i value estimated for each CYP inhibitor were in agreement with *in vivo* corresponding data and were more accurate relative to the prediction by the static model. Importantly, the discrepancy between *in vivo* K_i and *in vitro* K_i values was pronounced, possibly ascribed to nonspecific binding to liver microsomes that was well defined by lipophilicity.

For irreversible inhibition, prediction using a PBPK model has been challenged for DDI between 5-fluorouracil and sorivudine [146], triazolam and erythromycin [147], and midazolam and macrolides [141]. As already pointed out, CYP turnover rate ($k_{deg,CYP}$) is a key parameter that has an impact on the predictability. There has been difficulty to estimate the $k_{deg,CYP}$ of each CYP enzyme in the human liver in biological experiments, and hence, rat CYP data of 0.0005/ min was employed as human $k_{deg,CYP}$ in those studies.

DDI prediction has, in general, been performed under the following assumptions [141,147]: (i) substrate and inhibitor are orally administered according to the first-order rate constant and only undergo hepatic metabolism, (ii) unbound concentration in the hepatic vein and liver are equal due to a rapid equilibrium according to the well-stirred model, (iii) only unbound drug in the liver is subject to elimination, and (iv) disposition and metabolism of inhibitor are not altered by the inhibition of CYP3A4. In the prediction of triazolam and erythromycin interaction, when the K_I and k_{inact} values estimated in human liver microsomes or recombinant CYP3A4 were incorporated into a PBPK model, repeated administration of erythromycin at 333 mg three times a day for three days resulted in simulated reduction in hepatic CYP3A4 enzyme to 50–60% of the initial level [147]. Subsequently, the predicted 2.0- and 2.6-fold increase in triazolam AUC was derived from human liver microsomes and recombinant CYP3A4, respectively, which was similar to *in vivo* clinical data with a 2.1-fold increase. Similarly, for midazolam and macrolide interaction, simulation using the K_I and k_{inact} of macrolides estimated in human liver microsomes yielded a 2.9- to 3.0-fold increase in midazolam AUC after pretreatment with erythromycin, a 2.1- to 2.5-fold increase after pretreatment with clarithromycin, but little effect for azithromycin [141]. It should be noted that the

assessment of DDI in first-pass metabolism in the small intestine after oral administration is important to increase predictability, because the intestinal metabolism has reportedly had a great influence on the bioavailability of CYP3A4 substrates [7,145].

An advantage of the PBPK model beyond the static model in the DDI prediction is to estimate a change in the plasma concentration–time curves, including $C_{\max}$ and $t_{1/2}$, which would be associated with pharmacodynamics, in single and multiple ascending dose studies. Recently, advanced modeling and simulation tools combining *in vitro* data have increasingly been utilized to predict *in vivo* profile to optimize the dose regimen in a clinical trial to ensure that any interaction is appropriately measured. One such program is SIMCYP® (Simcyp Ltd., Sheffield, UK), which would give confidence to CL and DDI prediction [148,149].

12.5 CYP ENZYME INDUCTION

In vivo induction of CYP enzymes can result in significant clinical consequences with suboptimal pharmacological efficacy through the reduction in plasma concentration of comedicated drugs, and in the worst case, the increased formation of reactive metabolites. Clinical DDIs have been reported, with the therapeutic failures that comedication with CYP3A inducers lead to reduced ethinylestradiol levels from contraceptives, resulting in breakthrough pregnancies [150], and reduced cyclosporine level with organ rejection in transplant patients [151,152]. Induction activity on CYP enzymes has become one of the criteria for the selection of NMEs.

Studies on the induction have mainly been focused on CYP enzymes that are involved in the metabolism of a large number of drugs. CYP induction can occur as a result of either an increase in *de novo* synthesis of enzymes or a decrease in degradation associated with stabilization of the protein [153]. Regarding all CYP enzymes, excluding CYP2E1, which is subject to the stabilization [154], increased synthesis is common. Induction of CYP enzymes by drugs and other xenobiotics typically occurs via activation of receptors that regulate transcription. Induction of CYP1A1 and 1A2 is mediated through the aryl hydrocarbon receptor (AhR) [155]. CYP3A4 is induced through the pregnane X receptor (PXR), which is also involved in the induction of CYP2C9 and organic anion transporters [156]. Therefore, it is considered that CYP2C9 induction consistently tracks with the expression level of CYP3A4. PXR is reported to have a synergistic effect with the glucocorticoid receptor (GR), although the mechanism is not fully understood [157]. The constitutive androstane receptor (CAR) is a member of the gene superfamily of nuclear hormone receptors and a key transcription factor in hepatic CYP induction [158,159]. PXR and CAR are demonstrated to be responsible for coordinating the regulation of a large number of drug-metabolizing enzymes, including CYP isoforms (CYP2B6, CYP2C9, and CYP3A4) and transporter genes [160,161].

Since almost all *in vivo* hepatic events, including drug metabolism and induction, can be evaluated by the hepatocyte system, human hepatocytes in primary culture are currently utilized in the assessment of enzyme induction as the gold standard in preclinical DDI studies. However, due to the limited availability of high quality human hepatocytes and interindividual variability in response to inducers, alternative assay systems with at least moderate throughput have been developed for the lead optimization under pressure from project organizations: reporter gene assay for PXR and AhR, and other cell lines such as nontumorigenic immortalized human hepatocyte line, Fa2N-4 cells, and a new human hepatoma cell line, HepaRG. The current status

TABLE 12.4 Advantages and Disadvantages on Various Methods for Assessment of CYP Induction

Methods	Advantages	Disadvantages
Reporter gene assay	• Very high throughput • Cost effective	• No consideration of metabolic elimination of inducer • Poor correlation of $E_{\max}$ with hepatocyte • Assessment of transcription activation only
Hepatocyte	• Gold standard method • Possible existence of functions regarding induction mechanism, nuclear transporters, uptake transporter, efflux transporter, and drug metabolism • Determination of both mRNA and activity level • Results applicable to NDA • Evaluation of all process involved in induction	• Low throughput • High cost and labor intensive • Limited availability of high quality fresh hepatocytes • High interindividual variation
HepaRG	• Moderate throughput • Determination of mRNA and activity level • High expression of nuclear factors and transporters • A certain level of drug-metabolizing activities • EC_{50} and/or $E_{\max}$ correlated with hepatocytes	• Alternation of drug-metabolizing activities by culture condition with DMSO • A relatively large lot-to-lot variation in fold induction reported
Fa2N-4	• Moderate throughput • Similar response over the passage • EC_{50} correlated with hepatocytes	• Extremely low expression of CAR • Low drug-metabolizing activities • Low expression of hepatic uptake transporters

of CYP induction assay and its prediction is separately reviewed. The advantages and disadvantages of those assay methods and the *in vitro* inducers recommended by the FDA draft guidance are listed in Tables 12.1 and 12.4, respectively. In addition, *in vitro* parameters for CYP induction are summarized in Table 12.5.

12.5.1 In Vitro System for Evaluating CYP Enzyme Induction

12.5.1.1 Reporter Gene Assay. PXR was first identified as an orphan nuclear receptor responsive to pregnanes by Kliewer *et al.* [169], who found that PXR induces the CYP3A family. Furthermore, their group [170] demonstrated a differential activation profile in human pregnane X receptor (hPXR) and mouse pregnane X receptor (mPXR)

TABLE 12.5 *In Vitro* Parameters for CYP3A4 mRNA in Various Assay Systems of CYP Induction

	PXR Reporter Gene Assay		Hepatocytes		HepaRG Cells		Fa2N-4 Cells	
	EC_{50} (μ M)	$E_{\max}$	EC_{50} (μ M)	$E_{\max}$	EC_{50} (μ M)	$E_{\max}$	EC_{50} (μ M)	$E_{\max}$
Carbamazepine	51 ^a 0.9 ^{e,f}	8 14.1	60, 55.8 ^b 42 ^a	10.2, 34.3 23	50 ^c —	58 —	62 ^d 11 ^g	2.5 4
Clotrimazole	1.2 ^a 1.1 ^{e,f}	11 15.2	— —	— —	— —	— —	0.4 ^g —	4 —
Dexamethasone	100 ^a 2.6 ^{e,f}	12 52.3	43, 39 ^a —	71, 13 —	— —	— —	5 ^g —	3 —
Nifedipine	2 ^a —	14 —	16.6, 18 ^b —	104, 34.3 —	16 ^c —	12 —	17 ^d 8 ^g	8.3 13
Phenobarital	100 ^a 9.7 ^{e,f}	9 41.3	153, 159 ^b 142 ^a	14.4, 49 17	139 ^c —	22 —	222 ^d 205 ^g	5.5 12
Phenytoin	0.3 ^a 25.1 ^{e,f}	3 27.5	3.7, 24 ^b 18, 12 ^a	8.8, 15.8 15, 12	21 ^c —	30 —	44 ^d 74 ^g	5.2 10
Rifampicin	— 0.9 ^a	— 19	5.1 ^h 2.6, 0.57 ^b	16.9 14.9, 33	— 4.3 ^{i,j}	— 42.5	10 ^h 1.9 ^d	9.2 13
	1.7 ^{e,f}	54.4	0.1, 0.4 ^a	76, 33	0.8 ^c	83	4 ^g	36
	—	—	0.4 ^h	52.5	—	—	8 ^h	59.5
Ritonavir	1.4 ^a	18	—	—	—	—	0.2 ^g	8
Rosiglitazone	—	—	14.3, 13.6 ^b	5.4, 23.9	14 ^c	15	14.5 ^d	2.9
Troglitazone	NA ^{a,k} 0.2 ^{e,f}	3 17.1	2.0, 8.5 ^b 0.8 ^a	9.8, 23 8.7	— —	— —	3 ^d —	2.4

^aMcGinnity *et al.* [162].^bFahmi *et al.* [164].^cGrime *et al.* [204].^dRipp *et al.* [166].^eEl-Sankary *et al.* [163].^fhGR/hPXR cotransfected cells.^gKenny *et al.* [167].^hHariparsad *et al.* [168].ⁱKaneko *et al.* [165].^jMidazolam 1'-hydroxylation activity.^kNot appropriate to define.

in the reporter gene assay. That is, hPXR was activated by clotrimazole, rifampicin, and dexamethasone-*t*-butylacetate, which was in contrast to low activation of mPXR by clotrimazole and rifampicin together with high activation by pregnenolone 16 α -carbonitrile (PCN).

The reporter gene assay for PXR has been used as a convenient high throughput tool to identify compounds with the potential to induce CYP3A4. Successful results to classify inducers and noninducers based on PXR reporter gene assay were reported by Persson *et al.* [171]. Seven *in vivo* inducers, one weak *in vivo* inducer, and noninducers were evaluated by the luciferase reporter gene assay for PXR with xenobiotic responsive enhancer module (−7836/−7208) and the −362/+53 proximal promoter from CYP3A4 in the pGL3 basic vector. When unbound *in vivo* AUC was taken into consideration, the assay identified inducers and noninducers separately. For phenobarbital, phenytoin, and troglitazone, which are prototypic inducers, $E_{\max}$ value, expressed as percentage of maximum rifampicin induction, was 71.7%, 20.6%, and 45.0%, respectively; however, lovastatin showed no response in the assay. Similar results for the rank order of induction responses (rifampicin > phenobarbital > phenytoin) in PXR activation assay was reported by Luo *et al.* [172].

Since hPXR and human glucocorticoid receptor (hGR) would reportedly contribute to the transcriptional regulation of CYP3A4 gene by a large number of xenobiotics [173], *in vitro* reporter gene assay, in which plasmid of hPXR and hGR was transfected into human hepatocyte carcinoma cell line, HepG2 cells, was developed [163]. This system utilized CYP3A4 proximal promoter (−1087 to −57) linked to a secretory alkaline phosphatase (SEAP) reporter gene. To increase the efficiency of estimating EC_{50} (concentration at half the maximum induction ratio) values in the assay, it was attempted to decrease to four- from eight-point concentrations on the dose–response curve. The four-point assay with respect to well-known CYP3A4 inducers produced a similar dose–response curve to the eight-point assay, and the highest fold induction based on $E_{\max}$ in lovastatin, followed by simvastatin $\approx$ troglitazone > rifampicin > phenobarbital > phenytoin.

To evaluate CYP1A1 induction, a cell-based high throughput screening system is also available. Yueh *et al.* [174] and Chao *et al.* [175] established stable cell lines harboring a luciferase reporter gene integrated in artificial multiple xenobiotic response elements. Moreover, the reporter gene assay was developed using HepG2 cells stably transfected pTX.DIR and pSV₂-Neo [171]. On the basis of the luciferase activity associated with AhR activation, the EC_{50} value of lansoprazole, omeprazole, and indole-3-carbinol was estimated to be 5.9, 18.1, and 55.8 μ M, respectively, whereas activation was not pronounced for phenytoin, primaquine, rifampicin, and troglitazone. Noticeably, the liver slice system with the treatment of primaquine, however, produced 17-fold induction of CYP1A1 mRNA, and phenytoin, rifampicin, and troglitazone appeared to show the induction of CYP1A1 mRNA, although to a lesser extent.

The molecular mechanism of AhR-mediated activation has been extensively studied for CYP1A1; however, the mechanism for CYP1A2 remains unclear. Recently, Sato *et al.* [176] have set up a dual reporter gene assay that enables simultaneous and rapid determination of transcriptional activation of CYP1A1 and 1A2 genes using indicators of luciferase and SEAP activity, respectively. Given that transcriptional activation of CYP1A1 and 1A2 genes is reportedly regulated simultaneously through a common regulatory element existing between the genes that act bidirectionally [177], the stable cell lines contain 23-kb intergenic spacer regions alongside the reporter gene. The

reporter gene assay detected the induction of CYP1A2 by omeprazole, lansoprazole, and alendazole, although the extent of induction of CYP1A2 was lower than that of CYP1A1. Therefore, the assay with the stable cell line may be a useful assessment tool for induction of CYP1A2 in addition to CYP1A1.

On the basis of the survey of Pharmaceutical Research Manufactures of America (PhRMA) to its member companies [178], PXR reporter gene assay is used by a majority of PhRMA companies surveyed, but only 23% of the companies assesses CYP1A induction potential of compounds in the AhR reporter gene assay.

12.5.1.2 Human Hepatocytes. Human hepatocytes have molecular mechanisms underlying CYP induction from signal transductions via nuclear receptors, in addition to drug-metabolizing enzymes and transporters involved in liver uptake and efflux, and those features are a benefit over other assessment systems. Accordingly, human hepatocytes have been regarded as a reliable *in vitro* system and the gold standard for evaluating enzyme induction of NMEs [179,180].

FDA draft guidance for DDI studies has recommended the evaluation of CYP induction, before clinical study, by a change in CYP enzyme activities (and CYP mRNA) in the primary culture of hepatocytes treated with test compounds and positive controls [89]. Fresh and cryopreserved human hepatocytes are commercially available. Comparative assessment of induction response between both hepatocytes demonstrated that there is not a significant difference in fold induction of CYP1A2 and CYP3A4 by lansoprazole and rifampicin, respectively [181]. The fresh hepatocytes exhibited higher activities of CYP1A2 and CYP3A4 enzymes than the cryopreserved hepatocytes; however, the use of fresh hepatocytes in a timely manner is difficult. Furthermore, there is large interindividual variability in response to inducers in fresh hepatocytes as demonstrated by Kostrubsky *et al.* [179].

Large interindividual variation in the basal activities of testosterone 6 β -hydroxylation and ethoxyresorfin deethylation was reported by some investigators [179,182,183]. Interestingly, Kostrubsky *et al.* [179] made it clear that there was an inverse relationship between fold induction and basal CYP3A4 activity when either taxol or rifampicin was exposed in hepatocytes; that is, hepatocytes with a low basal CYP3A4 activity tended to provide a large extent of induction and vice versa. As a consequence, the variation was decreased for the level of CYP3A4 activity enhanced after the treatment of the inducer, being possibly associated with a feedback regulation. A small lot-to-lot difference in the induced CYP3A4 activity was suggested by Chang *et al.* [184]. Moreover, a weak relationship was pronounced for the $E_{\max}$ values between different lots of hepatocytes, but the EC_{50} values were well correlated [162]. To dissolve these issues in the fresh hepatocytes, cryopreserved hepatocytes precharacterized as high quality lot in terms of induction and drug-metabolizing activities would be preferentially applicable to the CYP induction study.

A comprehensive work using a large number of human hepatocyte lots (62 lots) was conducted by Madan *et al.* [182], who evaluated the extent of induction of individual CYP activity after incubation with either β -naphthoflavone, phenobarbital, or rifampicin. The highest extent of induction in the treatment of rifampicin was noted for CYP2C19 activity (37-fold, $n = 10$), followed by CYP2B6 activity (13-fold, $n = 14$), CYP3A4 activity (10-fold, $n = 61$), CYP2C8 activity (5.2-fold, $n = 4$), CYP2C9 activity (3.5-fold, $n = 10$), and CYP2E1 activity (2.2-fold, $n = 5$). CYP1A2 activity was induced by 13-fold after treatment of β -naphthoflavone ($n = 28$).

Ritonavir is not only an inducer but also an irreversible inhibitor of CYP3A4, and the induction activity of ritonavir was evaluated in both the PXR reporter gene assay and the hepatocyte assay [172]. Unlike mRNA level, testosterone 6 β -hydroxylation activity in hepatocytes was reduced below the basal level after the treatment of ritonavir, whereas ritonavir displayed a strong induction in the PXR reporter gene assay. Similar dual activities as CYP inducer and inhibitor were also reported for troleandomycin. Induction parameters derived from the PXR reporter gene and human hepatocyte assay were comparatively evaluated using 24 compounds, including 14 time-dependent inhibitors [162]. The EC₅₀ values of CYP3A4 mRNA in hepatocytes were correlated with those in the PXR reporter gene assay. On the contrary, the $E_{\max}$ values of midazolam 1'-hydroxylation activity in hepatocytes showed poor correlation with the corresponding data for mRNA, which was attributed to metabolic activity reduced by TDI in hepatocytes. As expected, the exclusion of time-dependent inhibitors from the compounds examined led to dramatic improvement of the relationship. Fahmi *et al.* [185] comparatively investigated usefulness of CYP3A activity and mRNA as the reliable marker in the assessment of CYP3A induction. It was pointed out that cutoff value based on fold increase in CYP3A mRNA is the best classification on induction potential relative to that in CYP3A activity. This suggests that the measurement of CYP mRNAs, in addition to CYP activities, is important to characterize the induction profile of compounds when detailed information on TDI is not available. Interestingly, quantitative protein determination method for CYP induction by LC/MS/MS was reported [186]. A high throughput LC/MS/MS approach quantified CYP protein itself by measuring the isoform-specific peptides released by enzymatic digestion of hepatocyte incubation, with high reproducibility. This approach detected small fold change in CYP isoforms, and the induction of CYP3A5 by phenobarbital in human hepatocytes was detectable. The protein determination may be an alternative approach in the assessment of CYP induction.

CYP induction study using hepatocytes is a labor-intensive and time-consuming process. The use of a cassette dosing approach in the measurement of CYP enzyme activity would lead to a decrease in the workload of the induction study. Mohutsky *et al.* [187] employed cocktail probe substrates that were composed of phenacetin (CYP1A2), diclofenac (CYP2C), and midazolam (CYP3A4) to evaluate alternation of CYP enzyme activities. The extent of induction of CYP activities was comparable between the single and cassette dosing approaches regardless of CYP inducer or hepatocyte lot. In current study on profiling CYP induction of statins in human hepatocytes, Feidt *et al.* [188] demonstrated applicability of cocktail assay with metabolic activities of seven CYP enzymes: CYP1A2 (phenacetin), CYP2B6 (bupropion), CYP2C8 (amodiaquine), CYP2C9 (tolbutamide), CYP2C19 (*S*-mephenytoin), CYP2D6 (propafenone), and CYP3A4 (atorvastatin).

12.5.1.3 HepaRG Cells. A human hepatoma cell line, HepaRG, derived from a hepatoma cellular carcinoma has been utilized to evaluate CYP induction activity. In differentiated HepaRG cells after seeded at low density, mRNA levels of CYP3A4 and activity of testosterone 6 β -hydroxylation were low in the absence of 2% dimethyl sulfoxide (DMSO), compared with the corresponding data in the presence of 2% DMSO [189]. The low activity in the absence of DMSO was remarkably induced by rifampicin treatment. Profiling of the mRNA and activity level of drug-metabolizing enzymes in

differentiated HepaRG cells was conducted by Kanebratt and Andersson [190]. The differentiated HepaRG cells at one day after removal of DMSO from the medium showed comparable mRNA levels to human hepatocytes. Relative to human hepatocytes, the metabolic activities toward midazolam, naloxone, and clozapine in HepaRG cells were similar, whereas the HepaRG cells exhibited lower activity for 7-ethoxycoumarin and dextromethorphan associated with low levels of CYP2E1 and CYP2D6. Various nuclear receptors such as AhR, CAR, and PXR were retained at a high level, regardless of the presence or absence of DMSO [189]. HepaRG cells that exhibited the expression of nuclear receptors alongside most of all drug-metabolizing activities would be suitable for CYP induction screening, although the activity levels may depend on the culture condition.

Evaluation of EC_{50} needs a widely ranged dose–response curve. To avoid problems with cellular toxicity and solubility in the higher inducer concentration range, a new score to evaluate induction activity at lower concentrations was introduced. The score was defined as an F_2 value, which was obtained from the concentration at a twofold increase from baseline for mRNA and enzyme activity levels, instead of EC_{50} values [191]. The obtained F_2 values were correlated with decreases in AUC of CYP3A4 probe substrate drugs after treatment with inducer.

Lot-to-lot variability in CYP3A induction assays was reported in HepaRG cells [165]. The coefficients of variation (CV%) of the E_{max} of rifampicin, phenobarbital, and carbamazepine were 61.0%, 138.4%, and 198.8% and of the EC_{50} were 95.8%, 88.6%, and 198.3%, respectively, in four different lots of HepaRG. Since the variation tended to be smaller at lower rather than higher concentrations in the dose–response curve, the use of rifampicin- or phenobarbital-normalized fold induction at lower concentrations successfully minimized the variation.

12.5.1.4 Fa2N-4 Cells. Fa2N-4 cells are nontumorigenic and originated from human hepatocytes immortalized by transfection with the SV40 large T-antigen [192]. Hariparsad *et al.* [168] extensively characterized basal mRNA expression profiles with respect to 64 drug disposition genes that included drug-metabolizing enzymes, nuclear receptors, and transporters in Fa2N-4 cells, compared with four batches of human hepatocytes. Fa2N-4 cells showed a higher expression in almost all transcription factors and coactivators/corepressors associated with PXR-mediated enzyme induction. CYP3A4 expression level in hepatocytes was variable between the lots and ranged from 0.3- to 18-fold of Fa2N-4 cells. It was pointed out that midazolam 1'-hydroxylation activity in Fa2N-4 cells was ~100-fold lower than that in hepatocytes [167]. Compared with hepatocytes, the expression level of PXR, AhR, and GR was higher in Fa2N-4 cells, but significantly, the expression level of CAR was two orders of magnitude lower in Fa2N-4 cells [168]. Fa2N-4 cells also tended to show a significantly lower basal expression of several major hepatic uptake transporters, including sodium/bile acid cotransporter 10A1 (NTCP), solute carrier organic cation transporter 22A1 (OCT1), OATP1B1, and OATP1B3. In particular, the expression level of OATP1B1/1B3 in Fa2N-4 cells was 50-fold lower than that in hepatocytes, indicating that there is high possibility that substrates of those transporters apparently show low induction potency in Fa2N-4 cells because of low intracellular concentrations. This has been evidenced by the relatively higher EC_{50} values of rifampicin, which is substrate to OATP1B1/3, in Fa2N-4 cells (1.9–8 μ M) than hepatocytes (0.1–2.6 μ M), as shown in Table 12.5.

Rifampicin was regarded as an outlier in the obtained good relationship when EC_{50} values for various CYP3A inducers were plotted between Fa2N-4 cells and hepatocytes [162]. Noticeably, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime, a CYP2B6 inducer, produced no induction in Fa2N-4 cells, which was in contrast to hepatocyte data with E_{max} of 6.8-fold. This difference was consistent to extremely low expression of CAR in Fa2N-4 cells.

The usefulness of Fa2N-4 cells to identify CYP3A4 inducer was evaluated using 24 compounds selected from induction profiles in human hepatocytes [166]. Fa2N-4 cells showed CYP3A4 induction exceeding twofold for all 18 positive controls and less than 1.5-fold for all 6 negative controls. In addition to CYP3A4, induction of CYP1A2 and CYP2C9 was evaluated in Fa2N-4 cells [193]. Treatment of β -naphthoflavone, rifampicin, or phenobarbital yielded similar fold induction to CYP1A2, CYP2C9, and CYP3A4 mRNA levels, relative to human hepatocytes. Furthermore, a cassette dosing approach with tacrine for CYP1A2, diclofenac for CYP2C9, *S*-mephenytoin for CYP2C19, and midazolam for CYP3A4 was employed to assess induction based on changes in CYP activities in Fa2N-4 cells [194]. A comparable dose–response curve was obtained in both the cassette dosing and single dosing approaches. Similarly, successful measurement of induction activities in the cassette dosing approach was conducted by Kenny *et al.* [167].

12.5.2 Prediction of *In Vivo* CYP Induction

The first investigation of quantitatively predicting effects of CYP1A and CYP3A induction on *in vivo* exposure of substrates to those CYP enzymes was conducted by Kato *et al.* [195], who used *in vitro* and *in vivo* data of various inducers such as rifampicin, carbamazepine, phenytoin, dexamethasone, phenobarbital, and omeprazole. *In vitro* EC_{50} and E_{max} values were calculated from an E_{max} model using an averaged unbound concentration of inducers in the incubation medium with hepatocytes. The magnitude of AUC increase in comedicated CYP1A2 or CYP3A substrates was predicted based on the difference in CL_{int} values before and after treatment of inducers using the E_{max} model in which unbound steady-state plasma concentration of inducers after repeated administration was incorporated. With respect to CYP3A, predicted and observed induction ratios were correlated, whereas the prediction of CYP1A induction by omeprazole was overestimated. This discrepancy in CYP1A induction would be partly because of the underestimation of omeprazole exposure in the incubation medium with hepatocytes.

Ohno *et al.* [196] constructed a robust and accurate prediction method based on the equation of $1/(1 + CR_{CYP3A4} \times IC_{CYP3A4})$, in which CR_{CYP3A4} is the ratio of the apparent contribution of CYP3A4 to oral CL of a substrate (i.e., f_m) and IC_{CYP3A4} represents the apparent increase in CL of substrate by coadministration of prototypical inducers to CYP3A4. IC_{CYP3A4} was calculated for seven inducers based on a reduction in AUC of coadministered simvastatin, a standard substrate of CYP3A4. Rifampicin was found to be the most potent inducer among the tested compounds, with an IC_{CYP3A4} of 7.7, followed by phenytoin (4.7) and carbamazepine (3.0).

Relative induction ratio (RIS) was proposed as an indicator to predict percentage decrease in AUC of CYP3A4 substrate by comedication of CYP3A4 inducers [166]. RIS can be estimated from *in vitro* induction data (E_{max} and EC_{50} values) in Fa2N-4 cells and human hepatocytes with efficacious free plasma concentration using the

following equation:

$$\text{RIS} = \frac{E_{\max} \times C_{\text{eff,free}}}{\text{EC}_{50} + C_{\text{eff,free}}} \quad (12.7)$$

where $C_{\text{eff,free}}$ is the unbound plasma concentration of inducers after a standard therapeutic dose. In case of CYP3A4 inducers (rifampicin, phenobarbital, carbamazepine, troglitazone, etc.), the RIS score estimated from *in vitro* parameters in Fa2N-4 and human hepatocytes was in good agreement with the decrease in AUC of coadministered CYP3A4 substrates such as midazolam or ethinylestradiol.

The dose–response curve using widely ranged inducer concentrations can be described using *in vitro* parameters such as $E_{\max}$ and EC_{50} . In some cases, those parameters cannot be estimated as a result of cellular toxicity or a solubility problem in the high concentration of inducers. As an alternative indicator, Kanebratt and Andersson [191] have proposed to use F_2 parameter, which is an inducer concentration giving twofold induction of CYP3A4 mRNA from baseline. Relationship between the AUC/F_2 (ratio of *in vivo* AUC of inducer to F_2) and percentage decrease in *in vivo* AUC of CYP3A substrate was investigated. Use of either total (bound + unbound) or unbound AUC showed a high relationship with the *in vivo* data, accompanied with a correlation coefficient (R^2) of 0.859–0.863. Kaneko *et al.* [165] demonstrated a large lot-to-lot variation in the $E_{\max}$ and EC_{50} of CYP3A induction in HepaRG cells. The variation in induction response appeared smaller at the low exposure of inducers or positive control than at the high exposure in the dose–response curve. To minimize the variation, an index value termed the *relative factor* (RF) was introduced and is defined by the ratio of the minimum concentration to cause induction response of the inducers tested to the positive control such as rifampicin or phenobarbital. It was revealed that the ratio of the *in vivo* unbound plasma concentration of inducers toward the RF values was a good indicator to classify into three categories: low, medium, and high potency.

In contrast to qualitative assessment using constant inducer concentration or AUC, quantitative prediction of the extent of induction of CYP3A4 was performed based on time-dependent mass balance of CYP3A4 protein calculated by the $E_{\max}$ model incorporating the data in reporter gene assay and plasma concentration profile of inducers simulated by the one-compartment model [197]. The predicted induction ratio was related to urinary metabolite ratio of 6 β -hydroxycortisol/cortisol before and after treatment of CYP3A4 inducers.

Prediction approaches have been developed for each mechanism of DDI: reversible, irreversible inhibition, and induction. The method that focuses on a single interaction mechanism is likely to, in some cases, produce misleading predictions of compounds with multiple mechanisms such as ritonavir characterized as an irreversible inhibitor and inducer of CYP3A4. Fahmi *et al.* [198] has developed a more comprehensive mathematical model for simultaneously evaluating the effect of multiple mechanisms of CYP3A4-related DDI. Utilizing this model, DDI arising via inactivation (e.g., erythromycin), competitive inhibition (e.g., ketoconazole), or mixed mechanism (e.g., ritonavir) was well predicted within the ranges reported in clinical studies. The geometric mean fold errors were somewhat decreased by the combined DDI model. The integrated prediction model produced a success rate of 88%, which was comparable to that predicted by DDI functions in the computational PBPK model such as SIMCYP® [199].

12.6 FUTURE PERSPECTIVE

A great need for efficient identification of NMEs with desirable pharmacokinetic profiles has enhanced the efficiency of lead optimization processes based on metabolic stability, CYP inhibition, and induction, coupled with a paradigm shift from throughput to quality. By this trend, the development of high quality screening systems that can be more relevant to *in vivo* humans is needed. In the fine tuning of chemical structures that have an important position in the optimization process to create NMEs, the utilization of human hepatocytes has enhanced the quality of the data in the study of metabolic stability, CYP inhibition, and induction, but the demand for high quality hepatocytes applicable to those studies heavily outweighs the availability. Si-Tayeb *et al.* [200] imply the possibility that human induced pluripotent stem (iPS) cells will be used as a source of hepatocytes for toxicological and drug metabolism studies. The use of iPS cells has required the establishment of culturing conditions using microengineering approaches that support expression of a full panel of phase I and II enzymes and transporters. More recently, an advanced technology with transient overexpression of hemeobox gene using adenovirus vector allowed for differentiation of human iPS cells to hepatocytes, which successfully displayed CYP3A4 induction activity by rifampicin. Efficient generation of the hepatocytes from iPS cells would show a high possibility of giving an evolutionary change in the CYP induction study [201].

Furthermore, chromosome engineering techniques, which are based on transfer of a large size of chromosome with a defined region, have enabled to produce humanized model animals. The human chromosome 7 region around CYP3A locus was cloned into the SC20 vector. It is interesting to note that transcript of CYP3A gene was also detected specifically in the liver and small intestine of SC20-transferred chimeric mice, which is coincident with the tissue specificity of CYP3A expression in humans [202,203]. There has been possibility that HAC-mediated gene expressions of human drug metabolism enzymes in transgenic animals produce animal models to study drug metabolism and predict DDI-associated events in humans. Those *in vitro* and *in vivo* assessment tools will be a breakthrough in pharmacokinetic studies in drug discovery and development.

It has been revealed that multiple mechanisms such as transporter-mediated liver uptake and efflux and drug metabolism influence CYP inhibition and induction. Accordingly, using those human-relevant *in vitro* data, the selection of NMEs with high developability among the candidates will be routinely conducted based on the predicted pharmacokinetic profiles and the evaluated possibility of DDI by comedication in humans through utilizing a whole-body PBPK model incorporating those multiple mechanisms.

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14 Monoclonal Antibody Analyses of Microsomal Human Drug Metabolism and Multifunctional Cytochrome P450

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14.1	Summary	1
14.2	Introduction	2
14.3	Monoclonal antibody (MAb)	2
14.4	Immunoblotting MAbs	5
14.5	MAb Properties	6
14.6	Single and combinatorial analyses	8
14.7	Drugs metabolized by a single P450	9
14.8	Additional MAb functions	13
14.9	Other methods of microsomal analysis	14
14.10	Multifunctional cytochrome P450	16
14.11	Synopsis and critique (drug metabolism)	17
14.12	Conclusion	18
	References	18

14.1 SUMMARY

Monoclonal antibody (MAb)-based methods are ideal reagents for cytochrome P450 (P450) research. The properties and molecular diversity of P450s make them extraordinarily suitable for investigation with MAbs. The P450s are present in multiple forms. Defining the role of each P450 isoform for drug metabolism in human liver microsomes (HLM) *in vitro* requires a method that is both highly specific and highly inhibitory to P450 activity and thus can identify and quantitate the contribution of single or several P450 isoforms that metabolize the drug. MAbs have been isolated that are inhibitory and specific to 13 human P450s. The MAbs may also be used to determine the role of each P450 isoform for a variety of nondrug metabolizing activities such as mutagenesis carcinogen S9 activation and binding to DNA and others described in the text.

*deceased.

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14.2 INTRODUCTION

Our laboratory has produced highly specific and inhibitory MAbs to human P450 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2C Family (8, 9, 18, 19), 2D6, 3A4/5, 2E1, and 2J2. The MAb system identifies drugs metabolized by a single P450, multiple P450s, and alleles of polymorphic P450s. The MAbs may be used to measure the expression of specific alleles of polymorphic P450s as well as the formation of specific optically active metabolites of racemic mixtures. The *in vitro* assay system uses HLM from different individuals for the metabolism of the drug. The MAbs are added either singly or several combinatorially to determine potential interactions in the metabolism of a drug by two or more P450s. MAbs display extraordinary specificity, far greater than that of polyclonal antibodies or chemical inhibitors, which ordinarily are considerably cross reactive. We have used MAb-based analysis to examine the role of different P450 isoforms for diazepam metabolism, taxol metabolism, coumarin hydroxylation, and dextromethorphan metabolism by 2D6 and 2C9.

14.3 MONOCLONAL ANTIBODY (MAb)

14.3.1 Monoclonal Antibodies: Production

MAbs are produced by the “hybridoma” technology [1,2]. Their discovery is a major achievement of modern biology. The hybridoma technology grew out of the clonal selection hypothesis of Burnet and colleagues [2], which states that each B-lymphocytes antibody-forming cell and its progeny is committed to the production of a single type of antibody molecule. The MAbs are chemically defined reagents that recognize a single antigenic determinant or “epitope” on a macromolecule [2]. This specificity continues for the lifetime of the cell and its progeny.

For the production of MAbs [3], myeloma tumor cells are fused with the isolated dissociated B-lymphocytes of the spleen from mice immunized with a particular immunogen. The fused cells or “hybridomas” exhibit unique and special character by combining the characteristics of the B-lymphocytes that are committed to the continuous and stable production of a specific antibody with the myeloma tumor cells imparting immortal properties to the hybridoma cells. The individual hybridomas are cloned and screened for the desired MAb. The screening is done by a variety of methods including binding assays, Western blots, and enzyme inhibition. The selected clone and its progeny produce identical antibodies directed to a single epitope. The selected clones are usually subcloned three times to assure monoclonality. These hybridomas, grown in cultures in a defined medium, produce the chemically defined MAbs free of contaminating cells and protein debris. Large amounts of MAbs can be obtained by injecting the hybridomas into the peritoneal cavity of appropriate recipient mice, where they grow as ascites tumors and produce large quantities of the specific MAb. The MAb-producing hybridomas are essentially immortal and can be frozen and stored or grown in culture indefinitely.

14.3.2 MAbAnalytic Systems: Liver Microsomes

Comprehensive reviews of the value and utility of MAbs for the study of drug metabolism have been published [4,5]. MAbs were first made to rabbit [6] and rat

TABLE 14.1 Monoclonal Antibodies (MAbs) to Human Liver Cytochrome P450s

cDNA Expressed Human P450	MAb	Ig Subtype	Enzyme Inhibition (%)	Substrate
1A1	1-7-1	Ig G ₁	>90	7-Ethoxycoumarin (7-EC), phenanthrene, B[a]P
1A2	1-599-16 26-7-5	Ig G ₁ Ig	None >90	— 7-EC, phenanthrene, B[a]P, phenacetin, <i>p</i> -nitroanisole
2A6	151-45-4	Ig G ₁	>90	Coumarin, phenanthrene, 4-nitrophenol, 7-EC
2B6	49-10-20	Ig G _{2b}	>90	Diazepam, testosterone, 7-EC, phenanthrene
2 C8, 9, 18, 19	1-68-11	Ig M	>90	See 2 C substrates below
2 C8	2B1-1-1	Ig G ₁	>90	Diazepam, phenanthrene, taxol
2 C9*1, *2, *3	763-15-5	Ig G ₁	>80	Tolbutamide, diclofenac, warfarin, mephenytoin, phenanthrene, diazepam
2 C9*2	763-15-20 292-2-3	Ig G ₁ Ig G ₁	None >90	— Diclofenac, tolbutamide
2 C19	1-7-4-8	Ig G ₁	>90	Mephenytoin, tolbutamide
2D6	512-1-8	Ig G ₁	>90	Bufuralol, dextromethorphan, debrisoquine
2D6	50-1-3 2D6-50	Ig G ₁ Ig G _{2b}	>90 >90	— Dextromethorphan, testosterone, alfatoxin, bufuralol
2E1	2D6-50 1-73-18	Ig G _{2b} Ig M	>90 >90	— Phenanthrene, toluene, <i>p</i> -nitroanisole, chlorzoxazone
3A4/5	2-106-12 3-29-9	Ig G ₁ Ig M	— >90	— Diazepam, testosterone, taxol, phenanthrene, alfatoxin B1 (cyclosporin 70–80%)
2 J2	275-1-2 6-5-20-8	Ig M Ig G ₁	None >90	— Arachidonic acid, terfenadine, ebastine, phenanthrene

P450s [7]. Table 14.1 shows the MAbs that are P450 isoform specific and are >90% inhibitory to the expressed target P450 activity. Some, but not all the MAbs also immunoblot the target P450 (Table 14.2). The amount of inhibition of drug metabolism *in vitro* by HLM with the addition of the specific MAb quantitates the contribution of the target P450 to the metabolism of the drug. The MAb method is in stark contrast to other complex analytic systems that are relatively nonspecific and currently being

TABLE 14.2 Immunoblotting MAbs

cDNA Expressed Human P450	MAb	Ig Subtype
1A1	1-599-16	Ig G ₁
1A2	26-7-5 ^a	Ig G ₁
2A6	151-45-4 ^a	Ig G ₁
2B6	49-10-20 ^a	Ig G _{2b}
2C8	281-1-1 ^a	Ig G ₁
2C9*1, *2, *3	763-15-20 ^b	Ig G ₁
2D6	512-1-8 ^a	Ig G ₁
2E1	2-106-12	Ig G ₁
3A4/5	275-1-2	Ig M

^aAlso inhibitory.^bAlso immunoblots 2C8.

used to study drug metabolism. Drugs metabolized by a single P450 isoform or metabolites formed from a single metabolic pathway may be useful as markers for identifying the target P450 *in vivo*. The MAb methodology is precise, simple, and highly reproducible. The MAbs, which are highly specific and highly inhibitory, may also be used to measure the specificity and the potency of chemical inhibitors.

14.3.3 Monoclonal Antibody and Human Liver Microsomes Analytic System

Figure 14.1 shows production and analytic system of MAbs. Specific inhibitory MAbs identify individual P450s responsible for drug metabolism in HLM *in vitro*. The MAb system analyzing HLM identifies the P450 isoforms that catalyze the metabolism of the drug substrate and quantitates the maximum contribution of each P450 for the metabolism of the drug. The MAb system uses saturating levels of both the MAb and the drug substrate using zero-order kinetics for the reaction, and thereby defining the maximum contribution of each P450 isoform to the metabolism of the drug. The system also identifies drugs that are metabolized by a single P450, two or more P450s, and polymorphic P450s. The drugs metabolized by a single P450 have potential for use as markers identifying the presence and the activity of the target P450 *in vivo*. The MAbs also identify the P450s catalyzing metabolism in alternative pathways, yielding metabolites that may be used as *in vivo* markers for specific P450s. The system can study polymorphic P450s, examine the metabolic consequences of an absent or deficient polymorphic P450 in individual microsomes, and identify other active nonpolymorphic P450s that can reduce the deleterious effect of the missing or deficient isoforms. The methodology of the MAb system is simple. Basal control levels of metabolism of the drug are measured, and the amount of inhibition of metabolism on the addition of the isoform-specific inhibitory MAb determines the quantitative role of each P450 for the metabolism of the drug. The MAb system of analysis of HLM metabolism is entirely self-contained, and the values obtained are the results of diverse elements such as interindividual differences in the amount and activity of the target P450 as well as factors such as lipid and cofactor content, P450 protein structural configuration, membrane–enzyme interactions, and other currently unknown factors. Table 14.1 is a list of the human cytochrome P450 enzymes for which MAbs have been made in the Laboratory of Metabolism, National Cancer Institute. These are MAb 1-7-1,

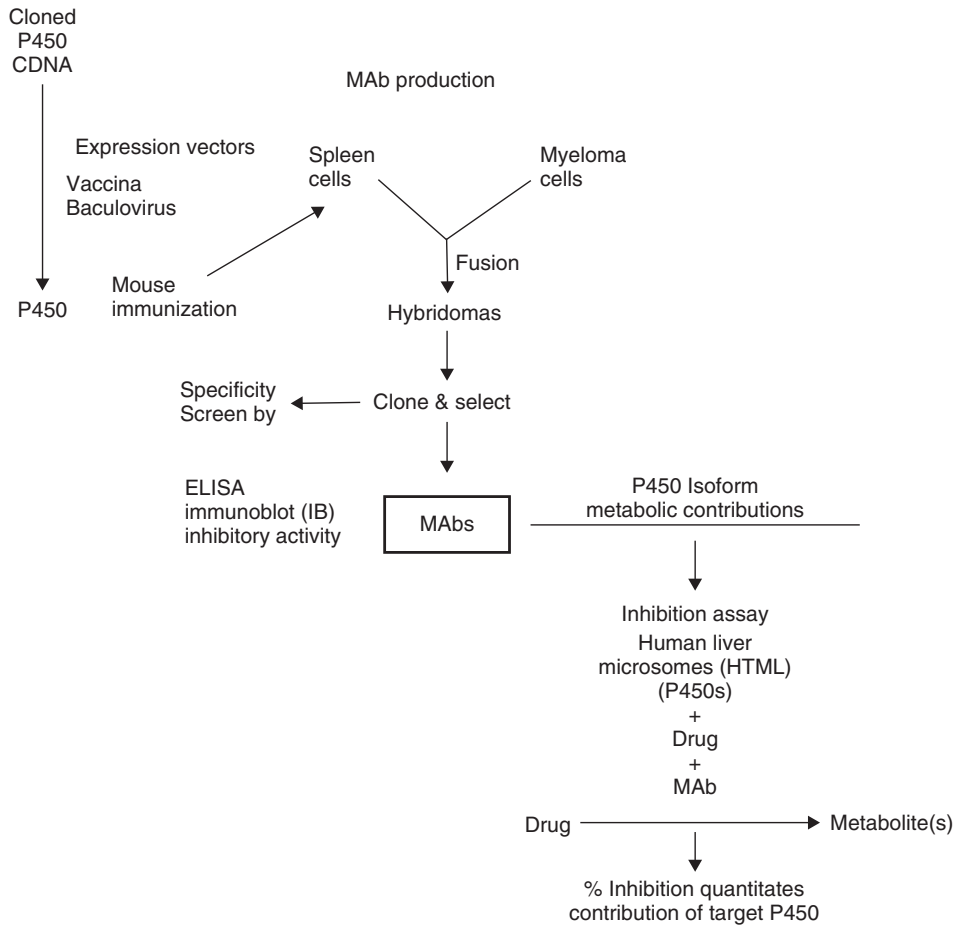


Figure 14.1 Monoclonal antibodies to human cytochrome P450s (preparation and assay).

which inhibits both 1A1 and 1A2 in rat livers but is specific only to 1A1 in human tissues [8]. The MAb 1-7-1 has also been used to phenotype 1A1 in different human tissues [9] and phenotype differences in single and twin human placenta [10]. Anti-P450 MABs have been made to 1A2 [11], 2A6 [12], 2B6 [13], 2C8, 2C9, 2C19, 2C [14,15], 2D6 [16,17], 2E1 [18], 2J2 [19], and 3A4/5 [20]. One of the MABs, 1-68-11, binds and inhibits each of the 2C8, 2C9, and 2C19 isoforms of the P450 2C family, indicating that they share a common epitope. Table 14.1 also shows the Ig type of each of the MABs and their inhibitory activity toward the expressed P450 isoform, which was >80–90%. The last column of Table 14.1 lists the substrates that were used to measure the inhibitory activity of each MAB to the expressed P450.

14.4 IMMUNOBLOTTING MABs

Table 14.2 lists nine MABs that immunoblot the target P450. There are large interindividual variations in the protein content as well as enzyme-inducible activity of the

different P450s. The immunoblotting MAbs can be used to determine the protein amount of a specific P450 in human tissues [13] and measure interindividual differences of different populations. The immunoblotting MAbs have also been used in immunohistochemical studies. A noninhibitory MAb, anti-P450 1B1, has been used for the immunohistochemical localization of 1B1 in breast cancer [21]. The MAb anti-rat P450 2E1 has been used for immunohistochemical studies of murine lung [22] and for studies on the effects of acetone and ethanol in murine liver [23]. Among other functions, P450 2J2 metabolizes arachidonic acid and is involved in calcium regulation in cardiomyocytes [19].

14.5 MAb PROPERTIES

14.5.1 Monoclonal Antibodies and Epitope Specificity

The specificity of the MAb resides in its precise ability to recognize and bind a specific epitope on the surface of a P450. Thus, an MAb may recognize only a single species of P450 if it contains a unique epitope. In this case, the MAb would be specific for only a single isozymic form of P450. AnMAb that recognizes an epitope that is present in more than one form of P450 will bind all of those forms of cytochrome P450 containing the common epitope. On theoretical grounds, a comprehensive MAb library will contain MAbs that recognize single unique forms of P450.

14.5.2 Monoclonal Antibody Allele Specificity

Table 14.3 demonstrates the allele specificity potential of a MAb. MAb 292-2-3 specifically binds to a single allele, P450 2C9*2(Arg₁₄₄Cys), of three expressed P450 2C9 allozymes (*1,*2,*3). This MAb does not bind the 2C9*1(Arg₁₄₄) wild type or the third allele P450 2C9*3(Ileu₃₅₉Leu) [24]. Of the three alleles of P450 2C9, the wild type and two polymorphic alleles have only a single amino acid substitution relative to the wild type and differ from the wild type by a single amino acid, and thus MAb

TABLE 14.3 MAb Allele Specificity

Allele		Tolbutamide	Diclofenac
2 C9*1 Arg ₁₄₄	Control	1.0 ^a	0.30
	+MAb	0.98	0.30
	% Inhibition	1.3	0.1
2 C9*2 Arg ₁₄₄ Cys	Control	0.85	0.42
	+MAb	0.03	0.02
	% Inhibition	96.4	95.1
2 C9*3 Ileu ₃₅₉ Leu	Control	0.22	0.35
	+MAb	0.20	0.30
	% Inhibition	9.3	14.2

MAb 292-2-3 inhibits only the single allele 2C9*2 arg₁₄₄cys catalyzed tolbutamide and diclofenac metabolism.

^aActivity (mmol product/min/nmole P450).

292-2-3 detects a single amino acid substitution. MAb 292-2-3 inhibits the P450 2C9*2 allozyme-catalyzed metabolism by 96% and shows no inhibition of the wild type P450 2C9*1 metabolism. The P450 2C9*2 allozyme-specific MAb does not bind to any of the other P450 2C9 isoforms, 2C8, 2C18, or 2C19, or any of the other human P450s, 1A1, 1A2, 2A6, 2B6, 2D6, 2E1, or 3A4/5. The MAb 292-2-3 inhibits the metabolism of tolbutamide, diclofenac, and phenanthrene by the P450 2C9*2 by >90% and does not inhibit the enzyme activity by the other two alleles or any of the other human P450 isoforms listed above. The MAb 292-2-3 can be viewed as a prototype of an ideal and extraordinary specific reagent for the detection and measurement of the metabolic role of highly related isoforms and polymorphic alleles of human cytochrome P450 [24].

14.5.3 Monoclonal Antibody Enantiomer Specificity

Phenprocoumarin is a potent anticoagulant in common usage and is hydroxylated by the P450s of the 2C family. One route of metabolism converts the drug to both the *S*-4-OH and to its enantiomeric *R*-4-OH (Table 14.4). The MAb specific to P450 2C8 inhibits the formation of the *R*-enantiomer and does not inhibit the formation of the *S*-enantiomer. The MAb to P450 2C9 also shows enantiomer specificity, inhibiting the *R*-4-OH formation and not inhibiting the *S*-4-OH formation [25]. The MAb anti-P450 2C9 also exhibited enantiomer selectivity. P450 2C9 catalyzes the demethylation of the endocrine disrupter pesticide methoxychlor, forming a chiral product [26]. The HLM generates primarily the *S*-mono OH form. The MAb anti-2C9 selectively reduces the formation of the *S*-form from 80% to 55%, thereby enantiomer selectively changing the ratio of the *S*- and *R*-enantiomers formed. This study also found that expressed P450 2C19 exhibited the highest level of activity for demethylation but was not inhibited by MAb anti-2C19 in HLM, thus indicating that P450 2C19 is not active in HLM. These unusual results suggest that with certain substrates (e.g., methoxychlor), there is no correlation between the expressed enzyme activity (in this case, P450 2C19) and its presence in HLM. MAbs have also been used to study the mechanism of toxicity of the chemotherapeutic agent ifosfamide, which is believed to be due to its conversion to nephrotoxic chloroacetaldehyde [27]. The study showed that metabolism of ifosfamide was catalyzed by P450 3A4 and 2B6, both of which are present in fetal, pediatric, and adult tissues. The P450 3A4 and 2B6 were found in the fetus as early as eight weeks of gestation. Thus, the MAb system can easily map the tissue distribution of P450 isoforms in fetal as well as adult tissue and help to clarify the mechanism of toxicity of chemical agents.

TABLE 14.4 MAb Enantiomer Specific Hydroxylation of Phenprocoumarin

Inhibitor	Product Formation/% Inhibition			
	[<i>S</i>] 4'OH	[<i>R</i>] 4'OH	[<i>S</i>] 6'OH	[<i>R</i>] 6'OH
MAb anti-2C8	32	<5	15	<5
MAb anti-2C9	31	<5	52	40
Triacetyloleandomycin	52	80	40	51

TABLE 14.5 Inhibition of 7-ECO Metabolism in HLM by the Addition of a Single Purified MAb Compared to its Inhibitory Effects when in Combination with Other Mabs^a

HLM (Activity ^b)	Single MAb		Combinatorial Addition of MABs	
	MAbs to P450	% Inhibition	MAbs to P450	% Inhibition
HL 80	Control	0	Control	0
(0.52)	2E1	26.1	2E1	33.5
	2A6	28.9	2E1 + 2A6	30.1
	2B6	31.5	2E1 + 2A6 + 2B6	23.9
	1A2	9.5	2E1 + 2A6 + 2B6 + 1A2	4.4

Abbreviations: HLM, human liver microsomes; MAB, monoclonal antibody.

^aPurified MABs from ascites.

^bActivity is in nanomole 7-OH coumarin formed/min/nmol total P450.

14.6 SINGLE AND COMBINATORIAL ANALYSES

Table 14.5 shows the MAb-based analysis of 7-ethoxycoumarin deethylase activity using purified MABs added singly to the reaction mixture compared to analysis with a combinatorial system. The values obtained using the single MAB addition for measuring the contribution of P450 2E1, 2A6, 2B6, and 1A2 corresponded well with values obtained for the same four P450s using the combinatorial analysis with MABs against each of the four P450s. The combinatorial system permits the concurrent analysis of the contribution of up to four different P450s. Table 14.6 is a summary of the interindividual differences in the activity of four different HLM comparing the single and combinatorial systems. There was wide variability in the basal activity of the different HLM samples. The largest approximate amount of activity was due to P450 2E1 and 2B6. P450 2B6 activity ranged from 11% to 50%, P450 2E1 from 16% to 57%, P450 1A2 from 7% to 22%, and P450 2A6 from 8% to 32%. Table 14.6 uses ascites fluid as the MAB source, which, in comparison, is similar to the data in Table 14.5 using the purified MABs. These values could not be determined by simply using the expressed P450 system for analyses. The enzyme activities exhibited by expressed P450 isoforms do not accurately reflect their activities in the HLM and obviously cannot be used to examine interindividual differences. However, the expression system is valuable for identifying P450s that exhibit enzymatic activity toward a given drug substrate. The presence of high activity of an expressed isoform may be a presumption but not a certainty indicating the presence of the isoform activity in HLM. Figure 14.2 shows a combinatorial analysis of diazepam hydroxylation to temazepam (TMZ). For this figure, the height of each column is compared to the height of the previous column. No difference in the height of the adjacent column indicated that the P450 isoform of the second column does not contribute to the reaction. The MABs anti-2B6 and anti-3A4 were combined for convenience with the previous knowledge that P450 2B6 is inactive for TMZ formation. P450 3A4 is the only isoform that exhibits metabolic activity for TMZ formation. The amount of activity exhibited by 3A4 ranges from 90% in HLM 80 to 70% in HLM 79. P450s 2C8, 2C9, and 2C19 exhibit no activity. In contrast, Fig. 14.3 shows the variable contribution in eight HLM of four P450 isoforms for the N-demethylation of diazepam to nordiazepam (NDZ). The large interindividual variation is reflected by the contribution of P450 3A4/2B6 activity, which ranges from

TABLE 14.6 MAb^a Single and Combinatorial Analysis of Ethoxycoumarin Deethylation (ECOD)

HLM	Single MAb		Combinatorial Addition of MABs			
	MAb to P450	Inhibition (%)	MAb to P450	Total Inhibition	% Contribution P450	
HLM75	Control		Control		Control	
	2E1	26	2E1	26	2E1	26
	2A6	28	2E1 + 2A6	58	2A6	32
	2B6	21	2E1 + 2A6 + 2B6	80	2B6	22
	1A2	22	2E1 + 2A6 + 2B6 + 1A2	98	1A2	18
HLM 76	Control		Control		Control	
	2E1	16	2E1	16	2E1	16
	2A6	22	2E1 + 2A6	42	2A6	26
	2B6	50	2E1 + 2A6 + 2B6	85	2B6	43
	1A2	14	2E1 + 2A6 + 2B6 + 1A2	96	1A2	11
HLM 77	Control		Control			
	2E1	57	2E1	57	2E1	57
	2A6	7	2E1 + 2A6	68	2A6	11
	2B6	20	2E1 + 2A6 + 2B6	79	2B6	11
	1A2	10	2E1 + 2A6 2B6 1A2	89	1A2	8
HLM 80	Control		Control		Control	
	2E1	22	2E1	22	2E1	22
	2A6	15	2E1 2A6	30	2A6	8
	2B6	43	2E1 2A6 2B6	78	2B6	48
	1A2	10	2E1 2A6 2B6 1A2	85	1A2	7

Abbreviations: 7-ECOD, 7-ethoxycoumarin deethylation; HLM, human liver microsome; MAb, monoclonal antibody.

Control Activity: HLM 75 (1.6), HLM 76 (4.6), HLM 77 (3.1), HLM 80 (1.4).

^aMAb in ascites fluid.

20% to 80%, and the large variable contribution from P450 2C9 ranges from 10% to 46% and 3–30% from P450 2C8. There is little or no contribution from P450 2C19. It is important that a significant number of samples of HLM be examined in these studies. These data are also summarized in Table 14.7.

14.7 DRUGS METABOLIZED BY A SINGLE P450

Taxol 3'-hydroxylation is catalyzed solely by P450 3A4/5, and there is relatively constant inhibition of 6-hydroxy formation by MAb anti-2C8 (Table 14.7), which indicated that there is relatively little interindividual variation in the nine HLM samples for taxol 6-hydroxylation catalyzed by P450 2C8. Table 14.7 shows that coumarin hydroxylation is catalyzed entirely by P450 2A6 and thus would be an excellent drug substrate marker for the *in vivo* presence of P450 2A6. The MAB system also determines the role of each P450 isoform for the metabolism of a drug through alternate metabolic pathways, such as is the case with diazepam and taxol. When a metabolite of a drug is the result of the activity or affinity of a single P450,

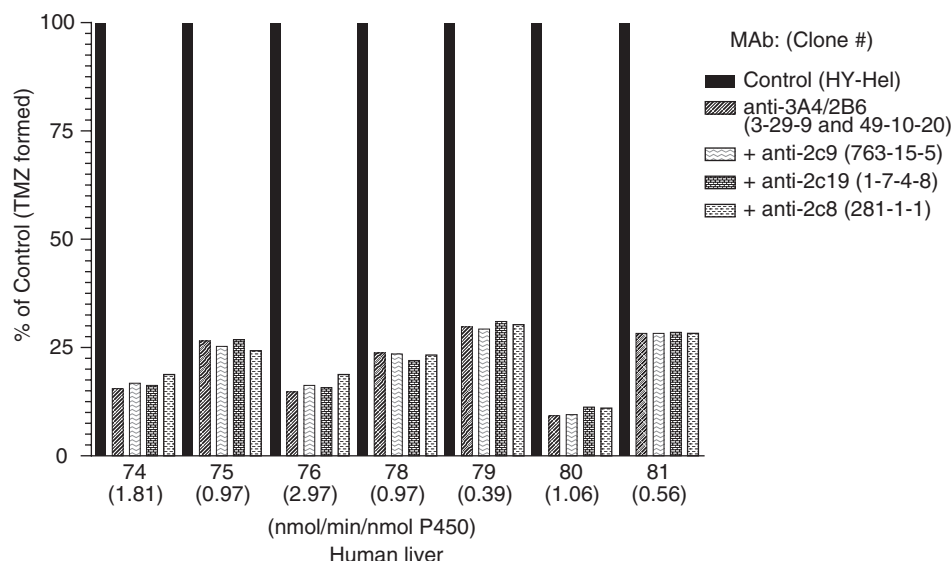


Figure 14.2 MAb combinatorial analysis of diazepam hydroxylation in human liver microsomes. The difference between each bar represents the amount of P450 activity contributed by the subsequent addition of the indicated MAb. Results are the average of duplicate incubations. Values in parenthesis are control activity.

the metabolite identifies the P450 isoform as a potential marker for the *in vivo* presence of the P450 isoform. Metabolite analysis for taxol 6-hydroxy and taxol 3-hydroxy formation catalyzed solely by P450 2C8 and 3A4 would be excellent markers for the *in vivo* presence of P450 2C8 and 3A4, respectively. Likewise, coumarin hydroxylation that is metabolized solely by 2A6 would be an excellent marker for 2A6 activity *in vivo*. There are two extensive reviews [28,29] on the use of probe drug substrates for the *in vivo* targeting of a P450 isoform for its role in the metabolism of a drug. A primary concern for this method is the specificity of the probe drug and the potential contribution of low activity isoforms to the reaction *in vivo*. In many cases, the specificity of the probe, substrate has not been determined for either the metabolism of the drug or the formation of marker metabolites arising from alternate markers must be responsible for essentially the entire metabolism of the drug, and minor P450s should not contribute to the reaction. Minor isoforms exhibiting activity for drug metabolism may cause a misreading of the identification of the *in vivo* target P450. Combinatorial analysis of diclofenac 4-OH formation indicates that the P450 2C9 is the primary and major catalyst for diclofenac hydroxylation. Minimal or very slight activity is shown by other members of the P450 2C family. Table 14.8 indicated that in 10 HLM, 83–88% of activity was due to P450 2C9, and 7–8% was contributed by P450 2C19. The activity of the expressed P450s for hydroxyl tolbutamide formation shows that two alleles of P450 2C9, 2C9*1, and 2C9*2, show high activity, with P450 2C9*2 exhibiting half the activity of the wild type. P450 2C19 exhibits moderate activity, and little or no activity is shown by the other expressed P450s—2C8, 2C18, and 2D6. Table 14.8 shows that in eight HLM, P450 2C9 contributes 77–82% of tolbutamide 6-hydroxylation activity with variable low contributions of about 5–10% from 2C8 and 2C19. Table 14.8

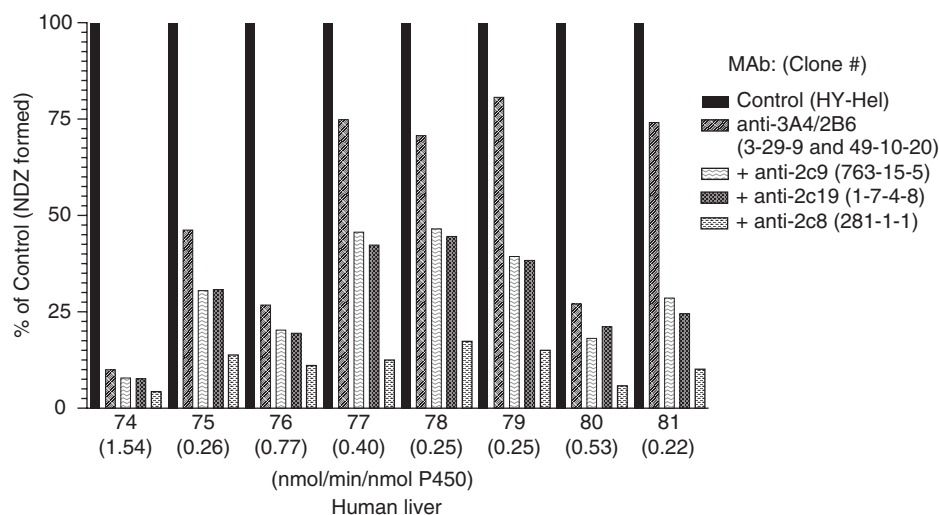


Figure 14.3 MAb combinatorial analysis of diazepam N-demethylation in human liver microsomes. The difference between each bar represents the amount of P450 activity contributed by the subsequent addition of the indicated MAb. Results are the average of duplicate incubations. Values in parenthesis are control activity.

TABLE 14.7 Monoclonal Antibody (MAb)-Determined Contribution of Individual P450 Isoforms to Metabolism in Human Liver Microsomes (HLM)

	% Contribution of P450 Isoforms (Amount of MAb Inhibition)				
	Diazepam ↓ Temazepam (OH)	Diazepam ↓ Nordiazepam (N-CH ₃)	Taxol ↓ 6-OH Taxol	Taxol ↓ 3-OH Taxol	Coumarin ↓ 7-OH Coumarin
MAb anti-1A2	0	0	0	0	0
2A6	0	0	0	0	>90 ₍₈₎
2B6	1–5 ^a ₍₆₎	6–23 ₍₆₎	0	0	0
2C8	0	3–30 ₍₈₎	83–91 ₍₈₎	0	0
2C9	0	2–46 ₍₈₎	0	0	0
2C19	0	0–4 ₍₈₎	0	0	0
2D6	0	0	0	0	0
2E1	0	0	0	0	0
3A4/5	75–84 ₍₆₎	14–45 ₍₆₎	0	73–90 ₍₆₎	0

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.

(), Number of HLM samples; 0, No activity of expressed enzyme.

^aValues <5% are not reliable.

also shows the MAb-based analysis of HLM for the P450-catalyzed metabolism of 7-ethoxycoumarin, phenacetin, diclofenac, *S*-mephenytoin, dextromethorphan, and bufaralol. *S*-mephenytoin metabolism was strongly and primarily catalyzed by the expressed P450 2C19 and only slightly by P450 2C9. Table 14.8 also shows that in seven HLM, P450 2C19 was the major catalyst. Significant but very low activity was

TABLE 14.8 Monoclonal Antibody (MAb)-Determined Contribution of Individual P450 Isoforms to Metabolism in Human Liver Microsomes (HLM)

	% Contribution of P450 Isoforms (Amount of MAb Inhibition)						
	7-Ethoxycoumarin	Phenacetin	Diclofenac	S ± Mephenytoin	Tolbutamide	Dextromethorphan	Bufuralol
	↓ 7-OH coumarin	↓ Acetaminophen	↓ 4-OH diclofenac	↓ 4-OH mephenytoin	↓ 6-OH tolbutamide	↓ Dextrophan	↓ 4-OH bufuralol
MAB anti-1A2	7–16 ₍₁₀₎	64–84 ₍₁₆₎	0	0	0	0	5–20 ₍₃₎
2A6	7–28 ₍₁₀₎	0–9 ₍₁₆₎	0	0	0	0	0
2B6	18–30 ₍₁₀₎	0	0	0	0	0	0
2C8	0	0	0 ₍₂₎	0	3–10 ₍₈₎	0	0
2C9	0	1–17 ₍₈₎	83–88 ₍₁₀₎	10–22 ₍₇₎	77–82 ₍₈₎	38–45 ₍₂₎	10–18 ₍₂₎
2C19	0	0–9 ₍₈₎	7–8 ₍₂₎	72–87 ₍₂₎	4–8 ₍₈₎	1–4 ^a ₍₂₎	8–27 ₍₂₎
2D6	0	0	0	0	0	35–91 ₍₁₇₎	9–70 ₍₂₁₎
2E1	16–60 ₍₁₀₎	0	0	0	0	0	0
3A4/5	0	0	0	0	0	0	0

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.

(), Number of HLM samples; 0, No activity of expressed enzyme.

^aValues >5% are not reliable.

contributed by 2C9. However, when the MAb anti-2C19 was added before the addition of the MAb anti-2C19, the 2C9 showed significant activity (20–25%). These results suggest that there may be competition for certain substrates by two or more P450 isoforms. Minor or moderate contributions of activity from P450 isoforms other than those responsible for the major amount of metabolism may seriously limit interpretation and cause a misreading of the role of different P450s *in vivo*. This is especially true when there is large interindividual variability in microsomal P450-catalyzed metabolism.

14.8 ADDITIONAL MAb FUNCTIONS

14.8.1 Polymorphisms

The first P450 polymorphism found to have clinical significance was the discovery of the 2D6 polymorphism. Dextromethorphan metabolism has been used as primary marker of P450 2D6 activity. Figure 14.4 is a combinatorial analysis of dextromethorphan metabolism in eight HLM. The MAb-based analysis indicated that in individuals polymorphically deficient in 2D6 activity, the P450 2C9 partially replaces the absent 2D6-catalyzed metabolism and catalyzes up to 50% of the total dextromethorphan metabolism. A parallel analysis of bufuralol metabolism with the same eight HLM in Fig. 14.4 showed that in the MAb anti-2D6-based analysis of the same eight HLM, in six of the eight HLM, 2D6 metabolized 80–90% of bufuralol metabolism. The two HLM deficient in 2D6 metabolism of dextromethorphan were also deficient in bufuralol hydroxylation. In these two HLM, 1A2 showed no activity, 2C9 contributed 15–25% activity, and 2C8 showed 10–40% activity. Specific and inhibitory MAbs have been used in a variety of studies. To cite several examples, MAbs have been used to identify the human P450s responsible for the *in vitro* formation of *R*- and *S*-norfluoxetine [30]. MAbs have also identified P450 2C8 and 3A4 as the principal enzymes involved in the metabolism of the insulin secretagogue repaglinide [31]. P450 2D6 and 3A4 were found responsible for hydrocodone oxidation [32]. A variety of environmental agents, including acylether, nicotine, *N*-nitrosoethylamine, and *N*-nitrosobenzyl-methylamine, are metabolized by P450 2A6 [33]. Propofol is metabolized by P450 2B6 [34]. P450s 3A4 and 2C9 are responsible for the metabolism of 17 α -ethinylestradiol [35]. It would be useful to have a comprehensive array of MAbs to all the enzymes of phase II drug metabolism. However, that would be difficult to attain because the conjugating enzymes UDPGT, GST transferase, and sulfotransferase all have numerous different isoforms, and phenotyping an individual with a large array of MAbs to each isoform would be very difficult.

14.8.2 Endobiotic Metabolism

Table 14.9 shows the metabolism of endobiotics and nondrug xenobiotics responsible for chemical toxigenesis. Table 14.9 shows that in 18 HLM samples, P450 3A4/5 was responsible for 72–86% of testosterone 6- β -hydroxy formation. Table 14.9 also shows an analysis of the metabolism of three xenobiotics. In 18 HLM samples, 32–60% of chlorzoxazone metabolism was catalyzed by P450 2E1 and 16–46% of nitroanisole metabolism was metabolized by P450 2E1. In 18 HLM samples, 74–86% of the

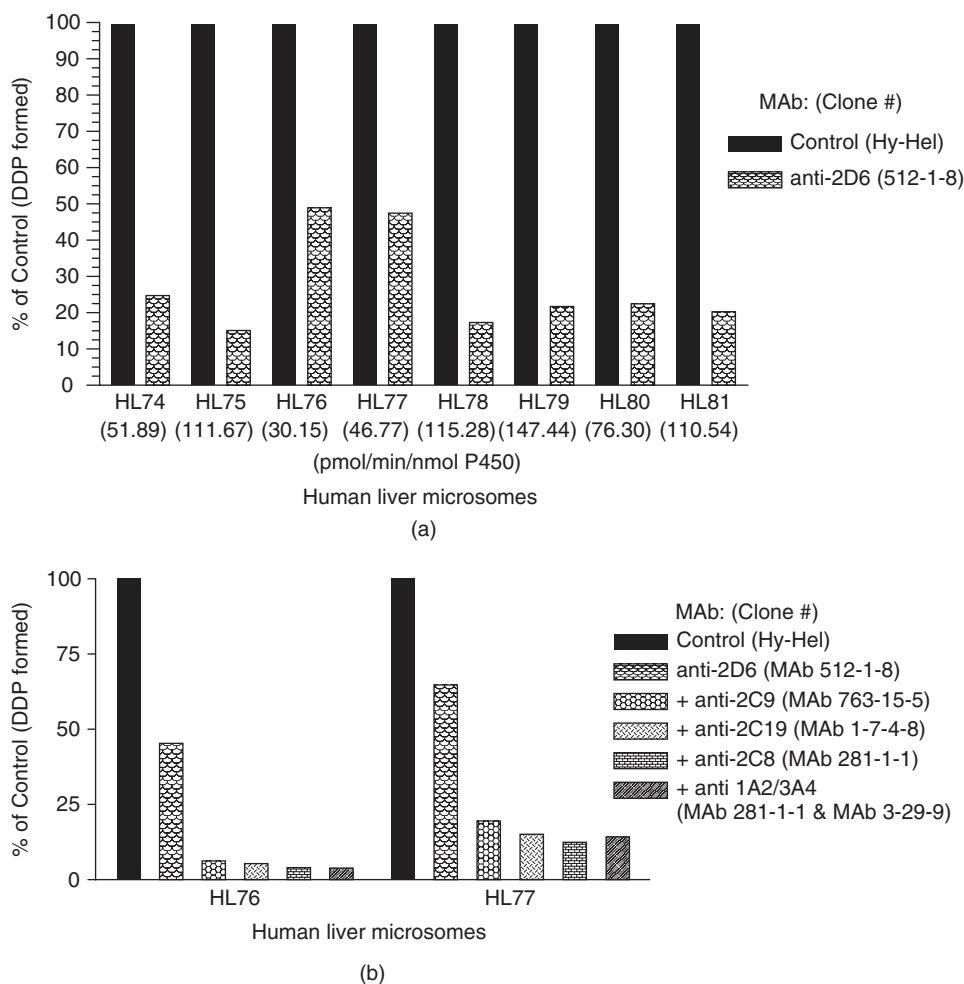


Figure 14.4 MAb analysis of dextromephorphane metabolism in human liver microsomes. Each bar represents the P450 activity relative to control after the subsequent addition of the indicated MAb. MAbs were added singly (a) or in combinatorial succession (b). Results are the average of duplicate incubations. Values in parenthesis are control activity.

metabolism of the potent carcinogen aflatoxin was metabolized by P450 3A4. The analysis of the metabolism of chlorzoxazone and nitroanisole was incomplete because the role of other P450s was not defined.

14.9 OTHER METHODS OF MICROSOMAL ANALYSIS

14.9.1 Specificity and Potency of Chemical Inhibitors

Chemical inhibitors have been used extensively to identify the role of different P450 isoforms in the metabolism of a drug. The chemical inhibitors are generally selective and do not display the specificity of the MAbs that is required to target and

TABLE 14.9 Monoclonal Antibody (MAb)-Determined Contribution of Individual P450 Isoforms to Metabolism in Human Liver Microsomes (HLM)

	% Contribution of P450 Isoforms (Amount of MAb Inhibition)			
	Testosterone	Chlorzoxazone	4-nitroanisole	Aflatoxin-B1
	↓ 6β-OH Testosterone	↓ 6-OH Chlorzoxazone	↓ 4-nitrophenol	↓ 3-hydroxylation
MAb anti-				
1A2	0	?	?	—
2A6	0	0	22–65 ₍₇₎	—
2B6	0	0	?	—
2C8	0	—	0	—
2C9	0	?	0	—
2C19	0	—	?	—
2D6	0	?	?	—
2E1	0	32–60 ₍₁₈₎	16–46 ₍₈₎	—
3A4/5	72–86 ₍₁₈₎	?	0	74–86 ₍₁₈₎

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.

(), of HLM samples; 0, activity of expressed enzyme; —, Not determined; ?, with expressed P450; not yet assayed with Mab/HLM.

quantitate the role of a P450 isoform responsible for a drug's metabolism. Chemical inhibitors have been used rather extravagantly, with little attention to their lack of isoform specificity. The MAb system can be used to assess both the potency and the specificity of a chemical inhibitor. The MAb-based analyses were used to evaluate the chemical inhibitors, ketoconazole, orphenadrine, and quinidine, which are commonly used as inhibitors of P450 3A4, 2B6, and 2D6, respectively. Ketoconazole at 100 μM inhibits the metabolism of phenanthrene by 80%, indicating the participation of P450s other than 3A4. The ketoconazole inhibitor does not identify all the other P450s responsible for phenanthrene metabolism. Quinidine is thought to be a P450 2D6-specific inhibitor, and debrisoquine metabolism is generally used as a P450 2D6-specific marker. However, a report found that debrisoquine is also metabolized by P450 1A1, and the metabolism is inhibited by quinidine [36]. Thus, neither ketoconazole nor quinidine is a specific inhibitor of P450 3A4 or 2D6, respectively. Orphenadrine is commonly used as a P450 2B6 inhibitor. The MAb system was used to measure the relative potency of orphenadrine compared to the specific inhibitory MAb anti-2B6. Phenanthrene metabolism was inhibited by 60% by the MAb anti-2B6, whereas 100 μM orphenadrine inhibited the reaction by only 25%, indicating its relatively poor inhibitory potency of orphenadrine compared to the inhibitory anti-2B6 MAb. Thus, the specific MAbs can be used to analyze both the specificity and potency of a chemical inhibitor. A different study examined the specificity of inhibition of expressed P450 with eight commonly used chemical inhibitors [37]. Ketoconazole and quinidine exhibited similar high inhibitory activity toward P450 3A4 and 1A1, sulphenazole showed selectivity for P450 2C9, and α-naphthoflavone (ANF) inhibited both P450 1A1 and 1A2. The remaining inhibitor selectivities were at best moderate. The major advantage of the MAbs compared to chemical inhibitors is the extraordinary specificity of the MAbs. Some of the chemical inhibitors, such as ketoconazole, can be highly selective

but not specific for a target P450. However, a major advantage of a highly selective chemical inhibitor, such as ketoconazole, is the ability to administer the inhibitor to the patient. That is not possible with the MABs because they cannot be administered *in vivo*. Thus, chemical inhibitors can be used to alter routes of metabolism and metabolic pathways that would be therapeutically advantageous.

14.9.2 Monoclonal and Polyclonal Antibodies

MABs derive their single-epitope specificity by being formed from a single MAB produced by a parent spleen cell. The MAB-producing spleen cells are immortalized by fusion with myeloma cells, producing hybridomas that maintain their single-epitope specificity. The hybridomas are stable and continuously produce identical MABs that can be used universally and are transferable to any laboratory as pure reagents. In contrast, polyclonal antibodies commonly display cross-reactivity because they are heterogeneous mixtures of monoclonals that, by definition, vary with each immunization, even when using pure immunogens. Undefined polyclonal antibodies resulting from independent immunizations require extensive purification and characterization to eliminate potential and undesirable cross-reactivity. The major advantage of polyclonal antibodies is the relative simplicity of their production compared to the isolation of highly specific and highly inhibitory MABs to target specific P450 isoforms. The hybridomas producing MABs are essentially immortal and produce identical MABs with an identical epitope-binding character. Experimental results with polyclonal antibodies from different laboratories are not comparable because the polyclonal antibodies are epitopically different and may have different cross-reactivity. Thus, the MABs are more reliable reagents than the polyclonal antibodies.

14.10 MULTIFUNCTIONAL CYTOCHROME P450

Cytochrome P450 is multifunctional and the MAB technology can be successfully applied to the study of a variety of diverse functions of P450 and within each system identify the P450 isoform responsible for the observed biological activity (Table 14.10). The MAB system can be used to map the protein amount and subcellular content and the activity of individual P450s in different human tissues. For each tissue or cell, the inhibitory MABs may define the amount of P450-specific enzyme activity present and the immunoblotting MABs measure the P450 isoform protein amount. The MAB system demystifies the "S9" used commonly for activation in the Ames mutagen detection system [38], an activation system previously described for the covalent binding of benzopyrene to DNA [39]. The activity of the "S9" is microsomal and a function of the heterogeneous mixture of P450 isoforms in the microsome-derived "S9" [39]. The MABs can identify those isoforms responsible for the "S9" activity. The MAB system identifies the individual P450s that catalyze the conversion of compounds to electrophiles that are mutagenic [40,41] or to active metabolites that covalently bind to DNA and protein. The MAB system identifies the P450 isoforms that convert environmental agents to mutagenic, carcinogenic, toxic, and immunotoxic metabolites. The MAB system may identify the function of each P450 in the metabolism, induction, and

TABLE 14.10 Multifunctional Cytochromes P450 and Monoclonal Antibodies

<i>Monoclonal Antibodies Identify P450 Isoform Content, Function and Expression</i>
Allyl(Allozyme)expression
S-9 Dymystification-mutagen activation
DNA covalent binding
Protein covalent binding
Tissue and developmental mapping
Cell culture expression
P450 in metabolism, induction, and inhibition
Conversion of environmental agents, natural products, and neutraceuticals to carcinogenic, mutagenic, toxic, and immunotoxic agents
Potency and specificity of chemical inhibitors
<i>Monoclonal Antibodies and Drug Metabolism</i>
Identification and quantification of drug metabolism by single, several, or polymorphic P450s
Identification of drugs and metabolites useful for <i>in vivo</i> phenotyping
Purification of proteins

inhibition of P450 activity by neutraceuticals, natural products, and other environmental chemical agents. The MAb system may also identify the expression of different P450 isoforms in cells grown in a variety of cell culture systems.

14.11 SYNOPSIS AND CRITIQUE (DRUG METABOLISM)

Different methods have been used to identify and quantitate the role of each P450 for the metabolism of a drug [42,43]. One method is the measurement of the enzyme activity of the expressed pure P450 toward specific substrates. The amount of substrate metabolized by the pure expressed enzyme determines its capability to metabolize the substrate, but it is often not a good indicator of a role of the P450 isoform for *in vitro* microsomal metabolism. The P450 expression method does not take into account different amounts of the P450 isoform protein present in HLM and its usual large interindividual variability. The HLM analysis of a P450 isoform activity depends on the P450 protein content and other factors, such as cofactor and lipid content, membrane and structural positioning, and potential for competition with P450 isoforms that may also metabolize the subject drug. Drug therapeutics and drug discovery would be promoted if drugs or combination of drugs were available or developed that would be designed specifically for highly reliable phenotyping of the patient. Knowledge of the patient's phenotype would greatly enhance the ability of the clinician to reduce adverse drug reactions and administer effective drugs or combination of drugs at the right dosage by having a precise knowledge of the patient's phenotype for the metabolism of the drugs. The MAb method is in stark contrast to other complex analytic systems that are relatively nonspecific and currently being used to study drug metabolism. The MAb system identifies and quantitates drugs metabolized by a single P450, multiple P450s, and polymorphic P450s. Drugs metabolized by a single P450 isoform or metabolites formed from a single metabolic pathway may be useful as markers for identifying the target P450 *in vivo*. The MAb methodology is precise, simple,

and highly reproducible. The MAbs, which are highly specific and highly inhibitory, may also be used to measure the specificity and the potency of chemical inhibitors.

The MAbs will prove useful for identifying and quantifying P450 proteins that vary under different conditions, which include nutritional and hormonal states, developmental stages, age, sex, and inducer exposure. A full library of MAbs to P450 could develop a P450 taxonomy and would greatly enhance the progress of P450 research.

14.12 CONCLUSION

This chapter evaluated the efficacy of the various methods used to examine the role of P450 isoforms for the metabolism of a drug. The conclusion of this analysis was that specific and potent inhibitory MAbs against the different P450 isoforms (such as those listed in Table 14.1), which are characterized for specificity and inhibitory potency and verified with HLM and the expressed P450, represents the most valuable approach and basically the only method necessary for a phenotyping study.

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15 Precision-Cut Tissue Slices: A Suitable *In Vitro* System for the Study of the Induction of Drug-Metabolizing Enzyme Systems

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15.1	Summary	1
15.2	Introduction	2
15.3	Clinical and toxicological implications of enzyme induction	3
15.4	Preparation and culture of precision-cut tissue slices	4
15.5	Induction of phase I enzyme systems by xenobiotics in precision-cut tissue slices	5
15.6	Induction of phase II enzyme systems by xenobiotics in precision-cut tissue slices	12
15.7	Cryopreservation of precision-cut tissue slices	15
15.8	Conclusions	19
	References	20

15.1 SUMMARY

Induction of the drug-metabolizing enzymes, in particular, cytochrome P450 (CYP) forms, is a major cause of drug–drug as well as herb–drug interactions that can lead to adverse side effects, which may prove fatal. As a result, an integral part of drug development is to ascertain whether it has the potential to act as an inducer of CYP and other enzyme systems in order to avoid serious drug interactions with coadministered drugs. Moreover, for both drugs and other chemicals, CYP induction may be associated with adverse toxicology including tumorigenesis.

A versatile *in vitro* model system that can be employed to evaluate the effect of chemicals on xenobiotic-metabolizing enzymes is the precision-cut tissue slice procedure. Slices from liver as well as extrahepatic tissues such as the kidney, lung, and

intestines have been utilized to identify metabolic pathways and assess the toxicity of chemicals, including their potential to induce xenobiotic-metabolizing enzyme systems. Many studies have utilized precision-cut rat liver slices to monitor the induction of enzymes belonging to the CYP1–CYP4 families. A wide range of chemicals have been studied using this *in vitro* system, including drugs, environmental contaminants, and phytochemicals. CYP form induction has been determined by measurement of appropriate enzyme activities (EAs) (either in intact slices or in slice homogenates/subcellular fractions), protein levels (e.g., by Western immunoblotting), or mRNA levels. Established inducers of various CYP enzymes upregulated CYP enzymes as would have been envisaged on the basis of *in vivo* studies reported in the literature. In human liver slices, the induction of a number of CYP forms including CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 has been demonstrated.

As with the induction of CYP forms, a wide range of compounds have been shown to induce phase II xenobiotic-metabolizing enzymes, including epoxide hydrolase, UDP-glucuronosyltransferase (UGT), sulfotransferase, and glutathione *S*-transferase (GST) forms, in cultured rat liver and lung slices, and human liver and intestine slices.

Finally, procedures have been developed for the successful cryopreservation of precision-cut liver slices from various animal species, which can also be used to assess the potential of xenobiotics to induce the xenobiotic-metabolizing enzyme systems.

15.2 INTRODUCTION

The use of precision-cut tissue slices is increasingly gaining acceptance and is utilized worldwide to provide answers in studies concerned with the metabolism and toxicity of chemicals [1–5]. The major “competing” *in vitro* system involves the use of isolated cells such as hepatocytes. However, precision-cut slices offer some important advantages over the use of isolated cells that cannot be ignored, and these are summarized below.

- Compared to single cell preparations such as hepatocytes, slices function as “mini” tissues, maintaining the functional heterogeneity of the parent tissue and permitting the study of zonal effects. Moreover, in tissues such as lung that has a heterogeneous cell population where drug or xenobiotic-metabolizing EAs are present only in some cell types [6], the use of slices allows the identification of cell-specific toxicity.
- The procedure for isolation of single cells employs digestive enzymes that remove cell-to-cell contact, thus preventing cellular communication and interactions, and lead to the collapse of tissue architecture. Such communication is preserved in slices and, furthermore, maintenance of tissue architecture in slices makes possible the use of morphological endpoints, such as histology and immunocytochemistry, in evaluating xenobiotic toxicity.
- Generation of slices using an appropriate tissue slicer (e.g., a Krumdieck or Brendel-Vitron slicer) is rapid and causes only minimal damage to the tissue and does not necessitate the use of detrimental proteolytic enzymes that are routinely utilized in cell isolation.

- The use of slices facilitates comparisons of the metabolism and/or toxicity between animal species, including human, and tissues, especially as slices can be successfully cryopreserved (see below), which would not be otherwise possible. Such cross-species comparisons can provide invaluable information, enabling the identification of animal species that would be appropriate surrogates for human in toxicological evaluation studies.
- Slices from different tissues may be cocultured so that, for example, the sequential metabolism and toxicity of xenobiotics can be delineated.

15.3 CLINICAL AND TOXICOLOGICAL IMPLICATIONS OF ENZYME INDUCTION

In an era where polypharmacy has become routine, it is inevitable that coadministered drugs were shown to interact with adverse or even fatal consequences for the patient. The induction of the drug (xenobiotic)-metabolizing enzymes by one or more administered drugs is a major mechanism underpinning many such interactions. In most instances, enzyme induction involves the stimulation of CYP forms that are present in the liver and extrahepatic tissues [7,8]. The most important microsomal CYP forms involved in the metabolism of therapeutic agents in human liver include members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A subfamilies [7–12]. Such drug interactions often involve CYP3A4, as this enzyme is the most active human CYP form in drug metabolism and, moreover, is the dominant form in the liver and intestine, the principal site and the first site of metabolism following oral drug intake, respectively. There are numerous examples of drug–drug interactions consequential to CYP induction [9–12]. Patients with transplanted organs and successfully stabilized on the immunosuppressant cyclosporin displayed signs of rejection after self-medicating with St. John's wort, a herbal remedy taken to treat mild depression [13]. It appears that a component(s) of this remedy elevates CYP3A4 activity, leading to enhanced cyclosporin metabolism resulting in suboptimal plasma levels of the drug and, consequently, tissue rejection. Since the norethindrone and ethinyl oestradiol components of the contraceptive pill are also deactivated by CYP3A4, intake of St. John's wort has also been associated with intracyclic bleeding episodes as a result of contraceptive failure [14,15]. The treatment of humans with the antibacterial agent rifampicin (RIF), which induces CYP3A and other CYP forms, has been shown to produce a number of clinically important drug–drug interactions [10–12]. These include cyclosporin (reduction in blood levels leading to transplant rejection), warfarin (reduced prothrombin time), oral contraceptives (unplanned pregnancies), and human immunodeficiency virus protease inhibitors such as ritonavir and indinavir (reduced systemic exposure leading to a diminished antiretroviral effect).

The treatment of rats and mice with therapeutic agents and other chemicals, which induce hepatic microsomal CYP forms may be associated in chronic studies with the formation of tumors in the liver and other tissues [12]. For example, CYP2B inducers can give rise to liver and thyroid gland tumors and exposure to CYP4A inducers can lead to tumor formation in the liver and testis. In these instances, the induction of rodent hepatic CYP forms represents just part of a pleiotropic response to such compounds. However, the induction of CYP forms can serve as a biomarker for

potential subsequent tumor formation, and hence it is important in the toxicological evaluation of new chemical entities (NCEs).

As a result of the potential clinical and toxicological consequences of CYP enzyme induction, studies to screen for such an effect form an integral part of drug development. Studies encompassing a large number of doses, that would not be feasible to conduct *in vivo*, can be readily performed *in vitro* using precision-cut tissue slices from laboratory animals and human donors.

15.4 PREPARATION AND CULTURE OF PRECISION-CUT TISSUE SLICES

Tissue slices can be prepared from the liver and extrahepatic tissues such as the kidney, lung, and different parts of the intestine [1–5]. For solid tissues, such as the liver and kidney, the first step is to prepare cylindrical cores (normally 8 or 10 mm in diameter) with a hand or preferably a motor-driven tissue coring tool. The tissue cylinders are then loaded into a suitable tissue slicer and slices of a defined thickness are produced. For liver slices, the optimal thickness has been established to be 200–250 μm , with limits of usable thickness ranging from 150/180 to 300 μm [1–3]. The optimal thickness of kidney slices is also around 200–250 μm . Tissue slice thickness can be estimated by a number of methods including determination of tissue slice weight or protein content, microscopic examination of freshly cut slices, use of a digital micrometer, and the microscopic examination of formalin-fixed tissue slice paraffin sections [1–3,16].

Commercially available tissue slicers include the Krumdieck and the Brendel-Vitron [1–3]. The Krumdieck tissue slicer is fully automated, with the tissue core being brought across a motor-driven oscillating blade that is submerged in a chamber containing a suitable slicing buffer. The buffer is circulated by a pump and the tissue slices are removed from the blade by a gentle flow of buffer into a glass collecting vessel. In contrast, the Brendel-Vitron slicer is cheaper and less mechanically complex than the Krumdieck slicer. However, it is only semiautomated and uses a rotating blade with the slicing buffer being both circulated and oxygenated by a gas supply. Both tissue slicers have certain advantages and a level of technical expertise is required [1–3]. One study has demonstrated that there was little difference between precision-cut rat liver slices produced with Krumdieck and Brendel-Vitron tissue slicers for studies of xenobiotic metabolism and toxicity [17].

With soft tissues, such as the lung and intestines, agarose is used to enable the preparation of tissue cores. For example, rodent lungs can be filled with a warm agarose solution at 37°C, which is then cooled to 4°C before preparing tissue cores and, subsequently, tissue slices as described above for liver slices [1,2,4]. Intestines can also be filled with warm agarose solution and then embedded in agarose in a core-shaped container to obtain solid cores for slicing [4,18,19]. The optimal thickness of intestinal slices is around 200–250 μm , whereas for lung slices the optimal thickness is around 500–700 μm [1–24].

A wide variety of incubation systems have been developed for precision-cut tissue slices [1–5,20]. Generally, the available incubation systems may be divided into two types, namely, surface culture (where the slices are dipped in and out of the culture medium) and submersion culture systems. One much used surface culture system is the dynamic organ culture system where the slices are floated onto a mesh support

(e.g., stainless steel or titanium) of a roller assembly, which is housed in a suitable vial (e.g., a glass scintillation vial) containing culture medium. The vial is then placed on a roller assembly housed in an incubator or is placed in a purpose-built incubator (e.g., a Vitron incubator). It has been shown that tissue slice viability is extended if the slices are placed on a nitrocellulose filter rather than directly on the mesh of the roller assembly [5,21]. Submersion tissue slice culture systems include the use of Erlenmeyer flasks and 6-, 12-, and 24-well tissue culture plates. For multiwell plates, slices can be simply immersed in culture medium or placed on mesh supports. Both the surface culture and submersion culture systems have advantages, and the choice of incubation system may depend on the particular application of tissue slices under investigation [1–5,20,22,23]. A microfluidic biochip system for the perfusion of liver slices has also recently been described [24].

A number of suitable media for tissue slice preparation and culture media for the incubation of tissue slices from both the liver slices and extrahepatic tissues have been described in the literature [1–4]. Culture media are often supplemented with hormones (e.g., insulin and a glucocorticoid) and bacterial and antifungal agents, the latter being essential to prevent microbial contamination during prolonged incubations. Tissue slices can be incubated under air or gas phases with a higher oxygen concentration, ranging from 40% to 95% [1–4].

15.5 INDUCTION OF PHASE I ENZYME SYSTEMS BY XENOBIOTICS IN PRECISION-CUT TISSUE SLICES

Developments in precision-cut tissue slice preparation and incubation techniques have permitted the culture of slices for several days, which is long enough for their use in xenobiotic-metabolizing enzyme induction studies [1–5,20]. As they contain all the cell types present in a tissue, precision-cut tissue slices can be used to study the effects of drugs and other chemicals on all the xenobiotic-metabolizing enzymes present in that tissue. The most important phase I xenobiotic-metabolizing enzymes comprise members of the CYP superfamily [7–12], whereas phase II enzymes include UGT, sulfotransferase, and GST forms (Section 15.6). This section considers the induction of CYP forms in liver and extrahepatic slices from animals and humans.

Most CYP forms are induced by receptor-mediated mechanisms, leading to an increase in gene transcription [8,10–12]. Important receptors involved in the induction of CYP1A, CYP2B, CYP3A, and CYP4A forms comprise, respectively, the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor alpha (PPAR α). These and other receptors are present in precision-cut tissue slices, thus permitting cultured slices from the liver and other tissues to be used in CYP form induction studies.

The induction of CYP forms in precision-cut tissue slices can be evaluated by monitoring CYP-dependent EAs, CYP mRNA levels, or CYP protein levels. CYP EAs can be determined either directly by adding the substrate to the culture medium or alternatively by removing the slices and preparing whole homogenate and/or subcellular fractions (e.g., postmitochondrial supernatant, microsomes) [25–34]. As xenobiotics and their metabolites may be retained in liver slices if the CYP substrate is added to the culture medium, some workers homogenize the slices in the incubation medium

and then analyze the slice/medium homogenate. However, for some substrates only the incubation medium needs to be analyzed. Because phase II enzymes will also participate in incubations with intact slices, it may be necessary to treat the slice/medium homogenate or medium with a β -glucuronidase/sulfatase preparation in order to quantify the total (i.e., free and conjugated) CYP metabolite(s) produced [23,35]. One study reported that the induction of CYP-dependent enzymes by β -naphthoflavone (BNF) and dexamethasone (DEX) could be detected in both intact liver slices and liver slice homogenates [36]. CYP mRNA levels are best determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) methodology (e.g., TaqMan®), although other less quantitative procedures have also been employed [27,29,34,37–53]. To normalise for RNA loading, levels of CYP mRNAs are often expressed as a ratio to a suitable control mRNA such as albumin, β -actin, or 18S rRNA. Several studies have examined the effects of xenobiotics on CYP proteins by preparing microsomal fractions and performing Western immunoblotting of selected CYP forms [25,31–34,37,52–60]. CYP proteins may also be determined by enzyme-linked immunosorbant assay (ELISA) procedures [27].

As with other *in vitro* systems, it is important to evaluate a range of xenobiotic concentrations and incubation times in order to ascertain whether the xenobiotic under investigation is an inducer of CYP forms in cultured precision-cut tissue slices. To determine the effects on CYP form mRNAs, only short incubation times may be required (e.g., 3–24 h), whereas to determine the effects on CYP EAs and proteins, longer incubation times (e.g., 24–72 h) may be necessary. In addition, it is imperative to ensure that the chosen concentrations of the xenobiotic studied are not toxic to the tissue slices. Suitable markers of tissue slice viability include leakage of potassium and enzymes (e.g., alanine aminotransferase, lactate dehydrogenase (LDH)), inhibition of protein synthesis, and levels of ATP and reduced glutathione [1–4].

Like hepatocytes [20], levels of CYP forms decline in cultured precision-cut rodent and human liver slices. For example, compared to freshly cut slices levels of CYP1A, CYP2B, CYP2C, CYP2D, CYP2E, CYP3A, and CYP3A4 forms and associated EAs have been shown to decline with time in cultured rat liver slices [25,32,54]. While levels of CYP forms also decline in human liver slices [30,33,34,51,54,55,57], one study demonstrated a differential loss with some CYP forms (e.g., CYP2D6, CYP4A11) being relatively stable over a 72-h culture period, whereas other CYP forms (e.g., CYP1A2, CYP2C19) exhibited a more rapid loss [33]. However, the decline in CYP forms in cultured liver slices does not preclude the use of liver slices as an *in vitro* model system to screen xenobiotics for induction of CYP forms. Indeed, a wide range of chemicals have been shown to induce CYP forms in cultured precision-cut liver slices from animals and humans. Such chemicals include drugs, environmental contaminants, and phytochemicals. Thus, like hepatocytes cultured liver slices can be employed as a model system to assess the potential of NCEs to induce hepatic CYP forms.

15.5.1 Studies in Liver Slices from Animal Species

The majority of studies conducted in animal species have been performed with precision-cut slices prepared from rat liver. Some examples of CYP induction studies in cultured rat liver slices are shown in Table 15.1. The induction of CYP1A, CYP2B, CYP3A, CYP4A, and other subfamily forms have been studied by determination of CYP EAs (intact slices and slice homogenates/subcellular fractions), protein (normally

TABLE 15.1 Induction of CYP Forms in Cultured Rat Liver Slices

Inducer ^a	CYP Form or Subfamily	Endpoint Studied ^b			References
		EA	Protein	mRNA	
ARO, BNF, PB	1A, 2B	✓	✓	—	25
BNF	1A	✓	—	—	26
TCDD	1A1, 1A2	✓	✓	✓	37
ARO, BNF, CIP, MCP, PB, WY	1A2, 2B1/2, 3A, 4A	✓	✓	—	54
BNF, DEX	1A1	—	✓	—	27
BNF, PB, TCDD	1A, 2B, 3A	✓	—	—	28
BNF, 7EC, 4Me7EC	1A1	✓	—	✓	29
BNF	1A1	—	—	✓	38
BP	1A1	—	—	✓	39
TCDD	1A1, 1A2	✓	✓	—	31
ARO, 3ICN, DIM	1A, 2B, 3A	✓	✓	—	58
PB	2B1	✓	✓	—	41
PCN	3A1	—	—	✓	42
ARO, PB, WY	1A1, 1A2, 2B1, 4A1	—	—	✓	43
DEX, PCN	3A	✓	—	✓	45
ARO, BNF, MCP, PB, PCN, WY	1A1, 1A2, 2B1/2, 3A1, 3A2, 4A1	—	—	✓	46
ARO, BP	1A	✓	—	—	61
DEX	3A	—	✓	—	62
CARB, CLOT, DEX, PB, PCN, others	2B1/2, 3A1	—	—	✓	47
BNF, DEX, PB, PCN	1A, 2B, 2C, 3A	✓	✓	—	63
BP	1A1, 1B1	—	—	✓	48
ISN, PB	2B, 2E1	✓	✓	—	64
BP	1A1, 1B1	✓	✓	✓	52
ERUC, SULF	1A2, 1B1, 3A2	—	✓	—	60
BNF, DEX, PB, PCN	1A, 2B, 2C, 3A	✓	✓	—	65
BP other PCHs	1A1, 1B1	✓	✓	✓	53
BUD, DEX, PCN	3A1, 3A2, 3A9	—	—	✓	66

^aInducers used were: ARO, Aroclor 1254; BNF, β -naphthoflavone; BP, benzo(*a*)pyrene; BUD, budesonide; CARB, carbamazepine; CIP, ciprofibrate; CLOT, clotrimazole; DEX, dexamethasone; DIM, 3,3'-diindolylmethane; 7EC, 7-ethoxycoumarin; ERUC, erucin; 3ICN, indole-3-acetonitrile; ISN, isoniazid; MCP, methylclofenapate; 4Me7EC, 4-methyl-7-ethoxycoumarin; PB, phenobarbital; PCHs, polycyclic hydrocarbons; PCN, pregnenolone-16 α -carbonitrile; SULF, sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WY, Wy-14,643.

^bEndpoints studied were determination of CYP enzyme activities (EAs), protein levels (e.g., Western immunoblotting), and mRNA levels.

by Western immunoblotting), and mRNA levels. Table 15.1 lists either the CYP subfamily studied or the particular CYP form examined if this was identified (e.g., by mRNA determination or Western immunoblotting).

A number of studies have established that CYP1A forms are inducible in cultured liver slices following treatment with agents such as Aroclor 1254 (ARO) (a polychlorinated biphenyl mixture), BNF, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),

polycyclic aromatic hydrocarbons (PCHs) including benzo(a)pyrene (BP) and phytochemicals including 3,3'-diindolylmethane (DIM), erucin (ERUC), and sulforaphane (SULF). Some studies [48,52,53] have also examined effects on CYP1B1 as, such as CYP1A1 and CYP1A2, this CYP form is also involved in the metabolic activation of carcinogens including PCHs and heterocyclic amines (cooked food mutagens) [8]. In one study, CYP induction by PCHs was shown to correlate with the potency of the compounds studied to bind to the AhR receptor [53]. Correlations were also observed between the molecular size of the PCHs studied and the induction in rat liver slices of CYP1A-dependent EA, CYP1A1 mRNA levels, and CYP1B1 mRNA levels. The induction of CYP forms by BP in cultured rat liver slices has been shown to be associated with increased levels of BP-DNA adducts [67].

The induction of CYP2B and CYP3A forms in cultured rat liver slices has been demonstrated in studies emanating from a number of laboratories. For example, the induction of CYP2B forms has been demonstrated after exposure of the slices to phenobarbital (phenobarbitone, PB), whereas DEX and/or pregnenolone-16 α -carbonitrile (PCN)-induced CYP3A forms [25,41,42,45–47,54,62–66]. Because of the high degree of similarity between CAR and PXR, a number of inducers function as activators of both nuclear receptors [10–12]. Thus, in some studies, in cultured rat liver slices, PB has been shown to induce CYP3A forms in addition to CYP2B forms, whereas DEX- and/or PCN-induced CYP2B forms as well as CYP3A forms [46,54,63,65]. In one study, a range of NCEs were shown to have variable effects on CYP2B1/2 (i.e., CYP2B1 and CYP2B2) and CYP3A1 mRNA levels in cultured rat liver slices [47]. Moreover, good correlations were observed between the potency of the NCEs to induce CYP2B1/2 and CYP3A1 mRNA levels in liver slices *in vitro* and the potency of these NCEs to induce CYP2B (by EA determination) and CYP3A (by Western immunoblotting) forms after *in vivo* treatment (oral administration to rats for eight days), thus highlighting the usefulness and validity of utilising precision-cut tissue slices to evaluate the potential of a xenobiotic to induce the CYP enzymes. In another study utilizing a series of structurally diverse compounds, a correlation was observed between the induction of CYP3A mRNA levels in cultured rat liver slices and the effects of the compounds in a rat PXR reporter gene assay [68].

While CYP1A, CYP2B, CYP2C, and CYP3A forms are primarily involved in the metabolism of drugs and other xenobiotics [7,8], the CYP4A subfamily forms are involved in the metabolism of endogenous chemical such as fatty acids. CYP4A forms are inducible in rodent liver and this is associated with hepatic peroxisome proliferation [10,12]. The induction of CYP4A forms has also been demonstrated in cultured rat liver slices [43,46,54]. In keeping with known *in vivo* effects, peroxisome proliferators have also been shown to induce peroxisomal fatty acid oxidizing EAs and, by ultrastructural examination, to increase numbers of peroxisomes in cultured rat liver slices [69].

One advantage of the precision-cut tissue slice technique is its ready application to studies of species-, tissue-, sex-, strain-, and age-related differences. In a study of age-related differences, using liver slices from young, adult, and old rats (aged 3, 9 and 24 months, respectively), a decrease in total CYP content and a loss of CYP3A inducibility by DEX were observed in old rats [62]. The treatment of liver slices from one-day-old rats with BNF, PB, DEX, and PCN was shown to result in the induction of CYP1A, CYP2B, CYP2C, and CYP3A marker EAs [63]. In a similar study, the effects of BNF, PB, DEX, and PCN on CYP-dependent activities in liver slices from 1-day-, 40-day-, and 1-year-old rats were investigated [65]. Overall, CYP EAs were

somewhat lower in liver slices from one-year-old rats, whereas CYP inducibility was more pronounced in younger rats.

As tissue morphology is retained in precision-cut tissue slices, the cell-specific and zonal effects of xenobiotics on CYP forms can be investigated. By employing immunocytochemical techniques, a number of studies have investigated the effect of xenobiotics on induction of CYP forms in different regions of the liver lobule. The majority of these studies have focussed on CYP1A forms. In untreated rat liver slices cultured for 24 h, CYP1A1 protein immunostaining was not detected but was detected after BNF treatment [70]. A centrilobular induction of CYP1A1 protein immunostaining was reported in rat liver slices after treatment for 24 h with BP [39] and for 24 h with TCDD followed by 72 h culture in control medium [31]. In another study, CYP1A1 protein immunostaining was not detected in liver slices cultured for 24 h in control medium, but following BP treatment CYP1A1 protein immunostaining was observed throughout the liver slice [48]. Furthermore, a low level of CYP1B1 protein immunostaining was observed in both control and BP treated liver slices. The effect of BNF, PB, DEX, and PCN on CYP1A1, CYP2B1, and CYP3A2 protein expression was assessed in liver slices from neonatal and adult rats [63,71]. In freshly cut slices from adult rats, only weak CYP1A1 protein immunostaining was observed, whereas strong CYP2B1 and CYP3A2 protein immunostaining was detected mainly in centrilobular and midzonal areas of the liver lobule [71]. After 24 h treatment with BNF, increased CYP1A1 and CYP2B1 protein immunostaining was observed, whereas PB increased CYP2B1 and CYP3A2 protein expression and DEX increased CYP3A2 protein expression. Similar results were obtained in studies with one-day-old rats except that an increase in CYP3A2 protein immunostaining was also observed with PCN [63].

Fluorescence confocal laser cytometry has also been used to investigate zonal differences in CYP form induction in liver slices from untreated (control) and BNF-treated rats [72]. An induction of 7-ethoxyresorufin *O*-deethylase (a marker of CYP1A induction) was observed in centrilobular hepatocytes of liver slices from BNF-treated rats, whereas no induction was observed with fluorescent substrates of CYP2B forms.

Compared with the rat, fewer studies have been performed using precision-cut liver slices from the mouse. However, as would be expected CYP form induction can be readily demonstrated in this species. CYP1A-dependent EA was induced in mouse liver slices by BNF and TCDD, with PB inducing CYP2A- and CYP3A-dependent EAs [28]. The treatment of mouse liver slices with BNF has also been reported to induce CYP1A1 and CYP1A2 mRNA levels, with CYP2B10 mRNA levels being induced by BNF, PB, and DEX, and CYP3A11 mRNA levels by DEX [49]. The induction of CYP3A12 and CYP3A26 mRNA levels by various compounds in cultured precision-cut dog liver slices has also been reported [73].

15.5.2 Studies in Human Liver Slices

Some examples of CYP induction studies in cultured human liver slices are shown in Table 15.2. The induction of CYP forms in a number of subfamilies has been investigated following treatment with drugs, environmental contaminants, and phytochemicals.

A number of studies have demonstrated the induction of CYP1A1 and/or CYP1A2 in cultured human liver slices following treatment with agents such as ARO, BNF, BP, and other PCHs, TCDD, omeprazole (OMP), DIM, ERUC, SULF, and phenethyl

TABLE 15.2 Induction of CYP Forms in Cultured Human Liver Slices

Inducer ^a	CYP Form or Subfamily	Endpoint studied ^b			References
		EA	Protein	mRNA	
ARO, MCP	1A2, 4A	✓	✓	—	54
RIF	3A4	✓	✓	—	55
TCDD	1A1	✓	✓	—	56
DIM	1A2	✓	✓	—	57
BNF	1A1	—	—	✓	40
CP, PB	2B6, 3A4	✓	✓	✓	34
BNF, PB, RIF	1A2, 2C19, 3A4	—	✓	—	59
OMP, RIF, TCDD, others	1A1, 1A2, 2C9, 3A4	—	—	✓	51
ERUC, SULF	1A1, 1B1	—	✓	—	60
BNF, PB, RIF	1A1, 1A2, 2A6, 2B6, 3A4, 3A5	✓	—	✓	74
BP, other PCHs	1A1	✓	✓	—	53
CDCA, DEX	3A4	—	—	✓	66
PEITC	1A1, 1A2, 2B6, 3A4	✓	✓	—	75

^aInducers used were: ARO, Aroclor 1254; BNF, β -naphthoflavone; BP, benzo(*a*)pyrene; CDCA, chenodeoxycholic acid; CP, cyclophosphamide; DEX, dexamethasone; DIM, 3,3'-diindolylmethane; ERUC, erucin; MCP, methylclofenapate; OMP, omeprazole; PB, phenobarbital; PCHs, polycyclic hydrocarbons; PEITC, phenethyl isothiocyanate; RIF, rifampicin; SULF, sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

^bEndpoints studied were determination of CYP enzyme activities (EAs), protein levels (e.g., Western immunoblotting), and mRNA levels.

isothiocyanate (PEITC). CYP1B1 protein levels have also been reported to be induced by the chemopreventive phytochemicals, ERUC and SULF [60].

Apart from CYP1A forms, in keeping with studies in cultured hepatocytes, a number of other human hepatic CYP forms can be induced in cultured liver slices. These CYP forms include CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 [34,51,55,59,66,74,75]. Compounds that are known to induce one or more of these CYP forms in human liver slices include chenodeoxycholic acid (CDCA), cyclophosphamide (CP), DEX, PB, PEITC, and RIF. For example, investigations from a number of laboratories have demonstrated that PB and RIF can induce CYP3A4 EA, protein levels, and mRNA levels in cultured precision-cut human liver slices [34,51,55,59,74].

The lobular distribution of CYP2B6 and CYP3A4 induction has been investigated in precision-cut human liver slices [34]. In untreated human liver slices, the distribution of CYP2B6 and CYP3A4 immunostaining was random and centrilobular, respectively. The staining intensities of both CYP2B6 and CYP3A4 proteins were increased following treatment with both PB and CP for 72 h, with no change in the zonal distribution of these two CYP forms being evident.

15.5.3 Studies in Extrahepatic Slices

While the liver is the major site of xenobiotic metabolism in mammals, both phase I and II xenobiotic-metabolizing enzymes are found in many extrahepatic tissues [4,7,8]. Moreover, some extrahepatic xenobiotic-metabolizing enzymes are known to

TABLE 15.3 Induction of CYP Forms in Cultured Rat and Human Lung, Kidney, and Intestinal Slices

Species, Tissue, and Inducer ^a	CYP Form or Subfamily	Endpoint Studied ^b			References
		EA	Protein	mRNA	
Rat lung—ARO, BNF, BP	1A1, 2B1/2	✓	✓	✓	76
Rat lung—ARO, BP, NIC	1A	✓	—	—	61
Rat lung—BP	1A1, 1B1	—	—	✓	48
Rat lung—BP	1A1, 1B1	✓	✓	—	52
Rat lung—BP, other PCHs	1A1, 1B1	✓	✓	—	53
Rat lung—ERUC, SULF	1A1, 1B1, 3A2	—	✓	—	77
Rat kidney—BP	1A1	—	—	✓	39
Rat intestine ^c —BNF	1A	✓	—	—	18
Rat intestine ^c —BNF, DEX, IR, 20MC, PB	1A, 2B15, 3A9	✓	—	✓	78
Rat intestine ^d —BUD, CDCA, DEX, 1,25(OH) ₂ D ₃ , PCN	3A1, 3A2, 3A9	—	—	✓	66
Human intestine ^e —BNF, DEX, PB, RIF	1A1, 2B6, 3A4	✓	—	✓	19
Human intestine ^f —BUD, DEX, 1,25(OH) ₂ D ₃	3A4	—	—	✓	66

^aInducers used were: ARO, Aroclor 1254; BNF, β -naphthoflavone; BP, benzo(*a*)pyrene; BUD, budesonide; CDCA, chenodeoxycholic acid; DEX, dexamethasone; IR, indirubin; 20MC, 20-methylcholanthrene; NIC, nicotine; 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; PCHs, polycyclic hydrocarbons; PCN, pregnenolone-16 α -carbonitrile.

^bEndpoints studied were determination of CYP enzyme activities (EAs), protein levels (e.g., Western immunoblotting), and mRNA levels.

^cRat small intestine and colon examined.

^dRat jejunum, ileum, and colon examined.

^eHuman proximal jejunum and colon examined.

^fHuman ileum examined.

be inducible. The lung and intestines are important sites of xenobiotic metabolism, as these tissues are the first sites of metabolism of inhaled and orally ingested xenobiotics, respectively. This section focusses on CYP induction studies performed in cultured lung and intestine slices, including investigations performed with human intestine slices.

A number of studies have examined the induction of CYP forms in cultured rat lung, kidney, and intestine slices. As shown in Table 15.3, the treatment of rat lung slices with ARO, BNF, BP and other PCHs, nicotine (NIC), ERUC, and SULF has been shown to induce CYP1A1, and in some instances, the induction of CYP1B1 has also been observed [48,52,53,61,76,77]. Apart from the induction of CYP1A1 and CYP1B1 in cultured rat lung slices, ARO was shown to have a weak effect on CYP2B1/2 mRNA levels [76] and SULF a weak effect on CYP3A2 protein levels [77]. In one study in cultured rat kidney slices, BP was shown to induce CYP1A1 mRNA levels [39].

The effect of xenobiotics on CYP forms in rat and human intestine slices has also been investigated (Table 15.3). Xenobiotic-metabolizing enzymes are not uniformly distributed throughout the intestinal tract, and there can also be regional differences in the effects of CYP inducers [18,19,66,78]. The treatment of rat small intestine slices with BNF, indirubin (IR), and 20-methylcholanthrene (20MC) and colon slices with BNF and 20MC has been shown to induce a CYP1A marker EA [18,78]. Following treatment with DEX, a CYP3A marker EA was increased in cultured rat small intestine slices but not in colon slices [78]. Treatment with PB increased CYP3A9 mRNA levels in both small intestinal and colon slices, and treatment with both PB and DEX increased CYP2B15 and CYP3A9 mRNA levels in colon slices. In another study, the effect of xenobiotics on CYP3A1, CYP3A2, and CYP3A9 mRNA levels in cultured rat jejunum, ileum and colon slices was examined [66]. Treatment with $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}_3$)-induced CYP3A1 mRNA levels in cultured rat jejunum, ileum, and colon slices and CYP3A2 mRNA levels in ileum slices. CYP3A1 mRNA levels were increased in rat ileum slices by CDCA, DEX, and PCN, whereas CYP3A9 mRNA levels were increased in jejunum and ileum slices by BUD, DEX, and PCN, with the former two also increasing CYP3A9 mRNA levels in colon slices.

Treatment with BNF increased CYP1A1 mRNA levels in both human proximal jejunum and colon slices [19]. In addition, both PB- and RIF-induced CYP2B6 and CYP3A4 mRNA levels, and DEX-induced CYP3A4 mRNA levels in human proximal jejunum slices. Significant increases in CYP1A (by BNF) and CYP3A (by PB and RIF) marker EAs were also observed in cultured human proximal jejunum slices. While some increases in CYP2B6 (by PB) and CYP3A4 (by RIF) mRNA levels were observed in human colon slices, these effects were not statistically significant. In another study, significant increases in CYP3A4 mRNA were observed in human ileum slices after treatment with BUD, DEX, and $1\alpha,25(\text{OH})_2\text{D}_3$ [66].

15.6 INDUCTION OF PHASE II ENZYME SYSTEMS BY XENOBIOTICS IN PRECISION-CUT TISSUE SLICES

Phase II enzyme systems are closely linked to the deactivation and detoxication of xenobiotics. Conjugation reactions with endogenous substrates, such as sulfate, D-glucuronic acid, glutathione, and certain amino acids, yield highly hydrophilic, readily excretable metabolites [79], although functional groups may also be methylated or acetylated to produce less hydrophilic compounds. Thus induction of these enzyme systems will impact on the metabolic fate and biological activity of chemicals. Toxicologically, a most important enzyme system is the GSTs, which catalyze one of the most important pathways of metabolism that allows the cell to defend itself from chemical insult. It utilizes the nucleophilic tripeptide glutathione, possessing a nucleophilic sulphur atom, to detoxify reactive electrophiles. Indeed, dietary phytochemicals considered to be potential anticarcinogens, such as isothiocyanates, indoles, and curcumin, owe their chemopreventive activity largely to their ability to stimulate conjugation of chemical carcinogens, or of their genotoxic metabolites, with glutathione.

On culturing, phase II activities in precision-cut tissue slices decline, but these enzyme systems appear to be more robust and are better maintained compared with CYP forms [23]. Substantial phase II activity remained in rat liver slices after a 24 h incubation and, in some cases, significant activity was retained even after a 72 h

incubation; for example, the specific activity of UGT, monitored using 2-aminophenol as substrate, did not change significantly following a 24 h incubation, and only a modest loss of activity was noted after 72 h [23]. In concordance, glucuronidation reactions, measured using *p*-nitrophenol, 4-methylumbelliferone, and 4-hydroxybiphenyl as substrates, were more stable than CYP enzymes in rat slices [80,81]. Similarly in human liver slices, form-specific changes were noted when CYP protein levels were determined [33], but glucuronidation of 7-hydroxycoumarin was well maintained [30]. Such observations imply that not only the functional integrity of the enzymes is maintained but also that the slices are capable of synthesizing the necessary cosubstrates for conjugation, such as UDP-glucuronic acid.

The regulation of phase II enzymes by xenobiotics has been the subject of numerous publications, studies being conducted largely *ex vivo* in many animal species, and *in vitro* using systems, such as freshly isolated hepatocytes and cultured cells. It is now established that phase II enzymes are also inducible, in particular, the UGTs, GSTs, and quinone reductase. However, it is evident that phase II enzymes are far less inducible compared with CYP forms, and this is also displayed in slices. Most of the studies conducted in precision-cut tissue slices focussed on the liver, but there is sufficient experimental evidence that induction can be achieved in other tissues. As in the case of CYP forms, two approaches may be used to determine induction of phase II enzymes in precision-cut tissue slices. The first involves incubating slices for a defined period of time, removing the slices, and incubating them once again in fresh medium supplemented with a substrate extensively metabolized by conjugation, the most frequently used being 7-hydroxycoumarin, which is readily conjugated with sulfate or D-glucuronic acid. Alternatively, following incubation of tissue slices with the potential inducing agent, the slices are harvested and subsequently homogenized and subcellular fractions (e.g., microsomes and cytosol) are prepared by differential centrifugation. This second approach is particularly useful when information is required for the modulation of individual forms of the enzyme of interest or for mechanistic studies. Once again, changes in enzyme expression can be determined at the activity level, protein level by immunoblotting, and mRNA by using techniques such as RT-PCR.

15.6.1 Studies in Liver Slices from Animal Species

In order to be able to detect and quantify the inductive potential of xenobiotics, incubation conditions must be first optimised to ensure that maximum induction is attained. One of the most critical factors is the length of incubation time of the tissue slice with the inducing agent. Of course, this will also depend on the biomarker employed to assess the inductive effect, such as mRNA levels or EA. Using the polycyclic aromatic hydrocarbon BP as the model inducing agent, it was revealed that maximum induction of GST activity, determined using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole as the substrate that is preferentially metabolized by GST α [82], was attained after 24 h incubation and was accompanied by a rise in the enzyme protein levels; no significant change in activity was detected at the earlier time points studied [52]. At the mRNA level, however, an increase was clearly evident as early as 4 h, this being the earliest time point investigated. A similar picture emerged when epoxide hydrolase, monitored using benzo[*a*]pyrene 4,5-oxide, was the investigated enzyme [52]. At the mRNA level, exposure to PB doubled UGT mRNA levels (UGT2B12) in rat slices, but the increase was seen only after incubation of 24 h but not at 2 or 6 h [81].

The next issue that merits discussion is the choice of concentrations of the potential inducing agent; a major attribute of the precision-cut system is that it allows the facile use of a range of concentrations to be investigated. Of course, the chosen range should reflect the concentrations of relevance to human, for example, total plasma, or tissue when available, concentrations following therapeutic intake of a medicinal drug, exposure to an environmental contaminant, or dietary intake of a phytochemical. An initial study should be undertaken in all cases to ensure that the chosen concentrations do not compromise the viability of tissue slices.

Studies employing a number of diverse inducing agents indicated that the upregulation of phase II enzyme systems is concentration dependent. When rat liver slices were incubated with a range of concentrations of BP and, subsequently, the slices were used to assess epoxide hydrolase activity using benzo[*a*]pyrene 4,5-oxide as substrate, a significant increase in activity was seen only at the 10 μ M concentration, and declined at higher concentrations [52]. Increases at the enzyme protein level were detectable at lower concentrations, and when mRNA levels were determined, a significant rise was also observed at relatively much lower doses (0.75 μ M); in both cases maximum elevation occurred at the 10 μ M concentration. Similarly, other PCHs capable of upregulating this enzyme caused a maximal response, either at the activity or protein levels, at about 5–10 μ M, with the response declining at higher concentrations; at the mRNA level, once again increases were evident at much lower concentrations (0.10–0.75 μ M) [83]. When GST activity, determined using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole as substrate, was monitored, a similar picture was observed following incubation of BP with rat liver slices, in that maximal rise in activity and protein levels was attained at about 10 μ M; in this case, a rise in mRNA levels was evident even at 0.1 μ M. The PCHs dibenzo[*a, h*]anthracene and benzo[*b*]fluoranthene also achieved maximal induction of activity and enzyme protein levels at concentrations of 5–10 μ M with increases in mRNA levels being manifested at much lower concentrations [84].

The value of slices for evaluating the upregulation of phase II enzymes can be illustrated by the use of isothiocyanates, a major class of chemopreventive phytochemicals present in cruciferous vegetables, whose mechanism of action involves detoxification of the reactive intermediates of carcinogenic chemicals [85]. Three such isothiocyanates, namely, ERUC, SULF, and PEITC, upregulated GSTs as well as quinone reductase (a phase II enzyme system that detoxifies quinones) in hepatic precision-cut rat liver slices [86,87].

15.6.2 Studies in Human Liver Slices

Available literature on the induction of phase II enzyme systems in human tissues is limited and largely confined to the liver. Following a 24 h incubation, four PCHs, namely, BP, dibenzo[*a, h*]anthracene, fluoranthene, and benzo[*b*]fluoranthene significantly elevated, in a concentration-dependent manner, epoxide hydrolase activity determined using benzo[*a*]pyrene 4,5-oxide as substrate; parallel increases in enzyme protein levels were also observed [83]. GST activity and expression, on the other hand, were elevated only by benzo[*b*]fluoranthene [84]. At the mRNA level, UGT1A1 and UGT1A6 were upregulated after a 24 h incubation with BNF, whereas when RIF or PB was used as the inducing agent, not all livers responded [74].

Incubation of precision-cut human intestine slices with BNF for 24 h increased UGT1A6 mRNA levels, but in colon slices, there was a very marked variability that

precluded attainment of statistical significance. Similarly, RIF increased mRNA levels in the intestine, but there was a wide variation in the colon. Incubation with quercetin led to a rise in UGT1A6 mRNA levels in the small intestine; a similar increase was seen in the colon but only based on a single human sample [19].

In studies aimed to establish the effect of isothiocyanates on GSTs and quinone reductase, upregulation of these enzymes being considered as an important mechanism of their chemopreventive activity, precision-cut liver slices from human donors were exposed to ERUC, SULF, and PEITC for 24 h [86,87]. In general, marked differences in response were noted illustrating that there are very pronounced differences in the response of human liver to isothiocyanates, raising the possibility that the chemopreventive effect of isothiocyanates may not be manifested in all individuals.

15.6.3 Studies in Extrahepatic Slices

The decline of phase II EAs following culture of precision-cut slices is not confined to the liver. In precision-cut rat lung slices, the glucuronidation of 1-naphthol and sulfation of 2-naphthol were very well maintained over a 72 h incubation period, whereas 50% of GST activity was retained; conversely, under the same conditions, CYP forms were not as successfully maintained [88].

When BP was incubated with precision-cut rat lung slices, a rise in epoxide hydrolase activity was noted only after a 24 h incubation, but an increase in enzyme protein could be observed after an 8 h incubation, although maximal induction necessitated a 24 h incubation [52]. When precision-cut rat small intestine slices were incubated with *tert*-butylhydroquinone, an increase in the glucuronidation of 7-hydroxycoumarin was observed after 24 h but not 5 h [78]. Collectively, these studies demonstrate that the complex signaling systems that regulate the induction of phase II enzymes remain intact and functioning even after a 24 h incubation. Clearly, the length of incubation time required to achieve maximal induction of phase II enzymes may be dictated by the nature of the tissue as well as the enzyme under investigation.

Surprisingly, in precision-cut lung slices exposed to BP, epoxide hydrolase activity was elevated only at 1 μ M, that is, a far lower concentration compared with liver, and this was also evident in the case of other PCHs [52,83]. Whether this difference in concentration reflects the higher metabolic breakdown of BP in the liver compared with lung, thus leading to a lower effective concentration of the inducing agent remains to be established. When precision-cut rat lung slices were incubated with ERUC and SULF, both isothiocyanates elevated quinone reductase and GST activities [77]. In slices prepared from rat small intestine and colon, PB, at a concentration of 4 mM but not 2 mM, enhanced glucuronidation, but not sulfation, of 7-hydroxycoumarin. At a concentration of 8 mM, glucuronidation was markedly impaired; similarly, sulfation decreased, and these effects were paralleled by a drop in viability as exemplified by lower intracellular ATP levels [78]. These studies help illustrate the importance of studying a range of concentrations and ensuring that these do not influence adversely slice viability.

15.7 CRYOPRESERVATION OF PRECISION-CUT TISSUE SLICES

When the validity of the precision-cut tissue system was established, it was inevitable that a lot of effort would be devoted into establishing conditions under which slices

could be cryopreserved so that their functional viability is preserved. This is a pivotal issue particularly with respect to human tissue, the supply of which is limited and unpredictable. In cases where human liver is made available but cannot be immediately used in its entirety, slices could be produced and cryopreserved for storage and subsequent use. Similar considerations apply to tissue from large animals such as dogs, monkeys, and food-producing animals. Cryopreservation would allow a comparison of the metabolic profile of a compound of interest to be investigated so that an appropriate surrogate animal can be chosen for toxicological studies. Moreover, successful maintenance of tissue slices through cryopreservation will inevitably lead to a reduction in animal use.

Considerable effort has been expended into defining conditions of cryopreservation that would maintain the functional characteristics of slices as near as possible to what is observed in fresh slices. For slice viability to be conserved, it is essential that the injury to cells that occurs during cooling and thawing processes is minimised. As a result, the objective is to prevent the formation of ice crystals, which are believed to be responsible for cell death following cryopreservation. Surprisingly, however, it appears that liver slices can survive cryopreservation, following rapid freezing, even when ice crystals are formed intracellularly [89], indicating that other factors may be responsible for the loss of viability. Three fundamental procedures have been adopted to achieve successful cryopreservation of tissue slices: (i) computer-controlled slow freezing to allow cellular dehydration by removal of water from the cells, so that ice crystals are produced extracellularly; (ii) rapid, or even ultrarapid freezing, a much simpler procedure that utilizes relatively lower concentrations of cryoprotectants; slices in the medium are placed in cryovials that are directly submerged to liquid nitrogen; and finally, (iii) vitrification, a process that deploys high concentrations of cryoprotectants, which in itself has the potential to cause toxicity, and employs flash freezing; it leads to the formation of amorphous rather than crystalline intracellular ice. A number of studies have been carried out aimed at defining the conditions that achieve the highest slice viability. To assess slice viability, various parameters have been used, including leakage of lactate dehydrogenase, protein synthesis, intracellular levels of potassium and ATP, histological evaluation, and metabolic functionality, the most frequently employed model substrates being testosterone and 7-ethoxycoumarin. This chapter focusses on metabolic viability when such data is available because of its paramount importance in studies of xenobiotic metabolism and toxicity.

All initial work concerned with cryopreservation emanates from the University of Arizona, where the preparation of precision-cut tissue slices was pioneered with the development of the Krumdieck automatic slicer [1,2]. The potential for cryopreservation was shown in studies employing liver slices from human and pig, an anatomically related animal species, using dimethyl sulfoxide (DMSO) as the cryoprotectant and assessing slice viability using intracellular potassium and protein synthesis as biomarkers [90]; the same procedure was also applicable to human kidney slices [91] as well as to other animal species such as dog and monkeys [91,92]. Vitrification by immersing human slices into liquid nitrogen, using 1,2-propanediol as cryoprotectant, was also demonstrated to be feasible in maintaining viable slices [93]. In these studies, the metabolic competence of the slices was evaluated using 7-ethoxycoumarin; the cryopreserved tissue could catalyze both the CYP-mediated oxidative deethylation of the substrate and the subsequent conjugation of the 7-hydroxycoumarin, demonstrating that the slices maintained the integration of phase I and phase II metabolism of

xenobiotics. However, metabolic integrity of human slices could also be achieved by rapid freezing of the slices in cryovials with DMSO serving as cryoprotectant [94]. In these studies, the metabolism of both exogenous, lidocaine and 7-ethoxycoumarin, and endogenous, testosterone, substrates was used to monitor metabolic function; the same metabolites were generated by the cryopreserved slices as with fresh slices, indicating that the metabolic pattern was not modulated as a result of cryopreservation. Better metabolic viability was reported when rat or human slices were frozen sandwiched between aluminum plates than when frozen in cryovials [95]. In a simplification of this method, the slices were frozen sandwiched between standard aluminum foil; the deethylation of 7-ethoxycoumarin was fully maintained in comparison with fresh slices [96].

More detailed studies were undertaken to better define cryopreservation conditions by comparing the freezing/thawing conditions and the importance of the cryopreservation medium. In these studies, it became evident that the critical aspects of the cryopreservation process were the freezing rate and the nature of the cryopreservation medium. On the basis of studies carried out utilizing rat liver slices, it was proposed that rapid freezing and the Williams Medium E, as compared with the University of Wisconsin organ preservation medium, should be applied to all cryopreservation protocols [97,98]. The optimum concentration of DMSO, itself a toxic chemical, has also been investigated [98]; these authors employed three different concentrations of DMSO, namely, 12%, 18%, and 30%, and observed that metabolic functionality, assessed using 7-hydroxycoumarin and testosterone as substrates, was best maintained at a concentration of 18%. Similar studies by other research groups led to the same conclusion [99]. Even after a six-month storage period, metabolic function of cryopreserved rat and human liver slices, determined using testosterone as biomarker, was well retained [100]; sulfation and conjugation of 7-ethoxycoumarin was fully retained in cryopreserved rat liver slices, but in human slices glucuronidation was halved. It has also been reported that the survival of cryopreserved rat slices post thawing could be extended if the slices were cryopreserved in Williams E medium by rapid freezing and using a high concentration of DMSO (30%). In these studies, metabolic competence was monitored using a number of substrates, but the effect was substrate dependent; for example, the glucuronidation of *p*-nitrophenol was only maintained for 2 h after thawing, whereas the glucuronidation of 4-methylumbelliferone was fully maintained for 24 h, implying that stability may depend on the nature of the enzyme monitored [101]. Whether DMSO concentration modulates the levels of enzyme protein and/or cofactor availability remains to be established. The effect of ultrarapid freezing with DMSO as cryoprotectant has also been studied. Once again metabolic function was dependent on the enzyme monitored; the CYP-mediated hydroxylation of midazolam was well maintained, whereas the conjugation of paracetamol (acetaminophen) with sulfate and D-glucuronic acid was markedly impaired [102]. Interestingly, the metabolic viability of slices, assessed by using testosterone and conjugation with glutathione, was inadequate when cryopreservation was controlled using a computer-controlled slow freezing procedure, which is successful in the case of hepatocytes [103]. Finally, as far as slice thickness is concerned, some researchers reported that it had no effect [97], whereas others observed that thin slices were better maintained, possibly as a result of inadequate diffusion of the cryoprotectant in the thick slices to secure uniform cryopreservation [98].

In a study aimed at assessing the viability of individual CYP forms, seven substrates were used to investigate the metabolic function of rat liver slices following cryopreservation; the rate of metabolism of these substrates, as exemplified by intrinsic clearance Cl_{int} , was not influenced by cryopreservation [104] indicating that it is unlikely that individual CYP forms exhibit different sensitivity to cryopreservation. When metabolic viability of liver slices from a number of animal species, including rat, man, and dog, was assessed, the conjugation of 7-hydroxycoumarin was well maintained after cryopreservation by rapid freezing, and testosterone hydroxylation even increased in the rat and mouse [105].

The vast majority of studies concerned with cryopreservation were conducted in liver slices, and it was anticipated that slices from other tissues would display similar behavior. Using a toxicological end point, namely the ammoniagenic effect induced by the antiepileptic drug valproate, it was established that human precision-cut renal cortical slices were very well preserved in comparison with fresh slices; similarly LDH leakage was unaffected but intracellular levels of ATP decreased [106]. In a recent study, the cryopreservation of rat liver and kidney slices by rapid freezing and vitrification was investigated; viability was assessed using intracellular ATP levels and histologically. As expected, liver slices were viable following rapid freezing but, in contrast, kidney slices were susceptible and viability was severely curtailed [107]. Vitrification of liver slices with VS4, a mixture of 1,2-propanediol, formamide, and DMSO, or VS3, a mixture of ethylene glycol, formamide, and DMSO, led to a marked loss in viability despite the fact that no ice was formed. Vitrification of slices from the renal medulla using VS4 was successful in almost completely maintaining viability but was not suitable for slices from the renal cortex. When vitrification was performed using VS3, kidney slices from both the cortex and medulla were well maintained; thus it would appear that kidney slices are more suited to vitrification. It would be informative to assess the metabolic function of the slices under these conditions. Clearly, one cannot extrapolate from one tissue to another even if they originate from the same animal species.

The ability of cryopreserved tissue slices to respond to inducing agents, that is, the viability of the enzyme synthesis apparatus, has not received much attention. Although cryopreserved precision-cut human slices could respond to the CYP1 inducer BNF, at least at the mRNA level, the effect was less pronounced compared to fresh slices [108]. Subsequent studies by the same workers revealed that the same agent caused upregulation of CYP1 activity level, assessed using 7-ethoxycoumarin, to the same extent in fresh and cryopreserved slices [101]. Elevation of CYP2B1 and CYP3A1 mRNA levels was also demonstrated, to a similar extent, in both fresh and cryopreserved rat liver slices incubated with PB and PCN, respectively [42]. Clearly, more extensive studies are warranted in this area not only to establish inducibility of CYP forms and other xenobiotic-metabolizing enzymes in cryopreserved slice but also to ascertain whether CYP-form-specific differences exist. Inducibility of phase II enzyme systems in cryopreserved slices has not been examined. Moreover, the impact of cryopreservation on the induction process in human liver as well as animal and human extrahepatic tissues are issues that merit further in depth investigation, bearing in mind their potential value.

It is abundantly clear that the success of cryopreservation is dependent on the tissue in question, the animal species from which it derives as well as the nature of the chosen biomarkers for assessing viability. Despite the considerable amount of published

literature in this area, there is no universally agreed and adopted protocol for cryopreservation of liver slices, but rapid freezing in DMSO appears to be effective in most cases.

15.8 CONCLUSIONS

Tissue slices have been used for many decades in biochemical studies, especially in the unraveling of the pathways of carbohydrate metabolism by, among others, Krebs and Warburg. However, because of the difficulty in generating viable slices of uniform thickness, this *in vitro* system was abandoned and was gradually replaced by alternatives, such as primary hepatocytes and cell lines. What reignited interest in this system is the construction, some 30 years ago, by Krumdieck and coworkers of a tissue slicer capable of producing viable thin slices of reproducible thickness, from a number of tissues, which led to the development of the precision-cut tissue slice technique [1–5,20]. The facile generation of tissue slices and the potential applicability of this technique to the use of human tissue attracted the attention of researchers in both academia and industry, and automatic tissue slicers (e.g., Krumdieck, Brendel-Vitron), incubation systems and other equipment were developed and became commercially available. In addition, the published literature describes suitable media for the preparation and culture of precision-cut tissue slices [3,4,20].

Precision-cut slices, as they maintain the cellular architecture of the tissue, function as “mini tissues,” and as such they represent more precisely the *in vivo* situation. A major advantage of the precision-cut tissue slice technique is that once suitable conditions for the preparation of tissue slices from one species (e.g., rat liver slices) have been established in a laboratory, the technique can be readily applied to tissue slices from other species. Moreover, while precision-cut tissue slices can be easily prepared from “solid” tissues (e.g. liver, kidney), procedures have also been established for the preparation of tissue slices from “soft” tissues such as the lung and intestine [1–5,20]. Furthermore, precision-cut slices can be successfully cryopreserved, which is of enormous benefit in human studies, since the availability of fresh human tissue is very limited.

Many studies have been performed with precision-cut liver slices and some studies have compared liver slices with other *in vitro* systems. For example, in a series of investigations with three drugs, namely, almokalant, carbamazepine (CARB), and selegiline, the ability of human liver slices to predict pathways and rates of drug metabolism was compared with human hepatocytes, liver microsomes, and cDNA-expressed CYP forms [109–111]. As both liver slices and hepatocytes contain a full complement of phase I and II xenobiotic-metabolizing enzymes, these systems were superior to liver microsomes and expressed CYP forms in predicting pathways of metabolism. However, human liver slices were less successful than hepatocytes in predicting the intrinsic clearance of the three drugs examined.

A number of studies described in this chapter have established that the induction of xenobiotic-metabolizing enzymes in precision-cut tissue slices concords with *in vivo* observations in animals. All classical established inducers of CYP forms and phase II enzymes have been investigated in tissue slices where they provoked the same response as observed *in vivo*, demonstrating the validity of the system. For human hepatic CYP form induction, cultured hepatocytes are considered to be the “gold standard” and are recommended by the US Food and Drug Administration (FDA) for *in vitro* studies

to assess the potential of a new therapeutic agent to induce hepatic CYP forms in humans [112]. Human hepatocytes have some advantages as, unlike human liver slices, both freshly isolated and cryopreserved human hepatocytes are readily commercially available and human hepatocytes can be cultured in a 96-well plate format, which permits the rapid screening of a large number of chemicals. However, as described in this chapter, precision-cut liver slices can also be employed to screen compounds for their potential to induce CYP forms in human liver.

In conclusion, precision-cut tissue slices are a valuable *in vitro* model system to assess the effects of NCEs on xenobiotic-metabolizing enzymes in the liver and other tissues, from animals and humans. This technique allows the facile and simultaneous comparison of the potential response of different tissues, emanating from one or more animal species, to drugs and other chemicals that would not be possible with other *in vitro* systems, or would have necessitated the use of many animals in *in vivo* studies.

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16 The Impact of Solubility and Dissolution Assessment on Formulation Strategy and Implications for Oral Drug Disposition

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16.1	Summary	1
16.2	Introduction	2
16.3	Rate-limiting-steps in oral drug absorption	3
16.4	Biopharmaceutical classification system (BCS) and biopharmaceutical drug disposition classification system (BDDCS)	9
16.5	Solubility and dissolution	15
16.6	Impact of solubility and dissolution on formulation strategy	38
16.7	Preclinical formulations for candidate profiling	49
16.8	Formulation and excipient effects on drug disposition	51
16.9	Conclusions and future perspectives	52
	Acknowledgments	53
	References	53

16.1 SUMMARY

For selecting the best drug candidate for development, discovery and development scientists need a profound understanding of the importance of drug solubility and dissolution to identify appropriate formulations/formulation strategies and to estimate formulation impact on drug disposition. This chapter provides detailed information on the importance of solubility and dissolution as rate-limiting steps for oral absorption and reviews formulation approaches to address them. The relevance of solubility and dissolution data for the categorization of compounds according to the Biopharmaceutical Classification System (BCS) and the Biopharmaceutical Drug Disposition Classification System (BDDCS) is highlighted, and the role of maximum absorbable

dose (MAD) as a simple tool for rough estimates of drug absorption is assessed. Also, common elements of solubility and dissolution protocols are presented, and methods addressing equilibrium, apparent, kinetic, and intrinsic solubility, as well as powder and intrinsic dissolution are evaluated. The importance of drug solid state properties for solubility, dissolution, and oral absorption is reviewed, and its early assessment and continuous monitoring in drug batches, assays, and formulations throughout discovery and development is stressed on several occasions in the review as important information to better estimate or reduce development risks. The usefulness of solubility and dissolution data for the identification of appropriate formulations and formulation strategies is discussed. A detailed description of available formulation approaches is provided, including improvement of drug dissolution rate and/or apparent solubility, drug solubilization approaches, and inhibition of drug precipitation. Finally, some general aspects of preclinical formulation strategies and the effect of selected formulations and excipients on bioavailability (BA), pharmacokinetics (PK), and pharmacodynamics (PD) of drugs are briefly discussed. Overall, this review highlights the importance of solubility and dissolution measurements and demonstrates that formulations can, unintentionally or intentionally, have a huge impact on drug disposition and candidate selection.

16.2 INTRODUCTION

Development of new drugs becomes increasingly complex and requires a close, cross-functional cooperation of a multitude of scientific disciplines. Many processes in drug discovery and development are now tightly interfaced and parallelized, and understanding each others terminologies, technologies, needs, and processes is essential and has a huge impact on the speed and efficiency of the drug development process and on the time to market.

After new molecular entities (NMEs) with desired pharmacological properties have been identified in discovery, their physical and chemical properties need to be systematically explored and optimized to rapidly select a “druglike,” “developable” lead compound [1–5]. Ideally, this lead compound combines potency, selectivity, no or acceptable toxicity, and a favorable PK profile into a single molecule. In parallel with the physicochemical characterization of drug candidates, appropriate initial oral and/or parenteral formulations have to be developed to support animal PK, PD, and toxicological studies. Both formulation type and selected pharmaceutical excipients in formulations can markedly affect the absorption, distribution, metabolism, and elimination (ADME) of potential drug candidates, hence, unreflected use of formulations in early PK studies may unintentionally alter the intrinsic PK characteristics of potential drug candidates with sometimes detrimental effects on candidate selection and drug development [6]. Therefore, scientists working in this phase and beyond must have a profound knowledge of not only the drug’s physicochemical properties relevant for development but also a mechanistic and conceptual understanding of the available formulation types and formulation constituents to select appropriate formulations, to interpret PK results correctly, and to actively modulate drug disposition when needed.

Traditionally, medicinal chemists have focused on ligand selectivity and specificity, and during lead optimization, compound properties were chemically modified until the desired PD response, PK profile, metabolic and chemical stability, solubility, and/or

permeability was achieved. Compounds were then handed over to development scientists to test their biopharmaceutical properties and, in case of development liabilities, they were expected to “fix” them, somehow, by formulation approaches. In the past, this often resulted in high attrition rates in later phases or increased development times and costs since compounds turned out to have unfavorable properties for development. Fortunately, this mindset has changed in most research organizations [7]. Development scientists are now becoming involved in drug discovery programs during *in vitro* and *in vivo* optimization itself and can bring in much earlier their thinking and understanding of success factors that are important along the pipeline. This close interaction at the discovery-development interface is expected to address developability criteria early on and to reduce the compound attrition rate during later development.

Despite these changes and efforts for multidimensional optimization of drug candidates, the “new” process does not necessarily yield a “perfect” candidate. Many different properties have to be balanced against each other in one molecule, and optimizing one property may actually compromise another one [5]. Finally, during or after this iterative optimization process, development scientists receive intermediate or final candidate molecules for formulation without an option to further alter the molecular structure according to their formulation needs; an exception is the generation of salts in case of acidic or basic drug molecules. At present development scientists usually first collect available information or generate new data on the most important biopharmaceutical properties of the compound, such as ionization constants, lipophilicity, chemical stability, compatibility, permeability, solubility, and dissolution. These data provide insight into potential compound liabilities and are used to identify suitable formulation options. Unfortunately, developers have limited options to rescue compounds with poor druglike properties by formulation. Metabolic instability and permeability issues typically require additional chemical optimization cycles and can, if at all, only partially be addressed by formulation approaches (Section 16.8). In contrast, developers have a long tradition of handling compounds with dissolution and solubility limitations and a broad range of enabling technologies exist to deliver even high doses.

This review focuses on the importance of dissolution and solubility for the overall *in vivo* performance of drug molecules. The principals and experimental methods for solubility and dissolution measurement are described and both traditional and high throughput (HT) screening (HTS) methods are presented, and their limits highlighted. The importance of dissolution and solubility data for the classification of drugs, the selection of appropriate formulation strategies, and the rational choice of formulation constituents are depicted with a focus on oral drug delivery strategies. Finally, the complex molecular interactions of formulated NMEs in different *in vitro* and *in vivo* surrounding media are discussed. This review does not address rational drug design, identification and optimization of chemical lead structures [8], the pros and cons of HTS strategies [9,10], and the impact of HTS on molecular weight and lipophilicity [11,12] since this has been the subject of several recent and detailed review articles.

16.3 RATE-LIMITING-STEPS IN ORAL DRUG ABSORPTION

Among the different routes of drug administration, the oral route is still the most preferred with respect to patient compliance and for commercial reasons. To achieve high oral absorption, drugs must go into solution, maintain the dissolved state in the

gastrointestinal tract (GIT), and, except for locally acting compounds, must permeate across the intestinal membrane to reach the circulation. Any of these steps may be rate limiting for oral drug absorption.

Membrane permeation is the first barrier for oral drug absorption. Under sink conditions (i.e., drug is immediately removed from the site of absorption and its concentration equals zero inside the membrane), the drug flux (J) (mass/area/time in $\text{mg}/\text{cm}^2/\text{s}$) through the intestinal wall is the effective permeability of the intestinal membrane (P_{eff})(cm/s) and the drug concentration at the intestinal wall (C_W)(mg/cm^3) [13].

$$J = P_{\text{eff}}C_W \quad (16.1)$$

The local mass absorbed per unit time, the absorption rate, depends on the effective surface area (A)(cm^2) and on C_W , which is determined by the available intestinal fluid volume (V)(cm^3) and the amount of drug (M)(mg).

$$dM/dt = P_{\text{eff}}(A/V)M(t) \quad (16.2)$$

The overall absorption rate from the entire intestine is then

$$dM/dt = \int \int_A P_{\text{eff}}C_W dA \quad (16.3)$$

where the double integral is over the entire absorbing surface.

To calculate the total mass of drug M absorbed at time t from the lumen, time- and position-dependent changes in surface area, permeability, and local drug concentration in solution have to be considered [14].

$$M(t) = \int_0^t \int \int_A P_{\text{eff}}C_W dA dt \quad (16.4)$$

This very general equation considers (i) increases in surface area exposed to drug after spreading of fluid in the GIT; (ii) regional differences in P_{eff} caused by differences in the morphology or expression of membrane transporters along the intestinal wall, by formulation effects on membranes, or by ionization of molecules; (iii) drug solubility changes due to variable luminal contents; and (iv) changes in fluid volumes by absorbed or secreted fluids or fluids administered along with the drug.

From Equation 16.4 it can be expected that maximal absorption rate occurs if drug concentration is maintained at saturation concentration (C_S) at the intestinal wall during the whole gastrointestinal (GI) transit, stressing the importance of solubility and dissolution for absorption. Based on this equation, a relatively simple approach to rapidly calculate the MAD (mg) in humans was introduced by Johnson and Swindell [15]

$$\text{MAD} = k_a C_S V t \quad (16.5)$$

where k_a (min^{-1}) is a first-order absorption rate constant and V is the small intestinal water volume (SIWV). The latter may be determined directly, or indirectly, as the slope of the linear relationship between the logarithm of the amount unabsorbed and t , when

TABLE 16.1 Input Parameters for the Calculation of the Maximum Absorbable Dose (MAD) (Eq. 16.6 and 16.7)

	Species			
Parameter		Rat	Dog	Human
Permeability k_a (min^{-1}) P_{eff} (cmsec^{-1} or cmmin^{-1})	High	$>5.02 \times 10^{-5}$ (cmsec^{-1}) (20×10^{-4} cmmin^{-1})	Jejunum/ileum = 0.044/0.11 (min^{-1})	0.0134 (min^{-1})
	Medium	$<4.2 \times 10^{-5}$ (cmsec^{-1})	Jejunum/ileum = 0.0012/0.018 (min^{-1})	0.003 (min^{-1})
	Low			0.0006 (min^{-1})
t (min)		120-180	120	180
V (mL)		3.2 (fasted) 7.8 (fed)	4 (per kg)	250 (500)
A (cm^{-2})		62	4 (per kg)	800
R (cm)		0.2	0.5	1.5
L (cm)		100	255	350

t , small intestinal transit time; V , small intestinal water volume; A , geometric surface area of the small intestine; R , radius of small intestine; L , length of small intestine

Reference and explanation can be found in Section 25.3.

Absorption rate constants k_a and P_{eff} may be converted using Equation 16.6

natural logarithms, that is, logarithms to the base e , are used. k_a can be estimated from *in vivo* P_{eff} by a volume (cm^3) to geometric surface (A) (cm^2) ratio of the intestine as follows:

$$P_{\text{eff}} = k_a(V/A) = k_a(\pi R^2 L / 2\pi RL) = k_a(R/2) \quad (16.6)$$

where R is the radius (1.5 cm) and L is the length (350 cm) of the human small intestine [4] (Table 16.1).

$$\text{MAD} = P_{\text{eff}} C_S 2\pi RLt \quad (16.7)$$

MAD calculations assume a constant permeability along the intestine, and reliable k_a values should be derived from human PK studies. Since such studies are usually not available in early phases, k_a may be derived from the correlations developed between P_{eff} values determined in humans (#640) and in cell lines [16,17] or may even be replaced in the future by parallel artificial membrane permeability assay (PAMPA) values for compounds with pure passive diffusion [18,19]. Gu *et al.* [20] proposed to use for human MAD calculation k_a values of 0.0006 min^{-1} , 0.003 min^{-1} , and 0.0134 min^{-1} for poorly permeable, moderately permeable, and highly permeable compounds [20], respectively; these correspond to Caco-2 P_{eff} of $<20 \text{ nm/s}$, $20\text{--}100 \text{ nm/s}$, and $>100 \text{ nm/s}$, respectively [4]. If validated Caco-2 models are available, human P_{eff} may be calculated for passively diffusing drugs from Caco-2 data; examples are the equations published by Parrott and Lavé [21] [$\log(\text{hum}P_{\text{eff}}) = 0.63 + 0.89 \times \log(\text{Caco-2}P_{\text{eff}})$] and by Sun *et al.* [4] [$\log(\text{hum}P_{\text{eff}}) = 0.7524 \times \log(\text{Caco-2}P_{\text{eff}}) - 0.5441$]. Slightly different k_a values for drug categorization have been suggested by other groups [4,15,22].

A comprehensive summary of measured human P_{eff} values for marketed drugs has been published recently [23]. Detailed descriptions of methods and comparisons of methods [17–19, 23,24] used to determine drug permeability *in vivo* in humans [25,26] and in animals [27,28] or *in vitro* in Caco-2 [29–31], Madin–Darby canine kidney (MDCK) [17,32], or PAMPA model [33,34] are beyond the scope of this review and can be found elsewhere.

The SIWV in man available for dissolving the drug is generally assumed to be approximately 250 mL [15,22,35]; however, this volume might be too conservative for *in vivo* solubility, and for MAD calculations, a volume of 500 mL has been suggested [36–38]. The latter value represents the average SIWV under fasting conditions in humans and could also be a compromise for the 250 mL and 900 mL volumes recommended by FDA guidelines for *in vitro* solubility and dissolution studies, respectively. The mean small intestinal transit time (SITT) (t) in man usually used for MAD calculations is 180 min [4,20]; however, 199 min [39], 240 min [40], 270 min [22] and 360 min [15] have also been reported. The effective available surface area, A , in human proposed by Yu [41] is 800 cm^2 ($11 \text{ cm}^2/\text{kg}$) ($L = 350 \text{ Cm}$; $R = 1.5 \text{ Cm}$); larger value has also been reported [42]. In MAD calculations, C_s is the solubility of the drug in any relevant medium. Since the small intestine is assumed to be the major site of absorption [43], solubility in aqueous buffer at pH 6.5 is used in most cases. However, other pH values have also been reported [20,44] and solubility values from different media such as simulated intestinal fluids (SIF) [45,46] might be more relevant for MAD calculations and for the *in vivo* situation than pure buffer systems [47].

For calculations in other species, MAD input parameters need to be adapted (Table 16.1). In rat, P_{eff} values for high permeability (HP) compounds are $>5.02 \times 10^{-5} \text{ (cm/s)}$ [48] and $20 \times 10^{-4} \text{ (cm/min)}$ [49] and for low permeability (LP) compounds are $<4.2 \times 10^{-5} \text{ Cm/s}$ [48]. In dog, the reported k_a for the medium and HP drugs atenolol (jejunum/ileum $0.0012/0.018 \text{ min}^{-1}$) and propranolol (jejunum/ileum $0.044/0.11 \text{ min}^{-1}$) may be used for guidance [50]. The SIWV volumes proposed are 3.2 mL and 7.8 mL for fasted and fed conditions, respectively [51], and in dog, the water volume administered with the dose, for example, 4 mL/kg , may be used [52]. Average SITT in dog and rat is 120 min [53–55] and 120–150 min [56,57], respectively. The proposed available surface areas for absorption in the small intestine in dog ($L = 255 \text{ Cm}$; $R = 0.5 \text{ Cm}$) [56] and rat ($L = 100 \text{ Cm}$, $R = 0.2 \text{ Cm}$) [49] are $4 \text{ cm}^2/\text{kg}$ [52,58] and 62 cm^2 [56], respectively. As summarized by Varma *et al.* [59], the fraction absorbed (Eq. 16.16) in human correlates well with that in rat and monkey, while dogs show poor correlation and in many instances overpredict human absorption [28]. Human P_{eff} may be calculated from rat P_{eff} using the equation $P_{\text{eff}}(\text{human}) = 11.04 P_{\text{eff}}(\text{rat}) - 0.0003$ [60].

MAD calculations should not be taken as absolute values. Moderate changes in SITT and SIWV do not significantly affect the outcome of a MAD analysis [22]. MAD may be used for rank ordering a series of potential drug candidates [4,44], for predicting food effects [20], for identifying potential reasons of low absorption to trigger additional chemical or formulation activities [22], or for setting certain target properties such as the minimum acceptable solubility for compounds in a project [22,43,61]. For example, an estimated human daily dose of 50 mg of a HP drug ($k_a = 0.03 \text{ min}^{-1}$) will need a minimum solubility of 0.037 mg/mL to be completely absorbed (Eq. 16.5) ($V = 250 \text{ mL}$; $t = 180 \text{ min}$). Limitations of the MAD are that supersaturation, precipitation,

pH-dependent solubility, and solubilization by bile salts/lecithin cannot be adequately modeled.

Even more important, drug dissolution is not considered as a rate-limiting step in MAD calculations. For HP drugs, both drug solubility and dissolution can limit the extent of drug absorption (Eq. 16.4, 16.5, and 16.7). When a dose, M_0 , administered to a SIWV exceeds C_S and drug dissolution rate (= supply) is higher than the uptake rate (= transport across the intestinal barrier), C_W equals C_S and absorption is limited by drug solubility. If dissolution rate is too slow to keep C_S , drug absorption is limited by drug dissolution ($C_W < C_S$). The amount of drug dissolved (m) per unit time (t) is proportional to the difference between C_S (concentration of drug in stagnant layer = equilibrium solubility) and the instantaneous concentration, C at time t , as described by Noyes and Whitney [62].

$$dm/dt = k(C_S - C) \quad (16.8)$$

where k is a constant.

Nernst [63] and Brunner and Tolloczko [64] modified the equation to include other important factors affecting the kinetics of dissolution:

$$dc/dt = DS/Vh(C_S - C) \quad (16.9)$$

where dc/dt is the instantaneous concentration, C at time (t), D the diffusion coefficient (molecular weight dependent, typically $4-8 \times 10^{-6} \text{ cm}^2/\text{s}$ [65]), S the surface area of particle available for dissolution, h the thickness of the stagnant or unstirred water layer (UWL) at the surface of particles, and V the volume of the dissolution medium. Under given experimental conditions, D and h can be regarded as constant and D/h may be replaced by k , also known as *dissolution rate constant* (cm/s). It is assumed that in most cases, a rapid equilibrium is achieved at the solid-liquid interface followed by the rate-controlling diffusion across a thin layer of solution, called *diffusion layer*, into the solution. The latter step is affected by temperature, solution viscosity and composition, degree of agitation, and drug particle size. Depending on the particle size, h may vary (Section 16.5.1.3)

Amidon *et al.* [14,66] have introduced three dimensionless key parameters, D_o , D_n , and A_n , that relate the discussed absorption limiting parameters permeability, dissolution, and solubility, respectively, to the administered dose (M_0).

$$D_o = \frac{M_0/V_0}{C_S} = \text{Dose number} \quad (16.10)$$

$$D_n = \frac{DC_S}{r_0} \frac{4\pi r_0^2}{\frac{4}{3}\pi r_0^3 \rho} t_{\text{res}} = t_{\text{res}} \frac{3DC_S}{r_0^2 \rho} = t_{\text{res}}/t_{\text{Diss}} = \text{Dissolution number} \quad (16.11)$$

$$A_n = \frac{P_{\text{eff}}}{R} t_{\text{res}} = \frac{t_{\text{res}}}{t_{\text{abs}}} = \frac{2P_{\text{eff}}\pi RL}{Q} = \text{Absorption number} \quad (16.12)$$

$$t_{\text{res}} = \frac{\pi R^2 L}{Q} = \text{mean residence time in intestine} \quad (16.13)$$

$$t_{\text{Diss}} = \frac{r_0^2 \rho}{3DC_s} = \text{time required for a particle to dissolve} \quad (16.14)$$

$$\frac{1}{t_{\text{abs}}} = k_{\text{abs}} = \frac{A}{V} P_{\text{eff}} = 2 \frac{P_{\text{eff}}}{R} = \text{the effective absorption rate constant} \quad (16.15)$$

where V_0 is volume available for dissolution, r_0 is the initial particle radius (an average of 25 μm might be used), ρ is the drug density (between 1 and 1.5 for most drugs, an average of 1.3 may be used), Q is the fluid flow rate, and R , L , A , and V are the radius, length, surface area, and volume of the intestinal tube. The dose number, D_o , considers the required dose M_0 (determined from PK and PD studies) and its solubility in the available SIWV, V_0 . If a compound has a high C_s and is administered at low M_0 , D_o is <1 and is not critical. However, if M_0 is high and C_s is low, D_o becomes >1 , and then both the time to completely dissolve drug particles (t_{Diss}) and the time available for absorption (t_{res}) have to be known to estimate, if M_0 can completely go into solution and will be available for absorption. This is expressed by the dissolution number D_n , the ratio between t_{res} and T_{Diss} . If D_n is <1 , not all drug particles will be dissolved during passage through the absorption window in the intestine, and hence drug absorption will be incomplete. Finally, the absorption number, A_n , represents the ratio of the radial absorption rate to the axial convection rate or, in other words, the ratio of the mean residence time of the drug in the intestine to the effective absorption rate constant. It describes the membrane permeation of the drug and provides the information if a drug has a sufficiently high P_{eff} to become completely absorbed during the time it passes its absorption window in the intestine. A_n is species specific since all its variables (R , P_{eff} , L , Q) are species specific. At $A_n = 1$, fraction dose absorbed is 86%, and at $A_n > 1$, complete absorption of a compound is expected, if dissolution and dose do not limit oral absorption [14,66].

In addition to solubility, dissolution, and permeability, drug metabolism is the fourth factor that can significantly limit oral drug BA. Oral BA of a drug should not be mixed up with oral absorption [67]. Oral absorption only refers to the movement of drug across the outer mucosal membrane of the GIT. In contrast, oral BA refers to the availability of the drug at the site of measurement, i.e., general circulation, or to the amount that becomes available at the site of action. Oral BA of a drug (F) can be described as

$$F = f_a x f_g x f_h x \quad (16.16)$$

where f_a , f_g , and f_h are the fractions of intact drug absorbed (f_a) that escape metabolism during passage through the gut wall (f_g) and traverse the liver (f_h) into the systemic circulation [68]. Thus, oral BA can be equal to or less than the fraction absorbed depending on drug metabolism and loss during oral absorption. Before extensive formulation screens are initiated to improve the oral BA of drugs, intestinal and systemic metabolism should always be considered as potential reasons for poor blood levels. This information is extremely important as guidance for the formulation scientist who has a wealth of options to work on compounds with solubility and/or dissolution issues, very few options to rescue compounds with permeability-limited absorption, and almost no tools to handle compounds with metabolic liabilities (Sections 16.6 and 16.8).

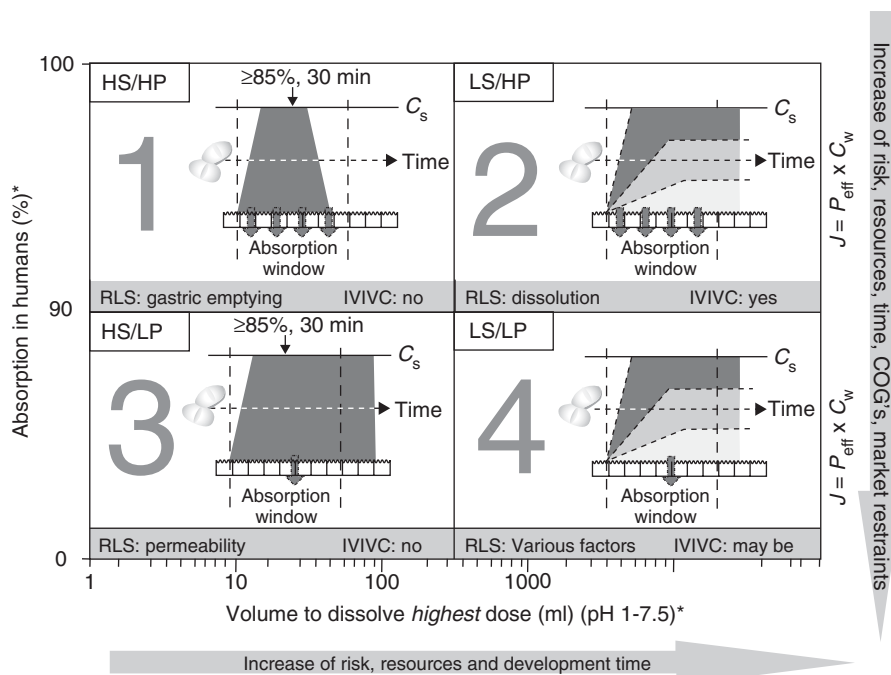


Figure 16.1 The Biopharmaceutics Classification System (BCS) after Amidon *et al.* [14] (section 16.4.1). RLS, rate-limiting step; HS/LS, high/low solubility; IVIVC, *in vitro*–*in vivo* correlation; HP/LP, high/low permeability; C_s , saturation concentration; C_w , concentration at gut wall; J , flux across membrane; COG, costs of goods; P_{eff} , membrane permeability; *FDA, EU, WHO, definitions differ.

16.4 BIOPHARMACEUTICAL CLASSIFICATION SYSTEM (BCS) AND BIOPHARMACEUTICAL DRUG DISPOSITION CLASSIFICATION SYSTEM (BDDCS)

The three fundamental parameters, dissolution, solubility, and permeability, which control the rate and extent of oral absorption, have been recognized by Amidon and coworkers, and they proposed a BCS to categorize drugs accordingly [14,66]. The BCS provides a scientific approach to classify drugs on the basis of solubility as related to dose, intestinal permeability, and dissolution properties of an oral immediate-release (IR) dosage form. It categorizes drugs into four classes, on the basis of high solubility (HS)/low solubility (LS) and high/low permeability (HP/LP) (Fig. 16.1). In 2005, Wu and Benet proposed an extended BCS, the BDDCS, which introduced first-pass metabolism in the intestine and/or liver as the fourth factor for the categorization of drugs [69,70].

16.4.1 BCS

The BCS provides a scientific basis for estimating the absorption of drugs and for establishing *in vitro*–*in vivo* correlations (IVIVC) according to Food and Drug Administration (FDA) guidance. The latter is used as a drug development tool to help sponsors

to request a waiver of *in vivo* BA and/or bioequivalence (BE) studies for highly soluble and highly permeable drugs in IR solid oral dosage forms. It sets the relevant criteria under which certain BE studies may be replaced by accurate *in vitro* dissolution studies. The current FDA guidance on biowaivers considers drugs to be highly soluble if the highest clinical dose dissolves in ≤ 250 mL of aqueous medium at pH 1–7.5 at 37°C [71]. It classifies drugs as highly permeable if the extent of absorption in humans is determined to be $>90\%$ of an administered dose. These HS and HP specifications thus correspond to a D_o of <1 (Eq. 16.10) and an A_n of >1 (Eq. 16.12). Since solubility is a static equilibrium parameter, drug dissolution from a solid dosage form is another important factor in the BCS and reflects the dynamic part of absorption. The role of the solubility to dose ratio for the dissolution and GI absorption kinetics of drugs has recently be re-examined by Papadopoulou *et al.* [38] who suggested physiologically based dissolution (D_I) (equal to D_n) and a permeability (P_I) (equal to A_n) indices for categorizing drugs into low ($D_I \leq 3$) and high ($D_I \geq 3$) solubility and low ($P_I \leq 3$) and high ($P_I \geq 3$) permeability drugs, respectively. According to FDA, for rapidly dissolving compounds, $>85\%$ of a dose has to dissolve in less than 30 min in ≤ 900 mL of the recommended pH 1.2, 4.5, and pH 6.5 buffers using USP apparatus 1 or 2. Under these conditions, D_n is <1 (Eq. 16.11), and the administered dose is expected to be completely dissolved and available for absorption. The European Medicines Agency (EMA) [72] and World Health Organization (WHO) [73] conditions differ slightly from FDA recommendations for BCS classification, a comparison of eligibility for BCS-based biowaivers can be found in Tubic-Grozdanis *et al.* [74]. Applications for biowaivers are not feasible if the drug (i) is not stable in GIT, (ii) has a narrow therapeutic range designation, (iii) is absorbed in oral cavity, or (iv) if excipients affect the rate or extent of drug absorption [71].

Based on these definitions, BCS class 1 compounds are drugs that have HS and HP, dissolve to $>85\%$ within 30 min, and are absorbed to $>90\%$ from the small intestine (Fig. 16.1–1). Accordingly, class 1 compounds have $D_o < 1$, $A_n > 1$, and $D_n < 1$ and are well absorbed. Examples of class 1 drugs are digoxin, metoprolol, and caffeine [75]. The dissolution of these drugs is so rapid that differences in the *in vitro* dissolution profile will not affect BA, and hence an IVIVC with dissolution is not expected. After dissolution, class 1 compounds are maintained in solution throughout the intestine and due to their HP (hum $P_{eff} > 2 \times 10^{-4} \text{ cm/s}^1$; (Caco-2: $>10^{-5} \text{ cm/s}^1$ [75]), complete absorption is expected within the absorption window in the intestine. In PK studies, the absolute amount of drug absorbed should increase with increasing doses. Deviations from linearity may result from drug instability in the lumen, presystemic metabolism, the formation of insoluble complexes, or active secretion from the gut wall. The rate-limiting step (RLS) for class 1 compounds is the gastric emptying rate. In the fasted state, emptying is extremely fast for water, with an overall average half gastric emptying time in human of 22 min and 12 min for a 50 mL and 200 mL volume, respectively [76]. More recent studies indicate considerably longer half gastric emptying times of 80 ± 22 min for caloric fluids and 144 ± 55 min for solids [77]. In animal, under fasted conditions, half gastric emptying time of water in rat is 8 min [78] and in dog, similar to human, 35–45 min has been reported for volumes smaller than 100 mL and 10 min for larger volumes [79,80]. Apart from volume, the rate of emptying is influenced in all species by many factors such as energy content, viscosity, density and particle size of gastric contents, temperature, and amount of acid in the duodenum [42,81].

BCS class 2 drugs are highly membrane permeable; however, they have solubility too low to be consistent with complete absorption. An administered dose (M_0) is no longer soluble in ≤ 250 mL of medium pH 1.2–7.5, and hence has $D_o > 1$ (Eq. 16.10). According to Equation 16.7, the MAD is equal to P_{eff} times C_S . Since class 2 drugs have lower C_S , drug absorption is usually slower than for class 1 compounds. Depending on the dissolution behavior of class 2 drugs, C_S may or may not be achieved *in vivo* in the intestine (Fig. 16.1–2). For rapidly dissolving drugs ($D_n > 1$), C_S is achieved and solubility at the gut wall (C_W) is maintained at C_S throughout the absorption window. In this case, drug solubility becomes the rate-limiting factor for oral absorption if the dose administered is higher than the absolute amount that can be absorbed ($M_0 \gg \text{MAD}$). Therefore, an increase in dose will not increase the MAD. It is already at its maximum, and hence an IVIVC is not expected. According to Yu *et al.* [41], typical parameters for solubility-limited class 2 compounds are $t_{\text{Diss}} < 50$ min (micronized drug), hum $P_{\text{eff}} > 2 \times 10^{-4}$ Cm/s (Caco-2 $P_{\text{eff}} > 10^{-5}$ Cm/s [75]), and $\text{MAD} < M_0$. Examples of class 2 drugs are griseofulvin, naproxen, and phenytoin [75]. For compounds with slower dissolution rates ($D_n < 1$), drug removed from the lumen by absorption is not replaced rapidly enough by the dissolving drug, therefore C_W is $< C_S$ and drug absorption is below its theoretical maximum (Eq. 16.7). Here, the RLS for drug absorption is dissolution, and IVIVC can be expected if the *in vitro* dissolution conditions are similar to the *in vivo* dissolution conditions. Gastric emptying time is less important than for class 1 drugs since the drug is exposed for a much longer time to gut wall. Drugs with dissolution-limited absorption typically have $t_{\text{Diss}} > 199$ min (unmicronized drug), hum $P_{\text{eff}} > 2 \times 10^{-4}$ Cm/s (Caco-2 $P_{\text{eff}} > 10^{-5}$ Cm sec⁻¹ [75]), and $\text{MAD} < M_0$. [41]. They show an increase in the absolute amount of drug absorbed with dose since the surface area of the particles available for dissolution increases (Eq. 16.9). Therefore, particle size can also significantly affect the fraction dose absorbed, and in particular, HP drugs dosed at D_n and D_o values around 1 are expected to show highly variable absorption with changes in particle size (Eq. 16.14) [66]. Other factors affecting the kinetics of dissolution are the drugs' diffusion coefficient, the thickness of the diffusion layer, and the volume of dissolution medium (Eq. 16.9). Since they may all be affected by changes in formulation or by the volume, viscosity, pH, and composition (surfactants, complexing agents, etc.) of the medium, the experimental design of dissolution media mimicking the *in vivo* situation for BCS class 2 compounds is challenging (Section 16.5.4).

Drugs in BCS class 3 have HS and LP, and therefore permeability is assumed to be the RLS in absorption (Fig. 21.1-3). Class 3 compounds have $T_{\text{Diss}} < 50$ min (unmicronized drug), hum $P_{\text{eff}} < 2 \times 10^{-4}$ Cm/s (Caco-2 $P_{\text{eff}} < 2 \times 10^{-6}$ Cm/s [75]), and $\text{MAD} < M_0$ [41]; examples are ranitidine and gancyclovir [75]. Class 3 compounds are expected to behave like oral solutions *in vivo* if $>85\%$ of an administered dose will dissolve *in vitro* in 30 min under the condition of the FDA guideline. However, in contrast to class 1 compounds where HP ensures sink conditions *in vivo*, this may not be true for class 3 compounds. The latter have low P_{eff} *in vivo*, and hence dissolved compounds may not be absorbed rapidly enough to create sink conditions *in vivo* for dissolution in the lumen and C_S may become an additional rate-limiting factor (Eq. 16.7). Therefore, more stringent *in vitro* dissolution conditions, $>85\%$ dissolved *in vitro* in 15 min, have been suggested by Yu *et al.* [36] for class 3 to minimize possible dissolution behavior anomalies [82]. The permeability of class 3 compounds often varies along the intestinal tract if drugs show pH-dependent permeability or are substrates

for active uptake or efflux mechanisms [83]. Drugs with an absorption window or a gradient in the permeability along the intestine may exhibit some variability in the rate and extent of absorption [area under the curve (AUC), maximal concentration ($C_{\max}$)], since physiological factors, food, and formulation constituents may influence the transit time through specific regions and/or modulate membrane permeability (Section 16.8) [84]. For class 3 compounds, IVIVC with dissolution is not expected since permeability is the RLS; the absolute amount of drug absorbed increases with dose. Nonetheless, in case of uniform P_{eff} values along the gut, biowaivers might be considered for rapidly dissolving dosage forms that contain common excipients in usual amounts [37,85,86].

If A_n and D_n are low, a drug is considered as a class 4 drug (Fig. 16.1–4). Both solubility and permeability are rate limiting, and compounds have significant oral delivery problems; examples of compounds are furosemide, Taxol, sulfasalazine, and sulpiride [75,85]. Because of their usually LS in the lumen, BCS class 4 drugs are often more susceptible to efflux transporters and gut metabolism than BCS class 3 drugs [59]. Various RLSs may thus contribute to the overall low oral absorption of class 4 compounds. Nonetheless, an IVIVC may still be established for BCS class 4 compounds that exhibit uniformly low P_{eff} along the intestine and slow dissolution rate. If drug dissolution is slow and C_w remains below C_s throughout the intestine, MAD will not be achieved (Eq. 16.5) and, similar to dissolution-limited BCS class 2 compounds, it might be possible to correlate *in vitro* dissolution with *in vivo* absorption.

16.4.2 BDDCS

The criteria for supporting HP of drugs according to FDA are difficult to achieve since they require human PK data, direct permeability measurement, and detailed information about permeability methodology. Because of this regulatory burden, little predictive use has been made out of BCS, in particular for class 2, 3, and 4 compounds. Therefore, in 2005, Wu and Benet [69] proposed a modified version of the BCS, the BDDCS, which categorizes drugs on the basis of their solubility and extent of metabolism. They suggested that if the major route of elimination for a drug is metabolism, it has HP, while if it is excreted unchanged by renal or biliary elimination, permeability is low. It is believed that metabolism represents a surrogate for drug permeability since only drugs that permeate can be metabolized. Metabolism is much easier to access than permeability by *in vitro* incubation studies with hepatocytes and *in vivo* animal and human studies, and thus drugs can be classified without extensive and expensive human permeability studies. Animals are often poor predictors of human metabolism; however, if drugs are not metabolized in animals, this may already be used to assign drugs to BDDCS class 3 or 4. For regulatory issues, Wu and Benet suggested to designate drugs with >90% metabolism as class 1 and 2 compounds [69]. For biowaivers, class 1 compounds should also meet the FDA criteria for rapid dissolution. Overall, the BDDCS predicts if drugs will be highly absorbed; however, it does not predict the extent of absorption [87].

In addition to metabolism, the BDDCS allows predictions of drug disposition and drug–drug interactions [87] (Fig. 16.2). For class 1 compounds, significant transporter effects on absorption are not expected. The HP permits rapid and unaided absorption from the lumen, and even if drugs are substrates for transporters *in vitro*, their HP will not give transporters sufficient access to them or, due to their HS, drug concentration in the lumen will be high enough to saturate them. AUC and oral BA will thus not be

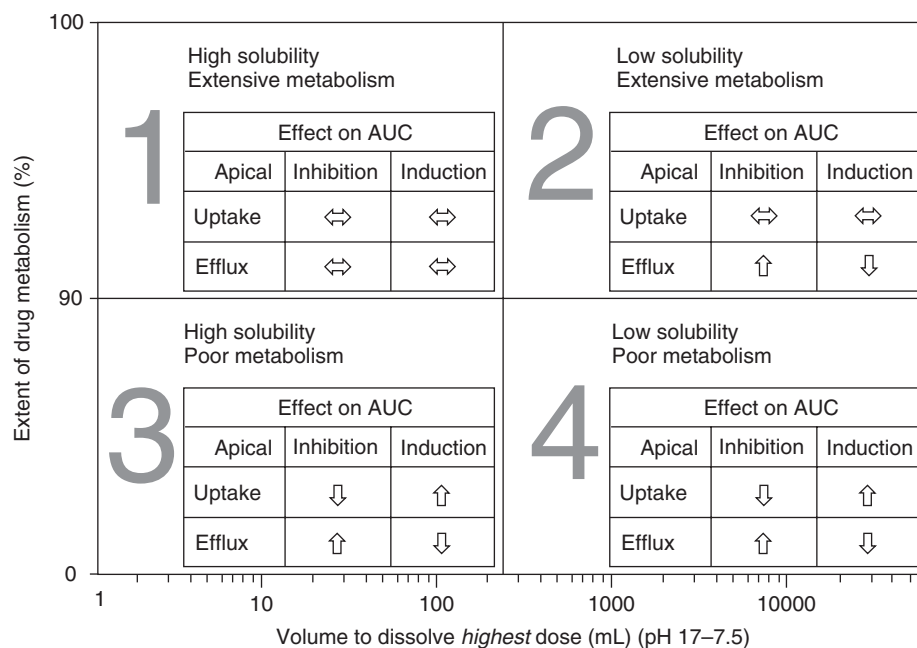


Figure 16.2 The Biopharmaceutics Drug Disposition Classification System (BDDCS) after Wu and Benet [69], and predicted effect (AUC) of inhibition or induction of apical uptake and efflux transporters on oral drug absorption [87] (Section 16.4.2).

influenced by transporters. Class 2 drugs also have HP per se, and hence inhibition or induction of transporters does not affect AUC. However, since LS limits their luminal concentration, saturation of efflux transporters is less likely, and hence inhibition and induction of efflux transporters may increase and decrease AUC, respectively. Drug pumped back into the lumen by efflux transporters will re-enter the enterocytes and thus increase the chance to exposure to metabolizing enzymes in the cell as described previously [88,89]. For class 3 and class 4 compounds, permeability is low and cellular uptake may need transporters, and even for the highly soluble class 3 compounds, intracellular concentration may be too low to saturate efflux transporters. Inhibition of efflux transporters or induction of uptake transporters will increase the AUC of drugs; vice versa, an induction of efflux and inhibition of uptake transporters will decrease it [87,90] (Fig. 16.2). Comparisons show that BDDCS classifies more compounds in class 1 than BCS, and hence more drugs might be eligible for biowaivers [70,90–92]. However, BDDCS has been introduced only recently and the correlation between BCS and BDDCS and their limitations are still under evaluation. Current topics discussed are compounds having HP but low metabolism [92] and how to make use of both BCS and BDDCS for drug classification in the future [87,90,92].

16.4.3 BCS and BDDCS in Drug Discovery and Early Development

BCS and BDDCS are useful tools in discovery and early development to identify RLSs, to rank order compounds, and to communicate potential higher formulation risks. In

later phases, the BCS is used as a regulatory tool to identify IVIVC and to obtain waivers for *in vivo* BA and BE testing according to FDA [93]. Waivers are designed to replace *in vivo* BE studies for IR products by *in vivo* dissolution studies, which can both reduce costs and improve the quality of medicines. Waivers were originally designed only for class 1 drugs; however, extensions to other classes have also been recommended [36,37,94,95].

In discovery, BCS is now also frequently used to identify potential compound liabilities early on and to design appropriate animal and human formulations [96,97]. With the introduction of HTS technologies for the characterization of the physicochemical properties of lead compounds around 1990, a shift in BCS classes is observed. Comparisons of marketed compounds and NMEs performed by several authors revealed a drop in class 1 compounds from 40% to ~20% and an increase in class 2 compounds from 30% to ~60% among the portfolio compounds of major pharmaceutical companies over the past two decades; class 3 and 4 compounds remained in the range of 10–20% [3,7,69,97–99]. This increase in class 2 drugs is presumably the result of the quest for higher binding efficiency, higher potency, and/or targets with properties unfavorable for oral absorption that ultimately led to larger and more lipophilic molecules with overall lower solubility. Lipinski and coworkers have summarized this trend in the famous “Rule of 5,” which indicates that molecules with MW >500, log *P* > 5, > 5 H bond donors (as a sum of NH and OH), and >10 H bond donors (N + O) are less likely to have good oral BA [1]; higher MW also means more rotatable bonds and rings [2]. Such compounds are also more prone to drop out during the development process [11] and to have poor absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties [100].

Decision making based on BCS classification in early phases first requires definition/identification of a drugs’ BCS class on the basis of solubility, dissolution, permeability, and dose information [96,97]. While the first parameter is relatively easy to assess in early phases, the others are less available. Experimental solubility can be determined by traditional shake flask methods or by more compound-saving miniaturized methods. Dissolution testing using standard USP dissolution methods is often not feasible in discovery because of limited compound availability; however, microscale assays have been introduced recently. Particle size measurement may be helpful for dissolution estimation and may be done by simple optical microscopy or by more sophisticated methods such as laser diffraction, centrifugal sedimentation, or scanning electron microscopy (SEM) as reviewed recently [101,102]. A detailed discussion of solubility and dissolution measurements can be found in Sections 16.5.3 and 16.5.4, respectively. As a rule of thumb, dissolution-limited absorption is seldom observed if drug solubility is >0.1 mg/mL [85]. In early phases, drug permeability in humans is not available and permeability estimations for BCS classification need to be derived from established correlations of human absorption values [4] with *in vitro* models (PAMPA [19], Caco-2 [24,32], MDCK [23]), *in situ* studies [27,49], or *in vivo* animal experiments [48]. Also often in this phase the clinical dose is not available and hence *D*_o cannot be calculated accurately. If experimental data are not available, *in silico* tools with different levels of complexity may also be used to predict oral BA [103,104]; however false positives are still possible since large and high quality datasets are often missing and compounds with poor and intermediate absorption are underrepresented in training sets. Commercially available tools for prediction are Stella[®] [105], GastroPlus[™] [106–108], PK-Sim[®] [109] Simcyp[®] [110], and the

TNO integrated software [111]; the pros and cons of absorption modeling have been reviewed in 2009 by Fotaki [112]. In recent studies, provisional BCS classification of marketed drugs was established on the basis of *in silico* calculation of solubility and permeability [91,113]. Here, compounds with a $\log P$ greater than metoprolol ($\log P = 1.72$) were categorized as HP drugs; however carrier-mediated uptake is not considered, hence incorrect classification is possible [92]. Moreover, Shugarts *et al.* [87] recently pointed out that BCS cannot be used for the prediction of drug disposition since the extent of absorption does not correlate with permeability in a number of cases. For example, the BCS class 2 compound talinolol has LS and a high extent of absorption; however, its measured permeability is low. Thus the FDA definition for HP and LP confounds permeability and extent of drug absorption.

In practice, NMEs do not necessarily have to meet BCS class 1 criteria to be developable. Many Class 2 and some class 3 and 4 compounds have also made it to the market. Actually, certain targets may even require properties unfavorable for oral absorption to be highly active [114]. These drugs may still be rescued by administration via a different route, and indeed, a comparison of oral and injectable drugs has shown that the latter tend to have higher molecular weight, more polar groups, lower lipophilicity, and more rotatable bonds than oral drugs [12]. However, it is also clear that the resources to develop drugs increase in the order of BCS class $1 < 2 < 3 = 4$ and that poor physicochemical properties are likely to increase time to market and the attrition rate of drug candidates [12]. Typically, BCS and BDDCS classification is based on experimental data; however, recently, computational models also became available that predicted them reasonably well [91,113,115–117]. These *in silico* tools will hopefully speed up the drug discovery and development process in the future and help to reduce costs. For marketed drugs, a number of provisional classifications have been published [69,91,98,118,119].

16.5 SOLUBILITY AND DISSOLUTION

The previous sections pointed out the importance and the interplay of solubility, dissolution, permeability, and metabolism in combination with the administered dose as potential rate-limiting factors for oral drug delivery. In most companies, a major proportion of new compounds in drug pipelines now tend to fall into BCS class 2 [97], and hence compounds exhibit LS and HP or, according to BDDCS, extensive metabolism (Section 16.4.2). The latter is usually addressed early on by *in vitro* microsomal or cellular assays or by testing in animals, and liabilities identified are corrected by medicinal chemists accordingly by structural modifications, if feasible. Thus for an increasing percentage of drugs, solubility and dissolution become rate-limiting factors for oral absorption and their exact measurement will set the stage for candidate selection and formulation development for compounds that have no permeability issues (BCS class 1 and 2). This section provides an overview of common definitions of different types of solids, solubility, and dissolution and review the experimental methods used to determine them. Several excellent reviews covering solubility [120,121], sparingly soluble ionizable compounds [122], salt formation and selection [123–125], polymorphs [126,127], cocrystals [128], and dissolution [129,130] provide the background for this discussion.

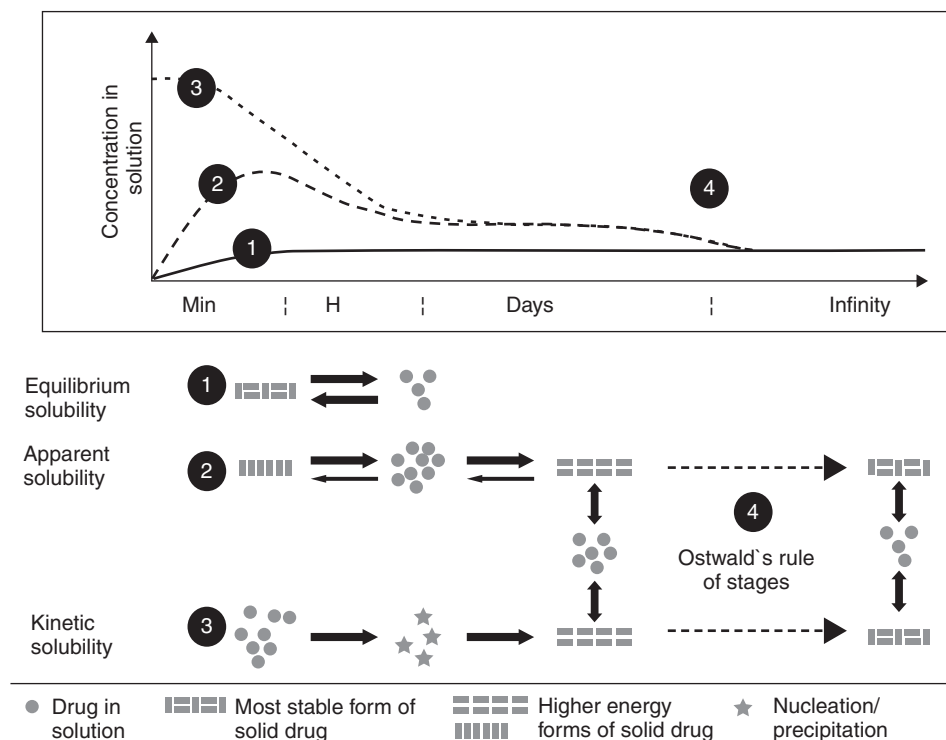


Figure 16.3 Schematic presentation of equilibrium, apparent, and kinetic solubility of drugs. Detailed explanations can be found in Section 16.5.1.1.

16.5.1 Definitions

16.5.1.1 Solubility. Solubility is fundamental in drug absorption, and both PK and PD can be affected by insufficient solubility. However, although solubility is a parameter that is conceptually easy to measure, unclear terminology may cause some misconceptions about solubility as pointed out by Sugano and colleagues [131]. This review basically follows their definitions.

Equilibrium solubility represents the concentration of the solute in its *saturated solution* C_S , where the saturated solution is in a dynamic equilibrium with an excess of undissolved solid compound (Figs. 16.1 and 16.3). As long as temperature, solid concentration, and solid form remain unchanged, the concentration of the solute is constant at equilibrium, since the rate of compound dissolution equals the rate of precipitation. At equilibrium, the dissolved molecule in solution can exist as monomer, dimer, higher self-aggregates, or complexes with larger molecules or be incorporated into micelles [131]. Time course measurements are required to confirm the equilibrium and to exclude transformation of solid into a more stable form. Equilibrium solubility is often regarded as being the “true” solubility; however, measured values can be affected by a multitude of compound properties and experimental artifacts (Table 16.1 in Ref. 120). The general solubility equation (GSE) of Yalkowsky and Banerjee may be used to estimate drug solubility from the octanol/water partition coefficient ($\log P_{OW}$) and

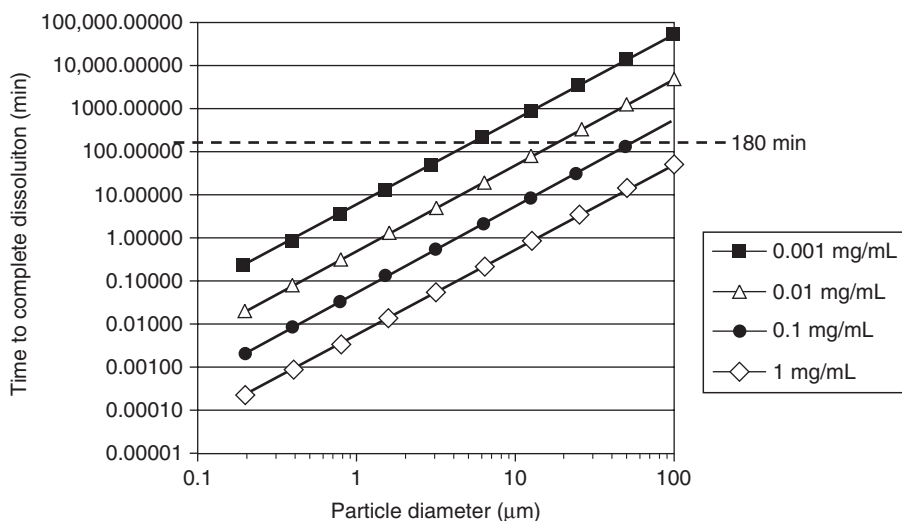


Figure 16.4 Relationship between drug solubility and time required to completely dissolve spherical particles of a given diameter (Section 16.5.1.3).

the melting point (MP) [132].

$$\log S = 0.8 - \log P_{OW} - 0.01(\text{MP} - 25) \quad (16.17)$$

Apparent solubility describes the apparent equilibrium reached after a long incubation time (typically several hours to a day) between drug in solution and a solid (Figs. 16.2 and 16.3). The solid is not necessarily in its most stable form and may be a powder or precipitated solid from for example, a concentrated drug solution in dimethyl sulfoxide (DMSO). Apparent solubility may decrease with time and is distinct from true equilibrium (thermodynamic) solubility, which is reached at infinite equilibrium time.

Kinetic solubility is the solubility measured immediately (minutes to a few hours) after the addition of a concentrated drug stock solution into an aqueous medium (Figs. 16.3 and 16.3). Therefore, kinetic solubility measures an antisolvent precipitation rather than solubility and represents the maximum solubility of the fastest precipitating species of a compound [121]; hence kinetic solubility is often significantly higher than equilibrium solubility. Kinetic solubility is strongly time dependent, and solubility values are not expected to be reproducible between different kinetic experimental setups (Section 16.5.3.1). In particular, precipitation from organic solutions may generate thermodynamically less favorable solid forms that may converted with time to more stable or to the most stable thermodynamic form according to “Ostwald’s Rule of Stages” [133] (Figs. 16.3 and 16.4)

Intrinsic solubility, S_0 , is defined as the solubility of the uncharged form of molecular species (undissociated, neutral) of an ionizable compound. Intrinsic solubility is determined at a pH where the compound does not dissociate.

Other commonly used terms are unbuffered/buffered solubility and unsaturated/supersaturated solutions. *Unbuffered solubility* means the solubility of a saturated solution of the compound in water at the final pH of the solution, and

buffered solubility is the saturated solubility in a pH-buffered system. In *unsaturated solutions*, compound concentration is below C_S . Under certain conditions, *supersaturated solutions* may also form, that is, drug concentration in solution exceeds C_S (Section 16.6.5). Brouwers *et al.* [134] has suggested to use the supersaturation ratio (S) and the supersaturation index (σ) for their characterization.

$$S = \frac{C}{C_S} \quad (16.18)$$

$$\sigma = \frac{C - C_S}{C_S} \quad (16.19)$$

where unsaturated, saturated, and supersaturated solutions have $\sigma < 1$ ($S < 1$), $\sigma = 0$ ($S = 1$), and $\sigma > 0$ ($S > 1$), respectively.

16.5.1.2 Solids. The solids used to determine equilibrium solubility can exist in several different solid states, such as amorphous form, hydrates, polymorphs, and solvates. *Amorphous solids* have no long-range three-dimensional order and are characterized by disordered, short-range arrangement between constituent molecules like in a liquid. Polymorphism in pharmaceuticals, describes the ability of a compound to exist in 2 or more crystalline forms. *Polymorphs* have the same empirical formula; however, they differ in the arrangement and/or conformation of the molecules in the crystal structure and typically have different lattice energies. In contrast, *solvates* have different empirical formulas and contain the substance and water (hydrates) or other solvent molecules in the crystals.

The solution process of a solid in a solvent can roughly be divided into three steps: first, a molecule is freed from the solute, second, a hole is created in the solvent, and third, the hole in the solvent is filled by a free solute molecule [121,127]. This process will continue until the energy required to break a molecule out of a solid matrix equals the energy returned from solvent–solute interactions. Since amorphous solids require less energy to release molecules from solids, they often have higher apparent solubility than crystalline forms. The solubility advantage between amorphous and crystalline form was predicted to be 10- to 1600-fold; however, the actually measured ratios were only in the range of 1.1- to 24-fold [135]. Nonetheless, much higher ratios may be achieved, and an experimental ratio of amorphous to crystalline form of 6000 has recently been reported for cinnarizine [136]. In contrast, the solubility difference among crystal polymorphs seems to be only in the range of 2- to 5-fold [126]. Experimental apparent solubility values higher than for the pure crystalline form may result from undetected amounts of amorphous substance [135]. As a rule of thumb, hydrates have a lower solubility in water and a higher solubility in solvents miscible with water than their corresponding anhydrides [137,138].

Amorphous forms are thermodynamically unstable and may crystallize. Therefore, if solubility studies start with metastable solids, phase transition to more stable forms may occur if the activation energy barrier can be overcome [127]. Real equilibrium solubility is achieved only when the energy of solvents and solute, to interact with themselves, and of solute and solvent, to interact with each other, is balanced. Thermodynamic solubility would then be the solubility of a saturated solution of the compound in equilibrium with the thermodynamically most stable polymorph at a given temperature and time.

16.5.1.3 Dissolution. Solubility represents a static phenomenon. In contrast, dissolution rate describes the speed at which certain solubility is reached. According to the Nernst–Brunner equation (Eq. 16.9), the dissolution rate is directly proportional to the saturation solubility C_S and the surface area S exposed to the dissolution medium and is expressed as the amount of compound liberated per time per area. During the dissolution of particles, the surface area available for dissolution constantly changes. Hixson and Crowell [139] derived an equation from the Noyes–Whitney equation that expresses the rate of dissolution based on the cube root of the weight of the particles [140]. When this model is applied to micronized particles, the change in particle radius with time is given by [127,140]

$$r^2 = r_0^2 - \frac{2DC_S t}{\rho} \quad (16.20)$$

where r is the radius of the particle at time equal to t , r_0 the initial particle radius, D the diffusion coefficient of the molecules dissolving from the particle, C_S the equilibrium solubility of the compound, and ρ the density of the particle (typical values are $D = 5 \times 10^{-6} \text{ cm}^2/\text{sec}$, $\rho = 1.3 \text{ g/mL}$). The equation assumes an aqueous diffusion layer around the particles comparable to or larger than the particle radius. The time required to complete particle dissolution ($r^2 = 0$) (T_{Diss}) may then be approximated from

$$T_{\text{Diss}} = \frac{\rho r_0^2}{2DC_S} \quad (16.21)$$

In Fig. 16.4, the time to complete dissolution is plotted against the particle diameter for four hypothetical compounds with different solubility. The graph shows, as a rule of thumb, that particle size reduction may not be needed for complete dissolution if the particle diameter (in micronmeter) is smaller than the aqueous solubility (in $\mu\text{g/mL}$). Moreover, compounds with $C_S > 0.1 \text{ mg/mL}$ in the pH range 1–8 and with particle diameter $< 50 \mu\text{m}$ are likely to completely dissolve during SITT (180 min), assuming sink conditions ($C \ll C_S$ in Eq. 16.8), spherical particles, and no particle agglomeration due to bad wetting properties [141]; on the contrary, compounds with aqueous solubility $< 0.1 \text{ mg/mL}$ often present dissolution limitations to absorption [142]. For other particle size ranges, different relationships may apply [130,143]. For example, in the small particle regime, experimental data indicate that dissolution time is proportional to the square of the diameter [144]. For dissolution simulation (Eq. 16.20), Sugano *et al.* [131] suggested to set the diffusion layer thickness (h), equal to the radius (r) for particles $< 30 \mu\text{m}$ and to use $h = 30 \mu\text{m}$ for particles with $r > 30 \mu\text{m}$. Reviews of dissolution modeling and comparison of dissolution profiles can be found elsewhere [130,145].

For ionizable compounds, the pH at the surface of the dissolving particles (pH_0) can deviate up to several units from the pH of the bulk solution since dissolving acids will lower and dissolving bases will increase pH_0 (self-buffering) [146–148]. This difference between pH_0 and the pH of the dissolution medium will rise with increasing intrinsic solubility (S_0) of compounds (=higher buffering capacity of compound) and with decreasing buffering capacity of the dissolution medium. The saturation solubility C_S of a compound at a particular pH can be written for monoprotic

compounds as

$$C_S = S_0(10^{\text{pH}-\text{p}K_a} + 1) \quad \text{or} \quad \log C_S = \log S_0 + \log^{\text{pH}-\text{p}K_a} + 1 \quad (16.22)$$

for an acid and as

$$C_S = S_0(10^{\text{p}K_a-\text{pH}} + 1) \quad \text{or} \quad \log C_S = \log S_0 + \log^{\text{p}K_a-\text{pH}} + 1 \quad (16.23)$$

for a base (equations for di- and triprotic molecules can be found in Ref. 149). As can be seen, a decrease (acids) or an increase in pH (bases) will decrease C_S at the surface of particles compared to C_S in the bulk. Therefore, both the use of bulk C_S in Equation 16.9 or of a buffer system in dissolution assays with a capacity above physiological conditions will overestimate the initial dissolution rate of a compound. A practical method to determine pH_0 was established by Serajuddin and Jarowski [150].

Similar to C_S , D is also inversely proportion to t_{Diss} (Eq. 16.21). The Stokes–Einstein equation describes the relationship of the diffusivity to the viscosity of the medium η , the temperature T in Kelvin, the radius of the drug molecule r_M , and the Boltzmann constant k_B

$$D = \frac{k_B T}{6\pi\eta r_M} \quad (16.24)$$

Diffusion through the UWL, also called *UWL permeability* (P_{UWL}), limits the intestinal permeability of highly permeable drugs as well as their diffusion away from the surface of particles during particle dissolution. P_{UWL} can be described by D , the diffusion layer thickness (h) coefficient, and the molecular weight (MW) and may be modeled by Takano *et al.* [52]

$$P_{\text{UWL}} = \frac{D}{h} = \frac{k_B T}{6\pi\eta r_M} = 16 \times 10^{-4} \times \left(\frac{180}{\text{MW}} \right)^{1/3} \quad (16.25)$$

Therefore, changes in compound diffusivity, for example, by an increase in molecular weight by incorporation into micelles or increases in viscosity by food intake, may significantly decrease P_{UWL} and hence the dissolution time of particles.

Since dissolution rate depends on the available surface area, S , direct comparisons of powders with different particle sizes are difficult to perform. Therefore, investigative studies in drug development are done using the *disk intrinsic dissolution rate* (DIDR) technique. Here, compound is compressed in a punch and die and only one planar face of the compressed powder disk is exposed to the dissolution medium. Under these conditions, the surface area exposed to the medium (typically 0.5 cm^2) [151] is regarded as constant and the DIDR is the amount of compound released from a unit area of the solid drug per time (mg/min/cm^2). According to the modified Noyes–Whitney equation (Eq. 16.9), the dissolution rate per unit area (= mass flux) is then directly proportional to C_S , since at the earliest stage, $C \ll C_S$. For good reproducibility, agitation intensity, dissolution volume, and dissolution vessel configuration need to be well controlled. Yu *et al.* [151] found good correlations between DIDR and BCS solubility class membership with 0.1 mg/min/cm as a class boundary unless the dose is extremely high or low or experimental conditions used for DIDR and solubility differ significantly.

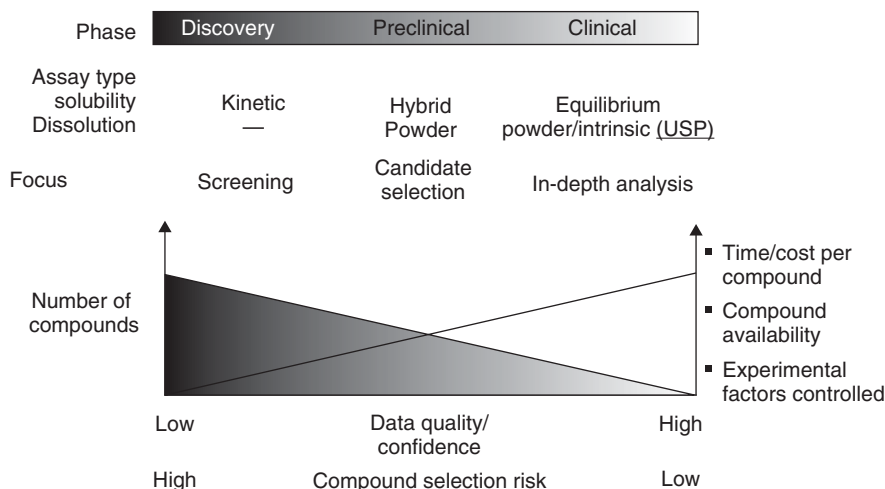


Figure 16.5 Quantitative and qualitative considerations for the setup of solubility and dissolution assays in different phases of drug development (Section 16.5.2.1).

16.5.2 Measurement of Solubility and Dissolution

16.5.2.1 Common Elements of Assays. The focus and setup of solubility and dissolution assays changes from discovery to preclinical and clinical development (Fig. 16.5). In discovery, the number of compounds to test is high and only small quantities of compound are available. Solubility assays address kinetic solubility and compound dissolution is usually not tested. The focus is on screening and rapid decision making. For the sake of speed, many important experimental factors such as solid state properties (crystallinity, initial and final polymorphic form, particle size, flowability, chemical stability, etc.) are not controlled, and hence the data quality or confidence into these data is often low and there is a higher risk for selecting compounds with inappropriate properties for development than in later phases [120]. In contrast, in later development phases (Fig. 16.5), compound availability is not an issue. There are usually only one to two compounds remaining that are extensively studied in highly controlled equilibrium solubility and compendial powder and intrinsic dissolution assays. The data generated in late development and clinics are of high quality and are used for regulatory documents. In between, in the preclinical phase, the focus is on candidate selection, and here both speed and quality have to come together. Compound availability may still be an issue, and although only a few compounds are investigated in more depth, some throughput is required to select in a reasonable time appropriate solvents, polymorphic forms, and/or salts for animal and human formulations and for the identification of long-term formulation options [120]. Solubility assays are either short time (<24 h) equilibrium or kinetic/equilibrium hybrid assays, and dissolution is performed with milled or unmilled powders. The initial and final solid state properties of compounds in the assays are also looked at and, compared to discovery, data quality and confidence into data increases and selection risk for the right candidate decreases. Nonetheless, the workflows for solubility and dissolution assays in all phases have a number of key elements in common as shown in Fig. 16.6. Compound and solvent have to be distributed and

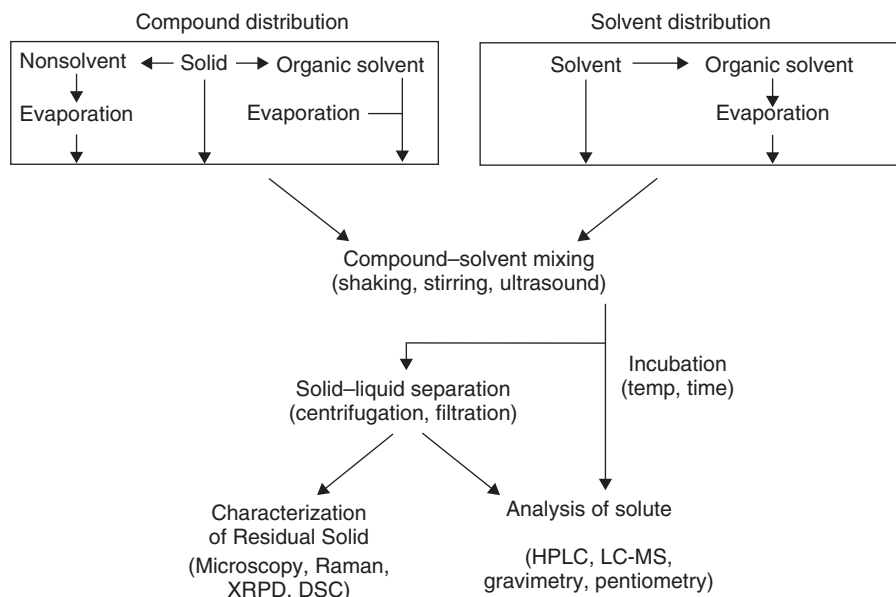


Figure 16.6 Key elements of (HT) solubility and dissolution assay workflows (Section 16.5).

mixed, and after an incubation step, depending on the assay, solid and liquid often have to be separated from each other before analysis.

16.5.2.2 Compound Distribution. For the distribution of one solid compound to one or to a few vials, manual distribution using a spatula and a fixed standard four- or five-digit balance for weighing is still the most popular and efficient method. It is rapid, works with a minimum amount of solid, does not need extra calibration of dispensing instruments, and is not prone to cross-contamination. However, if the focus is on medium to high throughput and on one-to-many or many-to-many distributions, appropriate approaches have to be identified that substitute balances and the labor-intensive and time-consuming weighing process. A number of companies offer automated powder dispensing systems, and the different dispensing techniques and associated challenges have been reviewed recently [120,152]. Most of these systems are expensive and designed for the development phase where compound availability is not an issue. In discovery, most compounds are available only in small amounts and significant compound losses cannot be tolerated. Here, different strategies have to be applied. Despite the known challenges in the handling and storage of DMSO stock solutions [3,153–155], DMSO stocks have become a de facto standard for compound distribution in discovery for various assays and are now also used for solubility screening. The compound is either added as DMSO stock directly to solvents or is used as solid starting material after DMSO evaporation of the stock solution under low pressure [120,156] (Section 16.5.3). DMSO may be replaced by other solvents such as acetonitrile [157], MeOH/DME [158], acetone [159], or acetone/EtOH [160]. In all cases, the solid starting material generated by either precipitation or evaporation is not characterized and may differ from the initial solid state properties. Viable, manual alternatives to DMSO stocks that do not change initial solid state properties are

compound distribution in suspension in a nonsolvent [161] and small-scale volumetric dispensing systems, such as the Titan 96 Well Resin Loader™ from Radleys Discovery Technology [162,163], the Biodot handheld DisPo powder dispensing pipettes (<http://www.biodot.com>), and the Gilson MICROMAN® positive displacement pipette-based “PowderPicking” technology [164]; reported lowest target masses delivered are 0.5 mg, 10 mg, 0.1 mg, and 0.5 mg, respectively.

16.5.2.3 Solvent Distribution and Solvent Selection. Solvent distribution is usually straightforward and not considered as a problem. Commercially available solutions for liquid handling range from simple manual pipettes to highly sophisticated, programmable, 96-tip, robotic systems. These systems are often already available in discovery settings and can be used for aqueous solvents without further modification. In contrast, organic solvents or highly viscous solvents are more demanding and may require specialized equipment or adaptation of aspiration/dispensing protocols as discussed recently [120]. A popular, low technology solution for the manual manipulation of more problematic solvents is the Gilson MICROMAN® positive displacement pipette. Another approach is the solvent casting method [159,160,165]. In this approach, the solvent is first mixed with an organic solvent to facilitated distribution and the organic solvent is subsequently removed by evaporation.

For solubility studies, the traditional solvents used in discovery are water and simple buffer systems. However, in particular, for the increasing number of BCS class 2 compounds (Section 16.4.1), solubility in these solvents often underpredicts the oral absorbability of compounds. Therefore, more biorelevant media, such as simulated gastric fluid (SGF), fasted simulated small intestinal fluid (FaSSIF), and fed simulated small intestinal fluid (FeSSIF), are now used already in early phases. These media often increase the apparent solubility of compounds and their dissolution rate, and they seem to provide a better basis for the ranking of compounds and for absorption simulations [40,166,167]. In early- and late-phase development, these media represent a de facto standard; however, the original media [166,167] are constantly developed further to improve their predictive power [168–170]. In the preclinical phase, a large variety of additional solvents is usually tested to generate a “solubility fingerprint” for each compound to support formulation selection and development; examples are solutions of surfactants, cyclodextrins (CD), or cosolvents in water, pure cosolvents and surfactants, medium- and long-chain mono-, di-, and triglycerides, and organic solvents [161]. Solubility values in the latter are required to support salt and polymorph screening, process and analytical chemistry, cleaning validation, and the preparation of solvent-based solid solutions [120,160,171].

For dissolution studies, 0.1 N HCl or Simulated Gastric Fluid USP without enzymes, pH 4.5 buffer, and pH 6.8 buffer are commonly used media for the BCS classification of drugs [71,172]. Other widespread dissolution media are FaSSIF and FeSSIF, which are used for establishing IVIVC and for predicting the oral absorption of BCS class 2 compounds. Dissolution media often contain surfactants, cosolvents, CD, or lipids to dissolve larger fractions of LS compounds in a reasonable time. Such media are used for formulation development, as control tools for manufacturing or for the determination of product stability in quality control and in research and development [173–176]. A decision tree for dissolution method development and for the selection of appropriate dissolution media for different categories of drugs has been proposed by Li *et al.* [177] based on the work from Dressman [178].

16.5.2.4 Mixing of Compound and Solvent. Experimental approaches to efficiently mix compound and solvent have been the focus of a number of reviews over the past years [120,179,180]. Depending on the compound and the solvent tested, mixing can be challenging and the method used may affect the outcome of an experiment. In solubility and powder dissolution studies, particles should be rapidly and homogeneously distributed in solvents to maximize the initial surface area exposed to medium. Continuous intense mixing will also decrease the height (h) of the UWL at the surface of particles, improve their wetting, and avoid particle (re)aggregation. This will ensure a high dissolution rate (Eq. 16.9) and the rapid reaching of saturation solubility. However, the high energy introduced by too extensive stirring or sonication may also induce partial or complete amorphization and result in an overestimation of drug solubility. Higher apparent solubility due to an increased surface area is also expected during stirring where particles may be “milled” between the wall of the incubation vessel and the stir bar, particularly in small scale approaches with high vessel surface to volume ratios [161]. On the contrary, insufficient mixing may lead to reduced particle surface due to bad wetting, aggregation, or floating of particles of LS compounds and incubation time might be too short to reach saturation solubility. These problems might be overcome by adding glass beads to deaggregate particles [179] or surfactants below their critical micelle concentration (CMC) for wetting [181] or small amounts of predissolved compound in an organic solvent to temporarily generate supersaturated solutions. In kinetic solubility studies, too intense shaking [182] or the insertion of stir bars [183] was found to decrease the measured solubility for LS compounds for unknown reasons. Other important factors to control during mixing and incubation are the avoidance of solvent evaporation and of “dead spaces” [161,163]. As pointed out recently, highly viscous solvents may need special considerations since they are difficult to mix and saturation solubility is reached later due to the slower diffusivity of molecules in these media [120]. When solubility and dissolution testing is applied in later phases to test product performance, stability, and formulation equivalencies or to establish BCS classification, clear specifications exist for both mixing and equipment. In this regulated environment, even a minor modification must be thoroughly evaluated during method development and validation before acceptance [71,99, 184–186].

16.5.2.5 Solid–Liquid Separation. Depending on the solubility and dissolution method used, solids need to be removed before a solute can be analyzed. This can be done by centrifugation or filtration. Centrifugation is a fast and easy method that is commonly used for solvents that are difficult to filter and reported results were comparable to those of filtration [158,187]; however, floating particles can cause problems [120,186]. In most situations, filtration is the method of choice. It is faster than centrifugation and filtration devices with largely different properties (filter material, surface area, pore size) and formats (single to 384 well) are commercially available for almost any application. Known problems with filtration are (i) compound losses by filter sorption of hydrophobic and poorly soluble compounds, (ii) incompatibility of some solvents with certain filter materials, and (iii) difficulties to filter highly viscous solvents. The latter two issues may be addressed by replacing filters by more resistant material [188] and by centrifugation at high g -forces to force the solvent through the filter [161]. Common approaches to address compound sorption to filters are prerinsing of filters with saturated compound solutions, discarding the

first fraction of filtrate, or using assay protocols that expose filters already during the incubation to saturated drug solutions such as the Whatman UniPrep[®] filter [156,189] or the SORESOS method [163]. Another approach might be the use of hydrophilic Polyvinylidene fluoride (PVDF) membranes for filtration, which showed the lowest sorption compared to other filters for the majority of compounds [120,156,190–193]; except for a recent study where hydrophilic polycarbonate filters were superior [182].

16.5.2.6 Solute and Solid Analysis. In a number of solubility and dissolution protocols, the compound in solution is directly measured by optical methods without removal of solids. In kinetic solubility studies (Section 16.5.3.1), increasing amounts of dissolved compound are added to the solute until precipitation occurs and the kinetic solubility is defined as the concentration preceding the point at which it occurs. The formation of precipitates is detected either indirectly by the increase in UV absorption or directly by a nephelometric turbidity detector [194,195]; in-depth discussions of the pros and cons of these techniques can be found elsewhere [153,196]. The solubility of ionizable compounds can also be directly measured by potentiometric methods, which detect a shift in pH values due to drug loss from solution by precipitation. Commercially available systems are the pSol Gemini [189,197] and the Cheqsol system [198]; their differences have been addressed briefly by Alsenz and Kansy [120]. In powder and intrinsic dissolution studies, the increase in drug in solution as a function of time may also be directly measured by *in situ* fiber optic dip-probe methods without removal of solids [199,200]. Miniaturized versions of these systems have recently been introduced and compared to USP methods [201–203]. Other less common systems for the detection of powder dissolution without prior separation use scattering of laser light from the particles in a liquid medium [204] and ultrasound [205].

In the majority of solubility and dissolution protocols, solutes are first separated from solids by filtration or centrifugation before analysis (Section 16.5.3.2). The compound in solution is then quantified by a UV plate reader [120,190,206], conventional high performance liquid chromatography (HPLC) analysis [120,207] or ultrafast HPLC systems [158,161,163]. In contrast to HPLC, the UV plate reader methods typically work only in the microgram to low milligram range, cannot differentiate between compound and degradation products, and may not work with solvents that have a high intrinsic background. Solubility in organic solvents may still be quantified by gravimetric analysis [188,208].

For the generation of high quality solubility and dissolution data, results have to be related to compound's solid state properties. Therefore, residual solids should always be analyzed for polymorphic changes with appropriate techniques such as X-ray powder diffraction (XRPD), Raman spectroscopy, infrared (IR) or terahertz spectroscopy. Some published protocols require a workup of samples before XRPD [209], others allow direct transmission XRPD analysis of residual solids on commercially available 96-well MultiScreen[®] solubility plates after filtration [163]. Direct measurements of polymorphic changes in slurry during or at the end of experiments is also feasible with Raman [163,188,210]. Optical microscopy with polarized light can be another option, however, it might be limited to the detection of drastic changes of solids such as amorphous–crystalline and crystalline–amorphous transformations or

significant changes in crystal shape and/or size [161,207]. A drying step may convert hydrates to the anhydrate.

16.5.3 Solubility Assays

The solubility assays applied in discovery and development combine the elements described in the previous section and the assays focus on the different key questions discussed in Section 16.5.2.1. Driving factors and considerations behind the development of specific assays are usually compound availability, throughput, costs, time, budget, available infrastructure, equipment, human resources, type of solubility addressed (kinetic vs. equilibrium), and quality of data. This results in largely different workflows and technologies used. Table 16.2 summarizes a number of them sorted by type of solubility addressed to better visualize common elements, differences, and trends among the various assays, the list being far from completeness.

16.5.3.1 Kinetic Solubility Protocols. Kinetic solubility assays are usually run in 96- to 384-well format and have medium to high throughput formats. The average test volume per assay is 0.1–0.3 mL and for a single data point, less than 1 mg of compound is required (in Table 16.2, mM values from publications were converted to mg/mL assuming an average compound MW of 500). Therefore, the solubility range addressed is ≤ 1 mg/mL and in most cases ≤ 0.3 mg/mL. The compound is added as solution in DMSO or in other organic solvents to the solvent of interest by robotic systems. After shaking for a few minutes to several hours at room temperature (RT), the compound in solution is quantified after filtration through 0.45- μ m filters by UV detection or indirectly by measuring precipitation with nephelometry. Precipitated solids and the final pH of solutions are not analyzed.

This setup of kinetic solubility assays is typical for discovery. It is adapted to other microtiter-plate- and DMSO-stock-solution-based automated screening protocols in this phase, and similar to other assays, the long-term stability of compounds in DMSO stock solutions can sometimes affect the outcome of studies [154,225]. Also, the organic solvent introduced into aqueous solutions in concentrations of 0.5–5% [120] can act as a cosolvent and enhance the solubility of some compounds [190,207,211,226]. Therefore, kinetic solubility is likely to overpredict thermodynamic solubility. Measured solubility values are expected to differ between different kinetic methods, since precipitation is a kinetic phenomenon that depends on a multitude of factors such as initial compound concentration in DMSO stock, time, temperature, mixing efficiency, and/or ability of compounds to stay in solution in supersaturated form. Nonetheless, kinetic solubility studies are suitable for the discovery stage for categorization or rank ordering of compounds, identification of solubility liabilities, comparison of hit-dose response values with apparent solubility values, guidance for chemists, and selection of development candidates [120].

The importance of equilibrium solubility values for decision making is increasingly recognized by discovery chemists. They realized that kinetic solubility neglects the solid state properties of a compound and that the promotion of drug candidates based on overestimated kinetic solubility values can have detrimental effects on any subsequent development plans and options [100]. Therefore, a number of protocols were established that tried to now address equilibrium solubility also without sacrificing the existing, compound-saving, rapid workflows established for kinetic solubility

TABLE 16.2 Summary of Representative Kinetic and Equilibrium Solubility Protocols

Name	Type	Format	Through- put	Volume (mL)	Compound/ Point	Compound Distribution	Evaporation	Maximum Solubility (mg/mL)	Solvent Distribution	Mixing	Time (h)/ Temperature (°C)	Solid/ Liquid Separation	Liquid Analysis	Solid Analysis	Final pH	Ref.
PISA	K	96	M/H	0.2	10 μ L 10 mM	DMSO	N	0.25	Ma/Ro	Sh	1.5/22	F	UV	N	N	206,211,212
	K	96	H	0.3	15 μ L 10 mM	DMSO	N	0.2	Ro	Sh	3/22	N	Neph	N	N	213
	K	384	H	0.05	2 μ L 10 mM	DMSO	N	0.08	Ro	Sh	—	N	Neph	N	N	195
	K	96	M/H	0.3	3 μ L	DMSO	N	≤ 0.5	Ro	Sh	1.5/22	F	UV	N	N	214
	K	96	M/H	0.05	10/100 mM 5 μ L	DMSO	N	10	Ro	—	0.2/22	N	Tensio- metry	N	N	215
	K	96	H	0.2	100 mg/mL 2 μ L 10 mM	DMSO	N	0.1	Ro	Sh	0.1/22	N	Neph	N	N	120
	K/E	96	M/H	0.2	1–10 μ L 200 mM	DMSO	N	1.5	Ro	Sh	24/22	F	UV	N	N	182
	K/E	96	M/H	0.2	2 μ L	DMSO	N	<1	Ro	Sh	24/22	N	Neph	—	N	194
	K/E	96	M/H	0.2	2 μ L	DMSO	N	<1	Ro	Sh	20/37	F	UV	N	N	216
	K/E	96	H	0.2	2 μ L 30 mM	DMSO	N	0.15	Ro	Sh	20/37	F	HPLC	Micro	N	207
DMSO- SP	K/E	96	M/H	0.1	100 μ L 5 mM	DMSO	Y	2.5	Ro	St	24/22	C/F	LC/MS	N	N	183
	K/E	96	M/H	0.15	150 μ L 10–40 mM	DMSO	Y	20	Ro	St	24/22	C/F	HPLC	N	Y	217
HT-Eq sol	K/E	1	M/H	0.25	25 μ L 10 mM	DMSO	Y	0.5	Ro	Sh	24/22	F	HPLC	N	N	156
LYSA	K/E	96	M/H	0.15	20 μ L 10 mM	DMSO	Y	<0.5	Ro	St/Sh/U	1, 2, 20/22	F	UV/ HPLC	N	N	120
	K/E	96	M/H	0.5	0.25 mL 0.5 mg/mL	MeOH/ DME	Y	0.25	Ro	St/U	24 or 24–48/22	C	HPLC	N	N	158
Solvent casting	K/E	96	H	0.2	0.01–0.05 mg	Org. solvent	Y	0.25	Ro	Sh	0.5–24/22	F	UV/LC	N	N	165
PASS	E	96	M	0.04–0.08	0.5–2 mg	Suspension in heptane	Y	100	Ma/Ro	St	24/22	F	UPLC	Raman, Micro	Y	161
	E	96	M/H	1	3 mg	Powder	—	3	Ma/Ro	Sh	17/22	F	UV	XRPD	Y	209

(continued overleaf)

TABLE 16.2 (continued)

Name	Type	Format	Through-put	Volume (mL)	Compound/Point	Compound Distribution	Evaporation	Maximum Solubility (mg/mL)	Solvent Distribution	Mixing	Time (h)/Temperature (°C)	Solid/Liquid Separation	Liquid Analysis	Solid Analysis	Final pH	Ref.
	E	96	H	0.2	3 mg	Powder	—	20	Ma/Ro	Sh+ glass beads	24/22	F	UV	N	N	157
SORESOS	E	96	M	0.2	20 mg	Powder	—	100	Ma/Ro	EoE	24/22	F	UPLC	XRPD, Raman	Y	163
	E	96	M	0.5	Cd	Powder	—	Cd	Ma/Ro	Sh	24/37	F	HPLC	N	N	52
	E	6	L	0.125-0.5	≤0.5 mg	Powder	—	4	Ma	Pump	24/22	F	HPLC	N	Y	218
	E	1	L	1	1 mg	Powder	—	1	Ma/Ro	St	168/22	F	HPLC	N	N	219
THESA	E	1	L-M	0.1	<1 mg	Powder	—	<10	Ma	Us	23/22	F	UPLC	N	Y	120
	E	1	L	0.05-0.14	Cd	Powder	—	Cd	Ma	-		N	DSC	N	N	220
	K/E	6	L	1	≤1000 mg	Powder	—	1000	Ma	St	10/10-7	N	Turbidity	N	N	221
	(E)	60	M/H	1	50-1000 mg	Powder	—	1000	Ro	Sh	3/22	N	Turbidity	N	N	222
	E	1	M/H		40 µL	Suspension	—	300	Ma/Ro	St	72/22	F	TGA	N	N	208
Mini SF	E	1-6	L	1-5	Cd	Powder	—	Cd	Ma	Sh	24/37	F	HPLC	N	Y	189
Small-scale SF	E	1	L	0.05-0.2	Cd	Powder	—	Cd	Ma	Sh	24/22		HPLC	N	Y	187
SF		1	L	4	Cd	Powder	—	Cd	Ma	EoE	24-72/25	C	UV	DSC, XRPD	N	223
SF	E	1	L	10	Cd	Powder	—	Cd	Ma	Sh	24/37	F	HPLC	N	N	40
SF	E	96	M/H	0.5	Cd	Powder	—	Cd	Ma/Ro	Sh	24/37	F	HPLC	N	N	52
PSol	I	1	L	20	1-10 mg	Powder	—	<0.5	Ma	St	3-10/22	N	Potent-iometry	N	Y	224
Chasing equilibrium	I/E	1	L	10	>2-5 mg	Powder	—	4	Ma	St	0.3-1.3/22	N	Potent-iometry	N	Y	198

C, centrifugation; H, high; M, medium; Neph, nephelometry; U, unstirred; Cd, compound dependent; I, intrinsic; Ma, manual; Ro, robotic; UPLC, ultrahigh pressure liquid chromatography; E, equilibrium; K, kinetic; M, microscopy; SF, shake flask; US, ultrasound; EoE, end over end; L, low; MS, mass spectrometry; Sh, shaking; UV, ultraviolet; F, filtration; LC, liquid chromatography; N, no; St, stirring; Y, yes.

determination (Table 16.2, K/E). Two strategies are followed: one extends the equilibrium time of existing kinetic solubility protocols and the other uses solids generated from dried stock solutions.

The first approach increases the time between addition of the compound stock solution to the solvent and measurement of drug solubility from originally minutes to hours to a day or more. The extended incubation time favors precipitation of compounds from supersaturated solutions, conversion of solids to thermodynamically more stable forms (amorphous to crystalline, hydrates, etc.), and/or equilibration between solid and compound in solution. Incubation at 37°C is expected to speed up these processes further. Now, in a few cases, residual solid is also inspected for crystallinity, at least by optical microscopy [207]. Since equipment and protocols (except for time) remain basically unchanged, solubility ranges tested and uncertainties regarding cosolvent effects on drug solubility are similar to pure kinetic approaches. The latter is addressed by the second strategy. The compound is still distributed dissolved in organic solvent; however, the solvent is removed subsequently by evaporation. This allows adding higher amounts of compound per well, and hence larger solubility ranges can be addressed. It also eliminates the potential effect of organic solvent on drug solubility. The incubation time is extended as well to a day or more, stirring is preferred for mixing, and drug in solution is analyzed by HPLC or LC-MS after removal of solids by filtration or centrifugation. Similar to the first approach, initial solids or solids at the end of the experiment are not analyzed. Therefore, it cannot be guaranteed that the polymorph relevant for development has really been formed during the experiments or that equilibrium solubility has been achieved.

16.5.3.2 Equilibrium Solubility Protocols. Equilibrium solubility protocols usually start from powders that are well defined with respect to polymorphic forms and impurities. The assay formats vary from single vials to 96-well microtiter plates, and powders are usually distributed manually using balances (Table 16.2). Viable alternatives to this tedious and slow process can be volumetric devices [163] or distribution as suspension in nonsolvents [161]. The amount of compound added per data point is between 1 mg and about 20 mg and should exceed the expected solubility of the compound to guarantee the formation of a suspension at the end of the experiment. The volume of solvents in most protocols is below 1 mL, and solvents are added manually or, for the higher throughput formats, by robotic systems. Samples are then mixed by various procedures for one to seven days at RT or at 37°C, and the solute is analyzed after filtration by UV or HPLC. The target solubility range is between 1 and 300 mg/mL. In addition to these assays, a number of less common methods have been reported that use temperature ramps and DSC, thermogravimetry, or turbidimetric methods to address some more specific questions regarding compound solubility or dissolution (for references, see Table 16.2).

The presumably most famous method to determine drug solubility is shake flask that is often regarded as the “gold standard” for determining equilibrium solubility. However, the experimental details of the method have never been exactly defined, and hence very different protocols exist (Table 16.2). Shake flask methods can thus differ tremendously in almost all experimental parameters, and even extremely important ones such as examination of final pH of the solution and of residual solid are not always considered. Consequently, measured solubility values should not be taken as absolute. Frequently, values cannot be linked to solid state properties or pH, and

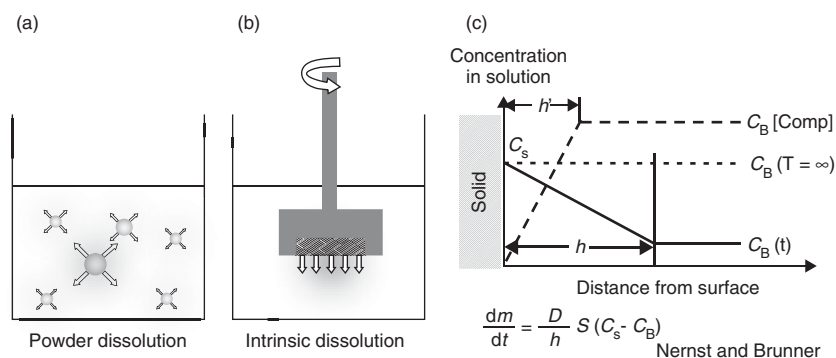
comparisons across different data set generated by the so-called “shake flask” methods may be problematic and error prone. Often, equilibrium solubility may also be affected by a variety of sometimes, unobvious factors [120]. Examples are the water content in oils, which decreases the solubility of steroids [227] or the overestimation of free drug concentration in filtrates due to nanoparticles or amorphous compound droplets passing filters. Moreover, some drugs form aggregates [228,229], nanofibers [230], or complexes with solvent constituents [231], and therefore, the real amount of free drug available, for example for absorption, will be much lower than expected. A number of partially and fully automated systems designed to measure equilibrium solubility are already commercially available [120]. Ideally, equilibrium solubility studies are performed with the most desirable physical form for development, which means with the most stable, crystalline polymorph with the highest possible purity. In most setups, equilibrium studies are used to confirm previous kinetic solubility results, to rule out potential artifacts, and to generate quality solubility data for formulation development, analytical development, and cleaning validation.

16.5.3.3 Intrinsic Solubility Protocols. The intrinsic solubility, S_0 , is the solubility of the uncharged species of a molecule. For neutral molecules, it is identical at all pH values; for ionizable compounds, it can be measured by potentiometric approaches (Table 16.2). In potentiometric titration, known volumes of strong acid or base are added to a vigorously stirred solution of test compound and the pH is recorded. Solubility is determined by the pH shift due to drug precipitation during the titration. The method is limited to ionizable compounds and does not take the solid state properties of precipitates into account. Potentiometric methods for the determination of solubility were first introduced by Avdeef [232], and an automated system, pSol, is commercially available [189,233]. The method is relatively slow but allows the generation of a pH/solubility profile with a single titration in about 8–24 h. A second method, termed *chasing equilibrium solubility* (CheqSol) was introduced by Stuart *et al.* [198] and is also commercially available [234,235]. This method enables concentrations of compounds to be established during the time course of precipitation and dissolution events. It allows determination of equilibrium, kinetic, and intrinsic solubilities within 20–80 min and has also been used for the investigation of supersaturation phenomena and for BCS classification of drugs [234,236] (Section 16.6.5).

16.5.4 Dissolution Measurement

Dissolution testing is an important tool in the development of drug characterization, BCS classification, identification of appropriate oral dosage forms, and BE testing. In contrast, dissolution testing in drug discovery is rare. A reason could be that standard solubility/permeability-type BCS representations do not readily show the importance of dissolution for the rate and extent of oral absorption, in particular for LS drugs, and hence it is neglected. However, this may result in wrong absorption predictions [e.g., MAD calculation (Section 16.3)] if calculations are solely based on equilibrium solubility and permeability. For example, a rapidly dissolving LS/HP compound (BCS class 2) with lower equilibrium solubility can exhibit a higher fraction absorbed than a slowly dissolving compound with higher equilibrium solubility (Section 16.4.1).

Shortage of candidate material can be another reason why dissolution is not tested in discovery. Traditional powder dissolution (Fig. 16.7a) and intrinsic dissolution testing (Fig. 16.7b) requires hundreds of milligrams of highly pure compound for single



Parameter		Affected by (examples)
m	amount of drug dissolved	
t	Time	
D	Diffusion coefficient	MW, complexation, soluble aggregates, temperature, viscosity
S	Effective surface area exposed to medium	Particle size and shape, particle agglomeration
h	Apparent stagnant diffusion layer thickness	Stirring rate, viscosity, particle size
h'	Effective stagnant diffusion layer thickness	'Reactive' media (e.g. ionization, complexation, buffering capacity)
$C_B [\text{Comp}]$	Concentration of 'complexed' drug in the bulk solution	
C_s	Saturation solubility at the surface of the solid	Salt form, crystal form, pH, pKa, 'reactive' media
$C_B(t)$	Concentration of drug in the bulk solution at time t	Sink conditions, volume of dissolution medium

Figure 16.7 Schematic presentation of (a) powder dissolution, (b) disc intrinsic dissolution, and (c) diffusion-controlled dissolution model according to the Nernst–Brunner equation. The table summarizes parameters that influence the amount of drug dissolved and gives examples for factors that affect them (Section 16.5.4).

measurements, which would then not be available for other, potentially more important experiments in this phase. Moreover, assays require large volumes (50–900 mL) of sometimes expensive biorelevant media, and assays are low throughput. Trials to reduce the volume and/or increase the throughput of dissolution assays are generally regarded with skepticism about the scalability of dissolution methods since dissolution measurement is affected by a large number of parameters (Fig. 16.7c) [201,237]. The most important ones can be derived from Equations 21.9, 21.21, and 16.25 and are briefly addressed subsequently.

The driving force for dissolution is the concentration gradient between the drug dissolved at the drug surface (C_s) and its concentration in the bulk at time ($C_B(t)$) (Fig. 16.7c). According to the equation (based on Eq. 16.9) in Fig. 16.7, the dissolution rate is highest when $C_B(t) \ll C_s$, which means under sink conditions where all dissolving compound is rapidly diffusing away from the surface of the solid drug. Such sink conditions are assumed when C_B is $<10\%$ C_s and are achieved in the USP methods by using large volumes of dissolution medium. However, for formulation

testing, large volumes may not always be favorable. Gu *et al.* [238] demonstrated that smaller, physiologically more relevant dissolution volumes predicted the BA of a salt of a weak base much better than the 1L standard approach, which masked the effect of formulation excipients on drug dissolution.

Most dissolution processes are diffusion controlled, and hence, agitation strength affects dissolution rate. Strong agitation reduces h , which is inversely proportional to the dissolution rate (Eq. 16.9). In particular, LS drugs may rapidly get close to their equilibrium solubility at the boundary layer and dissolution will be stopped or delayed without agitation [145,239]. The thickness of the boundary layer depends on the particle size. The smaller the particles are, the less affected the dissolution rate is by agitation, since the boundary layer thickness decreases and surface specific dissolution rates increase [130,131,143]. Thus, agitation intensities do not seem to affect dissolution rates of particles $<5\text{ }\mu\text{m}$ [131].

Dissolution is strongly affected by the composition of the dissolution medium. Ionic composition, pH, buffer capacity, and added excipients can influence the solubility and dissolution of a drug, and hence for the development of appropriate dissolution media, many factors have to be considered. In water, dissolving free acids lower and dissolving free bases raise the pH at the surface of the particle compared to the bulk solution and thus decreases their solubility C_S at the surface. According to Eq. 16.9, the drug then diffuses through the liquid UWL with the thickness h until $C_B(t)$ reaches C_S at $T = \infty$ (Fig. 16.7c). If a buffering species is added that shifts the pH to higher (for acids) or lower (for bases) values, it is expected that (i) the acid–base interaction at the interface will reduce the thickness of the UWL (h'), (ii) the larger difference between surface pH and pH in the bulk will act as a better sink, and (iii) C_S at the surface may increase. Together, these processes all favor a faster dissolution in the presence of buffers. Furthermore, the dissolution velocity often increases with buffer concentration [169,201] and the selected buffering species, for example, phosphate versus bicarbonate, can have a profound impact on dissolution rate and saturation solubility [240,241]. Neutral compounds are not affected by pH or buffering species. However, the dissolution rate of both neutral and charged drug species can be greatly modified by a number of excipients in dissolution media. Excipients are often added (i) to increase the solubility of less soluble drugs to measurable levels, (ii) to guarantee complete dissolution of poorly soluble drugs, (iii) to simulate *in vivo* conditions, (iv) to improve wetting of particles, (v) to deaggregate particles, and/or (vi) to provide sink conditions without increasing the aqueous sink volume. Water–cosolvent mixtures is one approach that can improve both drug solubility and dissolution [242]; however, dissolution testing of drug products may need special considerations [243,244]. Another common approach is to enhance solubility by adding micelle-forming surfactants. Micelles also “react” with free compound and thus reduce h' . They exert their effect on both ionized and unionized molecules, and the total solubility C_S is the sum of free solute form(s) and micelle-solubilized form(s). Compared to the often high enhancement in solubility, the increase in dissolution rate is usually much lower. The reason is that solutes in micelles diffuse slower than single molecules because of the much larger diameter of the micelles, hence micelles have a lower apparent diffusion coefficient. For example, the solubility of griseofulvin in 60 mM sodium dodecyl sulfate (SDS) increased ~ 107 -fold, whereas its dissolution rate rose only ~ 30 -fold. Also, the diffusivity of micelles dropped about 10-fold compared to that of free griseofulvin ($11 \times 10^{-6}\text{ cm}^2/\text{s}$) [245]. Similar examples have been reported for Danazol [246],

fenofibrate [247], and ketoprofen [248], the latter showing an even 30-fold decrease in diffusivity. Other reported factors affecting dissolution rate in the presence of SDS are changes in apparent pK_a value of ionizable compounds with increasing concentrations [249,250] and repulsive forces between charged micelles, which reduced micelle diffusivity and may need pH and ionic strength control [246,248]. To generate sink conditions, surfactants may be replaced by CD in dissolution media if the drugs form complexes with them [251]. As a result, solubility and dissolution rate increase [252] and diffusivity of complexes decreases; however, diffusivity velocity decreased only by a factor of two since complexes are smaller than micelles [253]. Combinations of surfactants and CD may result in more complex situations. Some surfactants compete with drugs for binding to CD [254,255], and this affects both the solubilization capacity of the medium [252] and the diffusivity of the various molecular species, the overall effect depending on the drug/cyclodextrin/surfactant ratio [256–258]. If viscosity enhancing agents are added, the diffusion layer thickness of particles is expected to increase and the diffusion coefficient to decrease. Dissolution of small particles seems to be less affected by viscosity enhancing agents than larger particles.

The surface area available for dissolution is another important factor in powder and solid dosage form dissolution studies. Particle size reduction is expected to increase surface area available for dissolution and particle aggregation to decrease it. Experimentally, particles with a size of $1.5\text{ }\mu\text{m}$ showed indeed a much faster dissolution than $12\text{-}\mu\text{m}$ particles [173], and agglomerates of furosemide [259] or oxazepam [260] exhibited decreased dissolution rates. *In vivo*, particle size reduction of felodipine (solubility in saline: 0.077 mg/mL) from $125\text{ }\mu\text{m}$ to $8\text{ }\mu\text{m}$ resulted in a 14-fold increase in AUC in dog [261].

In vitro dissolution results may be used as a surrogate to predict the *in vivo* performance of drugs and formulations, particularly of BCS class 2 and 4 compounds. In the GIT, bile salts and lecithin act as physiological surfactants that improve wetting and solubility of lipophilic compounds. Media designed to simulate this *in vivo* situation have been introduced to establish IVIV correlations [166], and efforts to further improve them are ongoing [262,263]. Compared to many surfactants with micelle aggregation numbers >60 [245], bile salt structures give values <10 [246], hence bile salts are expected to solubilize less compound. Lecithin added to bile salts (mixed micelles) leads to an improvement of the solubility and of the dissolution rate of some LS drugs [264]. However, the effective diffusivity of mixed micelles is approx 100-fold lower than in comparable taurocholate solutions since lecithin also increases the micellar diameter [264], the increase depending on the dilution [131]. Furthermore, the buffer capacity of previously used FaSSIF and FeSSIF media [166,168] were too high compared to values measured in human aspirates [265], hence self-buffering effects may have been underestimated with these media (Section 16.5.1.3). Recently, this was corrected by the introducing new media with lower buffer capacity [169].

Other applications for dissolution testing are the screening and characterization of salts [266], cocrystals [267,268], and polymorphic forms [269,270] for appropriate form selection for development. As described in Section 16.5.2.6, their solid state properties need to be carefully monitored at the end of the dissolution study since they may rapidly dissolve, form supersaturated solutions, and then precipitate as crystalline or amorphous material. Dissolution studies with cocrystals have recently been reviewed by Schultheiss and Newman [128]. Hydrate formation also needs to be carefully investigated since it can reduce drug dissolution rate [271] and solubility [138] and can affect

the outcome of *in vivo* oral absorption studies [270,272]; however, reduced solubility does not always correlate with reduced dissolution rates [138].

Another application of dissolution testing is the screening for excipients (polymers, surfactants, etc.). These studies are useful to identify formulations with enhanced dissolution rates, crystallization/precipitation inhibitors, or stabilizers, for example, for suspension formulations. In most of these studies, the effect of excipients on drug dissolution is tested after physical mixing [273], after cogrinding [274], or after preparation of solid dispersions or solid solutions by spray drying [275] or hot melt extrusion [273,276]. More recently, excipients have also been added directly to dissolution media or tested in slurry experiments [272,277,278]. Changes in solid form such as hydrate formation are either analyzed *in situ* [279–281] or at the end of the experiment [272].

Dissolution testing has become an important tool in development for testing of drug powders and dosage forms and is an official test used by pharmacopoeias [172,176]. Most of the testing is performed in quality control to confirm or test product consistency. In research and development, the focus is more on the prediction of the *in vivo* behavior of a drug or a drug product and on the identification of appropriate formulations. Therefore, a number of different testing protocols and apparatus have been designed to address the two types of dissolution methods, powder dissolution, and intrinsic dissolution. Some representative protocols are summarized in the following two sections and in Table 16.3.

16.5.4.1 Powder Dissolution. The dissolution tests listed in the USP are designed for oral solid dosage forms, and currently, there is no official powder dissolution test [176]. However, the general principles of these tests should also be applicable to powders, hence compendial apparatus are commonly used for powder testing. The most frequently used USP apparatus are shown in Fig. 16.8 including a short summary of their specifications and applications (see also www.sotax.com, www.erweka.com, www.varianinc.com, <http://www.distekinc.com>). A number of studies have addressed and compared their use and value for specific applications [176,237,282,290,291]. Details of the common elements of dissolution assays have been discussed before (Section 16.5.2).

Table 16.3 lists a number of representative protocols used for powder dissolution testing, the table being roughly sorted by volume of dissolution medium used per assay. The operational volumes of the rotating basket (USP 1 apparatus) and of the rotating paddle (USP 2 apparatus) are in the range of 500–900 mL (Fig. 16.8). Powder and medium are distributed manually, and throughput is usually low; however, fully automated systems with 10–15 dissolution test vessels are now commercially available [292,293] (see also www.sotax.com, www.erweka.com). Systems with scaled-down baskets and vessels are also available [237,294]; however, their use may be limited to LS compounds and low dose solid dosage forms in terms of maintaining sufficient sink conditions. The USP 3 (BioDis) (www.varianinc.com) and USP 4 (flow-through cell) can be used for dissolution testing in only one medium or in several media to imitate passage through the GIT (Fig. 16.8). The BioDis system uses cylinders sealed with membrane filters to retain powders or disintegrating particles inside the vessels when they move from one medium to another. The medium volume is in the range of 50–200 mL [286]. In the open loop flow-through cell, one medium is pumped through the dissolution cell and, if needed, is simply replaced by another. In closed

TABLE 16.3 Summary of Representative Powder and Intrinsic Dissolution Protocols

Name	Type	Format	Throughput	Volume (mL)	Compound/ Point	Compound Distribution	Evaporation	Solvent Distribution	Mixing	Incubation Time (h)/ Temperature (°C)	Solid/ Liquid Separation	Solid Characterized	Liquid Analysis	pH	Ref.
USP 1	PD	1	L	900	Cd	Powder	—	Ma	St	-/37	F	N	UV	N	282,283
	PD	1	L	900	Cd	Powder	—	Ma	St	—	N	N	UV turbidity	N	284
USP 2	PD	1	L	500	Cd	Powder	—	Ma	St	-/37	F	N	HPLC	N	285
	PD	12	L/M	500	Cd	Powder	—	Ma	St	-/22	N	N	UV dip-probe	N	199
BioDis	PD	1	L	200	Cd	Powder	—	Ma	St	6/37	N	N	UV	N	286
	PD	1	L	50	Cd	Powder	—	Ma	St	-/37	F	N	HPLC	N	40
Solvent casting	PD	70	M/H	4–10	1–2.5 mg	Org. solvent	Y	Ro	Sh	0.5–1/22	F	N	HPLC	N	159
Solvent casting	PD	96	H	0.2	0.01–0.05 mg	Org. solution	Y	Ro	Sh	0.5–24/22	F	N	UV/LC	N	160,165
μARCS	PD	384–3456	H	—	40 μL 5 mM DMSO	DMSO	Y	Ro	None	0.5/22	Y	N	LC/MS	N	287
USP Wood Apparatus	IDR	1	L	225–900	200–500 mg	Powder	—	Ma	St	-/37	F	N	HPLC	N	151,185
Mod USP4	IDR	1	L	1.5–20/min	150–200 mg	Powder	—	Ma	Pump	-/22	N	Raman	UV	N	280,282
	IDR	1	L	1/ min	5 mg	Powder	—	Ma	St	-/22	N	N	HPLC	Y	288
μDiss	IDR/PD	6	M	10–15	5 mg	Powder	—	Ma	St	-/37	N	N	UV dip-probe	N	201,203
MINDISS	IDR	96	M/H	0.25	2–5 mg	Powder	—	Ma	St	-/22	F	Y	HPLC	Y	289

F, filtration; LC, liquid chromatography; PD, powder dissolution; UPLC, ultrahigh pressure liquid chromatography; H, high; M, medium; Ro, robotic; UV, ultraviolet; IDR, intrinsic dissolution rate; Ma, manual; Sh, shaking; Y, yes; L, low; N, no; St, stirring

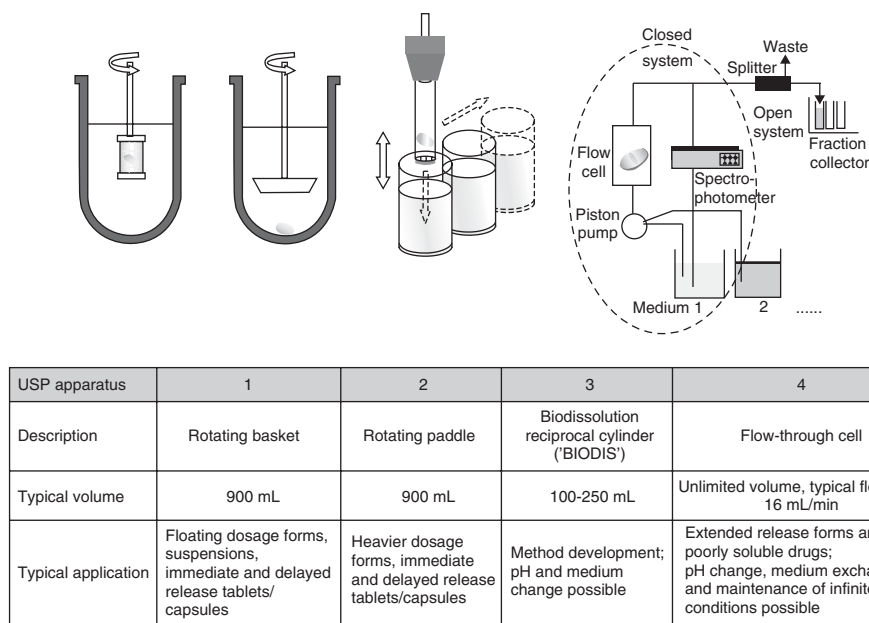


Figure 16.8 Schematic presentation of USP dissolution apparatus for the testing of solid dosage forms and description of typical volumes and applications (Section 16.5.4.1).

loop systems, the medium is continuously pumped through the cell and volumes can be as low as 15 mL [237,295,296].

Compound consumption in USP apparatus depends on the expected saturation solubility and is typically in the range of 100–500 mg per dissolution experiment. For most discovery setups, this is far too high and several small-scale dissolution systems have been established for screening. The focus of these studies is formulation and excipient screening; these studies are performed at RT, whereas compendial tests are run at 37°C. Protocols are adapted to the needs of discovery and early development, and compounds and excipients are distributed in organic solvents, which are subsequently removed by evaporation [159,165]. These protocols neglect the solid state properties of the drug after evaporation; however, they may be useful for identifying formulations that dissolve more rapidly or form supersaturated solutions and improve oral BA [160] (Sections 16.6.3 and 16.6.5). These applications may also be addressed by the high throughput microarrayed compound screening system (μ ARCS) [287]. About 40 nL of a 5 mM solution of compound in DMSO is spotted onto polystyrene sheets and dried. An agarose gel is placed on top of it, and the time-dependent diffusion of compound into the gel is monitored in excised gel pieces by LC-MS.

16.5.4.2 Intrinsic Dissolution. The measurement of intrinsic dissolution rate (IDR) is useful for the characterization of pure drugs, for mechanistic studies, and for comparing different drugs or solid forms of a drug. For these studies, compressed disks of drugs with a known diameter are produced using a punch and die, and then, a defined single surface area of the disk, typically 0.5 cm², is exposed to the medium. During the experiment, the surface area is taken as constant, and if all other conditions (volume,

temperature, agitation, pH, etc.) are also fixed, the IDR can be directly calculated from the amount of drug released per unit surface area and time (Eq. 16.9). Such comparisons are difficult to perform in powder dissolution studies since the initial particle size and particle size distribution are often not known and the surface area exposed to the medium constantly changes throughout the experiment (Section 16.5.1.3).

IDR is traditionally determined using the rotating disk system (USP Wood apparatus). It is similar to the USP 2 apparatus shown in Fig. 16.8, however, the paddle is replaced by a rotating holder carrying the die with the compressed drug disk (Fig. 16.7b). The die is inserted into the dissolution medium at a defined distance from the bottom and rotated at constant speed at 37°C [151]. However, in a modified system, the stationary disk system, the design of the USP 2 apparatus is kept, the die is placed at the bottom of the vessel, and the dissolution medium is moved over the disk by the paddle [185]. The volumes used are between 200 mL and 900 mL, and about 200–500 mg of drug is required per disk.

An alternative to USP 2-apparatus-based systems is the modified channel flow-through system. Between 150 mg and 200 mg of a drug is compressed with a single punch tableting machine, mounted into a tablet holder, and then placed into the center of a cell with an inlet and an outlet for the medium [282]. In dissolution studies, the medium is pumped through the cell with flow rates between 1.7 mL/min and 20 mL/min, and the dissolved compound is detected using a UV–VIS spectrophotometer. In a modified system, the cell is equipped with a quartz sight window to allow *in situ* Raman measurements for the direct identification of solid phase changes [280]. Another system combines a flow-through cell system with a rotating disk [288]. It requires only 5 mg of drug to make a compressed disk in a hole in a magnetic bar. The bar is inserted into a 0.3-mL chamber of a flow-through cell and rotates during the dissolution experiment.

Recently, Berger and colleagues [203] introduced a new, miniaturized rotating disk IDR measurement system that has gained considerable interest. It uses a Mini-IDR™ compression system (Heath Scientific, UK) to make a disk with a surface area of 0.07 cm² from about 5 mg of drug powder in a die [201]. The die is inserted into a Teflon carrier with an embedded stir bar and placed into a vial with 10–15 mL of medium. The carrier is rotated during the dissolution experiment, and the drug released is determined in a μ DISS ProfilerPLUS™ instrument (pION) using a fiber optic dip probe. Experiments are performed at 37°C in up to eight parallel channels. The data from the new miniaturized IDR instrument showed excellent correlation to literature values from traditional IDR measurements and suggests that IDR measurements can be scaled down to the needs of drug discovery without sacrificing data quality [201]. For LS compounds (IDR <0.1 mg/min/cm²), compound consumption may even be reduced further to 0.06 mg per experiment, since IDR may be calculated from powder dissolution experiments without prior knowledge of particle size and shape [202].

The miniaturized intrinsic dissolution screening system (MINDISS) also consumes only 2–5 mg of compound per disk [289]. Disks with a surface area of 0.031 cm² are made in custom-made holders and inserted into 0.25 mL of dissolution medium in 96-well microtiter plates. Similar to Caco-2 transport experiments, holders are transferred into new wells with fresh medium after a predetermined time to achieve sink conditions or disks are transferred to another medium to simulate GI passage. Samples are filtered, and the dissolved compound is finally quantified by ultrahigh pressure liquid chromatography (UPLC).

All miniaturized IDR protocols have the option of obtaining XRPD or Raman data on the initial disk and the remaining disk to identify solid-form modifications during dissolution studies. Although this is not routinely done, the correlations obtained between traditional rotating disk methods and the new compound-saving, miniaturized IDR methods are already excellent [201,288]. Therefore, dissolution as a rate-limiting step in oral absorption can and should now be addressed earlier and become an integral part of late discovery/early development selection strategies. Even if compounds do not yet have the required final purity, small-scale dissolution testing offers the opportunity to screen, compare, and select polymorphs, salts, and cocrystals for development much earlier and thus hopefully reduce later-stage issues due to inappropriate solid state properties.

16.6 IMPACT OF SOLUBILITY AND DISSOLUTION ON FORMULATION STRATEGY

16.6.1 General Considerations for Formulation Strategy

In principle, the very general equation (Eq. 16.4) [or the easier-to-approach MAD equation (Eq. 16.5)] and the Nernst–Brunner equation (Eq. 16.9) already describe all options formulators have to influence drug absorption. In theory, each and any of these parameters can be modified by formulation, although not to the same extent. Realistically, C_S and the dissolution rate are the two factors where formulators can do most to improve absorption since drug solubility can vary over six orders of magnitude ($\sim 0.1 \mu\text{g/mL}$ – 100 mg/mL) [43] and dissolution rate up to 400-fold, for example, in response to pH [151]. In contrast, permeability varies only about 50-fold (hum k_a ranges from 0.001 – 0.05 min^{-1}) [43] and physiology-related parameters (transit time, surface area exposed to drug, water volume, etc.) can, if at all, be influenced only within very small limits. Therefore, at least the solubility, dissolution, and permeability characteristics of a drug have to be known before one can decide on which drug delivery technology to use in which development phase for the delivery of a defined target dose to a specific species.

LP is still an unmet challenge and difficult to address by formulation approaches. Depending on the underlying reasons for poor membrane permeability, formulation may or may not increase the absorption of BCS class 3 and 4 compounds (Section 16.4.1). If low lipophilicity comprises absorption, transient opening of the tight junctions with excipients may improve absorption of polar compounds [84]. If efflux mechanisms and/or metabolism limit absorption, excipients inhibiting them may be used in formulations to enhance the extent of absorption [297] (Section 16.8). In both cases, the drug and excipient must be delivered to the site of absorption simultaneously and potential toxic effects related to the excipients have to be evaluated carefully. As an alternative, the solubility of drug in solution can be increased as discussed later (Section 16.8). This will increase the extent of absorption per se (Eq. 16.4) and above a certain level, saturate efflux mechanisms and/or metabolism, which will further improve absorption.

For the majority of compounds at present, solubility and/or dissolution are the limiting factors. In recent years, the percentage of LS lead compounds has significantly increased, and in many development pipelines, 60–70% of the new NCEs now fall into BCS class 2 (Section 16.4.3) [97]. Therefore, comprehensive assessment of solubility

and dissolution is essential. It serves as guidance for the formulation scientist and sets the stage for formulation development. Fortunately, solubility and dissolution liabilities can be addressed by a large variety of formulation approaches as is discussed below.

16.6.2 Decision Trees for Formulation Development

A considerable number of very different decision trees and flow charts have been published for formulation selection, the schemes considering largely different criteria for decision making. Some schemes cover only specific development phases [298–303], others focus more on when to apply standard, intermediate, or enabling technologies for drug delivery [298,304] or when to use specific technologies such as nanosuspensions [302,305], lipid formulations [306,307], or absorption-window-specific technologies [308]. Special formulation decision trees also exist for acute PK/PD studies and for long-term studies [298,299,309], and particularly, excipient effects on PK/PD studies [310] and the potential toxicity of some excipients in chronic studies need thorough considerations [299,311]. Depending on the route of administration, for example, intravenous versus oral [301,302,304,312,313], and the species, for example, human versus animal [96,304], decision trees may also differ. Decision trees purely built on physicochemical properties such as log D, solubility, IDR, solid state properties, or MP are rare [312]. More recently, the BCS classification scheme has been used for the selection and design of formulations [96,97]. The BCS (Section 16.4.1) combines dose with solubility, permeability, and dissolution and the derived parameters D_o , D_n , and A_n (Section 16.3) may be used as more easily accessible decision criteria for building up simplified decision trees [314,315].

The general issue with decision trees is that it tightly couples knowledge and the use of knowledge in individual decision paths, and hence all possible decision paths and criteria have to be present in the tree. If information is missing, decisions can not be made; examples are the missing criteria for high and low MP, log P, and dose in the nanosuspension scheme from Rabinow (Fig. 16.1 in Ref. 305) and the decision tree from Gardner *et al.* (Fig. 16.5 in Ref. 316), which cannot be followed if a compound does not crystallize. Moreover, many decision trees may require techniques or measured physicochemical properties that are not available everywhere or consume too much compound to be practicable. Therefore, the subsequent formulation section does not show yet another decision tree (that might not be applicable to the readers situation) but provides some basic insight and guidance to the reader on the rational behind *oral* formulation development to foster the setup of individual formulation strategies. Parameters that can be modulated by formulation are addressed, potential formulation techniques are presented, and the pros and cons of these techniques are discussed. Strategies to overcome permeability-limited absorption are not discussed as mentioned before (Section 16.6.1).

For scientists outside formulation departments, formulation development is often confusing. Formulation scientists have a multitude of options to combine formulation principles, technologies, and excipients to address specific delivery issue, and often, they forget to communicate the rational behind a formulation. In principle, the strategy in formulation development is very simple and consists of the following steps:

1. collect or measure the most important physicochemical properties of a drug
2. identify the rate-limiting steps in absorption (Section 16.3)

3. react accordingly by building up a formulation strategy that is adapted to the development phase, therapeutic area, target dose, species, and available formulation technologies and resources, and, not to forget,
4. keep an eye on potential excipient toxicity, on the overall formulation strategy for entry into human, and on potential backup formulation strategies.

Therefore, a rational, fact-based formulation development starts with the collection of a number of key parameters that need to be known from the very beginning, for example MW, log P, polar surface area (PSA), pK_a , solid state properties, solubility, IDR, chemical stability, and permeability. Also important are the details behind reported values; for example, the formulator should know which type of solubility (kinetic/equilibrium, final pH, amorphous/crystalline starting material, changes in solid state properties, etc.) or permeability (PAMPA, cell based, at which pH, etc.) was actually measured or how the compound behaved macro-/microscopically in the respective assays (floating, aggregation, oiling-out, initial complete dissolution then precipitation, etc.). In some cases, this may already give a clue on potential formulation strategies and technologies and avoid misinterpretations. The challenge for formulators is then to translate this information into appropriate formulations. For compounds with HP, formulation can modulate oral delivery at the levels of dissolution, solubility, and precipitation as shown in Fig. 16.9. The discussion of formulation principles will basically follow the idea of “spring” and “parachute” recently reviewed by Brouwers *et al.* [134]. In Section 16.6.3, solid formulations with increasing energy of the solid are presented, which address enhancement of dissolution rate and/or increase of apparent solubility of drugs. In Section 16.6.4, solution formulations increasing a drugs’ kinetic solubility are presented, and in Section 16.6.5, the principles of precipitation inhibition are discussed.

16.6.3 Formulation Strategies Improving Drug Dissolution and/or Apparent Solubility

Drug in solid dosage forms or in suspensions needs to dissolve first before it can be absorbed from the GIT. For rapidly dissolving compounds, the saturation concentration C_S in the GIT is reached almost instantaneously and the rate of absorption is then maximal (Eq. 16.4) (Figs. 16.1 and 16.9⓪). For HP compounds ($A_n > 1$) with $D_o \leq 1$ (BCS class 1) (Sections 16.3 and 16.4.1), the total dose will dissolve and will be absorbed within the absorption window ($D_n > 1$), whereas for compounds with $D_o > 1$ (BCS class 2), only a fraction of the dose will be absorbed (solubility-limited absorption). If dissolution is slow, $D_n < 1$, the compound will be absorbed faster than it dissolves, hence C_S will not be achieved and the extent of absorption will be lower (dissolution-limited absorption) (Figs. 16.2 and 16.9). In these cases, several formulation strategies can be applied to speed up drug dissolution rate (to push 2 to 1 in Fig. 16.9).

16.6.3.1 Modification of Surface Area. The first dissolution-enhancing approach is to optimize the exposure of available surface area of particles to the solvent. In suspensions, hydrophobic particles often agglomerate to reduce their surface energy, which will decrease the dissolution rate according to the Nernst and Brunner equation (Eq. 16.9) and introduce kinetic hindrance to the diffusion process. Agglomeration

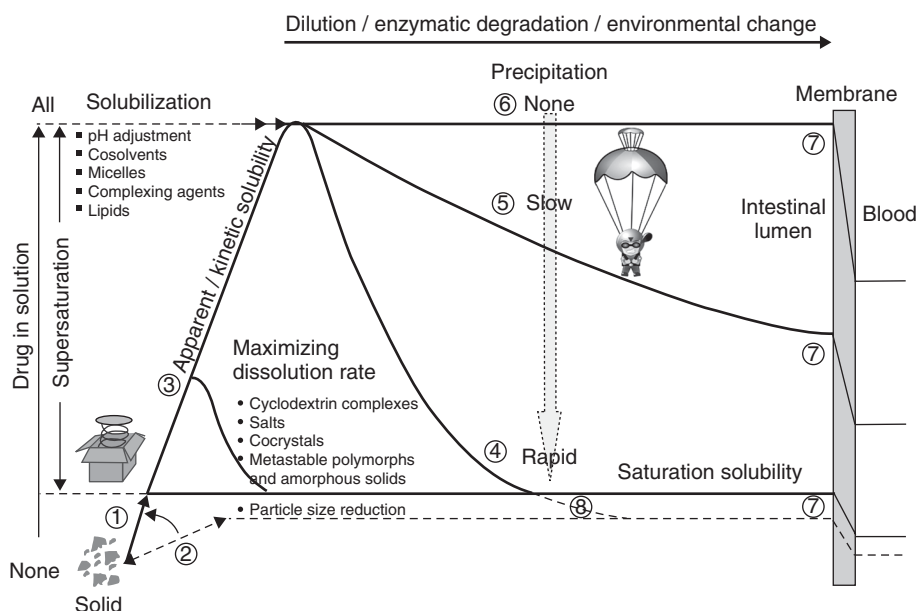


Figure 16.9 Drug concentration–time profiles for different formulation approaches. The schematic representation shows formulations maximizing drug dissolution rate (Section 16.6.3) and illustrates the “spring/parachute” approach for solubilizing formulations (Section 16.6.4) and for formulation inhibiting drug precipitation (Section 16.6.5).

can be inhibited by adding steric polymeric stabilizers such as polyvinyl pyrrolidones, pluronics, and cellulose derivatives or surfactants such as polysorbate 80, lecithins, cholic acid derivatives, or sodium lauryl sulfate to the suspension vehicle. A more in-depth description of the underlying mechanisms can be found in Refs. 305,317. In practice, on a small scale, homogenous suspensions may be obtained by intense stirring, which will support deagglomeration due to the shear forces. However, air incorporation should be avoided since air may form a cushion around hydrophobic particles and separate them from the solvent. For larger scale deagglomeration, short cycles in a Dyno® Mill or a Dispermat® may be used.

Particle size reduction is another method to improve the dissolution behavior of drugs (Section 16.5.1.3) (shift 2 to 1 in Fig. 16.9). With decreasing particle size, the surface area increases for a given mass, the diffusion layer thickness decreases, and thus the dissolution rate increases (Section 16.5.1.3). In addition, smaller particles are energetically unfavorable because of the higher interfacial energy and are subject to preferential dissolution. In some cases, their apparent solubility can be above C_S , leading to precipitation and growth at the surface of larger particles (i.e., Ostwald ripening) since bigger particles inversely have a lower dissolution rate and saturation solubility. The speed of the growth of large crystals at the expense of small ones due to Ostwald ripening decreases with decreasing saturation solubility of the drug, and Ostwald ripening may be circumvented by preparing suspensions with largely narrow particle size distribution. For spherical particles, the reduction in size is proportional to the increase in surface area, that is, a 10-fold decrease in size results in a 10-fold increase in specific surface area [177] and hence an only 10-fold increase in dissolution

rate may be achieved. Size reduction to the submicron range has found considerable interest for drugs that are difficult to formulate by other methods, and in particular, high dose, LS drugs with a high MP and a high lipophilicity profit from particle size reduction [305]. Such drugs, formulated as nanosuspensions, showed significantly increased oral absorption, for example, danazol [318], naproxen [319], and aprepitant [320]. However, a greatly improved dissolution rate *in vitro* does not always translate into higher absorption *in vivo* since other factors such as fasted and fed state or physicochemical properties of drug also seem to play a significant role [321]. Mucoadhesion, which may increase GI transit time, is discussed as another mechanism to explain the enhanced oral BA of nanosuspensions [305]. Compared to microsuspensions, nanosuspensions have several advantages: they often show (i) a higher BA, (ii) a less variable and less food-dependent exposures [319], (iii) a faster onset of action, and (iv) a more linear increase in exposure with dose and they can also be used for intravenous and subcutaneous drug administration with PK, often equivalent to that of a solution formulation [302]. Nanosuspensions, can be prepared at high weight-to-volume loading, for example, for toxicity studies, and since no cosolvents and only small amounts of excipients are required, the toxicity of vehicles is usually much lower than with other formulation approaches. However, because of the high surface energy, nanosuspensions are more prone to agglomeration, and thus, stabilization is very important to retain the large surface area. Miniaturized screening systems for their preparation and characterization and for the identification of surface-stabilizing excipients have been reported recently [322,323]. Standard techniques for producing micro-/nanosuspensions are media milling and high pressure homogenization, and both top-down (breaking down of bulk material) and bottom-up (building up from solution) technologies are applied. Extensive reviews discussing their advantages and disadvantages, the use of stabilizers, and the characterization of nanosuspensions are available in this area [319,324,325]. Small-scale systems for the preparation of 2–5 mL volumes of micro-/nanosuspensions for early phases PK/PD studies exist, for example the Mixer Mill from Retsch (www.Retsch.com) and the NanoMill® from Elan (www.elandrugtechnologies.com). The mechanical impact generated during milling in top-down approaches may cause chemical degradation and/or physical conversion of drugs. In particular, the latter is often ignored in early phases. Formation of new polymorphic forms, hydrates, or amorphous material with different dissolution behavior and apparent solubility during milling can have a huge impact on drug exposure and on the outcome of PK/PD studies. Nanosuspensions are also an option in clinical trials to improve exposure and to reduce food effects and variability.

16.6.3.2 High Energy Solids. Another option to improve drug dissolution is the use of higher energy (metastable) solid forms, which affects both the dissolution rate and the apparent solubility of drugs (Fig. 16.9, shift 2 to 1 and 2 to 3). In particular, amorphous forms have a higher energy state than the most stable crystalline form and, thus, their solubility and dissolution rate are higher [135]. However, if the apparent solubility exceeds the C_s of the most stable polymorph and supersaturated concentrations are formed, crystallization of amorphous material may occur. Crystallization at the surface is faster than in the bulk, and even a thin layer at the surface may already significantly slow down dissolution [281]. Thus the conversion rate of the metastable amorphous to the stable crystalline form has to be slower than the dissolution rate of the drug to take advantage of the amorphous form. Amorphous formulations without

stabilization are challenging with regard to reproducibility, since crystallization may occur at any time during manufacturing, storage, or *in vivo* absorption because of many factors such as agitation type and speed, medium composition, temperature, or surfaces properties. Also, amorphous forms often tend to adsorb moisture, which may lower its glass-transition temperature and facilitate recrystallization. Amorphous solids are often generated unintentionally in discovery chemistry programs, and exposures achieved with these materials are difficult to reproduce at a later time, even with the same batch. The manufacturing of pure amorphous material without stabilization in this phase is not common and not desirable; however, partially or completely amorphous solids may be generated during formulation preparation, for example, by the energy entry during wet milling. Therefore, monitoring of amorphous–crystalline transformations in suspensions, at least by polarization microscopy, should be performed to allow informed decisions, particularly if PK and PD results of amorphous and crystalline compounds are compared and used for candidate ranking. The gain in solubility using higher energy crystalline forms is usually less than with amorphous material and is typically in the range of <2 [126]. If they are used as starting materials, solid transformations are more difficult to follow and will require Raman or XRPD analysis. Nonetheless, this additional characterization can help (i) to identify polymorphism and hydrate formation issues early on, (ii) to improve the quality of data, and (iii) to better differentiate observed *in vivo* variability in exposure related to solid state changes in formulation from other factors [138,270,280].

16.6.3.3 Multicomponent Drug Solids. The dissolution rate of drugs may also be modulated by combining them with partner molecules, that is, by preparing salts, cocrystals, hydrates, cyclodextrin complexes, and solid dispersions/solutions. These approaches alter the chemical and physical solid state properties of drugs and are designed to increase their dissolution rate and, more than the previously discussed concepts, their apparent solubility (Fig. 16.9, ③).

16.6.3.3.1 Salts. Salt formation is limited to ionizable drugs, and salt selection largely depends on pK_a values. The selection of the “best” salt for development is not trivial, and for a discussion of criteria, the reader is referred to the book by Stahl [326]. For acidic drugs, sodium and potassium salts are often first considered; for basic drugs, the hydrochloride salts are the most common ones. As a rule of thumb, the pK_a difference between an acid and a base should be three or more to form stable salts. The solubility of a salt in aqueous medium is higher than that of a nonionized species, and when salts dissolve, they act as their own buffer and change the microenvironmental pH and solubility in the diffusion layer. Drug solubility is then governed by the solubility in the diffusion layer. Depending on the pH of the GI fluid, a drug may precipitate as nonionized fine amorphous or crystalline particles when it diffuses out of the diffusion layer. In many cases, this precipitate consists of prewetted fine particles with a very large surface area, which can rapidly redissolve when additional fluid becomes available. Therefore, a salt may achieve a higher exposure than the native drug [327]. In contrast, when the intrinsic solubility of a drug is very low, its unionized form may precipitate very close to the dissolving surface and coat the surface. This may prevent further dissolution of the salt or deaggregation of particles of a solid dosage form and hence result in lower exposure as shown for example, for sodium warfarin [328]. The formulation of a salt is often challenging since, in particular, in

suspension formulations for small animal species, salt may rapidly convert to the free form if inappropriate vehicles were selected.

16.6.3.3.2 Cocrystals. Pharmaceutical cocrystals are composed of at least two different components, the drug and a nontoxic cocrystal former, held together by freely reversible, noncovalent bonds, predominantly hydrogen bonds [329]. Cocrystals are a strategy for poorly soluble neutral compounds to improve their solubility and dissolution rate, similar to salt formation, regardless of ionizable groups [267,268]. It is important to screen carefully for appropriate cocrystal formers, to test both their solution stability and dissolution behavior, and to understand possible conversions of solids that could take place on contact with solvents. Coformers with lower aqueous stability appear to form cocrystals with better solution stability; however, there are still many uncertainties, and cocrystals were found to enhance, slow down, or not affect the dissolution rate of a drug at all. The screening for cocrystals is usually not done in discovery and often, only started in early development after other approaches to improve a drug's solubility, BA, or stability failed or after efforts to crystallize drugs by standard approaches were unsuccessful. Formulators should also be aware of unintentional cocrystal formation with excipients that sometimes occur in formulation, for example, with saccharin, benzyl alcohol, or parabens [330]. A comprehensive review summarizing the current status of cocrystal research and their characterization has been published recently by Schultheiss and Newman [128].

16.6.3.3.3 Hydrates. Solvates are crystalline drug solvent adducts. Adducts with water, hydrates, are a common phenomenon, and almost 50% of the pharmacopeial compounds, salts, and nonsalts are known to form hydrates [331]. Unidentified hydration or dehydration processes may result in unexpected and variable behavior of drugs and drug products during processing and storage and affect a number of factors such as chemical and physical stability, BA, wetting, dispersibility, or compactibility. Unintentional hydrate formation is often an issue in early phases of development when aqueous suspension vehicles are used for animal dosing, when the preparation/storage conditions of drugs are not well controlled or when water-based processes such as wet granulation or spray drying are used for drug processing. In many cases, the solubility and/or dissolution rate of hydrates is lower than that of the anhydrate [126], and hence exposures in *in vivo* studies can drop significantly [270]. These issues are presumably more important for LS than for HS compounds, and it is important to address hydrate formation early on; stable hydrates may already be identified in early phases by simple, longer term, slurry experiments in water with subsequent Raman or XRPD analysis of the solid.

16.6.3.3.4 Cyclodextrins. Improved dissolution of LS drugs and an increase in apparent solubility can also be achieved by complexation of drugs with CD (Fig. 16.9). CDs are (α -1,4)-linked oligosaccharides containing typically 6-10 α -D-glucopyranose units. They take the shape of a truncated cone or torus with the hydroxyl functional groups oriented to the exterior and the skeletal carbons and ethereal oxygens to the interior of the cone. Thus a hydrophobic cavity is formed in the CDs, which can take up poorly soluble molecules. The efficiency of these inclusion complexes depends on the size of the cavity and the properties of the guest molecule. Guest and host molecules are in a dynamic equilibrium, and complexation efficiency can, positively or

negatively, be affected by excipients or constituents of intestinal fluids as mentioned in Section 16.5.1.3 and reviewed by Brewster and Loftsson [251]. CDs often also increase the apparent solubility of compounds as discussed later (Section 16.6.4.3), the solubility profile depending on the type of complex formed and on its stoichiometry [332]. In addition, CDs can improve chemical and physical stability, mask bad taste or odor, reduce volatility, convert oils or liquids into solids, or reduce side effects. Complexes can be prepared by a variety of methods, including kneading, evaporation, and freeze drying [333]. In case of solid CD–drug complexes, drugs are molecularly dispersed and have no own crystal structure. The solid complexes may be crystalline or amorphous. The exterior of CD–drug complexes is hydrophilic, and typically, they dissolve very rapidly. Since CDs then keep the drug in solution for an extended period, an increase in drug absorption is often observed in their presence [333–335]. On the contrary, CDs are also reported to reduce drug permeability both *in vitro* and *in vivo*, although they markedly decrease UWL permeability [231]. Therefore, a trade-off exists between the increase in solubility and the decrease in permeability, and hence, the overall *in vivo* effect of CDs on absorption will also presumably depend on the local CD concentration and the binding constant of the complex.

16.6.3.3.5 Solid Solutions and Dispersions. In solid solutions and solid dispersions, an LS drug is embedded in an inert solid hydrophilic polymer carrier such as Polyethylenglykol (PEG), Poly-Vinyl-Pyrrolidon (PVP), Polyvinyl acetate (PVA), Hydroxypropyl Methycellulose (HPMC), or sugars to improve its dissolution rate and/or apparent solubility. In solid dispersions, drug is distributed as fine colloidal crystalline or amorphous particles in the polymer. In contact with aqueous media, the drug is released as very fine particles with a high surface area, which facilitates a rapid dissolution. In solid solutions, the drug is mixed with the polymer at the molecular level to form a real solution. In contact with water, the drug is then dissolved parallel to the polymer. Drug–polymer dispersions or solutions can be prepared by dispersing/dissolving drug in molten polymer or in a common solvent followed by resolidification of the melt or by solvent evaporation, respectively. Known challenges with these techniques are heat-labile drugs, which can not be used in a melt process, or the difficulty to identify a common solvent for both the hydrophilic carrier and the hydrophobic drug. Other common preparation techniques are physical mixing of the polymer and drug [273] and cogrinding [274]. The stability of such systems can be an issue. Depending on drug load, storage conditions, and formulation composition, dissolved compound in solid solutions or completely or partially amorphous drug generated during preparation of solid dispersions may crystallize with time in the matrix. Therefore, appropriate polymers have to be selected with care to avoid transformations during storage. Although screening systems for solid dispersion in discovery became available recently [160], these formulation approaches are typically only used in special cases in early and late development if other, less risky approaches fail to achieve a sufficient exposure.

16.6.4 Solution Formulation Strategies

All formulations described in the previous section contain the drug in solid form. This section summarizes formulation approaches that present drug in the dissolved state to the GIT, and hence no dissolution step is required for absorption (Fig. 16.9). Solubilization is achieved by pH adjustment, cosolvents, cyclodextrins, surfactants

and lipids, or combinations thereof. These approaches are often preferred in discovery and early development phases as the influence of the initial solid state properties of compounds on PK/PD studies can be neglected, and hence it is believed that compounds are easier to compare. Here, it is often overlooked that these approaches address kinetic solubility (Section 16.5.3.1) and that compounds may precipitate on dilution of the formulation or digestion of formulation constituents in the GIT. The overall *in vivo* performance of a drug would then be governed by both its precipitation kinetics and the dissolution rate of its precipitate; the latter depending on the kind of precipitate (amorphous or crystalline), the particle size, and the particle distribution (finely dispersed or agglomerated). Therefore, it is important to evaluate the precipitation tendency of drug formulations in artificial intestinal media (simulated gastric fluid (SGF), FaSSIF, FeSSIF) as well as the nature and dissolution behavior of the precipitates before drug candidates can really be compared and ranked. This is not always feasible in early phases; however, scientist should be aware of these issues.

16.6.4.1 pH Adjustment. Adjustment of pH can be used to solubilize compounds with ionizable groups. The solubility of these compounds is usually strongly pH dependent, and once their pH–solubility profile has been determined, it can be estimated whether or not a given dose can be given orally as solution by this approach. The pH of the final solution can be in the range of 3–8 for oral delivery. For the extreme pH values, high buffering capacities should be avoided, since this may cause irritations in the GIT because of unphysiological pH values, in particular, after repeated dosing. *In vivo*, solutions of weak bases may precipitate in the small intestine if they are not absorbed rapidly enough and the concentration of the unionized form exceeds their intestinal solubility. Similarly, weak acids may precipitate at low pH in the stomach; however, there is a high chance that they dissolve again further down in the GIT at higher pH. *In vitro*, tests are available to study this behavior [285].

16.6.4.2 Cosolvents. Cosolvent systems are another popular approach for oral delivery of drugs in solution, which may also be used for parenteral formulations. In particular, in early PK studies, LS compounds are often solubilized in cosolvents such as ethanol, PEG300, PEG400, propylene glycol, glycerin, Dimethylacetamide (DMA), N-Methyl-2-pyrrolidone (NMP), or DMSO [301]. Cosolvents are used alone, are mixed with water with or without pH adjustment, or are combined with cyclodextrins or surfactants. Combinations of cosolvents are also used to reduce vehicle toxicity. In addition to their solubilizing activity, pure cosolvents may also increase the stability of drugs, for example, against hydrolysis [336]. Uncontrolled precipitation may occur on dilution of cosolvent formulations with aqueous media, and a semilogarithmic relationship exists among total drug solubility, drug solubility in water, and the cosolvent volume fraction [301]. The increase in the extent of oral absorption that is often observed with cosolvent formulations may be explained by both the generation of temporary supersaturated solutions and the high energy precipitates (Section 16.6.3.2). Ideally, the precipitates should consist of prewetted, finely dispersed, and amorphous solids with very small particle sizes to achieve maximum dissolution rates.

16.6.4.3 Solution Formation Using Cyclodextrins. Solid inclusion complexes of drugs and cyclodextrins have been discussed before in Section 16.6.3.3.4. Drugs that interact with CDs may also be formulated as solutions for oral or parenteral

administration. Many drugs favorably bind to β -CDs; however, since its solubility is limited, usually, complexes with the more soluble hydroxypropyl- β -cyclodextrins (HP- β -CDs) and sulfobutylether- β -cyclodextrins (SBE- β -CDs) are preferred for solutions [251]. The effect of CDs on the oral absorption of a drug will depend on amount administered, binding strength, dilution, and presence of molecules competing for binding. If a drug is bound strongly to CDs and dilution in the intestine is low, lower amounts of free drug may be available for absorption than in situations in which the drug rapidly dissociates from the CDs or is displaced, for example, by bile salts. However, in the latter case, the risk for drug precipitation also increases.

16.6.4.4 Micelles. Surfactants added to solids at concentrations below their CMC are often used to improve drug wettability, that is, to reduce the interfacial tension between the drug and dissolution medium (Section 21.6.2.1). Above their CMC, surfactants form micelles, which can solubilize poorly soluble drugs in water [337]. In micelles, the hydrophobic tails of the surfactants point to the inside, and depending on their polarity, drugs may get inserted into various regions of the micelles. Common surfactants used for solubilization are nonionic (e.g., Tweens, Spans, Solutol HS-15, VitE-TPGS, Pluronic F68), anionic (e.g., sodium lauryl sulfate), cationic (e.g., cetyltrimethylammonium bromide), or zwitterionic (e.g., phospholipids) [301]. Above CMC, drug concentration in solution usually increases linearly with surfactant concentration. Inversely, drug solubility decreases on dilution of micelle formulations, and thus drug may precipitate in intestinal fluids. In general, the lower the CMC is, the more stable the micelles are and hence the more they can be diluted before micelles break apart. Therefore, nonionic surfactants with low CMCs are often better solubilizers than ionic surfactants. Cosolvents may be combined with surfactants to improve solubility [338]. For ionizable compounds, pH control may also be used to further enhance drug solubility and/or the drug load of micelles. However, especially for ionic surfactants, pH, ionic strength, and counter ions need to be controlled carefully to optimize micellar solubilization. The overall gain in oral drug absorption with micellar solutions will depend on the degree and duration of supersaturation that can be achieved *in vivo* (Section 16.6.5). However, absorption might also be reduced because of the lower diffusivity of drug-loaded micelles as discussed in Section 16.5.4.

16.6.4.5 Lipids. Many poorly water-soluble, lipophilic drugs may be formulated in lipidic drug delivery systems, and extensive reviews are available in this area [306,307,339,340]. Lipid formulations mimic to some extent the solubilization of drugs by food and can reduce or even eliminate the difference in drug absorption between fed and fasted subjects. Ideally, they maintain drug in solution during dispersion and digestion before absorption. Lipid formulations may include a broad variety of excipients ranging from triglycerides, mono- and diglycerides, lipophilic surfactants, hydrophilic surfactants, to cosolvents. Their *in vivo* performance depends on the dispersion and dilution of the formulation and on the influence of digestion on drug solubilization. Pouton suggested the Lipid Formulation Classification System (LFCS) to identify the factors that are likely to affect performance *in vivo*. Type I represent pure oil formulations that can only be used for highly lipophilic drugs. They are nondispersible, and digestion is needed for drug release. Type II formulations additionally contain water-insoluble surfactants (hydrophilic-lipophilic-balance (HLB) <12). They are water-insoluble self-emulsifying drug delivery systems (SEDDS), which form

turbid oil-in-water dispersions with particle sizes >100 nm without losing solvent capacity. Digestibility is not crucial for drug release but is likely to occur. Depending on the composition, viscous liquid crystalline gels may form at the oil–water interface. Type III systems are SEDDS or self-microemulsifying drug delivery systems (SMEDDS), which contain water-soluble surfactants ($\text{HLB} > 12$) and/or cosolvents. They form clear or almost-clear dispersions on dilution with particle of size <100 nm, and digestion is not crucial for drug absorption. Smaller particles have a larger surface area, which facilitates digestion and a more rapid and uniform drug release. Type III formulations can be further segregated into Types IIIA and IIIB, the latter representing more hydrophilic systems with higher amounts of hydrophilic surfactants and cosolvents and less oils. Type IV formulations contain no oil and consist of water-soluble and/or water-insoluble surfactants with or without cosolvents. On dispersion, these systems form micellar solutions, a loss of solvent capacity is likely, and hence this type bears a high risk of drug precipitation. Digestion is not required for drug absorption. Type IV systems are often used for drugs that are hydrophobic but not lipophilic. Cosolvents are added to facilitate dispersion and to reduce variability and irritancy caused by high local concentrations of surfactant.

The design of lipid formulations is not trivial. First, a vehicle has to be identified, which completely dissolves the target dose. Then, *in vitro* dilution studies have to be performed in simulated intestinal media to study the phase changes of the formulation and to estimate the fate of the drug. If precipitation occurs, the advantage of lipid formulation is largely lost. Relevant dilution ranges to look at may differ depending on the target species. To estimate the likelihood of precipitation, equilibrium solubility studies can be an alternative to these kinetic solubility studies. Here, compound solubility is measured in various dilutions of the formulation [341]. For formulations that require digestion for drug release, *in vitro* lipolysis studies should also be performed. Other questions to address are the importance of lipid particle size for absorption, the desired release kinetics, and the ability of certain lipids to support lymphatic transport [306]. Since dispersibility, dilution factors, and digestibility of formulation may vary in different species, the performance of formulations may differ [339].

16.6.5 Formulation Strategies Inhibiting Drug Precipitation

Most formulation strategies described before are designed to maximize the concentration of the drug at the site of absorption, thereby increasing the absorption rate (Eq. 16.4). As shown in Fig. 16.9, formulations either increase the dissolution rate of the drug to maintain C_s (shift from 2 to 1) and/or increasing its apparent or kinetic solubility above C_s (3) to enhance the concentration gradient across the intestinal membrane (7). If the local drug concentration exceeds C_s in the GIT, supersaturated solutions are formed (Section 16.5.1.1). These systems are inherently thermodynamically unstable, and thus the drug may rapidly (4), slowly (5), or not at all (6) precipitate during the intestinal transit, which can lead to variable absorption. Precipitation reduces free drug concentration, and if it happens at the surface of a dosage form, dissolution of additional drug may be prevented. In the worst case, precipitation may even decrease drug solubility below C_s , for example, if drug precipitates as agglomerate of large particles with slow dissolution rate (8). The risk for drug precipitation increases with the supersaturation ratio (Section 16.5.1.1) and it decreases with the viscosity of the medium. Supersaturated solutions can only be created from forms of the drugs that have a higher

energy than the thermodynamically most stable crystalline form. These high energy forms work like a “spring” (Fig. 16.9). A “spring” can be a higher energy solid (e.g., amorphous form, salts, solid dispersions) (Sections 16.6.3.2 and 16.6.3.3) or a solution (e.g., cosolvents, lipids) (Section 16.6.4). Higher energy forms are usually introduced by the formulation; however, they may also result from physiological processes. An example is a weak base that completely dissolves at the acidic pH of the stomach, then forms a supersaturated solution at the higher pH values in the small intestine, and eventually precipitates again as base [285]. Supersaturation enhances the absorption rate (Eq. 16.4) of drugs in all BCS classes (Section 16.4.1), since concentrations higher than C_S will always increase the driving force for molecules for transit into and across biological membranes (7). Once achieved, intraluminal supersaturated states have to be maintained for a period sufficient for absorption. The challenge for formulators is to identify appropriate pharmaceutical excipients that hinder (6) or slow down (5) nucleation and crystal growth and act as “parachutes” or precipitation inhibitors for drugs [134,342,343]. Precipitation inhibitors can act at different levels. They may increase drug solubility or medium viscosity, bind to the interface between liquid and solute aggregates (decrease nucleation), adsorb to the medium–crystal interface (hinder crystal growth) and/or change the solvation at crystal–liquid interface (affect integration of drug molecules into crystal) [134]. Commonly used classes of excipients are polymers (e.g., HPMC, PVP, PEG), surfactants (e.g., SDS, Cremophor RH40), and cyclodextrins (e.g., HP- β -CD, SBE- β -CD), which are also regularly used in high energy solid (Section 16.6.3.3) and solution formulations (Section 16.6.4) to enhance dissolution rate and/or solubility. Box and Comer [235] suggested that slowly precipitating and dissolving acids, bases, and ampholytes with high MPs of free form (“chasers”) may be better candidates for supersaturating systems than fast-precipitating, slowly dissolving bases with low MP (“nonchasers”). A number of solvent quench approaches are now available for *in vitro* screening of crystallization inhibitors [134,301,342,344] and several methods have recently been reviewed by Dai [345]. The impact of excipients on solubility may also be tested in any of the solubility assays discussed in Section 16.5.3. The design of predictive *in vitro* assays is challenging since a number of factors, such as drug, inhibitor, and medium (volume, pH, etc.), affect their outcome; however, often, excellent IVIVCs have been obtained [342,346–348].

16.7 PRECLINICAL FORMULATIONS FOR CANDIDATE PROFILING

The development of nonclinical formulations for dosing in animal is usually very pragmatic and dictated by the physicochemical properties of the compound, dosing route, dose, species, type of study, and time pressure of the project. If feasible, it starts with aqueous solutions with or without pH adjustment, cosolvents, and/or surfactants (Section 16.6.4). Compounds that are not soluble enough may be dosed in lipidic systems if properties and dose are compatible (section 16.6.4.5). For solutions, the precipitation behavior on dilution needs to be tested to avoid side effects after parenteral dosing or reduced BA after oral administration (Section 16.6.5). If a dose cannot be completely solubilized, suspensions and nanosuspensions are used as an alternative; the latter may also be used for parenteral dosing (Section 16.6.3.1). To avoid surprises and to aid interpretation, the properties of the initial and final (or precipitated) solid should be monitored, for the reasons discussed before. Many decision trees have been

developed for formulation selection (Section 16.6.2), and measurement of solubility (Section 16.5.3) and dissolution (Section 16.5.4) are key elements in most of them. In many instances, combinations of the formulation approaches discussed in Section 16.6 are used to achieve synergistic effects; however, a scientist should be aware of potential interactions in such systems, for example, loss of solubilization capacity by binding of surfactants to cyclodextrins [256] or reduction of preservative concentration by cocrystal formation [330]. Since formulation strategies depend on drug properties, dose, and species, and, for oral absorption, on the identified rate-limiting steps (Section 16.3), approaches may change with each and any of these factors. Rat and mouse are usually dosed with solutions or suspension, while larger species may also receive capsules. Important aspects for all formulations are sufficient chemical stability of the drug in the vehicle, optimal *in vivo* exposure at the target site, accurate dosing, no vehicle effect on PD parameter [300,310,324], and minimal toxic effects of the vehicle alone. Food effects [349–351] and species physiology also need to be considered. Comprehensive summaries of GIT physiology are available for human [352], cynomolgus monkey [353–356], rat [51,56,357], mouse [51], and dog [349,358]. Reports comparing several species [42,54,58,299,359] or only two species are available, for example, human–dog [28], human–cynomolgus monkey [360], human–rat [361], dog–cat [54] or rat–mouse [51].

The development of suitable formulations for toxicology is challenging since doses are high and excipient amounts often need to be high for sufficient and dose-linear exposure. An overview of commonly used excipients for preclinical PK, PD, and toxicity studies and use ranges can be found in Ref. 298,299,301,303,311. Scientists should always be aware of the maximum tolerated dose of excipient(s), and in short-term studies, as a rule of thumb, the amount of an excipient in formulations should not exceed one-twentieth of its LD₅₀ value (lethal dose for 50% of those exposed to a toxic agent). In chronic studies, other criteria may apply. Two smaller sets of commonly used non-clinical vehicles have been published by Maas *et al.* [298] and Neervannan [299], and an extensive list is available from Gad *et al.* [311]; the latter also includes maximum tolerated use levels by species, route, and duration of study, with accompanying dose-limiting toxicity. Excipients and vehicles for parenteral administration have been discussed in detail by Strickley [313]. Furthermore, guidelines have been suggested that recommend for common laboratory species, the maximal dosing volumes for different routes of administration as well as the volumes for blood sampling [311,362].

Researchers should be aware of the fact that the criteria for formulations for animal and human studies may differ and that right first-time formulations are rare [96,304]. One reason is the dose range required for testing, which is often several-fold higher in animal than in human and may necessitate different formulation approaches. Another one can be formulation related, for example, capsules cannot be administered to mice. Often, timelines also play a role, and enabling technologies such as polymer-stabilized solid solutions are only feasible in later development phases. In many instances, microsuspensions with crystalline solids are a useful starting point to estimate the developability of a simple human formulation since they represent a disintegrated dosage form such as tablet or capsule. If they give a sufficient exposure, clinical formulation development is usually not an issue. If solutions are used in early studies, results should be reaffirmed as soon as possible using characterized suspensions.

16.8 FORMULATION AND EXCIPIENT EFFECTS ON DRUG DISPOSITION

Both rate and extent of oral absorption of potential drug candidates can be significantly influenced by solid state properties, type of formulation, and excipients used, as discussed in previous sections. As a result, the intrinsic PK and PD characteristics of a drug may be altered unintentionally by influencing its absorption, distribution, metabolism, and/or elimination [6,297,308,310,363–365]. An in-depth discussion of all formulation- and excipient-related effects is far beyond the scope of this review. However, a few important aspects are highlighted below to increase the awareness of pharmaceutical scientists for formulation effects on drug disposition, to aid in the interpretation of drug metabolism and pharmacokinetics (DMPK) and PD results, and to reduce the risk of incorrect drug candidate classification.

For compounds with dissolution-limited absorption, an increase in dissolution rate by any of the formulation principles described in Section 16.6.3 is expected to result in an earlier peak of absorption and a larger AUC if C_s can be maintained throughout absorption. If drugs exhibit solubility- or permeability-limited absorption (Section 16.3), an increase of apparent/kinetic drug solubility in the GIT by formulation (Sections 16.6.3, 16.6.4, and 16.6.5) is likely to increase the $C_{\max}$ because of the higher concentration gradient across the membrane (Fig. 16.9). An increase in AUC is expected if the supersaturated state can be maintained, for example, by precipitation inhibitors (Section 16.6.5). In contrast, the benefit of a solution formulation (Section 16.6.4) may be completely lost on drug precipitation. If drug precipitates, for example, as low energy, crystalline, large, or aggregated particles, exposure may even be lower than with a defined suspension of finely distributed small particles. In general, particle size and polymorphic form of a drug in suspensions may significantly impact $C_{\max}$, $T_{\max}$, and AUC [261,286,366]. Suspensions of the same drug prepared from amorphous or crystalline forms [367] or from different polymorphic forms [366] or salts [327,367] may show completely different PK profiles. If this substantial impact of solid state properties on drug disposition is ignored for the ranking of preclinical drug candidates, an inappropriate compound might be selected for development.

Increases in intestinal drug solubility may also affect absorption by other mechanisms. If drugs are substrates for absorptive transporters, a less than dose-proportional increase may be observed with increasing doses of completely solubilized drug due to a saturation of the active transporter. On the contrary, drugs that are substrates for efflux transporters (e.g., P-glycoprotein (P-gp)) and/or for metabolic enzymes (e.g., CYP3A4) may show a more than dose-proportional increase in drug absorption with increasing concentrations since these mechanisms will be saturated above a certain level [368].

Furthermore, a number of excipients commonly used in solubility-enhancing formulations (e.g., VitE-TPGS, Cremophor EL, Tween 80, Solutol HS15) are also reported to enhance drug absorption by direct inhibition of efflux transporters and/or metabolic enzymes [6,300]. However, depending on drug properties and surfactant used, surfactant micelles may also trap drugs and reduce their absorption [364,369]. For example, Cremophor EL decreased digoxin absorption and shifted $T_{\max}$ to later times, while VitE-TPGS increased its absorption and decreased $T_{\max}$ [370], although both surfactants enhance drug solubility and inhibit P-gp. In many cases, it is difficult to distinguish between the direct effects of excipients on transporters or metabolic enzymes and their

indirect effects on drug solubilization. In later development phases, controlled-release delivery systems may be used to modify SITT, to deliver drugs to specific regions of the GIT, or to bypass metabolism or efflux pumps [74,308]. Similar to the oral route, surfactants may also influence the disposition of solubilized drug after parenteral administration, and huge differences have been reported, for example, in metabolism, excretion, and distribution of drugs bound to Tween 80 or Cremophor EL micelles [364].

Other commonly used formulation constituents may also alter drug absorption and drug disposition. For example, CDs may reduce oral BA if the administered CD concentrations are too high [6,231]. CDs may also influence GI motility and liver function at higher doses [310]. Similarly, cosolvents (e.g., PEG400, DMSO, ethanol), HPMC, and fat emulsions may change GI, renal, and/or liver parameters after application of a certain threshold [6,310,371,372]. Therefore, vehicle-dependent side effects should be known, and particularly in PD studies, the influence of placebo vehicle on the measured parameter should be evaluated; for example, SB β -CD, Tween 80, and HPMC were reported to decrease blood glucose after oral or parenteral administration [299,310], an effect that might be detrimental in projects hunting for glucose-lowering drugs.

Food adds further complexity to oral absorption studies. A number of postprandial changes in the GIT may affect the rate and extent of drug absorption, for example, slower gastric emptying time, pH shifts in the GIT, saturation of uptake/efflux transporters or of metabolism by food components, and enhanced dissolution/solubilization by increased bile flow, lipids, and lipolysis products. Extensive reviews of the different mechanisms for food–drug interactions and of related key physicochemical parameters of drugs in the frame of BCS and BDDCS classification (Sections 16.4.1 and 16.4.2) can be found elsewhere [20,314,368,373,374]. Lipid formulations may often have effects similar to food and may be used to target highly lipophilic drugs to the lymphatic system, to bypass liver first-pass metabolism, to increase absorption, or to modulate PK parameters [300,306,375].

Overall, the effects of formulation and food on absorption *in vivo* are clearly complex and may be multiple. Effects on drug disposition are more likely in early development programs where typically larger amounts of excipients are administered and studies are often less well controlled. Scientist should be aware of the fact that compound ranking or selection based on PK and PD data generated with different compound batches, formulation principles and/or compositions, administration volumes, or nutrition conditions is prone to errors. Therefore, a systematic, standardized approach for formulation preparation, administration, and data reporting should be established to allow a more realistic comparison of drug candidates and to reduce the overall risk for selecting the wrong candidate for development. There is probably no “universal” vehicle that can be used for all compounds or for all studies; however, a rational study design and selection of excipients and formulations will certainly help to minimize vehicle-dependent side effects and wrong decisions.

16.9 CONCLUSIONS AND FUTURE PERSPECTIVES

The awareness of scientists for preclinical formulation aspects has certainly increased in most pharmaceutical organizations. Discovery scientists now start to understand the impact of solid state properties and of formulation on the outcome of animal studies

and on preclinical attrition rate, and development functions recognize that putting more resources into early discovery can avoid problems and delays downstream in drug development. Consequently, development scientists are now more engaged already during early discovery stages to identify, address, and overcome compound liabilities together with their discovery colleagues to increase the overall chances for success. This process is more data driven than ever before, and an early identification of potential compound limitations such as dissolution, solubility, permeability, or metabolism is essential. Particularly, the growing number of BCS class 2 compounds necessitates thorough measurement of drug solubility and dissolution early on; protocols of different complexity and for different scales are now available. For scientists in pharmaceutical industry, a profound understanding of the advantages, disadvantages, and limitations of the various methodologies and awareness of the different types of solubility and dissolution is a must. It is the basis for a meaningful interpretation of solubility/dissolution data and for a conscious decision on which drug delivery technology to follow or to develop. Furthermore, awareness of the impact of the solid state properties of a compound (initial or created by precipitation) and of the formulation (type and/or excipients used) on PK/PD properties (e.g., $C_{\max}$, $T_{\max}$, AUC) will reduce the risk to misinterpret data from *in vivo* studies, to select the wrong candidate for development, or to eliminate a potentially promising candidate because of low exposure. In this context, the BCS and BDDCS are useful for both discovery and development to categorize or rank-order compounds and to communicate potential development risks to the organization. If the required human dose estimate for BCS/BDDCS is not yet available, MAD may be used in a first instance for compound ranking and for estimating required technologies and developability. Overall, conscious efforts should be made to control solid state properties already from the beginning and whenever feasible, “simple” suspension formulations should be applied in nonclinical studies early on, since they are closest to a disintegrating tablet in later phases. Enabling formulations such as solid solutions, cosolvent solutions, or lipidic formulations may mask compound liabilities if they are used too early or at random, and they should only be applied to improve exposure if the rate-limiting steps in drug absorption have been understood and formulation type and excipients have been selected accordingly. Today’s drug discovery and development process is complex and challenging for scientists in the respective functions. However, in the future, scientists will clearly need to expand their knowledge beyond their traditional core competencies, and only with the combined efforts of both discovery and development scientists, companies will be able to identify efficacious molecules, to transform them into customized drug products for testing in animals and humans, and to bring them to the market in a timely manner.

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17 Integrated Approaches to Blood–Brain Barrier

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17.1	Introduction	1
17.2	Important parameters govern brain penetration	2
17.3	Methodologies in assessing brain penetration	4
17.4	Retroanalysis for Pfizer clinical candidates	15
17.5	Conclusions	21
17.6	Future prospects	23
	Acknowledgments	23
	Abbreviations	23
	References	24

17.1 INTRODUCTION

The central nervous system (CNS) is one of the most challenging therapeutic areas and many brain diseases still do not have adequate treatments. The major hurdles of discovering new CNS drugs, particularly in the area of neurodegeneration, are that there are few animal disease models and drugs must cross the blood–brain barrier (BBB) to reach the therapeutic targets.

The brain is highly protected anatomically to physical injury by the skull and to chemical insult by two major barriers, namely the BBB and the blood–cerebrospinal fluid barrier (BCSFB). The BBB is formed by the endothelium lining of the cerebral capillaries, while the BCSFB is formed by the epithelium of the choroid plexus (CP) [1]. CP consists of many capillaries, separated from the ventricles by choroid epithelial cells. The BBB and BCSFB represent physical and biological barriers that restrict and regulate the penetration of compounds from blood into and out of the brain intracellular fluid (ICF), interstitial fluid (ISF), and cerebrospinal fluid (CSF), respectively, and maintain the homeostasis of the brain and CSF microenvironments. Therefore, unlike in systemic circulation, the pharmacokinetics (PK) of a drug in brain is complicated by multiple compartments created by the BBB and the BCSFB. A great amount of research went into understanding the properties of BBB and BCSFB. The relationship between drug concentrations in blood, ISF, and CSF can be delineated into a three-compartment model (Fig. 17.1).

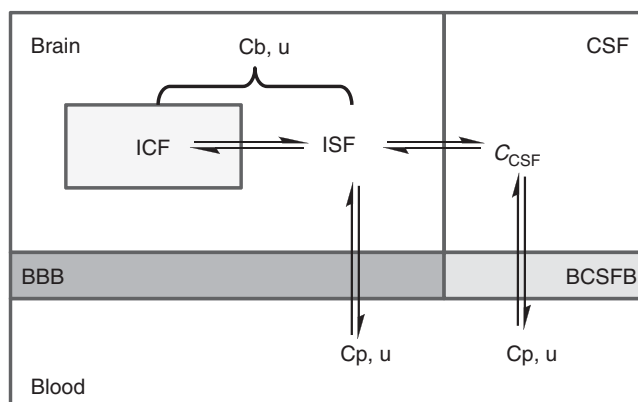


Figure 17.1 A three-compartment model illustrating unbound drug transfer between plasma, brain (ISF and ICF), and CSF. BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; $C_{p,u}$, plasma unbound drug concentration; $C_{b,u}$, brain unbound drug concentration; C_{CSF} , CSF drug concentration.

For many physiological membranes, the unbound drug concentrations are equal on both sides at steady state. However, at the BBB and BCSFB, the contributions of active influx transporters, efflux transporters, and tight junctions between brain endothelial cells can cause, for some compounds, the unbound drug concentrations to be unequal across the BBB or the BCSFB. Metabolism within the CNS (very rare) and transport to CSF via ISF bulk flow may also contribute to disequilibrium of unbound drug concentrations in the blood, brain (ISF and ICF), and CSF compartments.

From a PK and pharmacodynamic (PD) perspective, the purpose of assessing brain penetration of a drug is to understand drug exposure at the target cellular locale. According to the free drug hypothesis, only unbound drug at the target cellular locale is available for the pharmacologic interaction [2]. The relative importance of unbound drug concentrations in different brain compartments depends on where the relevant target receptors are situated. If the drug is actively transported across the cell membrane, ICF concentrations could be expected to differ from brain ISF concentrations.

In this chapter, we discuss the critical elements for brain penetration and methodologies in assessing brain exposure. Retroanalysis on the prediction success for brain penetration of Pfizer CNS drug candidates and lessons learned, as well as screening strategies and future prospects in the BBB will be highlighted.

17.2 IMPORTANT PARAMETERS GOVERN BRAIN PENETRATION

The fact that CNS-targeted drugs require good brain penetration is unequivocal. However, what is unclear is which parameters should be used to define “good” brain penetration. Ultimately, it is the free drug concentration at the site of action in the brain that exerts pharmacological activity *in vivo* [2]. There are three important parameters that characterize brain penetration: rate, extent, and brain tissue binding [1]. Rate is how fast compound molecules enter the brain, extent is how many in the brain at steady state, and brain tissue binding is how many molecules are bound to brain tissue compared with unbound in solution [3].

For the extent of brain penetration, the total brain concentration-to-total plasma concentration ratio (also known as total B/P or K_p) is a common parameter used to describe and evaluate brain penetration. A compound is deemed “brain penetrant” if its total B/P is >0.04 , because the cerebral blood volume is approximately 4% of the total brain volume. However, a ratio significantly >1.0 is often observed [4]. Does this indicate that drug molecules are being actively taken up into the brain? No, active uptake is not the case for a majority of drugs. Instead, the higher total B/P ratio is often owing to a significantly higher nonspecific binding of drug molecules to brain tissue than to plasma protein. An experiment conducted by Maurer *et al.* [5] explicitly revealed how nonspecific bindings can confound brain penetration assessment if it is based on the total B/P ratio. In the study, 32 structurally diverse CNS drugs were used. Their total B/P ratios were found to range from 0.06 to 24 after subcutaneous administration in mice [6]. Their nonspecific bindings in mouse plasma and brain homogenate were determined using equilibrium dialysis methodology, and not surprisingly, after corrected with binding factors, their free brain concentration-to-free plasma concentration ratios (also known as free B/P or C_{bu}/C_{pu} or $K_{p,uu}$) were significantly lower, ranging from 0.06 to 3.4. This indicated that nonspecific binding is a significant component of the total B/P parameter.

A high value of total B/P may mislead CNS researchers into the believing that a drug is being actively taken up into the brain and reaching high brain exposure levels, while, in reality, total B/P is merely driven by the different binding in plasma and brain, as indicated by fraction unbound in plasma ($f_{u,plasma}$) and fraction unbound in brain ($f_{u,brain}$).

Theoretically, free B/P would not exceed 1.0 when no active influx transporters are involved. Therefore, in CNS drug discovery, it is prudent to use the free B/P as the parameter to assess the extent of brain penetration, to avoid driving medicinal chemistry efforts down fruitless path of changing nonspecific binding. The free B/P represents the net extent of drug penetration across the BBB, which includes the effects of transporters, without being confounded by unspecific binding [1]. Therefore, free B/P is a better parameter to measure the extent of brain penetration compared with total B/P.

The rate of brain penetration is important for CNS drugs that require rapid onset. It is important to recognize that the rate of drug entering the brain across the BBB is not equal to the extent of transport/distribution to the brain, as the rate determines how fast the drug enters brain, not how much has entered at steady state. Rate across BBB affects whether distribution equilibrium among the various compartments can be established quickly. Rate is determined by permeability of the BBB, which is the composite of passive diffusion, efflux transport, and influx transport. The more permeable the BBB is to the drug, the quicker the drug will reach the brain tissue, but it is not permeability alone that is important regarding drug delivery to the brain. The brain PK of a centrally acting drug, and thereby its associated PD, are the result of a combination of influx and efflux processes and the extent of distribution of the drug within the brain tissue.

Another important parameter is unbound volume of brain ($V_{u,brain}$). $V_{u,brain}$ describes the relationship between the total drug concentration in the brain and the unbound drug concentration in brain ISF. It is a useful measure of drug distribution in the brain parenchyma. $V_{u,brain}$ is the ratio of total drug concentration in the brain and the unbound drug concentration in brain ISF. Since brain ISF concentration is often

converted from total brain concentration and fraction unbound of brain tissue ($f_{u, \text{brain}}$). $F_{u, \text{brain}}$ is inversely proportional to $V_{u, \text{brain}}$ [1].

17.3 METHODOLOGIES IN ASSESSING BRAIN PENETRATION

17.3.1 *In Vivo* Approaches

17.3.1.1 Preclinical Neuro PK Studies. On the basis of the understanding of multiple brain compartments, as discussed above, and the importance of nonspecific binding in the brain, efficient *in vivo* neuroPK (neuropharmacokinetics) (i.e., brain PK) studies are conducted in animals as part of CNS drug discovery [4,7]. NeuroPK helps define the relationships between drug concentrations in the CNS compartments and understand the mechanism-based pharmacology, the exposure (PK) and response (PD) relationships. The species selected for neuroPK is based on the species used for efficacy and toxicology studies. Plasma, brain, and CSF are collected to assess drug distribution in the various compartments. The neuroPK information provides temporal brain intercompartmental compound concentrations to guide dose and time point selection for pharmacology studies in preclinical species. Even though neuroPK studies may employ any dosing route, subcutaneous administration is the most preferred approach since it bypasses first-pass liver metabolism and has low experimental variability. Occasionally, for compounds with intrinsically low rates of CNS penetration, significant delay in reaching distribution steady state may be of concern, in which case an intravenous infusion regimen can be applied [8]. In a typical rodent neuroPK study, animals ($N = 3/\text{time point}$, preferably at least two dose levels, for example, 1 and 10 mg/kg) are euthanized at specific time points post dose, and plasma, CSF, and brain are collected for exposure analysis. Time-concentration profiles of the compound in plasma, CSF, and brain are generated.

These data reveal important PK parameters providing insight into the extent of compound CNS penetration and rate of elimination from each compartment. Knowing matrix-specific diffusion and elimination rates is important as compounds may have intercompartmental concentration-related delays and/or much longer half-lives in brain versus CSF or plasma. Such phenomena are key to understand the mechanism of hysteresis observed in exposure-PD response. Furthermore, free drug exposures in each of the compartment, plasma, CSF, and brain, can be calculated by combining area under the curve (AUC)-derived exposures for each neuromatrix and compound-specific $f_{u, \text{plasma}}$ and $f_{u, \text{brain}}$ values, determined by equilibrium dialysis studies using plasma and brain homogenate, respectively (Eqs. 17.1 and 17.2). Under normal physiological condition, CSF is low in protein. Therefore, the concentration measured in CSF is, in general, considered approximately free drug concentration:

$$\text{Free drug exposures in plasma: } AUC_{p,u} = AUC_p \cdot f_{u, \text{plasma}} \quad (17.1)$$

$$\text{Free drug exposure in brain: } AUC_{b,u} = AUC_b \cdot f_{u, \text{brain}} \quad (17.2)$$

Ratios between $AUC_{p,u}$, AUC_{CSF} , and $AUC_{b,u}$ provide important insights into the extent of compound distribution among these brain compartments. The ratio of $AUC_{b,u}:AUC_{p,u}$, presuming $C_{b,u}$ and C_{ISF} are equivalent, provides the concentration relationship across the BBB. The ratio of $AUC_{\text{CSF}}:AUC_{p,u}$, on the other hand, gives an

TABLE 17.1 Methodologies in Predicting Free Human Brain Concentration

Methods	Description	Comments
Human receptor occupancy (RO)	$RO = C_{bu}/(C_{bu} + K_i)$	Human RO can be obtained from imaging studies (e.g., PET)
Human CSF	Sampling CSF in human	CSF can overpredict free brain concentration for efflux transporter substrates
Animal neuroPK and human unbound plasma	$C_{bu} = [C_{bu}/C_{pu}]_{rat} \times C_{pu}$	C_{bu}/C_{pu} is often preserved across species, except for sheep
Human unbound plasma	$C_{pu} = C_p \times f_{u,p}$	When distribution equilibrium is established, typically for high permeable compounds that are not efflux transporter substrates

idea of the extent of partitioning at the BCSFB. These two parameters are important in defining whether there is distribution equilibrium among these neurocompartments, which is critical in understanding preclinical efficacy and safety and in predicting brain penetration in human. Whereas, the ratio of $AUC_{b,u}:AUC_{CSF}$ defines the compound concentration relationship between ISF and CSF. This ratio is very important, particularly for a compound that demonstrated distribution disequilibrium at the BBB and/or at the BCSFB, because, for these compounds, plasma will overestimate drug exposure in brain ISF. If CSF exposure is determined in the clinic, human brain ISF exposure may be estimated from this using the rodent-derived relationship assuming an identical ratio across species (Table 17.1). For CNS compounds, careful characterization of these three exposure ratios increases the chances of adequate compound delivery to the pharmacologic target site and understanding cross-species exposure-response relationships.

17.3.1.2 CSF as a Surrogate for Brain Exposure. The use of CSF concentration as a surrogate marker for the unbound drug concentration in the CNS has become a more common practice for assessing CNS exposure. This is because there is a close relationship between the brain ISF and CSF, and CSF sampling is relatively simple and straightforward both in preclinical experiments and clinical settings. However, it is important to recognize, as aforementioned, that the brain consists of multiple compartments created by two distinct barriers (BBB and BCSFB) and that many factors are involved in the transport of drugs from plasma into the brain and the distribution within the brain. Systemically administered drugs can reach CSF either directly via passage across the BCSFB or indirectly by passage across the BBB followed by diffusion/convection transport from the ISF to CSF. Therefore, the usefulness of CSF as a surrogate for measurement of unbound drug concentration in the brain has been critically questioned [9]. From the PK/PD perspective, to determine whether CSF can serve as a surrogate for assessing the drug exposure at the pharmacological target site within the CNS and the dynamics of drug concentration–effect relationship, the critical issue is whether CSF concentration is in equilibrium with the target biophase concentration.

CSF, an essentially aqueous fluid free of drug binding proteins, circulates throughout the ventricles within the brain and the subarachnoid space surrounding the brain and

spinal cord. Because of its physically close proximity to the brain, drug concentration in the CSF is sometimes used as a surrogate of free drug concentration in the brain [10]. However, there are several important aspects of CSF worth special consideration when interpreting drug concentration in CSF as a surrogate exposure at the CNS target site.

First, drug distribution in CSF may be uneven, because CSF is not a well-stirred compartment. Unlike systemic circulation, where it takes <1 min to circulate blood throughout the body, the movement of CSF is much slower. The turnover rate of CSF is about 5–7 h. Drug concentration in CSF is expected to be significantly different depending on the site of CSF sampling and the route of administration. Remarkable differences (approximately 10-fold) in drug concentrations between ventricular and spinal CSF have been reported after intraventricular or lumbar injection [10]. The apparent differences in CSF drug kinetics between the ventricular and lumbar sites is not a surprise when one considers that the flow through the subarachnoid space receives only a fraction of the CSF flow originating from the ventricles. Furthermore, exchange of drug between CSF and the cord tissue bordering the descending spinal subarachnoid space probably exerts influence on the composition of the spinal CSF. These considerations definitely raise a serious question as to the usefulness of lumbar CSF as an indicator of drug availability and disposition in the brain.

Second, drug concentration in CSF is influenced by BCSFB at CP as well as drug concentration in ISF. The total volume of the CSF in adult humans is approximately 150–200 mL [11]. Various studies have suggested that about two-thirds of CSF is formed at the CP, and one-third of CSF enters the macroscopic spaces from brain ISF [12]. The BCSFB, separating blood from CSF, is formed by the epithelial cells of CP and their tight junctions. Unlike the BBB, the capillaries supplying the CP are fenestrated. Similar to the BBB, a number of influx and efflux transporters have been identified at the BCSFB. However, their locations and functions are somewhat different than those at BBB [13]. It has been reported that P-gp (MDR1 and ABCB1) localizes subapically at the CP epithelium, with drug transport in the direction from blood to CSF. In contrast, at the BBB, P-gp localizes at the luminal membrane of brain endothelial cells and pumps drugs out of brain. At BCSFB, other efflux transporters localize and function differently as well. Several groups of researchers showed MRP1 (ABCC1) localized basolaterally, conferring an opposing basolateral-to-apical drug-transport barrier. The localization of MRP1 suggests that MRP1 is an efflux transporter pumping drugs out of the CP, which is functionally different from P-gp at BCSFB. Understanding of the role of the BCSFB in transporting drugs into the brain is emerging and has been gradually gaining more attention over the years.

Despite the complexity of CSF physiology and PK, CSF sampling remains an important part of neuroPK study, which is a valuable tool for assessing drug CNS penetration in preclinical studies, and more importantly, projecting human CNS penetration (Table 17.1). Since CSF is not a homogeneous fluid, it is recommended that, in animal studies, CSF sampling be performed through a catheter inserted into the cisterna magna (in close proximity to the brain). In addition, drug distribution into CSF may be delayed from plasma due to low membrane permeability. Comparison of CSF and corresponding plasma unbound drug concentration based only on a single time point could be misleading. Therefore, it is highly desirable to assess CNS exposure in animals by comparing the AUCs of unbound drug concentration in CSF, brain, and plasma. As mentioned in the previous section, a well-conducted preclinical neuroPK

study will define drug distribution kinetics among CNS compartments and provide insights in designing a clinical study to assess human brain exposure. For compounds that exhibited distribution equilibrium among neurocompartments, free drug concentration in plasma can be used as a surrogate for brain exposure. For those that exhibited impaired brain penetration, CSF can be used as surrogate of drug brain concentration, as plasma will overestimate free drug exposures in brain. However, it is prudent to assess unbound drug concentration in the brain and use the ratio of $AUC_{b,u}:AUC_{CSF}$ to define the relationship between ISF and CSF, as equilibrium may not always be established between these neurocompartments.

In summary, CSF drug concentration can be used as a useful surrogate for assessing unbound drug concentration at the target site within the brain. However, CSF drug concentration is not always an accurate surrogate for all drugs, especially for transporter substrates. Preclinical neuroPK can be used to design clinical studies to assess CNS target exposure in humans (Table 17.1).

17.3.1.3 Microdialysis. According to the free drug hypothesis, unbound drug in brain ISF is in direct contact or in equilibrium with that at the site of action [2]. *In vivo* intracerebral microdialysis is a valuable technique to determine biophase free drug concentrations and distribution in the brain as a function of time. The technique involves implantation of a thin dialysis probe into a selected area of the brain. The probe consists of an inlet tube, a semipermeable membrane, and an outlet tube. To mimic the function of a capillary blood vessel, the thin dialysis probe is continuously perfused with an artificial physiological solution. Drug molecules diffuse across the membrane along the concentration gradient (in the direction of the lower concentration) into or out of the selected tissue. A basic principle of microdialysis is that only unbound drug can freely diffuse through the semipermeable membrane [14]. Depending on the physicochemical properties of the drug, the concentration measured with the probe may not reflect the concentration in the ISF but is a function of the probe perfusion rate, blood flow, and diffusion in the tissue surrounding the probe. Therefore, *in vivo* recovery of the probe for a specific compound will need to be incorporated to translate the concentration measured in the dialysate to the unbound drug concentration in the tissue [15].

Microdialysis has been used to monitor biophase concentrations of antiepileptic drugs in animals and humans. Walker *et al.* [16] investigated the rate of lamotrigine penetration into plasma, CSF, and hippocampal and frontal cortex extracellular fluid compartments following systemic administration in nonepileptic rats. Following intraperitoneal injection, the ISF AUC of lamotrigine to total serum AUC ratio (0.4 ± 0.01) was similar to the free serum AUC to total serum AUC ratio (0.39 ± 0.01), and did not differ between hippocampus and frontal cortex. On the other hand, the CSF AUC to total serum concentration AUC ratio was 0.6 ± 0.05 . Overall, the unbound concentrations of lamotrigine in three different compartments, serum, CSF, and ISF, were in a reasonably good agreement, supporting the concept of free drug hypothesis. In human, an intracerebral microdialysis study was also conducted during surgery by Scheyer *et al.* [17], who collected dialysate from the hippocampus of refractory epilepsy patients and determined phenytoin level in the dialysate. The steady-state extracellular phenytoin concentrations corresponded closely to the unbound plasma concentration, indicating free drug distribution equilibrium between brain ISF and plasma.

Although the microdialysis technique was developed more than two decades ago, it is primarily used for determination of neurotransmitters and not drug concentrations in the brain in the pharmaceutical industry. The main limitations of this technique include high resource requirements, low throughput, and special surgical skills to set up the experiment. In addition, many compounds in the discovery stage are very lipophilic, and it is difficult to apply microdialysis technique to study these compounds because of high nonspecific binding to the dialysis devices and tubing. Importantly, this method cannot be used routinely to measure the ISF concentration in the clinic for ethical reasons. Because of these constraints, alternative methods, such as CSF sampling and neuroPK studies combined with brain homogenate binding, have been used to estimate brain ISF exposure. The accuracy of using unbound brain concentration determined by a neuroPK/brain homogenate binding method and CSF exposure as a surrogate for brain ISF concentration was compared with those obtained by microdialysis in rat brain [8]. The results supported the use of neuroPK/brain homogenate approach or CSF as a surrogate for the ISF free drug concentration in drug discovery and also confirmed the distribution disequilibrium between brain, CSF, and plasma for efflux transporter substrates and low permeability drugs.

17.3.1.4 PET Imaging. Imaging is an attractive technique to obtain information on brain drug uptake in humans due to the noninvasive nature of the approach. There are a number of methods for imaging compounds in the brain, including positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). Before PET and SPECT, MRI was used to obtain PK information of drug in brain [18]. It suffered from low spatial resolution; therefore, its value for quantitative assessment was limited. PET and SPECT both use radioactive tracer materials and detect γ -rays. However, the tracer used in SPECT emits γ -radiation that is measured directly, whereas PET tracer emits positrons that annihilate with electrons up to a few millimeters away, causing two γ -photons to be emitted in opposite directions. A PET scanner detects these emissions “coincident” in time, which provides more localized information of radiation events and higher resolution images than SPECT. Therefore, PET is a better quantitative method for assessing drug brain distribution. SPECT scans, however, are significantly less expensive than PET scans, in part because they are able to use longer lived more easily obtained radioisotopes than PET. PET is a nuclear medicine imaging technique that produces a three-dimensional image or picture of functional processes in the body. The system detects pairs of γ -rays emitted indirectly by a positron-emitting isotopes (^{11}C , ^{13}N , ^{15}O , or ^{18}F), which is incorporated into the drug of interest. Typically, the isotopes are made in a cyclotron, and because of their short half-lives, they need to be rapidly incorporated into drug immediately before administration. Once administered to a patient, the label can be imaged noninvasively using a PET scanner. To study drug brain distribution, there are two main approaches using PET. The first is the direct approach, where the drug itself is labeled. The second is the indirect approach, where a labeled receptor ligand is administered and then unlabeled drug of interest is administered. The time course of receptor occupancy will be measured using the PET scanner as the drug displaces the ligand from the receptor.

Verapamil, a calcium channel blocker, has been used as a PET tracer to assess P-gp-mediated transport at the BBB in human, since it is a P-gp substrate and can also be straightforwardly labeled with ^{11}C . A human volunteer study utilizing [^{11}C]-verapamil

as a model P-gp substrate and cyclosporine A (CsA) as a P-gp inhibitor was conducted by Sasongko *et al.* [19]. Results showed that the inhibition of P-gp activity at the human BBB was modest, with $\sim 90\%$ increase in [^{11}C]-verapamil distribution into the brain in the presence of $\sim 2.9\ \mu\text{M}$ steady-state blood CsA concentration. These results are dramatically different from those obtained in the P-gp knockout (KO) or chemical KO mouse or in rats administered with a high dose (e.g., 50 mg/kg) of CsA, where ~ 10 -fold increase in [^{11}C]-verapamil distribution into the brain was observed when compared with the wild-type mouse [20]. The observed discrepancy in the verapamil–CsA interaction at the BBB between rodents and human is likely due to differences in blood CsA concentrations. The PET imaging study provided evidence for the first time that P-gp activity at the human BBB is measurable and that it is only modestly inhibited by one of the most potent and Food and Drug Administration-approved P-gp inhibitors, cyclosporine. Kinetics of drug brain uptake must be interpreted with reference to concurrent blood drug concentrations and it is difficult to define the blood kinetics of a compound using PET alone. Solid-phase extraction (SPE) and high performance liquid chromatography (HPLC) analysis was used to determine the amount of radioactive verapamil and its metabolites in the plasma [19]. However, for a drug such as verapamil, which has a $t_{1/2}$ in the range of 6–8 h, the plasma concentration–time profile of ^{11}C -labeled parent tracer would fail to predict the drug's PK parameters as the drug's elimination phase cannot be captured during the short time course of the PET experiment. For such drugs, a combination of PET imaging for assessment of drug tissue distribution with accelerator mass spectrometry (AMS) for plasma PK analysis can be particularly powerful. Verapamil brain distribution kinetics was determined in human in a microdosing study combined with PK profiling in plasma using AMS after administration of a mixture of (*R*)-[^{11}C]verapamil and (*R/S*)-[^{14}C]verapamil [21]. Each subject underwent PET scan of 60-min duration after receiving a microdose of labeled verapamil. The time–concentration profile of (*R*)-verapamil in human brain was demonstrated by reconstruction of the PET data, while concentration in plasma was profiled using HPLC with a radiometric detector, chromatographically separating parent drug and metabolites.

PET imaging also provided the opportunity to study species difference of P-gp transporter at the BBB by measuring brain concentrations and brain-to-plasma ratio of three labeled P-gp substrates, [^{11}C]verapamil, [^{11}C]GR205171, and [^{18}F]altanserin in rats, guinea pigs, monkeys, and humans [22]. Pronounced species differences were found in the brain and brain-to-plasma concentrations of these P-gp substrates, with higher brain distribution in humans, monkeys, and minipigs than in rats and guinea pigs. The brain-to-plasma ratio of [^{11}C]GR205171 was almost ninefold higher in humans compared with rats. These findings suggested species differences should be considered when extrapolating data obtained in animals to humans. Compounds found to be P-gp substrates in rodents are likely to be substrates in higher species as well, but the ratio of brain penetration, even corrected with binding factors, may not be reliably extrapolated from animals to humans. Despite these reported species differences, an in-depth study has revealed that the rodent is a suitable model for CNS penetration (Section 17.4.1).

The advantages of PET are obvious as it is not only noninvasive, which makes it attractive for clinical applications, but it also allows drug concentrations and/or receptor occupancy to be followed with high spatial (several millimeters) and time resolution. Thus, it is possible to monitor drug concentrations or effects in discrete structures in the brain. However, on a practical level, a disadvantage of PET is that it requires expensive

infrastructure (i.e., both a cyclotron and PET scanner in close proximity). PET also requires clever chemistry (i.e., dedicated personnel) to incorporate labels rapidly into compounds of interest before the label has decayed excessively. The other limitation of PET is that, in general, it is unable to discriminate between bound and unbound drug or parent compound and metabolites. Because of these reasons, there has been less use of PET for animal studies during the discovery stage, but there is now a growing trend for its use in drug development with the availability of commercial small animal PET scanners [23].

17.3.2 *In vitro* Methods

17.3.2.1 BBB Permeability. Passive permeability is an important parameter for assessing the rate of CNS penetration, since most small molecules enter the brain by transcellular passive diffusion. The rate of brain penetration plays a critical role for drug candidates that require rapid onset of pharmacological effects (e.g., anesthesia). It is also pivotal in terms of time necessary to achieve distribution equilibrium among the various neurocompartments (e.g., brain, CSF, and plasma).

In situ brain perfusion is the gold standard method for measuring BBB permeability *in vivo* [24,25], since it is quite reliable to determine the rate of brain penetration. In the brain perfusion studies, animals were anesthetized. The test compounds were infused in a perfusate to the external carotid artery for about 30 s [26]. At the end of the experiment, animals were sacrificed and the brains were homogenized and analyzed with liquid chromatography–mass spectrometry (LC-MS). Although it is useful, the *in situ* brain perfusion assay is not commonly applied in the pharmaceutical industry, because it does not provide the free drug concentration in the brain directly, which is most important for pharmacological activity. Instead, *in vitro* BBB permeability assays or calculated properties are more frequently employed to estimate the rate of brain penetration. There are two major *in vitro* BBB permeability methods: (i) parallel artificial membrane permeability assays (PAMPA-BBB) and (b) cell-based monolayer permeability assays [e.g., MDCK apparent permeability (P_{app}) from apical to basolateral].

PAMPA is a high throughput assay for passive diffusion originally developed to mimic intestinal membrane permeation and predict oral absorption [27–29]. The assay was further expanded to predict BBB permeability (PAMPA-BBB) using brain lipids as the artificial membrane [30,31]. PAMPA-BBB permeability values correlated well with *in situ* BBB permeation values obtained from brain perfusion experiments in rodents [32,33]. Because PAMPA-BBB has good predictability of the rate of brain penetration and is high throughput with low cost, it is a valuable tool as first-tier screening in drug discovery for BBB permeability.

Cell monolayer transport assays with primary cells or cell lines are also commonly used as models to assess BBB permeability, such as MDCK, LLC-PK1, Caco-2, and brain microendothelial cell (BMEC) [34]. Among the various cell-based *in vitro* assays, MDCK cells are most commonly used [26,34–36] to predict BBB permeability. Although MDCK cells are not of cerebral origin, the MDCK permeability assay has clear advantages over other cell types: easy cell culture, low maintenance, tight cellular junctions, low endogenous transporters, and low metabolizing enzymes. Recently, a

MDCK-LE (low efflux) cell line has been developed to further minimize the potential interferences from endogenous transporters on passive permeability measurement [37].

A comparison study on PAMPA and MDCK was conducted using 31 structurally diverse marketed CNS-active drugs, one active metabolite, and seven non-CNS-active compounds [38]. In general, the two assays gave a good correlation. Compounds with $P_{app} > 5 \times 10^{-6}$ cm/s in PAMPA or MDCK had a good BBB permeability *in vivo*. Importantly, most successful marketed CNS drugs have moderate to high passive permeability. Additionally, *in silico* models have been quite successful in predicting passive permeability across the BBB based on molecular properties and they can be applied effectively in early drug discovery [39,40].

17.3.2.2 Brain Tissue Binding. As discussed above, free drug concentration at the site of action is the species that exerts pharmacological activity and the free brain/plasma ratio ($C_{b,u}/C_{p,u}$) is an important parameter to evaluate the brain penetration potential of drug candidates. Several methods have been developed to measure free brain concentration, including microdialysis, CSF sampling, and a combination of neuroPK and brain tissue binding (Section 17.3.1). In drug discovery and development, it is a common approach to obtain free drug concentration in the brain by a combination of neuroPK and brain tissue binding studies, and the free C_{max} and AUC are obtained using Equation 17.2. The approach has been shown to have a good correlation with direct microdialysis and indirect CSF measurements in determining free drug concentration in the brain *in vivo* [5,41,42].

There are two methods that are commonly used for brain tissue binding measurements, which use (i) brain slice [41,43–45] or (ii) brain homogenate [46–49]. Brain slices preserve the cellular structures (cell membrane, influx and efflux transporters, and ICFs) and are considered physiologically more relevant. However, even with the difference between brain slice and brain homogenate, the two methods have a good correlation in measuring f_u values, especially when cytosolic pH partition is corrected for basic compounds [44,50]. It appears that the nonspecific binding to lipophilic components in the brain is the dominant mechanism for brain tissue binding, and that the presence of intact structural elements plays a less significant role in determining brain binding. One advantage of using brain homogenates over brain slices is that they are readily available from vendors and can be stored frozen and thawed right before experiments. High throughput 96-well format dialysis devices have been developed and can be used to determine f_u using brain homogenates (e.g., HTD 96 from HTDialysis [51] and RED from Thermo [52]). The devices are engineered to minimize nonspecific binding to the plastic wells and dialysis membranes to shorten the time required for compounds to reach equilibrium. The advantage of the equilibrium dialysis device is that nonspecific binding does not affect f_u determination, which is particularly important for highly lipophilic compounds. The good predictability of *in vivo* free drug concentration and the ease of use make brain homogenate binding one of the most widely employed methods for determining fraction unbound in brain tissue. While plasma protein binding is often species dependent, brain tissue binding has been shown to be species independent [53]. One can use a single species (e.g., rat) to estimate brain binding of all other species, including human in drug discovery. This greatly increases efficiency and reduces cost. Brain tissue binding has been shown to be independent of regional brain tissues [54], and the brain regions in the study included

CP, striatum, hippocampus, motor cortex, cerebellum, and thalamus [54], suggesting that no regional brain binding studies are necessary.

There are many misconceptions among drug discovery project teams regarding drug binding *in vivo*, including the perceived value of increasing fraction unbound through structure modification [2]. The rational of this misconception is to increase the free drug concentration at the site of action by manipulating fraction unbound. This approach is scientifically unsound. Changing fraction unbound will not affect free drug concentration *in vivo* for orally administrated drugs. Project teams should not attempt to increase fraction unbound to improve free drug exposure at the target or use fraction unbound to rank order compounds. Fraction unbound should only be used to calculate the free drug concentration by multiplying it to the total drug concentration obtained from *in vivo* neuroPK studies. For this reason, f_u data alone can be misleading and one does not need to measure f_u , unless *in vivo* total drug concentration or AUC exposure data has been measured.

17.3.2.3 Transporters. Measurement of the interaction of drugs with BBB transporters is critical in predicting the brain penetration potential of drug candidates. Many influx (solute carrier) and efflux (ATP-binding cassette, ABC) drug transporters in the BBB (Fig. 17.2) and the BCSFB have been reported [55]. More than 200 drug transporters in human BMECs were profiled using real-time polymerase chain reaction (PCR) [56]. The expression of human P-gp (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) are the highest in human BBB based on mRNA level [56]. Recently, 114 drug transporter protein levels were quantitatively determined in human brain microvessels using proteomics approaches with LC-MS/MS. The results showed that human P-gp and BCRP are the most abundant transporter proteins at the BBB, which is consistent with the mRNA levels [57]. On the basis of the protein level, human BCRP is slightly higher than P-gp in brain microvessels in the ratio of about 4:3. The human BCRP protein level is higher than mouse Bcrp, while human P-gp is lower than mouse P-gp in the BBB [57]. Transporter protein levels have also been reported for monkey with P-gp and Bcrp being the two major transporters in

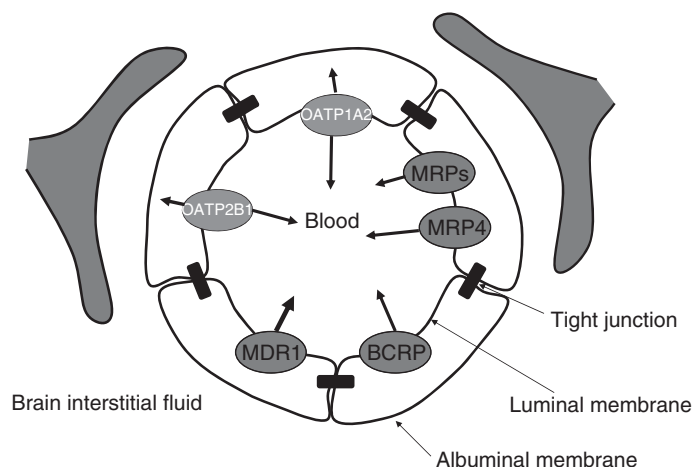


Figure 17.2 Drug transporters expressed at human blood–brain barrier.

the BBB. Monkey has slightly higher amounts of Bcrp compared with human [58]. Though human BBB BCRP has a slightly higher amount than P-gp, P-gp has been the dominant efflux transporter at the BBB to reduce compounds from entering the brain. P-gp and BCRP have a large substrate overlap and many BCRP substrates are P-gp substrates, and only a small number of compounds are BCRP-specific substrates. This may account for why P-gp seems to play a more important role than BCRP in limiting brain penetration of drugs *in vivo*. P-gp and BCRP transporters have a synergistic effect in restricting their dual substrates from entry into the brain, which is expected based on individual kinetic contribution to BBB efflux [59–62]. This has been applied in designing drugs that target peripheral tissues, where brain penetration can cause unwanted side effects. Besides P-gp and BCRP, other efflux transporters, such as MRP4, can pump certain compounds out of brain. However, the *in vivo* significance of other efflux transporters in the BBB is not well defined. Uptake transporters at the BBB, such as OATP1A2, OATP2B1, LAT1, and MCT1, can enhance transport of some drugs into the brain. L-DOPA, gabapentin, and pregabalin are examples of drugs that are active influxed into the brain by the LAT1 transporter [63]. It has been reported recently that OATP1A2 is a potential transporter to increase active uptake of certain drug candidates into the brain [64]. A positively charged amine atom is required for efficient OATP1A2-mediated uptake, and the uptake rate is in the order of tertiary > secondary > primary amines [64]. Uptake rate also improved with increased van der Waals volume, but not with Clog *P* [64]. The *in vivo* relevance of OATP1A2 in enhancing the CNS penetration has yet to be demonstrated.

Several *in vitro* BBB models with primary brain endothelial cells or immortalized brain endothelial cells have been developed. These models tend to have leaky junctions between the cells and downregulation of BBB transporters. Although they are of brain endothelial cell origin, the leaky junctions, low transporter expression, and high cost make them unsuitable for use on regular bases to support of drug discovery programs. Instead, the most common approach to assess transporter substrates and inhibitors is to use transfected cell lines that express selected transporters, for example, MDR1-MDCK, BCRP-MDCK, OATP1A2-HEK, and others. Cutoff values are established to differentiate substrates from nonsubstrates, typically based on empirical evaluation of compounds with known substrate properties. *In vitro* transporter data based on cell monolayer transport studies have a good correlation with *in vivo* results from transgenic animals. A study on P-gp efflux for a set of structurally diverse drugs showed that MDR1-MDCK data had a good correlation with *in vivo* efflux ratio obtained from P-gp KO mice versus wild type [6,38]. P-gp efflux activity is usually conserved across various species due to a high sequence homology of the protein (80–96%, rat, mouse, dog, and monkey). More than 3000 compounds were tested at both human MDR1-MDCK and mouse Mdr1a-MDCK transwell assays *in vitro*, and a good correlation ($R^2 = 0.92$) of efflux ratios was observed [38]. However, for certain structural classes, species difference in P-gp efflux has been demonstrated [65]. Cell lines transfected with transporters (e.g., P-gp and BCRP) from various animal species can be used to evaluate the different transporter activities from the different species. Human MDR1-MDCK showed to have a better prediction of human P-gp effect on BBB penetration than using P-gp KO mice, due to its human origin and high sensitivity without added complexity from various *in vivo* factors. Though the value of P-gp KO is to understand whether P-gp plays a major role in limiting brain penetration in mouse has been demonstrated, its predictive value to human P-gp interaction is limited compared with transfected

cell lines with human origin. Transporter information is widely used by project teams to guide structural modification, develop SAR (Structure-Activity Relationship), select compounds for *in vivo* studies, and diagnose *in vivo* observation.

P-gp found at the BBB reduces entry of toxic materials into the brain. Impaired P-gp function due to generic polymorphism, disease state, or aging may raise the levels of toxic compounds in the brain and increase the risk of developing certain neurodegenerative diseases. For example, decreased expression of P-gp in the BBB vasculature has been found in patients with advanced Parkinson's disease [66], Alzheimer's disease [67], and Creutzfeldt–Jakob disease [68]. In AD, P-gp upregulation in early pathogenesis was suggested as a compensatory mechanism to increase the capacity of detoxification, while at later disease stages, P-gp expression was lost due to brain cell degeneration [67]. Decreased BBB P-gp function seems to be a late event in neurodegenerative disorders and could enhance continuous neurodegeneration [66]. On the other hand, P-gp activity was found to increase in patients with chronic schizophrenia under treatment with antipsychotic drugs. The increased P-gp activity might be a factor for drug resistance in schizophrenia, induced by the use of antipsychotic agents [69]. Additionally, P-gp activity at certain BBB regions decreased during aging, which increased the risk of developing brain diseases [70,71].

17.3.3 *In Silico* Predictions and Simulations

17.3.3.1 Computational Models. A number of CNS models have been developed to predict brain penetration and they are most useful for library design and prediction before synthesis [72,73]. Models predicting passive permeability across the BBB have been developed using molecular descriptors related to lipophilicity, hydrogen bonding capacity, charge, and molecular weight (MW) [74], as well as descriptors [39], such as $\log D$, van der Waals surface area of basic atoms, and polar surface area (PSA). Models for active or facilitative transporter processes are less mature owing to insufficient quality data necessary for model development. The rules for good CNS penetration are $\text{PSA} < 60\text{--}70 \text{ \AA}^2$, $\log D$ between 1 and 3, and $\text{MW} < 450$, and rules for non-Pgp substrates are $\text{N} + \text{O} \leq 4$, $\text{MW} < 400$, and $\text{base } \text{p}K_a < 8$ [75–77].

17.3.3.2 CNS Multiparameter Optimization. To increase the survivability of CNS drug candidates, multiparameter optimization (MPO) was developed to shift the neuroscience medicinal chemistry to a design space with a higher probability of success [78]. Six calculated parameters were utilized to understand the interplay among the different physicochemical properties and to create a design tool focusing on aligning the CNS drug-like attributes. The six parameters in CNS MPO are lipophilicity ($\text{Clog } P$), distribution coefficient at pH 7.4 ($\text{Clog } D$), MW, topological polar surface area (TPSA), number of hydrogen bond donors (HBD), and basicity of the most basic center ($\text{p}K_a$) [79]. The CNS MPO scoring function offers great advantages over simple hard cutoff rules or using single parameter for optimization. The CNS MPO enables exploring medicinal chemistry design space through a holistic assessment approach to accelerate the identification of compounds with increased probability of success [80].

17.3.3.3 PBPK Models for BBB. Physiologically based pharmacokinetic (PBPK) models for BBB are based on the anatomical and physiological structure of the body and certain biochemistry. They are powerful tools to quantitatively predict brain exposure time course, brain-to-plasma ratio, and other important PK parameters *in vivo* [81]. Commercial BBB PBPK software is recently made available [82]. One of the challenges facing BBB PBPK models is to integrate transporter activity into the prediction. Several human BBB transporter proteins have been quantified using LC-MS [57]. The information, in conjunction with the level of transporter proteins in transfected cell lines, can provide a scaling factor to predict CNS penetration effectively.

Recently, *in vivo* disposition of 11 P-gp substrates in mouse brain was predicted using a PBPK model that incorporated the P-gp protein level in mouse brain capillaries and in transfected cells, P-gp *in vitro* activity, and drug unbound fractions in mouse plasma and brain [83]. For most of the compounds, the predicted brain-to-plasma concentration ratios and unbound brain-to-plasma concentration ratios were within three-fold of the observed *in vivo* values. With the progress in transporter proteomics in human BBB, the brain distributions of P-gp substrates in human would be predicted from the P-gp protein levels, *in vitro* activity, and drug unbound fractions as well. However, it is a challenge to validate the accuracy of the model in predicting human brain disposition, since the unbound human brain concentration is often unknown, and CSF and CNS pharmacology activity is unlikely to be in equilibrium with the free brain concentrations due to transporter interactions. The advances in imaging technology (e.g., PET) will be very helpful to validate the BBB PBPK models in humans.

17.4 RETROANALYSIS FOR PFIZER CLINICAL CANDIDATES

For drugs that have a site of action within the CNS, brain penetration is essential in accessing the site of action to deliver their pharmacological benefits and having a balanced efficacy and safety profiles. Even though all drugs do penetrate brain to some extent, drugs that have impaired brain penetration will have reduced exposures in brain and require proportionally higher doses to achieve therapeutic exposure at the CNS target site. Therefore, within CNS drug discovery, it has been recognized that having “good” CNS penetration is an added requirement for selecting promising drug candidates. However, in contrast to the established approaches of predicting systemic human PK parameters [84], it is not so obvious how to predict whether a compound will exhibit good CNS penetration in human.

Multiple factors contribute to the uncertainty around predicting human CNS penetration. First, how to measure brain penetration in human has always been a challenge. The easily accessible measurement site, blood, does not always reflect brain exposure. Despite close proximity of CSF to the brain, the value of CSF can be limited in predicting the drug concentration at the target site biophase in the CNS [12] (see above discussions). Intracerebral microdialysis and PET imaging are much improved techniques; however, their applications in CNS drug discovery and development are limited as intracerebral microdialysis is highly invasive, while suitable radiolabeled PET ligand may not be readily available for the novel drug targets being pursued. Furthermore, the limited human CNS exposure data have greatly hindered the progression

of predicting human CNS penetration, because multiple iterations of prediction refinement is the only way to advance predictive ADME science, as evidenced by success in the area of human PK prediction [85].

Nevertheless, the pharmaceutical industry has invested a significant amount of research in understanding factors influencing CNS drug disposition and, subsequently, in developing comprehensive tools to select candidates that have the best potential of being developed as successful CNS drugs. The preclinical tool box encompasses *in vitro* assays (e.g., membrane permeability, P-gp efflux transporter using MDR1-MDCK, and many others are being developed) and *in vivo* assays (e.g., neuroPK in rats, dog model for CSF sampling, and P-gp KO mouse). These are routinely used for CNS drug candidate selection. Questions have been raised regarding the predictability of these assays and confidence in using preclinical information in prediction of CNS penetration in humans. A retrospective analysis was conducted using 32 Pfizer proprietary clinical candidates, mostly with CNS indications, to address these important questions.

17.4.1 Preclinical and Clinical Data Used for Retrospective Analysis

To enable retrospective analysis, 32 Pfizer clinical candidates were selected that had either (i) CSF drug concentrations measured in humans or (ii) free drug concentrations in brain derived using receptor occupancy (from humans who had undergone clinical PET imaging studies), as well as *in vitro* receptor binding affinity, K_i . These 32 clinical candidates covered a wide range of CNS targets, including transmembrane G-protein-coupled receptors (GPCR), neurotransmitter gated channels, and ion channels. Their preclinical *in vitro* and *in vivo* data packages were collected, which included mainly membrane permeability, P-gp efflux, and brain penetration information from studies conducted in animals, mostly rats and mice, and a few guinea pigs or dogs. Compounds, which were shown as P-gp efflux substrates *in vitro* and/or exhibited brain penetration impairment *in vivo*, were also subjected to brain penetration studies in P-gp KO mice versus wild-type mice to confirm the role of P-gp in limiting their brain penetration [6].

These compounds were put into three groups (Fig. 17.3) based on their preclinical information by asking two questions: (i) Does the compound have a P-gp efflux liability and have low membrane permeability? (ii) Does the compound have good CNS penetration in animals? Since compounds in the data set are optimized clinical candidates, most of them have reasonably good membrane permeability. Therefore, the answer to the first question was essentially based on P-gp efflux liability. For the second question, free drug-based neuroPK methodology (see above) was applied to assess CNS penetration in animals. Compounds were considered as exhibiting interneurocompartamental distribution equilibrium when free drug exposures, in terms of AUC, were within 2.5-fold among plasma, CSF, and brain [5]. Compounds that showed both good *in vitro* and *in vivo* CNS penetration properties, meaning not having P-gp efflux liability and demonstrating good CNS penetration in animals, were categorized as group I compounds, also called *well-behaved* CNS compounds. On the other hand, compounds identified as having P-gp efflux liability and also preclinically showing impaired brain penetration were categorized as group III compounds. The remaining were put together as group II compounds, which had conflicting *in vitro* and *in vivo* findings, being either a P-gp efflux substrate or not showing distribution equilibrium among plasma, CSF, and brain compartments in preclinical species.

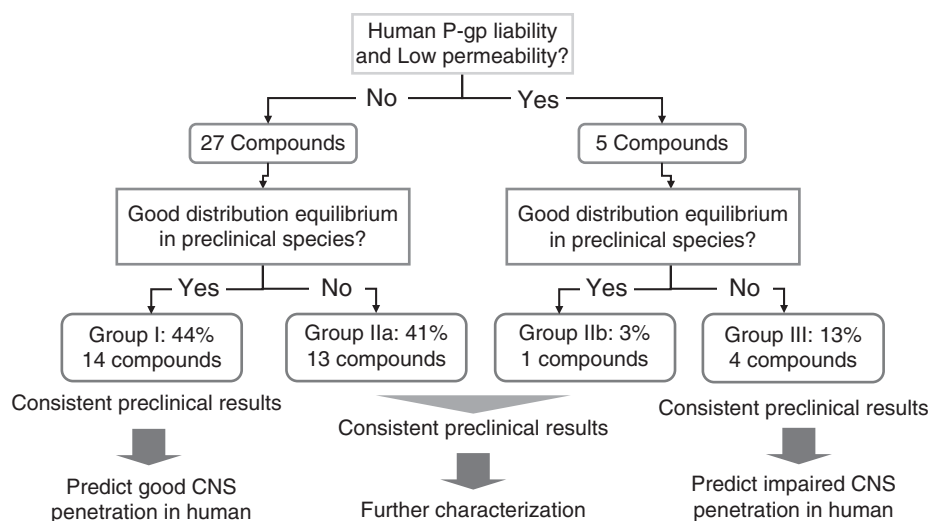


Figure 17.3 Road map of categorizing Pfizer proprietary clinical candidates ($n = 32$) into three groups based on their preclinical information. Group I (“well-behaved” CNS compounds): consistent *in vitro* and *in vivo* preclinical data suggesting good CNS penetration; group II: conflicting *in vitro* and *in vivo* preclinical CNS penetration data; and group III: consistent *in vitro* and *in vivo* preclinical indication impaired CNS penetration.

17.4.2 Human CNS Penetration Predicted from Preclinical Data

Human CNS penetration of these compounds revealed predictability of preclinical information (Fig. 17.4). For group I, or “well-behaved” CNS compounds, all of them exhibited good CNS penetration in human based on distribution equilibrium between CSF exposure and plasma or the fact that observed receptor occupancy could be predicted from unbound plasma in human and *in vitro* binding affinity. Furthermore, when

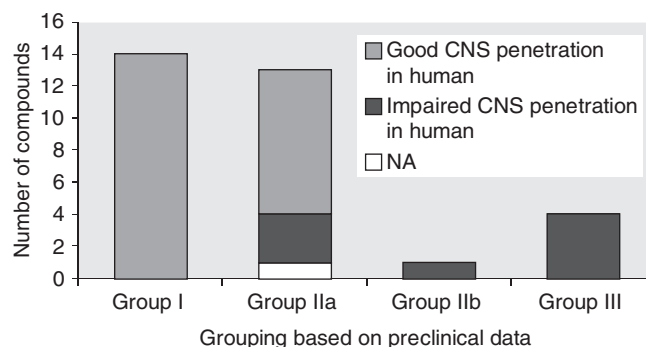


Figure 17.4 Correlation of preclinical and clinical CNS penetration of 32 Pfizer proprietary clinical candidates. Grouping was based on preclinical information (Fig. 17.3). Human CNS penetration assessment was based on human CSF/unbound plasma ratio using a cutoff of 2.5, human receptor occupancy data or efficacy outcome.

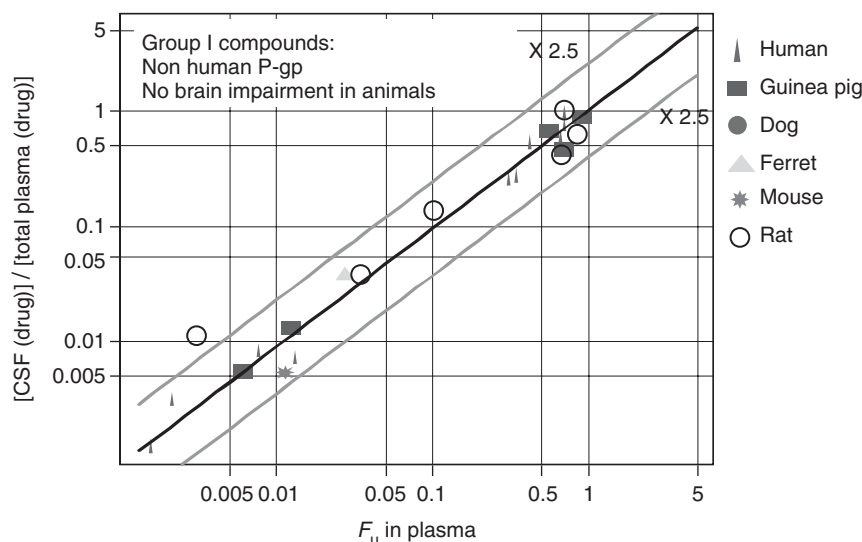


Figure 17.5 Correlation between $f_{u,\text{plasma}}$ and CSF/plasma ratio for group I compounds across multiple species. For compounds that exhibit free drug distribution equilibrium between CSF and plasma, unity line correlation is expected. The plot shows that majority of group I compounds are within 2.5 boundary of the unity line, indicating distribution equilibrium between CSF and plasma compartments.

plotting $f_{u,\text{plasma}}$ against the CSF/plasma ratio, essentially comparing CSF concentration with unbound plasma concentration, a majority of group I compound's data, across multiple species, are within the 2.5-fold boundary of the unity line (Fig. 17.5), indicating that for group I compounds, human CSF concentration data does not provide extra value over plasma free drug concentration data as a surrogate for CNS target exposure. This has a significant implication for CNS drug development, as this means for “well-behaved” CNS compounds, unbound plasma concentration is as good as CSF concentration in assessing PK/PD relationships at the CNS target site. Moreover, CSF sampling can be avoided, meaning significant clinical development cost saving and reducing unnecessary safety risk for clinical trial participants.

Group III compounds also had consistent *in vitro* data and *in vivo* preclinical CNS penetration data, with both data sets projecting unfavorable human CNS penetration. As expected, for these compounds, clinical data based on human CSF or receptor occupancy was consistent with preclinical prediction, all the group III compounds had impaired CNS penetration in human (Fig. 17.4). Furthermore, cross-species comparison of the CSF/unbound plasma concentration ratio indicated that a magnitude of disequilibrium between CSF and plasma compartments (i.e., CSF/unbound plasma ratio) is not consistent across species even when differences in plasma protein binding were accounted for. Though these compounds still penetrate brain, what makes their clinical development difficult is that it is very hard to get an accurate estimate of the actual concentration at the target biophase. As an example, one of the group III compounds, CP-615003, a potent subtype-selective GABA_A partial agonist, was once brought into clinical development as a psychotherapy, but discontinued due to lack of efficacy [86].

The compound was known to have P-gp efflux liability, and preclinical CNS penetration studies also indicated impaired brain penetration. Nevertheless, the compound was brought forward into clinical development, because it was believed that sufficient target receptor occupancy could still be achieved based on projected CSF exposure in human. However, clinical PET imaging clearly demonstrated an underachieved receptor occupancy in human. Intracerebral microdialysis in rats conducted afterward revealed that this is due to a further distribution gradient from CSF to ISF. For the mechanism of the drug, exposure in the ISF drove the downstream pharmacological effect. It was shown that there was a 43-fold difference in unbound drug exposure between ISF and plasma, compared with only an 8-fold difference between CSF and plasma.

Similar to the preclinical observations, human CNS penetration of group II compounds has mixed results (Fig. 17.4). The large percentage of compounds in group II in the data set (~44%) reflects the difficulty of evaluating and predicting CNS penetration. It was noted that possible species difference in P-gp efflux contributed to the different outcomes between preclinical and clinical CNS penetration [65]. Also, transporters, other than P-gp, were found to play a role in uptake or efflux of compounds into or out of brain, respectively (e.g., BCRP [55,57]). From a technical perspective, accuracy in determining extremely high nonspecific binding (>99.9%) may also confound result interpretation from preclinical neuroPK studies. On the clinical side, for compounds that exhibit delayed distribution kinetics among neurocompartments, single time point CSF sampling may not represent steady-state CNS penetration in human. These findings underscore the necessity of further research in the area of CNS drug disposition. Furthermore, it is important to understand the time course of drug disposition in CSF, and its relevance to target biophase, when designing clinical trials incorporating a CSF sampling end point for assessing CNS target exposure in human.

17.4.3 Predictability of Physicochemical Properties and Preclinical *In Vitro* and *In Vivo* Studies

The predictability of the four most commonly used preclinical assays, namely *in vitro* human P-gp, rat neuroPK, dog CSF PK, and P-gp KO mice studies, were evaluated, based on their correlation with human CNS penetration observed in the clinic (Fig. 17.7). Results indicated that all the human P-gp substrates from the analysis had impaired CNS penetration in human, confirming the critical role of P-gp efflux in drug CNS disposition. In contrast to the expectation that the dog CSF model is a better predictor for human CNS penetration than rat neuroPK, comparable human predictability was observed between the two models, suggesting rat neuroPK as the choice for efficient preclinical evaluation. Interestingly, P-gp KO mice had some “false positive” and some “false negative” prediction for human CNS penetration. These were found to be due mostly to species differences in P-gp efflux between human and mouse and to involvement of other efflux transporters, such as MRP4. Nevertheless, the P-gp KO mice study is still a useful tool to identify whether observed brain distribution impairment in rodents is primarily driven by P-gp.

Brain-penetrant CNS drugs are known to have certain physicochemical attributes [7]. Physicochemical properties of the 32 drug candidates were profiled and their correlations with human CNS penetration were sought (Fig. 17.6). Results confirmed that physicochemical properties, such as MW, lipophilicity (log *P* and log *D*), and PSA,

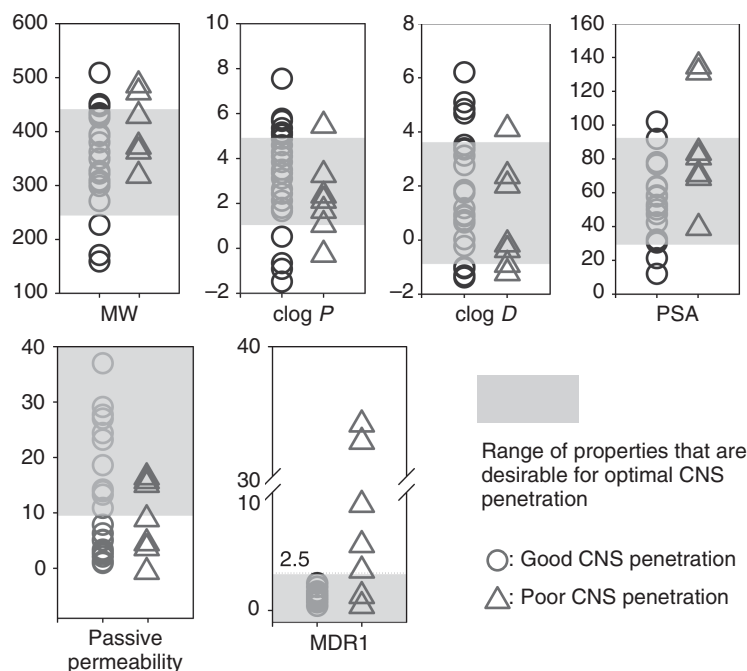


Figure 17.6 The roles of physicochemical properties and *in vitro* properties in predicting human CNS penetration. Clog *P*, Clog *D*, and PSA (polar surface area) were calculated using ACD Lab software (www.acdlabs.com). Passive permeability was assessed using a variant of MDCK cell line that does not express P-gp. MDR1 efflux ratio was obtained from transwell assay using MDR1-MDCK cell line.

are important determinants of CNS penetration. However, none of these should be used alone or as hard criteria when selecting CNS-penetrant compound (Section 17.3.3.2). One interesting observation is that the human P-gp assay appears to be the best assay to identify compounds that will have brain distribution impairment in human, as all the P-gp substrates in the data set exhibited distribution disequilibrium between plasma and brain/CSF ratios in human. However, the opposite is not true, as not being a P-gp substrate did not guarantee good CNS penetration in human (due to possible low passive permeability or efflux by other transporters).

17.4.4 Summary of Retroanalysis Results of Pfizer Proprietary Clinical Candidates

The outcome of retrospective analysis, wherein the predictability of preclinical assays for human CNS penetration was evaluated using 32 Pfizer proprietary clinical candidates, was encouraging (Fig. 17.7). When the human P-gp assay and rat neuroPK characterization (free drug partitioning between plasma, brain, and CSF) show consistent results, the two preclinical assays are effective in prediction of human CNS penetration with high confidence. When there is no human P-gp liability and good CNS penetration in rats (equilibrium of free drug partitioning between plasma, brain, and CSF), good CNS penetration in humans is predicted. More importantly, for

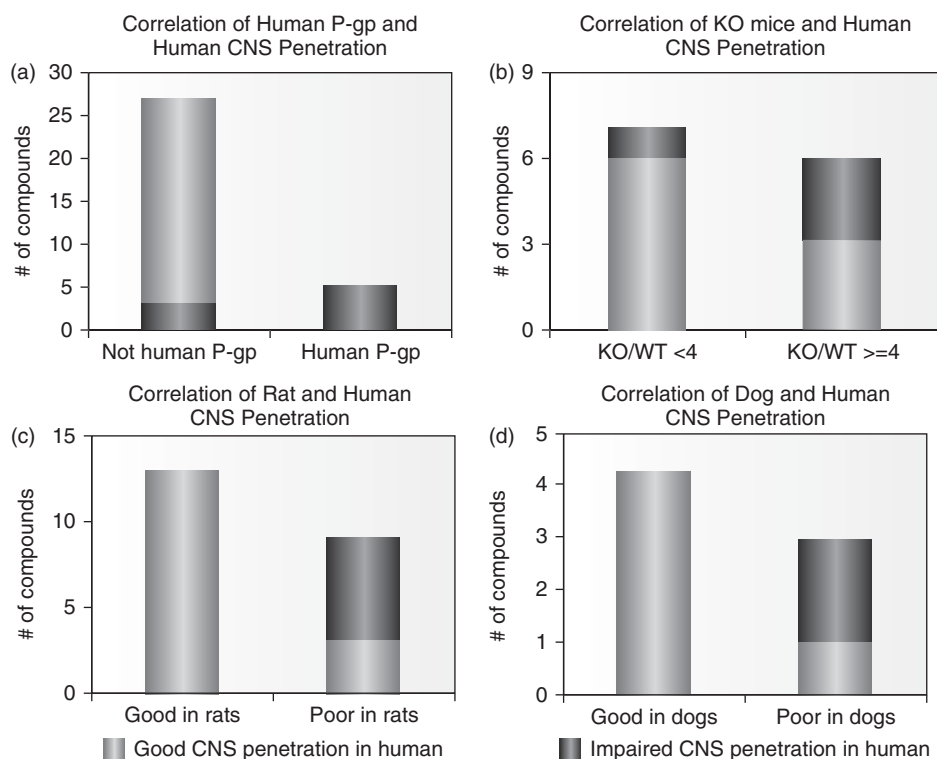


Figure 17.7 Predictability of preclinical assays to human CNS penetration, assessed by correlating results from human P-gp (MDR1/MDCK), P-gp KO mice model, rat neuroPK, and dog CSF model, respectively, to outcome of human CNS penetration observed in clinical trials by either human CSF exposure or receptor occupancy by PET. (a) Correlation of human P-gp and human CNS penetration, (b) correlation of KO mice and human CNS penetration, (c) correlation of rat and human CNS penetration, and (d) correlation of dog and human CNS penetration.

these compounds, the free drug exposure in plasma reflects the CNS target exposure in human. On the other hand, when there is human P-gp liability and impaired CNS penetration in rats (impaired drug partitioning between plasma, brain, and CSF), poor CNS penetration in humans is predicted. For these compounds, the free drug exposure in plasma will overestimate the CNS target exposure. In addition, the results of this retrospective analysis clearly underscore the need for further research in areas, such as transporters and brain PBPK, to better understand the physiological and molecular mechanisms influencing brain penetration and to predict human CNS penetration for compounds that exhibit less than favorable CNS penetration properties.

17.5 CONCLUSIONS

Integrated *in silico*, *in vitro*, and *in vivo* approaches have been developed to predict brain penetration of drug candidates. A BBB screening strategy is shown in Fig. 17.8

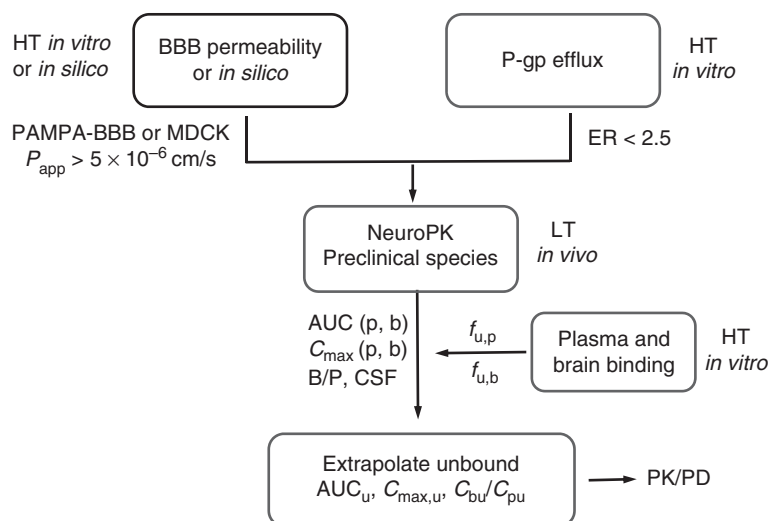


Figure 17.8 BBB screening paradigm in CNS drug discovery. *Source:* Modified from Ref. 3.

as an example to evaluate and predict brain exposure of drug discovery compounds. The key take-home messages and learning about BBB are summarized as follows:

- Use $K_{p,uu}$ (C_{bu}/C_{pu}) to evaluate the extent of brain penetration for drug candidates. Total brain/plasma ratio (i.e., B/P) is mostly due to nonspecific binding and does not reflect the potential of compounds to cross the BBB.
- $K_{p,uu}$ is usually preserved across species for nontransporter substrates, which enable prediction of human free brain drug concentration using unbound human plasma concentration.
- For compounds that exhibit distribution equilibrium in preclinical animals and have good passive permeability and no transporter efflux *in vitro*, free drug concentration in the plasma is a good surrogate for free drug concentration in the brain for humans. CSF measurement does not add extra values for the “well-behaved” (group I) compounds.
- CSF is a valuable surrogate for free drug concentration in the brain. However, for efflux transporter substrates, CSF tends to overestimate free drug concentration in the brain.
- Fraction unbound in brain tissue is useful to calculate free drug concentration in the brain. However, it will not affect the unbound brain concentration for orally administered drugs. Fraction unbound should not be optimized through structural modification or be used to rank order compounds.
- P-gp and BCRP are two major efflux transporters in the BBB and they work synergistically to eliminate compounds from the brain. Substrates of efflux transporters demonstrate significant brain impairment. Rodent-based neuroPK studies tend to overestimate efflux activity of human BBB, and monkey is a better preclinical model to predict CNS penetration for human.

- Drug–drug interaction due to inhibition of P-gp or BCRP at the BBB is unlikely at clinical relevant doses, owing to the relatively low free drug concentration compared with K_i for most inhibitors.

17.6 FUTURE PROSPECTS

Our understanding of the BBB has advanced significantly over the years. Strategies and methodologies continue to evolve to help us discover new medicines to treat CNS diseases. We expect to see more imaging techniques applied to early drug discovery programs to guide in-depth understanding of drug–target interactions and mechanisms of actions. The BBB transporter field will continue to expand and the resulting data will be incorporated into building PK/PD models and developing predictive tools. Uptake transporters and carriers will be utilized effectively to enhance influx of drugs into brain. Novel brain delivery technologies will advance to bring impermeable compounds into the brain, such as innovations targeting transporters [87] and nanoparticles [88]. Biologics will be delivered to the brain and be available to treat brain diseases. An example of the current approaches is to use molecular Trojan horses to deliver biologics to the brain [89,90]. More BBB PBPK models will be developed and widely applied to accurately predict brain PK.

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ABBREVIATIONS

ABC	ATP-Binding Cassette
AUC	Area Under the Curve
AUC _b	Brain Area Under the Curve
AUC _{b,u}	Unbound Brain Area Under the Curve
AUC _p	Plasma Area Under the Curve
AUC _{p,u}	Unbound Plasma Area Under the Curve
AUC _u	Unbound Area Under the Curve
BBB	Blood–Brain Barrier
BCRP	Breast Cancer Resistance Protein
BCSFB	Blood–Cerebrospinal Fluid Barrier
B/P = K_p	Brain-to-Plasma Ratio Based on Total Drug
$C_{\max}$	Maximum Concentration
$C_{\max,u}$	Unbound Maximum Concentration
$C_{bu}/C_{pu} = K_{p,uu}$	Free Brain-to-Plasma Ratio
CSF	Cerebrospinal Fluid
CNS	Central Nervous System
CP	Choroid Plexus

f_u	Fraction Unbound
$f_{u, \text{plasma}} = f_{u, p}$	Fraction Unbound of Plasma
$f_{u, \text{brain}} = f_{u, b}$	Fraction Unbound of Brain Tissue
ICF	Intracellular Fluid
ISF	Interstitial Fluid
$K_p = B/P$	Brain-to-Plasma Ratio Based on Total Drug
$K_{puu} = C_{bu}/C_{pu}$	Unbound Brain-to-Plasma Ratio
MDCK	Epithelial Madin-Darby canine kidney cell line
MDR	Multidrug Resistance Protein
MPO	Multiparameter Optimization
MRI	Magnetic Resonance Imaging
MRP	Multidrug-Resistance-Associated Protein
NeuroPK	Neuropharmacokinetics
PAMPA	Parallel Artificial Membrane Permeability Assay
PD	Pharmacodynamics
PET	Positron Emission Tomography
PK	Pharmacokinetics
SPECT	Single Photon Emission Computed Tomography
V_u	Unbound Volume of Brain

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18 Why Do We Need *In Vivo* Models in Drug Metabolism?

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18.1 Summary	1
18.2 Introduction	2
18.3 Oral absorption	3
18.4 Distribution	8
18.5 Excretion and elimination	13
18.6 Perspective on the use of <i>in vivo</i> models of drug metabolism	21
References	23

18.1 SUMMARY

The use of preclinical *in vivo* models is integral to drug metabolism science. They are used to predict human pharmacokinetics in terms of oral absorption and distribution, including CNS penetration and drug clearance. In addition, *in vivo* models are essential in ensuring compound safety through qualification of human circulating metabolites in toxicology species.

The challenge in the use of *in vivo* models to predict human pharmacokinetics comes in the significant species differences in physiology and active processes (metabolism and transport) between animals and humans. Rat appears to be an appropriate model for the prediction of human oral absorption. For simple passive processes, such as volume of distribution and passive renal secretion, simple allometric scaling shows reasonable predictivity between animals and humans. For an indication of CNS penetration, the rat model has the greatest potential, although species differences in CNS penetration are known. However, when active processes, such as metabolism, biliary excretion, and renal secretion, are considered, species differences in rate and extent can significantly complicate extrapolation to human. These differences also preclude the use of preclinical models for the examination of human drug–drug and drug–food interactions.

As drug metabolism science progresses, much greater use of scalable human *in vitro* assays and *in silico* prediction should replace the use of animals in drug metabolism

studies. Until that time, the use of *in vivo* animal models will remain essential in the drug metabolism field, although great care in their use should be exercised.

18.2 INTRODUCTION

A major aim of drug metabolism science is the description of the journey of a drug molecule through the body. It is important to understand how a molecule moves from its site of administration to the site of action, how long it remains there, and how it is eliminated from the body. These are termed the *absorption, distribution, metabolism, and elimination* (ADME) properties of a drug. It is the story of how the body deals with a drug molecule.

Since the inception of drug metabolism science as a separate discipline during the 1950s, scientists have developed a sophisticated understanding of how a drug molecule is handled by the body and, in many cases, can modulate the physicochemistry of a series of molecules to produce more desirable ADME properties (such as a longer elimination phase half-life) [1–4]. An understanding of the proteins that modulate the metabolism and pharmacokinetics of a molecule has been developed [5–8]. In addition, *in vitro* methods have been introduced, which further the understanding of drug metabolism and allow assessment of large numbers of compounds without recourse to *in vivo* preclinical models.

However, the use of *in vivo* animal models to understand drug disposition remains a key part of the drug discovery and development process. Wherever possible, a drug metabolism scientist has an ethical duty to replace *in vivo* experiments in preclinical species with *in vitro* and *in silico* approaches. However, with the current state of the science, a truly *in vitro* only approach to describe the properties of a molecule is not available. Thus, animals have to be used. The ultimate goal should be to replace all animal experiments with *in vitro* ones. However, until that time, the drug metabolism scientist is strongly recommended to follow a paradigm illustrated in Fig. 18.1, whereby

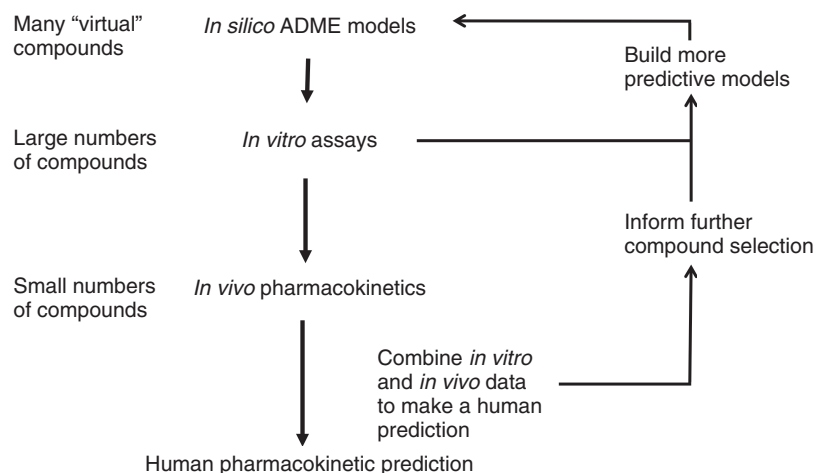


Figure 18.1 Proposed ADME screening funnel that drives to reduction in animal use.

large numbers of potential drug molecules are triaged using *in silico* and *in vitro* methods with only few compounds progressing to *in vivo* experiments.

From a pharmaceutical industry perspective, most drugs are aimed at the treatment of human disease. Thus, understanding ADME properties in humans is a key part of bringing a drug to the pharmaceutical market. A typical drug discovery and development programme (if successful) will take more than a decade to complete. Typically, medicinal chemists will synthesize thousands of molecules as putative drugs and the research process will reduce these to one or two for detailed scrutiny. During the development phase, the human safety and efficacy of these molecules are fully evaluated for presentation to regulatory authorities (such as the FDA). Drug metabolism science plays a significant role in the entire research and development process, with an interesting change in emphasis during the process. In early research, the aim is to identify pharmacologically active molecules with appropriate human ADME properties, without recourse to human *in vivo* experiments. Once the single compound has been selected, the drug metabolism effort tends to become descriptive with emphasis on a detailed understanding of the ADME profile of that molecule. In both phases, the use of *in vivo* models of drug metabolism is essential. However, there are significant species differences in the way drug molecules are handled from an ADME perspective. These species differences need to be well understood to enable correct decisions to be made.

The animal species used for drug metabolism *in vivo* experiments tend to depend on the phase of the project and the questions posed. In the research phase, the vast majority of animals used are rodents. *In vivo* pharmacology models tend to be in mice and rats and a significant proportion of drug metabolism effort can be expended in support of these models to understand PKPD relationships. As stated previously, the major aim of a research project is to select compounds with desirable ADME properties. Again, the *in vivo* model of choice is the rat, due to its relative availability and low cost. However, other nonrodent species (such as dog and nonhuman primate) are used during this phase. Whatever species is used, it is vitally important to understand the extrapolation of any pharmacokinetic property from the animal species to human. In the development phase, there is a regulatory requirement to complete toxicology studies in one rodent and one nonrodent species. Again the species most often employed are rat and dog or nonhuman primate. However, carcinogenicity studies are completed in mice and reproductive toxicity studies in rabbit. During the development phase, drug metabolism experiments are designed to support toxicology, ensuring that any chemical species derived from the drug in humans (i.e., metabolites) are also exposed to animals during toxicity studies (i.e., there are no human-specific metabolites). Thus, understanding of the metabolite profile of a molecule in mouse, rat, dog, rabbit, and nonhuman primate is important.

18.3 ORAL ABSORPTION

18.3.1 Oral Absorption and Bioavailability in Human

Most human drugs are administered at sites remote from the site at which they are designed to act. For patient ease of administration, the oral route is preferred. Orally administered drugs are most often given in tablet or capsule formulations. In order to be absorbed into the body, these solid formulations must disintegrate in the stomach and release their cargo of drug into aqueous solution in the gastrointestinal (GI) tract fluid.

The human GI tract is designed to be the organ of food digestion and nutrient absorption. The first organ encountered following oral administration is the stomach. This is where food digestion begins and can be a very acidic environment (pH 1–5) depending on the fed status of the individual. The major human drug absorbing site is the small intestine. This part of the GI tract is made up of three separate segments: the duodenum, jejunum, and ileum. In the small intestine, the pH returns to near physiological (pH 6.5–7.4). These segments contain a number of adaptations designed to facilitate the absorption of nutrients (and drug molecules), including luminal folding, villi, and microvilli, which increase the surface area for absorption. Finally, the lower part of the human GI tract is the large intestine (or colon), where the majority of the water involved in food digestion is reabsorbed before defecation.

Once a drug is in solution, it must pass across the GI tract wall to be absorbed into the body. There are two major routes of passage for drug molecules to pass across the GI tract wall: paracellular and transcellular (Fig. 18.2).

The major cellular barrier between the GI tract lumen and the blood is the gut wall epithelial cell or the enterocyte. To be absorbed, the drug must cross the enterocyte barrier. Between each enterocyte, there are very small tight junctions with aqueous pores through which low molecular weight compounds can be absorbed [9]. The estimated sizes of these aqueous pores in human small intestine vary from 5 to 13 Å, and there may be several size populations [10,11]. Drugs of small molecular weight can pass through these aqueous pores and are considered to be paracellularly absorbed. An example of such a drug is cimetidine [12], which is ~60% absorbed in human due to its molecular weight of 252 despite its hydrophilicity (cLog $D_{(7.4)}$ of -0.05). Owing

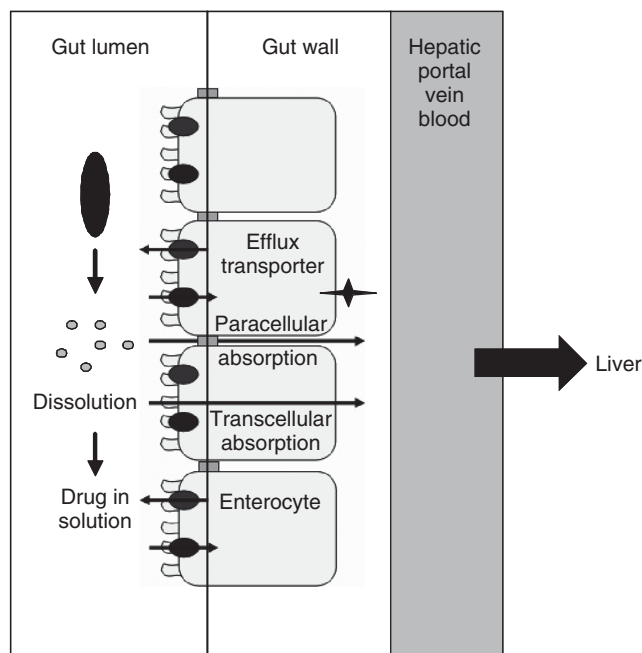


Figure 18.2 Schematic of the oral absorption process indicating paracellular and transcellular absorption.

to the size constraints and the relatively low surface area covered by these pores, the paracellular route is only a minor route of absorption for drug molecules. In addition, the aqueous pores become smaller lower down the GI tract and as such paracellular absorption from the colon is unlikely.

The major route of drug absorption is the transcellular route. To be absorbed by this route, the drug must dissolve in the apical membrane of the enterocyte and cross the cell to the basolateral membrane, cross that to enter into the blood. Drugs absorbed by the transcellular route are also subject to a battery of drug transporters that can either facilitate the absorption of the compound or act as a barrier to absorption by effluxing the compound back into the gut lumen. In addition, the gut wall cell contains many drug-metabolizing enzymes that can irreversibly remove the drug on passage through the cell.

Once a compound has passed through the gut wall, either by the paracellular or transcellular routes, it is considered to be absorbed into the body. However, it has one further barrier to overcome before it appears in the systemic circulation. All the blood from the gut passes into the hepatic portal vein, which flows directly to the liver. The liver is the major site of drug clearance and as such can exert a profound effect on the systemic concentrations of a drug in a process called *hepatic extraction*. The extent of hepatic extraction will depend on the physicochemistry of the drug and the affinity for drug transporters and metabolic enzymes. Owing to first-pass hepatic extraction, it is entirely possible that a compound can be completely absorbed from the GI tract but show no systemic concentrations due to complete removal by the liver. The extent to which a compound overcomes all the barriers between oral administration and the systemic circulation is called the *oral bioavailability*. It is a composite of the fraction absorbed from the GI tract and the fraction removed on first passage through the liver.

18.3.2 Animal Studies to Calculate Oral Absorption and Bioavailability

In the research phase, much emphasis is placed on the assessment of oral absorption in animal species as a predictor of human oral absorption. The most simple method to estimate oral absorption is by completion of an intravenous versus oral cross-over experiment in the species of choice. Following intravenous administration, all the dose is placed in the systemic circulation. Thus, the area under the plasma/blood concentration versus time curve (AUC) represents the complete dose. For an oral dose, the drug must dissolve in the GI tract contents and cross the GI tract wall (absorption). All the blood from the gut flows into the hepatic portal vein, which flows directly to the liver. The liver is a major drug extracting organ and, in order to reach the systemic circulation, the drug needs to avoid this potential “first-pass” extraction. Consequently, the AUC following an oral dose represents the amount of drug absorbed and the amount escaping first-pass extraction.

If it is assumed that clearance of a drug is solely by the liver, then the first-pass extraction of that drug can be estimated from the clearance of that drug and the hepatic blood flow in that species. Once the hepatic first-pass extraction is estimated, the AUC assuming complete oral absorption can be calculated. The ratio of the observed AUC to that expected following complete oral absorption represents the fraction of the dose absorbed in that species.

A more direct estimate of the fraction of an oral dose absorbed can be determined using the hepatic portal vein experiment. In general, this tends to be completed in

rodent species as it requires sampling of blood from the hepatic portal vein, thus eliminating the need to estimate hepatic first-pass extraction. Basically, the amount of dose absorbed can be estimated using one of two potential equations:

$$\text{Dose absorbed} = Q \times (\text{AUC}_{\text{portal}} - \text{AUC}_{\text{systemic}})$$

$$\text{Dose absorbed} = \text{Cl} \times \text{AUC}_{\text{portal}}$$

where Q is the hepatic portal vein blood flow and Cl the clearance of the drug.

18.3.3 Animal Studies as Models of Human Oral Absorption

The projection of extensive oral bioavailability in humans is often a key goal in pre-clinical drug discovery projects. Consequently, selecting compounds with appropriate oral absorption potential is important. There are significant differences in physiology between the human GI tract and those of animals used in *in vivo* drug metabolism experiments, which need to be understood for successful decision making. The use of animals for predicting human oral absorption has recently been reviewed [13].

18.3.3.1 Rat. The rat is the most studied preclinical model of human oral absorption. There are a number of physiological differences between the GI tracts of human and rat [14,15]. The pH in the rat duodenum tends to be slightly higher than that in human [15]. Clearly, the length and diameter of the human small intestine are higher than that in rat and there is significantly less unstirred water layer (the fluid nearest the gut wall epithelium) in humans than rats [14]. In addition, there are significant differences in terms of the bile system. Bile production in rat is some 16-fold higher than in human [16,17]. In addition, rat bile constantly flows into the GI tract, where it may have a solubilizing effect on low solubility agents. In contrast, all bile production in humans is stored in the gallbladder to be released following feeding. All these characteristics may lead to issues in terms of extrapolation of oral absorption between rat and human. However, in terms of aqueous pores, rat and human are similar with respect to size and distribution, which may suggest that the rat would be an appropriate model for paracellular absorption in humans [18,19].

Rat and human express similar amounts of drug transport proteins at the gut wall. Cao *et al.* [20] showed that several transport proteins (such as PepT1, MRP2, and GLUT5) are similarly expressed in rat and human intestine, whereas others (such as MDR1 and MRP3) are present in both species but at different expression levels. Indeed, the correlation between duodenal expression level of a large number of transport proteins in human and rat exhibited a strong r^2 value of 0.5687. There was a weak correlation between the rat and human expression level of intestinal drug-metabolizing enzymes.

Overall, Chiou and Barve [21] found a strong correlation between oral absorption in rats and humans for 64 drugs, which suggests that the rat is an appropriate model for human oral absorption, in the majority of cases.

18.3.3.2 Dog. A similar analysis of the dog suggests that it is not an appropriate model for human oral absorption under certain circumstances and should be used with caution in the research phase. The correlation between oral absorption in dog and human for 43 drugs was weak [22]. There are a number of potential physiological reasons for this.

In common with the rat, the intestinal pH is higher in the dog than in human. There is a higher bile salt secretion rate and a higher bile salt concentration that may drive to improved oral absorption of poorly soluble compounds [23,24]. In addition, the villus length in the dog small intestine is longer than that in human. Finally, total GI transit time is more rapid in dog than human. All these factors may play a role in the poor correlation between dog and human oral absorption. However, the major driver of this difference may well be the paracellular pathway.

The aqueous pore pathway in the dog assumes greater significance due to larger aqueous pores than human [19]. Many low molecular weight and polar compounds are absorbed by the paracellular route. In humans (and rats), there is a significant molecular weight cutoff with compounds >300 molecular weight exhibiting poor oral absorption by this pathway. However, in dogs, the molecular weight cutoff is higher, leading to significantly greater oral absorption for some compounds in the dog. For example, acyclovir (MW 225) shows <30% absorption in rat and human, whereas in dog, absorption is estimated at 85%. Similarly, atenolol (MW 266) is completely absorbed in dogs, but 22% and 55% absorbed in rat and human, respectively.

Unlike the rat, there appears to be limited information on expression of drug transporter proteins and metabolizing enzymes in the gut wall of the dog. However, species differences in oral absorption do not seem to be limited to paracellularly absorbed compounds in the dog. This is exemplified by the NK₂ antagonist UK-224,671 [25]. This compound would appear to be transcellularly absorbed as a molecular weight of 544 is unlikely to be absorbed through the aqueous pore pathway. It is a P-glycoprotein (P-gp) substrate [26], and this limits its transcellular absorption potential in mouse, rat, and human. However, in dog, the oral bioavailability is 55%, suggesting much greater absorption in this species.

18.3.3.3 Monkey. The use of monkey to predict human oral absorption has not been extensively studied. However, genetic and physiological similarities suggest that monkey may well be an acceptable model. The gastric pH is most like human than any other preclinical species. Gastric emptying time is similar to that in humans, whereas small intestinal transit time is similar to dog [27]. There is a paucity of data on expression levels of drug transporters and metabolizing enzymes in monkey gut.

Chiou and Buehler [28] have shown a good correlation between monkey and human oral absorption for 43 drugs, despite a range of solid dosage formulations being employed. Examination of solid formulations (tablets or capsules) is perhaps where the use of the monkey is most appropriate, since such formulations cannot be given to rats and the dog appears not to be a good model for human absorption. However, the balance of cost and ethics of monkey use needs to be strongly considered and use of this species should be strictly limited to fewer compounds at later stages of drug research.

18.3.4 Modeling the Effect of Food in Animals

A major consideration for human oral absorption is the effect of food on the absorption profile of a drug. The effect of food on the oral absorption of drugs in humans has been extensively reviewed for both general therapeutics [29,30] and for anticancer agents [31].

Food can have profound effects on both the rate and extent of drug absorption for certain compounds. For example, coadministration with food reduces the oral exposure of drugs such as doxazosin, verapamil, and zidovudine. In contrast, the absorption of felodipine, itraconazole, and phenytoin is increased by food. In addition, the rate of absorption is decreased by food for drugs such as diclofenac and paracetamol. Finally, there are significant numbers of drugs (including diazepam and amlodipine), where food has no effect on oral exposure in humans [30].

There are a number of potential reasons for these food effects on oral exposure. Food increases bile acid secretion and gastric retention, which will have an enhancing effect on poorly aqueous soluble agents and a potentially delayed effect on absorption rate. It may also increase hepatic blood flow such that high hepatic extraction drugs show reduced exposure after oral administration.

Given the potential variety of outcomes with food and the significant differences in GI physiology, modeling of human food effects in animals needs to be completed with great care. The rat should be ruled out as a model to predict food effects in humans due to its continuously high bile flow. It is also very difficult to achieve a truly fasted rat as on withdrawal of food, rats will tend to eat bedding or fecal pellets, meaning that there is always material in their stomachs. Dogs are not an appropriate model of human oral absorption in general as shown above. However, there are some accounts of the use of the dog in this context in the literature. For example, it has been shown that the food-driven 33–46% decrease in oral AUC of hydralazine in humans is adequately predicted by a food reduction in AUC of 63% in the dog [32]. However, celecoxib shows hardly any change with feeding in humans but a three- to fivefold increase in systemic AUC in the dog [33]. Finally, due to genetic and physiological similarities, the monkey may be an appropriate model, but there are very few examples of its use for studying food effects in the literature. It would appear that with state-of-the-art physiologically based pharmacokinetic models available (reviewed elsewhere in this book), the *in silico* approach to measure food effects in humans has largely superseded the preclinical study.

18.3.5 Oral Absorption Summary

Great strides have been made in the recent past in predicting human oral absorption in the research phase. Compounds with appropriate physicochemistry for oral absorption and acceptable *in vitro* absorption profiles will continue to be taken forward to pre-clinical animal species for oral absorption and bioavailability determination. The most appropriate species for this investigation is the rat based on its correlation with human oral absorption. The dog is not an appropriate model of human oral absorption and should be used with great care and a mechanistic understanding of the absorption process. Monkey may be an appropriate model but should only be used on rare occasions when solid dosage forms need to be investigated.

18.4 DISTRIBUTION

Distribution describes the reversible movement of a compound from the systemic circulation into tissues. It is dependent on the physicochemistry of the molecule driving a balance between plasma protein binding and tissue affinity [34].

18.4.1 Extrapolation of Animal Distribution to Human

The prediction of human volume of distribution has been extensively reviewed by Obach [34].

Since tissue distribution is driven by plasma protein binding and tissue affinity, extrapolation of the human volume of distribution from animal studies tends to be relatively straightforward. In general, tissue distribution is a passive process and tissues tend to show no real species differences (made of cells and membranes).

Therefore, it is possible to predict the human Vd of a particular drug using allometric (body weight) scaling [35]. The allometric exponent tends to approximate to unity [36,37]. However, in the early phase of drug research, the requirement for pharmacokinetics in three species to perform true allometric scaling can be limiting. Thus, many groups have tended to search for a single species that is as predictive for human Vd.

Obach *et al.* [38] examined several methods of prediction of human Vd. They found that allometric scaling of Vd (based on total drug) gave a relatively poor prediction of human Vd (53% of compounds with ± 2 -fold of actual human Vd). This improved significantly (77% within ± 2 -fold) if the unbound parameters were used. In addition, direct scaling of dog Vd corrected for species differences fraction unbound performed as well (81% within ± 2 -fold).

Other groups have examined alternative species for single species extrapolation of human Vd. Ward and Smith [39,40] used a correlation analysis to show that the monkey is an appropriate predictor of human Vd, whereas rat and dog showed less predictivity, based on total drug parameters. The same group recognized that the monkey may not be the most appropriate species to use in early drug research and have proposed incorporation of physicochemical rules to determine the likelihood of extrapolation errors from preclinical species [41]. Finally, Caldwell *et al.* [42] have shown that the extrapolation of rat Vd can exhibit a reasonable approximation of human Vd (average fold error 1.85 for 144 compounds with 84% of compounds with ± 3 -fold of actual human Vd), although this analysis does not factor in any species differences in fraction unbound between rat and human.

In summary, extrapolation of preclinical species Vd values is likely to provide a reasonable approximation of human Vd, especially when species differences in plasma protein binding are taken into account.

18.4.2 Free Drug Hypothesis

As described above, drug distribution is generally a passive process where there are no major barriers to drug diffusion into tissues. In these cases, eventually a steady-state situation arises, whereby the unbound concentration in blood reflects the unbound concentration in the tissues. Thus, for a large number of drugs, the unbound concentration in blood is a surrogate for the unbound concentration at the target. This is termed the *free drug hypothesis*.

18.4.3 Barriers to Passive Drug Movement

Within the body, there are a number of significant barriers to the passive movement of compounds between the blood and the tissue of interest. These include the

blood–eye and the blood–testis barrier. However, by far, the most important barrier is the blood–brain barrier (BBB).

The BBB acts as a highly selective barrier to the passage of compounds into the CNS. It is formed by the endothelial cells of the blood vessels supplying the brain. Unlike blood vessels supplying many other tissues, these cells form extremely strong tight junctions that effectively mean that the aqueous pore pathway does not exist. In addition, the membrane phospholipid make-up may be different from other endothelial cell membranes with a more rigid structure leading to increased difficulty in passive permeation. Finally, the cells express significant levels of efflux transporters and metabolic enzymes that are capable of removing compounds back into the blood on passage across the BBB.

By far, the most studied efflux transporter at the BBB is P-gp. Chen *et al.* [43] have extensively reviewed P-gp and P-gp knockout mice. The role of P-gp in preventing BBB penetration has also been extensively reviewed. The presence of P-gp as an efflux protein makes the BBB a formidable barrier for the passage of drug molecules. Molecules that are slow to permeate the endothelial cell membrane can be effectively removed and placed back into the blood by P-gp (and other efflux transporters). This has been exploited in the H1 antagonist area to provide drugs that show limited CNS penetration and thus the potential to exert a peripheral effect without the sedation side effects associated with central H1 antagonism. For example, fexofenadine is a potent H1 antagonist that is used in the treatment of allergic rhinitis. It is believed to be a substrate for P-gp and other drug transporters [44], and it is this aspect that prevents its entry into the brain and drives its lack of sedation activity versus earlier H1 antagonists [45,46].

In contrast, many drugs need to enter the CNS to exert their effects and so understanding brain penetration is a key feature for many drug research projects. This understanding often relies heavily on preclinical animal models.

18.4.4 Evaluation of CNS Penetration Preclinical Species

The evaluation of the potential of a compound to enter the CNS begins with the understanding of physicochemistry and the use of *in vitro* models of cell permeability. Promising compounds arising from these models are likely to be progressed to rodent studies. The major issues facing such studies are the route of administration and evaluation of which matrix to measure. Rodents are used because the end point is often cerebrospinal fluid (CSF) or brain tissue.

It is important to measure brain penetration, while plasma concentrations are as stable as possible. Following intravenous bolus administration, often the plasma concentrations decline rapidly during the early time points. Thus, brain concentrations may lag behind plasma concentrations at early time points, leading to an underestimate of CNS penetration (Fig. 18.3a). Following oral administration, the concentrations tend to rapidly rise and then fall as the compound is cleared. By far, the most appropriate route is the intravenous infusion to steady state (Fig. 18.3b), where the compound is infused at a constant rate for several hours, such that there is a steady rise in the plasma and potentially brain concentrations to a plateau, where the rate at which the compound is dosed is equivalent to the rate at which it is cleared.

The most simple measurement made in CNS penetration studies is the total concentration in the brain relative to the total concentration in the plasma (the brain to

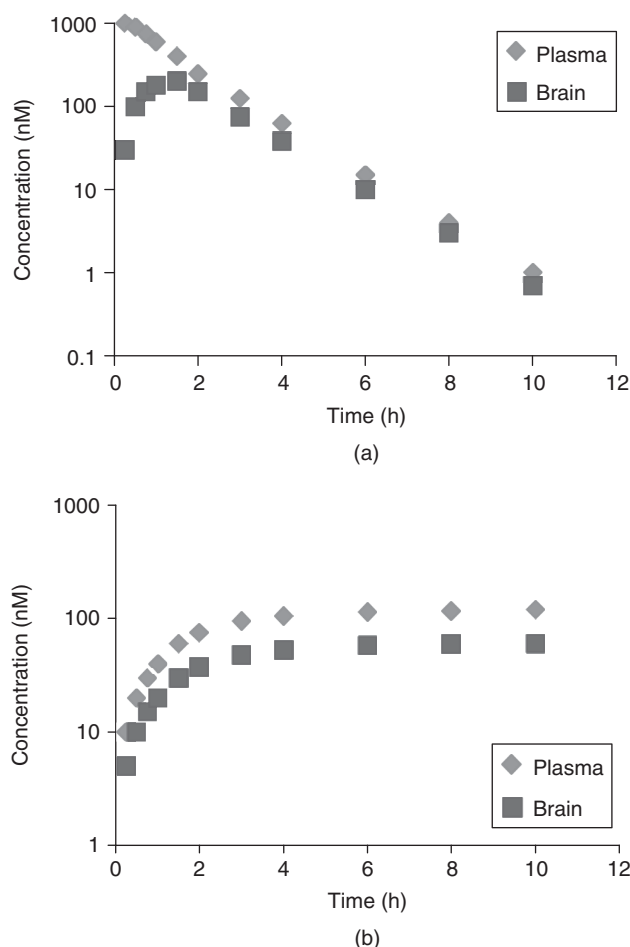


Figure 18.3 (a) Schematic of plasma and brain concentrations following an intravenous bolus administration assuming relatively slow CNS penetration. (b) Schematic of plasma and brain concentrations following intravenous infusion to steady state.

plasma ratio). Thus, at the end of the experiment, the animal is sacrificed and the brain removed. It is then homogenized and the brain homogenate analyzed for drug concentrations. This experiment assumes that binding to the brain tissue is the same as the binding to plasma proteins, and consequently, the free concentrations are equivalent. However, there are several issues with this approach. First, brain tissue is highly lipophilic and binding of lipophilic molecules is likely to be higher than that in the plasma. Thus, a compound with a brain to plasma ratio of greater than one, may reflect higher binding to brain tissue with the potential that unbound concentrations are low. Thus, a high brain to plasma ratio does not necessarily mean that the compound will have a therapeutic effect. Conversely, many lipophilic acid molecules bind tightly to plasma proteins with potentially less binding to brain tissue. Thus, total measurements may well underestimate the CNS penetration of such molecules.

The other potential issue with this experiment is that it is not possible to completely remove the blood from the brain before sacrifice. Since blood represents a small proportion of the volume of the brain tissue, compounds with high blood concentration may appear to have significant brain penetration.

Other groups have determined the extent of binding of a compound to brain homogenate and used this to calculate an unbound concentration in the tissue. This can be related to unbound concentrations in the plasma and is a more relevant measure of brain penetration and potential to achieve therapeutic concentrations in the CNS [47]. Thus, it is important to consider unbound concentrations in the CNS to fully define CNS penetration. The unbound concentration to measure would be the extracellular fluid (ECF), but this is a very difficult matrix to sample. Many groups have attempted to use microdialysis to sample ECF, but in the main, these have been hampered by the need for considerable surgical skill and the time-consuming experiments needed to quantify recovery of the probes. Another surrogate of the ECF is the CSF. This is a largely protein-free fluid that fills the ventricle in the brain. It is relatively easy to sample in the rat and can be compared to the unbound concentration present in the blood. The downside of this measurement is that the CSF concentrations may not necessarily reflect the ECF concentration, since there is a barrier between the CSF and the brain, which also expresses efflux transporters.

The other major issue with CNS penetration experiments in rodents as a model of human CNS penetration is that it is difficult to obtain good estimates of the human CNS penetration of a compound. Clearly, it is not possible to sample brain or complete microdialysis in humans, and the collection of CSF is challenging. The most appropriate method to achieve a measurement of CNS penetration in humans is to complete a PET imaging study, which requires significant investment and has not been completed on large numbers of compounds. Therefore, overall, it is very difficult to compile good data sets of CNS penetration in human for comparison with rodent experiments.

As a “rule of thumb,” if a compound readily penetrates the CNS in a rodent, it is likely that it will readily penetrate the human CNS, making rodent studies the *in vivo* model of choice for CNS penetration. However, the issue with the use of the rodent is that there may be species differences in the efflux transporters at the BBB. In a recent study, species differences in the brain penetration of three P-gp substrates were examined by PET methodology [48]. This group found that verapamil, GR-205171, and altanserin penetrated the CNS of humans and monkeys more readily than rat brain. In addition, this species difference remained when P-gp was inhibited by administration of cyclosporin, suggesting that other efflux transporters or a unknown active process may be responsible for this difference.

18.4.5 Summary of Preclinical Models of Drug Distribution

The potential for a drug to distribute into tissues is an important consideration in the study of drug disposition. Distribution is a largely passive process determined by the physicochemistry of the drug. Tissues are largely made up of cells with membranes and so lipophilic molecules are more likely to distribute into tissues.

There are no major species differences in the composition of tissues. Therefore, preclinical species are appropriate models of drug distribution in humans. The most appropriate extrapolation to human involves the allometric (body weight) scaling of

the unbound parameter (i.e., corrected for any species differences in plasma protein binding) using an allometric exponent of 1.

The free drug hypothesis suggests that under equilibrium conditions, the unbound concentrations in the plasma reflect those in the tissues. Thus, if a research project is using an animal model of pharmacology, then the measured unbound concentration in plasma giving the desired effect should be close to the target efficacious concentration in humans (assuming no pharmacological potency differences).

The free drug hypothesis may not hold when the pharmacological target is behind a barrier to passive drug movement (such as the BBB). In this case, the ability of the drug to cross the barrier and the unbound concentrations in the tissue are of prime importance. In the absence of strongly validated preclinical models of human CNS penetration, rodent CNS penetration is likely to reflect that in human, so long as unbound concentrations are considered.

18.5 EXCRETION AND ELIMINATION

The human body is constantly renewing itself by turnover of proteins and other macromolecules. Similar turnover processes apply to drug molecules. Any administered drug is subject to removal from the body by a variety of processes. The removal of drug-related material (compound and metabolites) from the body is called *elimination*. The excretion of drug-related material is similar but usually refers to the organ that removes the drug (usually the liver and the kidney).

Drugs are removed from the body at different rates, depending on the rate at which they are acted on by excretion and elimination processes. The quantitation of the rate of removal of a drug from the body is an important consideration and is captured by the pharmacokinetic concept of clearance.

18.5.1 Allometric Scaling of Clearance

It follows that human clearance is an important parameter to be able to predict from preclinical information. This is because it in large part determines the exposure of a compound for a given dose.

The most widely used method of prediction of human clearance from preclinical pharmacokinetic information is allometric scaling. This attempts to relate the clearance of a molecule to the body weight of the species investigated. The basis of this scaling dates back to an early paper [49], which suggested that metabolic rates could be related to body size using an allometric exponent of ~ 0.75 . Boxenbaum [50] developed this further by showing that physiological parameters such as liver weight and hepatic blood flow can be correlated to body weight with allometric exponents of 0.8–0.9.

The rationale behind simple allometric scaling is that clearance can be related to body weight using the following equation:

$$\text{Clearance} = a \times \text{body weight}^x$$

where a is a constant and x is the allometric exponent.

Thus, a plot of log clearance (mL/min) versus log body weight (kg) would be a straight line with a slope being the allometric exponent (Fig. 18.4).

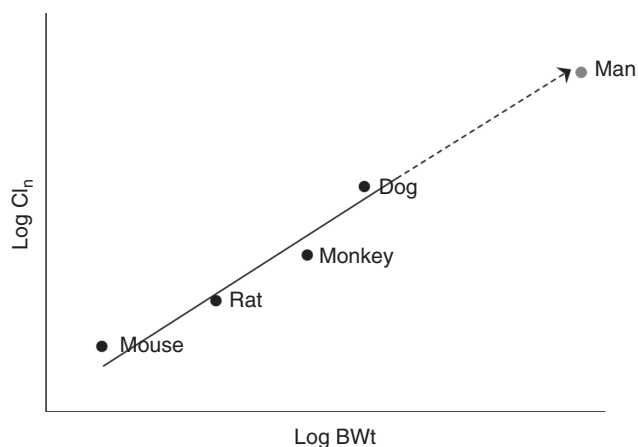


Figure 18.4 Principle of simple allometric scaling.

Using this method, the clearance of a compound in human could be predicted from extrapolation of the line to human body weight.

The success rate of simple allometric scaling in prediction of the human clearance of 102 molecules has been evaluated by Tang and Mayersohn [51]. The allometric exponents ranged from 0.26 to 1.2, although the majority were in the range 0.6–0.8. Overall, the average prediction error (APE) for this compound set was 254%. However, the authors noted that the application of simple allometry could be prone to some highly significant individual errors, in some cases over 1000%. Furthermore, they noted that the majority of these large errors seemed to occur in the low clearance (with respect to hepatic blood flow) cohort. Indeed, the low clearance cohort showed an APE of 598%, whereas the high clearance cohort showed an APE of only 47%. Their analysis suggested that a compound was more likely to be mispredicted to a large extent when the *cLog P* was >2 and the species difference in plasma fraction unbound was >5 . They rationalized this finding on the basis that for high clearance agents, the major contributor to that clearance by the liver or kidney was the hepatic or renal blood flow. Since blood flows scale well allometrically, so will high clearance agents. In contrast, the major determinant of low clearance is the unbound intrinsic clearance of the molecule for the clearing process (likely to be metabolism or active transport). For these clearance pathways, as will be shown later, significant species differences exist that will introduce error to the allometric extrapolation. This would fit with the overall suggestion that passive processes are likely to scale well allometrically, whereas active processes are prone to species differences and therefore error in extrapolation.

In addition to the potential for significant prediction errors, there are a number of issues with simple allometry for the prediction of human clearance. First, it requires pharmacokinetic studies in at least three species and so it is not especially amenable to compound selection in early drug research. Second, it is nonmechanistic and does not take into account the clearance mechanism of a particular molecule nor the potential for species differences in the rate of active processes. Finally, although the initial idea was that clearance would scale with an exponent similar to that with which physiological processes scale, the exponents for simple allometric scaling of clearance can show

significant deviation from 0.7 to 0.8. Indeed, Mahmood and Balian [52] argue that there is no reason why the allometric exponent for a particular drug should be 0.7–0.8. These authors have shown that human clearance prediction is improved by the incorporation of factors depending on the allometric exponent. When the allometric exponent is between 0.55 and 0.70, simple allometry predicts most accurately. For exponents of between 0.71 and 0.99, the maximum lifespan potential should be incorporated, and for exponents above 1, clearance multiplied by brain weight is more accurate. For a more in-depth analysis of the use of allometry with the rule of exponents, the reader is directed to several papers by Mahmood [37,53].

To address the requirement for pharmacokinetic studies in multiple species, several groups have proposed methods of human clearance prediction using scaling from one single species. One of the most comprehensive evaluations has examined the hepatic blood flow method [39–41], whereby the human clearance is predicted from the product of the animal clearance and the ratio of the human and animal hepatic blood flow. For a series of 103 compounds, the monkey hepatic blood flow predicted human clearance more successfully than rat or dog or the use of simple allometric scaling. Although these authors concluded that use of the monkey method was quantitatively more predictive than other species, they recognized that use of the monkey in early drug research projects is problematic. For this reason, other authors have proposed the use of single species allometric scaling from rat using a fixed exponent of 0.75 as a pragmatic and reasonably predictive method for the prediction of human clearance [42,54].

The use of fewer species and the incorporation of fixed exponents single species scaling is highly controversial [55,56]. This author argues that allometric exponents have no physiological relevance. The use of a fixed exponent of 0.75 is misleading and prone to significant prediction error. As shown previously, the preferred method is multiple species allometry using the rule of exponents.

Irrespective of the literature controversy, it appears that the use of preclinical species to predict human clearance will continue. This is despite the fact that no universal method appears to have the required degree of accuracy to predict differences between two closely related compounds [57]. In addition, many of these methods can produce some highly misleading human clearance predictions (probably as a result of species differences in active clearance processes).

18.5.2 Clearance Processes

There are a number of ways a drug can be eliminated from the body. The major routes are by metabolism, via the bile and via the urine. In a survey of the top 200 marketed drugs [58], by far, the predominant route of elimination was by metabolism. The second major route was in the urine, with only small numbers of compounds being eliminated in the bile.

18.5.2.1 Metabolism of Drugs. In the same review [58], the enzymes responsible for the metabolism of the top 200 marketed drugs were investigated. By far, the most predominant enzyme responsible for metabolism is the cytochrome P450 (CYP) family. The next most prominent drug-metabolizing enzymes are the UDPGlucuronyl transferases (UGTs), followed by more minor contributions from aldehyde oxidase (AO), monoamine oxidase, and sulfotransferases.

18.5.2.2 Cytochrome P450 (CYP) Metabolism. The CYPs are a superfamily of drug-metabolizing enzymes that, in general, catalyze the oxidation of drug substrates. In the main, they cause the hydroxylation of carbon atoms and the dealkylation at heteroatoms such as nitrogen or oxygen. They are expressed in many tissues, but the greatest expression occurs in the liver.

The CYPs comprise a family of several hundred isoforms, but by far, the most important human drug-metabolizing CYPs are CYP3A4, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP1A2. Each has its own structure substrate metabolism relationship.

18.5.2.3 Preclinical Models of CYP Metabolism. There are a number of distinct species differences in the structure and function of the CYPs. The same isoforms are not expressed across the preclinical species [59].

Table 18.1 shows the significant differences in the expression and substrate specificities of CYP isoforms between human and animal species. These marked species

TABLE 18.1 Species Differences in the Expression of CYP Isoforms

CYP Subfamily	Species Extrapolation	Inhibition	Induction
CYP1A	Strong conservation across species	Human inhibitor furafylline shows variable inhibitor profile in preclinical species	Omeprazole induces in human but shows variable induction in animals
CYP2A	Significant species differences in substrate specificity	NA	Different inducers in animals and humans
CYP2B	Different isoforms with different substrate specificities	Similar inhibitor profile across species	Phenobarbital induces in all species
CYP2C	Sex-dependent expression in rats. Poor expression in dogs. Significant substrate specificity differences between animals and humans	Inhibition by sulfaphenazole in human, monkey, and dog. Limited inhibition in rat	Inducible in humans
CYP2D	Polymorphic expression in human	Inhibition by quinidine in human, monkey, and dog but not rodents. Inhibition by quinine in rat, dog, and monkey but not human	Not inducible
CYP2E	Relatively well conserved across the species	Similar inhibitory profiles	Inducible by ethanol and acetone
CYP3A	Markedly different substrate specificities across the species	Species differences in inhibition	Species dependency in induction

differences (depending on the isoforms in question) could lead to major differences in clearance by CYPs across the species. Given that a large proportion of marketed agents are cleared by CYP metabolism, it is perhaps not a surprise that allometric scaling based on body weight shows moderate predictivity, with the potential for large extrapolative errors [51]. For these substrates, human liver microsomal scaling may be a more appropriate clearance prediction method.

In addition, there are striking species differences in the CYP inhibition and induction profiles. For these reasons, CYP induction or drug–drug interaction (DDI) studies in animal species as predictors to human are not recommended.

18.5.2.4 UDPGlucuronyl Transferases. The UGTs are a family of drug-metabolizing enzymes that catalyze the addition of glucuronic acid at phenol, alcohol, acid, and nitrogen functions. There are a significant number of isoforms expressed in human liver, including UGT1A1, UGT1A3, UGT1A6, UGT1A9, UGT2B7, UGT2B10, and UGT2B17. In addition, there is significant expression of UGTs in human intestine that can contribute to reductions in oral bioavailability due to gut wall first-pass glucuronidation.

As for the CYPs, each UGT has its own structure substrate metabolism relationship. Unlike CYPs, there has been limited research into the SAR of UGTs. However, some species differences in glucuronidation are beginning to emerge in the literature. For example, the major human urinary metabolite of afloqualone is an *N*-glucuronide that is not present in the urine of animals. *In vitro* studies suggest that the rate of glucuronidation is significantly higher in human liver microsomes than in microsomes from rat, dog, and monkey liver [60]. In addition, mycophenolic acid was glucuronidated at a higher rate in human liver microsomes than in rat liver microsomes [61]. Finally, there is a significant species difference in the oral bioavailability of raloxifene (39% in rat and 2% in human). It has been suggested that this is driven by a 33- to 72-fold difference in the rate of 4-hydroxy glucuronidation between human and rat intestinal microsomes, leading to a much greater intestinal first-pass extraction in humans.

In common with clearance by CYP enzymes, these potential species differences in glucuronidation will complicate extrapolation of human pharmacokinetics from animals. Thus, for such active metabolism processes, allometric scaling may be seriously flawed.

18.5.2.5 Aldehyde Oxidase. Of the more minor enzymes contributing to metabolism of drugs, AO potentially exhibits the most marked species differences. This enzyme is a cytosolic molybdenum-containing protein that tends to oxidize substrates on heterocyclic nitrogen-containing groups.

There are a number of documented observations of significant species differences in AO expression and activity. These include a potential null polymorphism in Sprague-Dawley rats [62] and the total absence of activity in the dog [63]. In those species where AO is expressed, there appears to be significant differences in activity with monkey showing greater activity than rat [64]. Sahi *et al.* [65] suggest that the rank order of AO conversion of vanillin to vanillic acid is monkey > mouse > human > rat.

These species differences in activity follow through to *in vivo* pharmacokinetics. The clearance of SB-2777011 (an AO substrate) was low to moderate in rat and dog, leading to moderate oral bioavailability [63]. However, in monkey, clearance approximated to hepatic blood flow and oral bioavailability was 2%. *In vitro* investigations

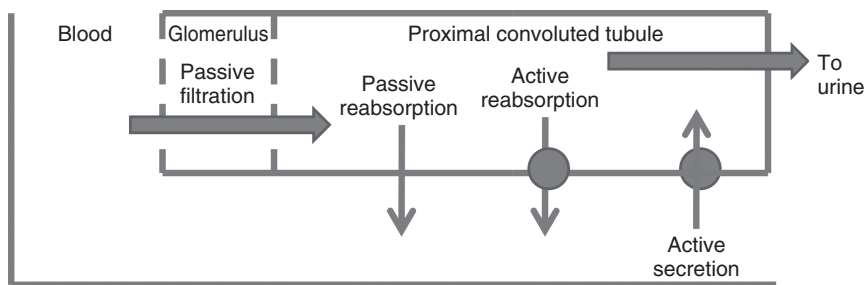


Figure 18.5 Schematic of renal excretion at the nephron.

suggested that human liver cytosol metabolized SB-277011 at an equivalent rate to that of monkey, suggesting the potential for low human oral bioavailability.

Overall, when AO is the principal contributor to the metabolism of a compound, care must be taken in the choice of species for human clearance prediction. The absence in dog and the low and polymorphic expression in rat suggest that these species are not suitable models for human AO clearance.

18.5.2.6 Renal Elimination. A second major human route of elimination is in the urine. Renal elimination of drugs has four distinct contributors (Fig. 18.5):

- Passive filtration of unbound drug at the glomerulus
- Transporter-mediated renal secretion into the urine from the blood
- Extent of passive renal reabsorption from the urine
- Transporter-mediated renal reabsorption from the urine

The amount of a drug excreted unchanged in the urine will depend on the balance of these four activities.

18.5.2.7 Passive Processes. Blood flows into the Bowman's capsule in the kidney where it is subject to filtration. Large components such as blood cells and plasma proteins do not pass through the filter and are retained in the blood. Small molecules (including drugs) that are not bound to plasma proteins are filtered into the proximal convoluted tubules.

For a compound that is not subject to active processes, the amount excreted in the urine will depend on how much of the drug returns back into the blood by passive diffusion across the epithelial cell of the proximal convoluted tubule. This, in turn, depends on the physicochemistry of the drug. Lipophilic drugs are likely to permeate across the membranes and be reabsorbed, leaving very little in the urine. Polar drugs have difficulty permeating membranes and consequently remain to be excreted in the urine. A good example of a passively renally cleared compound is a gabapentin, which shows essentially complete renal excretion at glomerular filtration rate (GFR; clearance 9.5 mL/min/kg) in rat [66] and human (clearance 1.8 mL/min/kg) [67].

Since the latter type of compound tend to be minimally plasma protein bound, their clearance tends to relate to the rate of blood flow to the glomerulus (i.e., GFR). Human GFR is ~ 1.5 mL/min/kg; consequently, the human clearance of gabapentin is

close to 1.5 mL/min/kg. In addition, because this is a passive process, it relates to GFR across the species. Since blood flows are allometrically related, the clearance of such molecules in preclinical species is often an accurate predictor of the human clearance.

A similar relationship holds for compounds that are predominantly passively renally cleared but show a significant degree of passive reabsorption. This is exemplified by fluconazole. Urinary excretion of unchanged fluconazole is the predominant route of elimination across all species. Fluconazole is also highly passively permeable and a constant 80% of the filtered material is reabsorbed from the proximal convoluted tubule. Thus, the renal clearance of fluconazole is ~20% of GFR across the species and scales extremely well allometrically from animals to human [68].

18.5.2.8 Active Processes. Some drugs are substrates for active transporters in the kidney. They can be extracted from the blood and effluxed across the proximal convoluted tubule epithelium by these drug transporters. Such compounds have renal clearances above that which is possible by GFR alone. A good example is lamivudine with 70% of the dose excreted unchanged in the urine in humans with a clearance of 20 mL/min/kg (i.e., greater than GFR [41]).

Conversely, some compounds are substrates for active reabsorption from inside the proximal convoluted tubule back into the blood. Such compounds show predominantly urinary excretion, but their clearance values tend to be significantly less than possible by GFR.

The kidney transporters are beginning to be elucidated and include organic anion transporters (OATs), organic cation transporters (OCTs), and organic anion transport proteins (OATPs) [7,8]. However, the preclinical transporters are not yet fully elucidated and (as with the CYPs) there are likely to be significant species differences in expression and activity. Therefore, allometric scaling of the clearance of such molecules may be problematic, suggesting that preclinical models may not be good predictors of human active renal excretion.

18.5.2.9 Biliary Elimination. Hepatobiliary elimination is a three-step process, most of which is driven by active transport. The first stage is uptake into the hepatocyte usually by drug transport proteins (such as OATPs) at the sinusoidal membrane of the hepatocyte. The compound is transferred across the hepatocyte, avoiding metabolism, to the biliary canalicular membrane. Active efflux into the bile is completed by biliary canalicular transporters, such as MRP2 and BCRP.

There are a number of species differences in the biliary elimination process. First, as with all active transport processes, there are species differences in the drug transporters involved, both in terms of expression and structure activity relationships. For example, it is thought that MRP2 expression at the rat canalicular membrane is significantly greater than that at the human canalicular membrane, whereas dog and monkey expression is similar to human [69]. In addition, the physiology of the rat biliary system is significantly different from human (and other species). Rat bile flow is some 16-fold higher than human [70]. In addition, rats do not have a gallbladder and bile continually flows into the gut lumen. In humans and higher preclinical species, the bile is stored in the gallbladder and is released into the gut lumen on feeding.

For these reasons, the rat tends not to be a good model of human biliary elimination. Some compounds can show high hepatic extraction in rat due to biliary elimination, which does not always translate to similar hepatic extraction in humans. A good

example of this is susalimod [71], where allometric scaling between preclinical species predicted relatively well, but there was a significant overprediction of human clearance (predicted 1.8 mL/min/kg vs actual 0.07 mL/min/kg). Indeed, allometric scaling of clearance by biliary elimination is fraught with difficulty and various scaling factors have been suggested [70].

The other major difficulty with the study of biliary elimination in humans is access to the bile. Sampling of bile in humans requires surgical cannulation of the gallbladder and so examples of true biliary elimination are limited. In addition, compounds with biliary elimination in rat tend to show significant hepatic extraction in this species. Simple allometric scaling of these types of compounds is likely to predict extensive clearance in humans, and so they may be selected out of the compound optimization process. Thus, these aspects may well explain the relatively few examples of biliary eliminated molecules in the top 200 marketed drugs list [58]. As transporter science evolves and understanding of biliary elimination is improved, this situation may change.

18.5.2.10 *In Vivo Models of Human Clearance.* A major driver for completion of *in vivo* pharmacokinetic studies in preclinical species is the prediction of human clearance. The most appropriate experiment is the intravenous administration of the compound followed by extensive blood or plasma sampling. The dose used is important as clearance can be saturable, so the dose needs to be in a similar range to that expected in humans. The plasma or blood clearance can be related to plasma or blood concentrations as shown previously. Comparison of blood clearance with hepatic blood flow will give an indication of the hepatic extraction of the compound (especially if the drug is solely cleared by the liver). The blood clearance (or more appropriately the unbound clearance in blood) can be allometrically scaled (as described previously) to gain a prediction of the human clearance of that molecule.

A further advantage of the preclinical *in vivo* pharmacokinetic study is the ability to collect other matrices and examine the fate of circulating metabolites of the compounds. The measurement of unchanged compound in urine will give an indication of the renal clearance. When compared with the GFR, such experiments yield the potential for a compound to be passively filtered or actively transported to or from the urine.

The determination of biliary elimination of unchanged drug cannot be determined from a simple *in vivo* pharmacokinetic study. The collection and measurement of bile requires surgical cannulation of the bile duct. For this reason, the rat is most often the species of choice. Pharmacokinetic studies can be conducted in bile duct cannulated rats, which has the advantage of relating plasma clearance to the biliary excretion rate. An alternative experiment that can be used to examine biliary elimination is the isolated perfused rat liver (IPRL). This has the advantage of enabling rat hepatic extraction as well as the biliary excretion of unchanged drug and metabolites to be determined. A good example of the use of the IPRL is shown in the examination of the disposition of 5,6-dimethylxanthenone-4-acetic acid, where unchanged drug accounts for 28% of the administered dose with the acyl glucuronide accounting for >50% of the dose [72].

Given the species differences in clearance and the variety of potential mechanisms by which a compound can be removed from the body, the use of preclinical species to model DDIs is fraught with difficulty. The choice of DDI perpetrator for any compound would be difficult, as would be the dose at which it should be administered. These facets are well established for humans and can be modeled using *in silico* techniques. Therefore, the study of DDIs in preclinical models is not recommended.

18.5.3 Metabolism and the MIST Guidance

One of the most important aspects of the use of *in vivo* models of drug metabolism comes in the drug development phase. It is essential that drugs are safe for administration to humans. This is achieved through rigorous toxicology testing in preclinical species. In these studies, it is extremely important that not only the unchanged drug is qualified in humans but also the metabolites of that drug. Thus, it is essential to show that any compound-related material that is exposed to humans has also been exposed to at least one toxicology species with no toxicological consequences. In general, this is achieved by administration of radiolabeled drug to the preclinical species used in toxicity testing. Assuming that the site of radiolabeling has been chosen appropriately, any circulating or excreted drug-related material will also be radiolabeled, and these components need to be identified and quantitated. Indeed, Metabolites in Safety Testing (MIST) guidance from the FDA and subsequent ICH M3 guidelines require any metabolites constituting more than 10% of drug-related material circulating in human plasma is qualified in each toxicology species. Thus, these types of *in vivo* studies represent a major component of the ADME package for a drug submission.

18.5.4 Summary of Preclinical Models of Clearance/Elimination

The clearance of a drug is an important parameter in humans as it relates dose to exposure. There are many ways by which a drug can be removed from the human body. Many drugs are cleared by metabolism with a significant proportion removed by active or passive renal excretion. In addition, biliary elimination of unchanged drug is another potential route. The active processes require intervention from proteins (enzymes and transporters) within the body. There are a number of species differences in terms of expression and structure activity relationships. This makes extrapolation of human clearance from preclinical animal models difficult.

Allometric scaling has been moderately successfully used to predict human clearance. However, it is likely that allometric scaling will work very well for passively cleared compounds and those that are cleared at close to hepatic blood flow (as these are dependent on blood flow and scale well with body weight). The issue comes in the extrapolation of clearance by active processes, where species differences will confound scaling on the basis of body weight and potentially lead to large errors in extrapolation.

At present, extrapolation of human clearance from preclinical *in vivo* models remains an appropriate method for most clearance pathways. The exception is CYP clearance where scaling of human liver microsomal experiments should be more appropriate. As drug metabolism science moves on, further scalable human *in vitro* models of clearance should be advanced that will reduce the need for *in vivo* animal models in this area.

18.6 PERSPECTIVE ON THE USE OF *IN VIVO* MODELS OF DRUG METABOLISM

The use of *in vivo* models has been integral to the progression of drug metabolism science. These models are used extensively to predict the human disposition of potential drug molecules and to support the progression of compounds through drug development, especially in support of toxicology studies. These *in vivo* models are likely to

TABLE 18.2 Summary of the Utility of Animal Models of Drug Metabolism for Predicting Human Drug Disposition

ADME Property	Model Species	Comments
Oral absorption	Rat correlates with human	Rat is an appropriate model for transcellular and paracellular absorption
	Monkey correlates with human	Paracellular pathway in dogs is larger
	Dog has a poor correlation with human	
Effect of food on oral absorption	No appropriate model for human	Rat physiology is different (bile flow). Difficult to fast rats. Dog precluded by physiology differences and paracellular pathway. Use <i>in silico</i> models
Volume of distribution	Scales well from all species, especially if fraction unbound considered	Passive processes scale well from preclinical species
CNS penetration	Rodent models appear best for human prediction. Must use unbound fraction in brain or CSF. Emphasis on which matrix to measure in rodent and human	<i>In vitro</i> systems should be used to triage compounds before <i>in vivo</i> evaluation
Metabolic clearance	Significant species differences. Will scale well for clearances approaching hepatic blood flow. For low clearance agents may be prone to errors due to it being governed by intrinsic clearance	For CYP substrates, human liver microsomal scaling may be a more appropriate predictor of human clearance. As scalable human <i>in vitro</i> systems become available, the use of animal models for human metabolic clearance prediction should reduce
Renal clearance	Passive renal clearance scales well from any preclinical species. Active secretion or reabsorption likely to be prone to species differences	For passive renal clearance, human clearance of unbound drug should approximate to human GFR. Prediction of active process should benefit from identification and expression of the transporters involved
Biliary clearance	Active process. Likely to be species differences. Physiology differences	Prediction of biliary clearance will continue to be problematic until human scalable <i>in vitro</i> assays become available
Drug–drug interactions	Likely to be poor predictivity due to species differences in clearance	DDI studies not recommended in animals. CYP DDIs are well predicted using <i>in silico</i> models

continue to be used into the future, but as drug metabolism science evolves, there needs to be continual scrutiny on their effective use. Every scientist who uses animals in his/her research has an ethical responsibility to minimize that use and replace with suitable *in vitro* alternatives. Drug metabolism has many future opportunities to do this.

A perspective of the current use of *in vivo* models of drug metabolism to predict human disposition is reviewed in Table 18.2.

It is clear that there are many issues with prediction to human and a number of approaches (*in silico*, *in vitro*, and *in vivo*) can be taken. For example, on the whole, for prediction of human oral absorption, it appears that the rat is a good model and indeed the rat can be used successfully for the prediction of volume of distribution and several clearance processes, especially passive processes. When active processes contribute to clearance, species differences complicate extrapolation to human and predictivity is not at the level required. Finally, drug–food and drug–drug interactions are complicated by physiology and species differences and preclinical *in vivo* experiments are not recommended.

As drug metabolism science evolves and the human proteins modulating drug disposition are discovered and evaluated, there will be the opportunity to replace animal use with scalable *in vitro* assays. As this continues, drug metabolism scientists need to continually reassess the appropriate use of *in vivo* models of drug metabolism.

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19 Utility of Genetically Modified Animal Models for Drug Metabolism and Drug Transporters

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19.1	Summary	1
19.2	Introduction	3
19.3	Nomenclature of different animal models	3
19.4	Cytochrome P450	14
19.5	Epoxide hydrolases	24
19.6	Oxidases	28
19.7	Esterases	34
19.8	UDP-glucuronyl transferase	36
19.9	Sulfotransferase	40
19.10	Nuclear hormone receptor (PXR, CAR, FXR, and PPAR)	41
19.11	Drug transporters in disposition	50
19.12	Conclusion	61
	References	62

19.1 SUMMARY

There are a variety of genetically modified animal (GEMA) models available to gain insight into the role that drug-metabolizing enzymes (DMEs), nuclear transcriptional factors, and transporters play in the disposition of drugs and in the regulation of endogenous substrates. These systems often work in concert to eliminate compounds from the body and are sometimes associated with drug-induced toxicities.

A GEMA for drug metabolism research is typically a mouse, whose genome has been changed by genetic engineering modifications. There are several approaches commonly used to generate genetically modified mice. The first method involves pronuclear injection of a human gene into a single mouse embryo cell, where the introduced gene will randomly integrate into the mouse genome, creating a transgenic animal. This method creates “knock-in” animals. A second method involves modifying the mouse embryonic stem (ES) cells with a DNA fragment containing an alteration in the mouse target gene, such as the deletion of all or part of the gene, or insertion of foreign genes, such as an antibiotic-resistance gene. The modified stem cells that contain the altered genomic DNA are selected by DNA sequencing and are then injected into mouse blastocysts. This method creates the “knock-out” (KO) animal models. Alternatively, partial replacement of mouse cells with human cells (i.e., hepatocytes) has also resulted in chimeric animals that exhibit human drug disposition characteristics.

Therefore, animal models described herein fall into several types: (i) KO/null, (ii) humanized knock-in of gene of interest and KO of mouse gene, and (iii) chimeric. Each of these models is described in greater detail in the chapter within the context of drug metabolism, nuclear transcriptional factors, and drug transporters.

For DMEs, GEMA models for phase I and II enzyme families have been reported, and salient features of the models used to elucidate roles, mechanisms, and toxicities are reviewed. In a number of studies using GEMA models, implications for new insights into disease states or drug development are also discussed. Models for phase I enzymes pertaining to cytochrome P450 families (CYP1A, CYP2D6, CYP2E1, and CYP3A), epoxide hydrolases (EHs) (soluble and microsomal), aldehyde oxidase, monoamine oxidase (A and B), alcohol and aldehyde dehydrogenases (ALDHs), flavin-containing monooxygenases, and cholinesterases [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] are reviewed in this chapter, as well as the phase II enzymes uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT) and sulfotransferases.

A variety of GEMAs have been generated to evaluate the role of ligand-activated transcriptional factors in gene regulation for pregnane-X-receptor (PXR), constitutive androstane receptor (CAR), peroxisome-proliferator-activated receptor, farnesoid X-activated receptor (FXR), and aryl hydrocarbon receptor (AHR). The generation of GEMA models with either the mouse gene KO or human gene knock-in and multiple gene KO models provide a wide range of *in vivo* tools to evaluate how these transcriptional factors work individually or in concert to regulate the expression of specific genes that control the differentiation, development, homeostasis, and metabolism of organisms.

Drug transporters are an evolving field with many proteins involved in the distribution of drugs and endogenous substrates. Many of the GEMA models are KO animals that have facilitated the association of transporters to drug distribution across tissues and tissue barriers of the gastrointestinal tract, placenta, testes, and brain. For transporters that lack human orthologs such as the organic anion transporting polypeptide (OATP), humanized animal models are ideal for exploring the contribution of human transporters toward the pharmacokinetics and disposition of new drug entities before dosing in humans.

Overall, the intent of this chapter is to provide an overview of the variety of GEMA models available to investigate drug metabolism, pharmacokinetics, and the role of transcriptional factors involved in the regulation of genes that govern the expression of DMEs and transporters.

19.2 INTRODUCTION

The body requires a state of homeostasis such that everything coming in must go out. Perturbations in this balance results in the organism accumulating compound, in concentrations that may reach toxic levels. Absorption, distribution, metabolism, and excretion (ADME) describes the way a compound passes through the body. Most compounds are not sufficiently polar (water-soluble) to be excreted without being metabolized. For these, the body may increase water solubility through the processes of phase I or phase II metabolism. Phase I metabolism is often mediated by oxidative processes involving cytochrome P450 and flavin monooxygenase (FMO). Parent compound and metabolites can either be eliminated directly from the body through the action of transporters or undergo further metabolism by phase II conjugative enzymes such as uridine 5'-diphosphoglucuronosyltransferase (UGT), sulfotransferase (ST), or glutathione-S-transferase (GST).

There is a tremendous degree of complexity and variation between DMEs and transporters in mammalian species. As such a chemical can be metabolized to a greater extent in preclinical species such as rats and mice as compared to humans. This is true for transporters in which species differences are commonly observed in the transport of compounds. This leads to difficulties when using typical rodent models to study the metabolism, transporter and biological activities of drugs, and then extrapolating this data to humans. Considerable progress has been made in the cloning and characterization of recombinant drug-metabolizing and transport proteins to understand substrate and inhibitor specificities. Only recently have genetic studies been employed using mammalian systems to assess DME and transporter function in animals, involving either targeted disruption of specific genes, or transgenic or humanized rodent (mouse or rat) models.

There are numerous GEMA models generated for drug metabolism and transporter proteins that have provided greater characterization and mechanistic understanding of proteins that contribute to the disposition of drugs. The generation of new models is constantly evolving and expanding. The authors have focused on GEMAs that have been widely studied and acknowledge that this chapter is not inclusive of all models. Several tables have been generated, which capture the GEMA models available to study DMEs and transcriptional factors involved in the upregulation of genes and drug transporters (Tables 19.1–19.3).

19.3 NOMENCLATURE OF DIFFERENT ANIMAL MODELS

GEMA models are a useful *in vivo* tool for evaluating the functions of genes. A transgenic animal is defined as an animal in which specific sequences of DNA from another species have been incorporated into the host genome, causing a disruption which effectively eradicates the activity of the targeted gene. The modified gene is known as the *transgene*. The mouse is the typical species in which to generate transgenic animal models. Thus, a transgenic animal can be defined as one having any specific, targeted genetic modification [1]. There are several transgenic animal models available to evaluate DMEs and transporters, comprising KO/null, chimera, and humanized/knock-in models.

TABLE 19.1 Genetically Modified Animal Model and Application: Drug-Metabolizing Enzymes

Gene Target	Model	Application	Section
Cytochrome P450 1A2	CYP1A knock-out mouse model Cyp1a2 ^{-/-}	Pharmacokinetics and pharmacodynamics of 1A2 substrates degradation of bilirubin	19.4.2.1
	CYP1A humanized mouse model hCYP1A1_1A2 Cyp1a2 ^{-/-}	Pharmacokinetic and human risk assessment studies, involving any drug or environmental toxicants that are 1A2 substrates	19.4.2.2
Cytochrome P450 2D6	CYP2D6 humanized mouse model hCYP2D6Cyp2d6 ^{-/-}	Pharmacokinetics of 2D6 substrates impact of compounds on 2D6 expression hepatic and no hepatic metabolism of endogenous/exogenous 2D6 substrates	19.4.3.1
Cytochrome P450 2E1	CYP2E1 knock-out/knock-in Cyp2e1 ^{-/-} hCYP2E1	CYP2E1-induced hepatotoxicity toxicity of acetaminophen (APAP) <i>in</i> <i>vivo</i> metabolism of benzene and benzene-induced toxicity	19.4.4.1
	CYP2E1 humanized mouse hCYP2E1 Cyp2e1 ^{-/-}	Toxicity of acetaminophen (APAP) and ethanol	19.4.4.1
Cytochrome P450 3A4	CYP3A humanized mouse hCYP3A4Cyp3a ^{-/-}	Pharmacokinetics of 3A4 substrates hepatic and intestinal metabolism of 3A4 substrates	19.4.5.1
	CYP3A chimeric-HL mouse uPA ^{+/+} /SCID	Pharmacokinetics of 3A4 substrates and induction of CYP3A4	19.4.5.3
Microsomal epoxide hydrolase	Microsomal epoxide hydrolase knock-out mEH ^{-/-}	Evaluate polycyclic hydrocarbon carcinogens and detoxification pathways	19.5.1.1
mEH and sEH	Chimeric-HL mice	Chimeric-HL model described for mEH and sEH	19.5.1.2
Epoxide hydrolase soluble	Soluble epoxide hydrolase knock-out sEH ^{-/-}	Evaluate role of sEH on blood pressure and renal arachidonic acid metabolism	19.5.2.1
	Soluble epoxide hydrolase humanized mouse	Model utilized to evaluate the role of sEH in the brain in relation to cerebral blood flow and protection from ischemic injury	19.5.2.2

Aldehyde oxidase	AOX chimeric-HL mouse UPa+/+/SCID	Investigate species-dependent metabolism of <i>N</i> ¹ -methyl nicotinamide by AOX	19.6.1.1
Monoamine oxidase	MAO-A knock-out mouse Mao-A—/—	Evaluate the role of MAO-A in the detoxification and regulation of amines for normal biological function	19.6.2.1
	MAO-B knock-out mouse Mao-B—/—	Evaluate the role of MAO-B in the detoxification of neurotoxin <i>N</i> -methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	19.6.2.2
	MAO-double knock-out mouse Mao-A/B—/—	Examine the developmental phenotypic changes when both monoamine oxidases are absent	19.6.2.3
Alcohol dehydrogenase	ADH knock-out mouse model Adh1—/—, Adh3—/—, and Adh4—/—	Investigated role of murine Adh enzymes in ethanol and retinol metabolism	19.6.3.1
Aldehyde dehydrogenase	ALDH knock-out mouse model Aldh2—/—	Investigate <i>in vitro</i> metabolism of methoxyacetaldehyde in mitochondrial fractions from Aldh2—/— mice. Evaluate inhalation toxicity of acetaldehyde. Investigate effects of subacute ethanol toxicity. Investigate effects of ethanol exposure on DNA and Cyp2E1 expression	19.6.3.2
Flavin monooxygenase	FMO knock-out mouse model Fmo1—/—	Evaluate contribution of Fmo1 to pharmacokinetics of imipramine	19.6.4.1
Acetylcholine esterase	AChE knock-out mouse model AChE—/—	Investigate role of AChE in VX toxicity. Evaluate effects of AChE-inhibiting drugs rivastigmine and donepezil on AChE levels for Alzheimer's disease therapy	19.7.1.1
Butyrylcholinesterase	BChE knock-out mouse model BCHE—/—	Investigate effects of AChE inhibitors donepezil and (–)-huperzine A in AChE and BChE—/— mice for Alzheimer's disease therapy. Evaluate toxicity of cocaine in BCHE—/— mice, wild type and heterozygotes mice	19.7.1.2

(continued overleaf)

TABLE 19.1 *(continued)*

Gene Target	Model	Application	Section
UDP-glucuronyl transferase	UGT1A knock-out mouse Ugt1 ^{−/−}	Evaluate the physiological significance of Ugt in development. Evaluate the role in bilirubin metabolism and metabolism of drug	19.8.1
	UGT1A knock-in mouse Tg-UGT1	Examine the functional and regulatory properties of human UGT1A1	19.8.2
	UGT1A knock-out/knock-in mouse Tg-UGT1	Examine human-specific drug conjugation. Improve human UGT1A-dependent drug clearance	19.8.3
	UGT chimeric-HL mouse UGT1A1, UGT1A9, UGT2B7	Study human glucuronidation	19.8.4
Sulfotransferase	SULT1E1 knock-out mouse model Sult1E1 ^{−/−}	Investigate reduced fertility in Sult1E1 ^{−/−} mice due to high estrogen levels and reduced COX-2 expression	19.9.1
	Natural SULT knock-out mouse brachymorphic mouse	Investigate the bioactivation of safrole and potential carcinogens in mouse liver	19.9.2

TABLE 19.2 Genetically Modified Animal Model and Application: Induction

Gene Target	Model	Application	Section
Pregnane X receptor	PXR knock-out mouse model Pxr ^{-/-}	Evaluate the role that Pxr plays in regulation of genes (<i>CYPs</i> , <i>UGTs</i> , <i>ST</i> , <i>P-gp</i> , <i>BCRP</i> , and <i>OATPs</i>). Pharmacokinetics of drug-metabolizing and transporter proteins. Bile salt regulation	19.10.1
	PXR humanized mouse model hPXR ^{Pxr} ^{-/-}	Evaluate the role of human PXR in regulation of drug-metabolizing and transporter genes and these genes in disease states. Pharmacokinetics of drug-metabolizing and transporter proteins	19.10.1.1
Constitutive androstane receptor	CAR knock-out mouse model Car ^{-/-}	Evaluate the role that Car plays in regulation of genes (<i>CYPs</i> , <i>UGTs</i> , <i>GST</i> , <i>MRPs</i> , and <i>OATPs</i>). Pharmacokinetics of drug-metabolizing and transporter proteins. Regulation of bilirubin homeostasis. Role in metabolic disorders	19.10.2.1
	CAR humanized mouse model hCAR ^{Car} ^{-/-}	Evaluate the role that human CAR plays in gene regulation (<i>CYPs</i> , <i>UGTs</i> , <i>GST</i> , <i>MRPs</i> , and <i>OATPs</i>)	19.10.2.2
Peroxisome-proliferator-activated receptor	PPAR knock-out mouse Ppar ^α ^{-/-} , Ppar ^{β/δ} ^{-/-} , Ppar ^γ ^{-/-}	Investigate role in control of CYP2B family in bioactivation of carcinogens. Evaluate PPARs' role in lipid metabolism, glucose homeostasis, and adipocyte differentiation and inflammation	19.10.3.1
	PPAR humanized mouse hPPAR ^α ^{Pparα} ^{-/-}	Evaluate species differences and role of human PPAR ^α in regulating CYP4A in starvation, diabetes, and fatty acid metabolism	19.10.3.2

(continued overleaf)

TABLE 19.2 *(continued)*

Gene Target	Model	Application	Section
Farnesoid-X-activated receptor	FXR knock-out mouse Fxr ^{-/-}	Evaluate role in regulation of bile acids, lipid, and glucose homeostasis	19.10.4.1
Pregnane X receptor and constitutive androstane receptor	Combination models hPXRCar ^{-/-} , hCARPxr ^{-/-} , Pxr ^{-/-} Car ^{-/-} , hPXRhCAR	Evaluate cross talk between PXR and CAR in gene regulation, determine which genes require both or only one transcriptional factor	19.10.5
Aryl hydrocarbon receptor	Ahr knock-out model Ahr ^{-/-}	Investigate regulation of P450 enzymes involved in bioactivation of aryl hydrocarbon aromatic carcinogens. Role of enzymes involved in reactive metabolite formation	19.10.6.1
	AHR humanized mouse hARHArh ^{-/-}	Investigate human AHR-induced gene response in the presence of toxins	19.10.6.2
	AHR chimeric mouse	Rat <i>Ahr</i> gene contains mutation rendering it TCDD resistant. Enables mechanistic evaluation of Ahr-mediated toxicities	19.10.6.3

TABLE 19.3 Genetically Modified Animal Model and Application: Transporter

Gene Target	Model	Application	Section
P-glycoprotein	MDR1 knock-out mouse Mdra/b—/—	Evaluate the role of P-gp in the GI for absorption, liver and kidney for biliary and renal clearance and brain penetration	19.11.1.1
	Dog MDR1-deficient model	Investigation of the role P-gp in a nonrodent model	19.11.1.2
Breast cancer resistant protein	BCRP knock-out mouse Bcrp—/—	Evaluate the role of P-gp in the GI for absorption, liver and kidney for biliary and renal clearance and brain penetration	19.11.2.1
Multidrug resistance protein	MRP1 knock-out mouse Mrp1—/—	Explore the role of Mrp in anticancer-mediated cytotoxicity and drug resistance	19.11.3.1
	MRP2 knock-out mouse Mrp2—/—	Evaluate the role of Mrp2 in the GI, liver, and kidney in the efflux of drug conjugates	19.11.3.1
	Rat Mrp2-deficient TR and Eisai hyperbilirubinemic	Examine the role of Mrp2 in the efflux of conjugated drugs and bilirubin	19.11.3.2
	MRP3 knock-out mouse Mrp3—/—	Evaluate role of Mrp3 in the efflux of glucuronide-conjugated drugs	19.11.3.3
	MRP4 knock-out mouse Mrp4—/—	Evaluate role in the renal clearance of anticancer and antiviral drugs. <i>in vivo</i> model to examine Mrp4 in protecting brain from cytotoxins	19.11.3.4

(continued overleaf)

TABLE 19.3 (continued)

Gene Target	Model	Application	Section
Organic anion transporting polypeptides	OATP knock-out mouse Oatp1b2 ^{-/-}	Examine the <i>in vivo</i> role of Oatp in the hepatic uptake of compounds	19.11.4.1
	Multiple OATP knock-out mouse Oatp1a/1b ^{-/-}	Understand role of two Oatps in the <i>in vivo</i> disposition of drugs	19.11.4.2
	Humanized OATP mouse hOATP1B1	Understand role of human OATP in the hepatic uptake of compounds as species differences among OATPs exists	19.11.4.3
Organic cation transporter	OCT1 knock-out mouse Oct1 ^{-/-}	Investigate the pharmacological and physiologic role of Oct1 in GI, liver, and kidney	19.11.5.1
	OCT double knock-out model Oct1/2 ^{-/-}	Examine if both Oct1 and Oct2 contribute to disposition and excretion	19.11.5.2
Organic anion transporter	OAT knock-out model Oat1 ^{-/-} , Oat3 ^{-/-}	Elucidate role Oat1 and Oat3 in the disposition of compounds	19.11.6.1
Bile salt export pump	BSEP knock-out model Bsep ^{-/-}	Role of Bsep in the biliary excretion of bile salts	19.11.7.1

19.3.1 Knock-Out (Null) Models

The original pioneering work in developing techniques for generating transgenic KO animals was done by Mario Capecchi, Martin Evans, and Oliver Smithies, who were recognized with the Nobel prize in “Medicine or Physiology” in 2007. The production of a null/KO animal model (Fig. 19.1) involves isolating ES cells from a male brown mouse with a normal gene [2–4]. A copy of the mutated gene containing

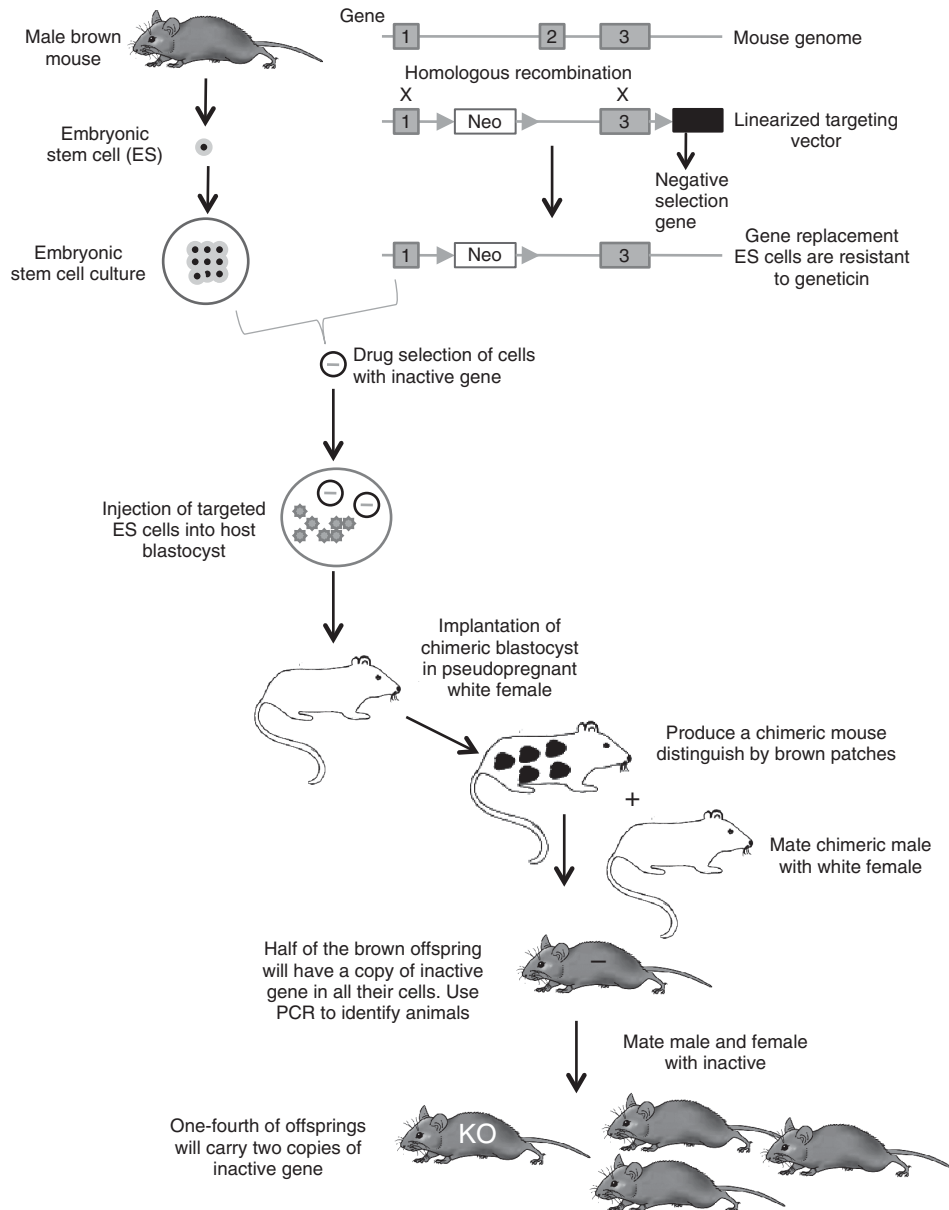


Figure 19.1 Diagram to construct knock-out animal model. (See color insert.)

a drug resistance marker gene such as phosphoglycerine kinase-neomycin (PGK-neo) is inserted into a selected region of the cDNA, generally downstream from the mouse promoter enhancer sequence of the target gene. The transgene is then isolated from the plasmid of the vector and microinjected into the pronuclei of white fertilized mouse eggs. The addition of *PGK-neo* disrupts normal gene expression. The altered DNA sequence will undergo homologous recombination to incorporate the altered target gene with the PGK-neo cassette into the mouse genome. By transplanting stem cells containing the inactive target gene into a white mouse embryo, a chimeric animal is formed that has regions of cells that grew from white mouse cells and regions that grew from brown stem cells. Some of the cells that have the inactive target gene may develop into reproductive cells. The germline chimeras are easy to identify because they have both brown and white patches on their fur. Among the offspring, half will be brown containing a copy of the inactive gene in all their cells. These mice have one normal and one inactive copy of the target gene. The heterozygote mice [identified by polymerase chain reaction (PCR)] are then bred. A quarter of the offspring will have two copies of the “knocked out” or inactive target genes.

19.3.2 Humanized Mouse (Knock-In and Knock-In/Knock-Out Mouse)

The formation of a humanized mouse follows similar cloning and isolation procedures as that used to make the KO mouse (Fig. 19.2). The human cDNA of the target gene is isolated and cloned downstream of the liver-specific mouse promoter/enhancer sequence in a plasmid vector. The transgene is then isolated and microinjected into the pronuclei of fertilized mouse eggs. The injected eggs are transplanted into the oviduct of a female KO target gene mouse. The KO mouse will lack the species target gene, enabling only the ortholog human gene to be expressed, this mouse is called *knock-in/knock-out*. However, if the human transgene is injected into the oviduct of a female normal mouse, the offsprings will contain both endogenous and human transgene, it is simply called a *knock-in*. The mice are screened by PCR to identify individuals carrying the human gene.

19.3.3 Chimeric-HL

19.3.3.1 (*uPA*+/*+/SCID*) Mouse. A single animal organism with genetically distinct cells from two different zygotes is commonly referred to as a *chimera*. Chimeric mice with partially humanized liver were first described in 2001 and for purposes of this chapter will be referred to as *chimeric-HL*. The animals generated were immunodeficient transgenics that express urokinase-type plasminogen activator (uPA) under the transcriptional control of an albumin promoter, rendering it hepatotoxic. Dandri *et al.* [5] reported that the liver (uPA)/recombinant activation gene-2 mice could be repopulated by ~15%, with human hepatocytes. Similarly, Mercer *et al.* [6] then showed a liver portion in *uPA*+/*+/SCID* mice could be repopulated with human hepatocytes. Given that these studies were used to investigate the hepatitis virus, a low replacement value was likely to be sufficient to achieve their purposes. In contrast, studies for human ADME would necessitate a higher degree of repopulation.

In this regard, Tateno *et al.* [7] generated chimeric-HL *uPA*+/*+/SCID* mice, in which the mouse livers could be replaced to more than 90% with human hepatocytes. The *uPA*+/*+/SCID* mice at 20–30 days after birth were injected with human

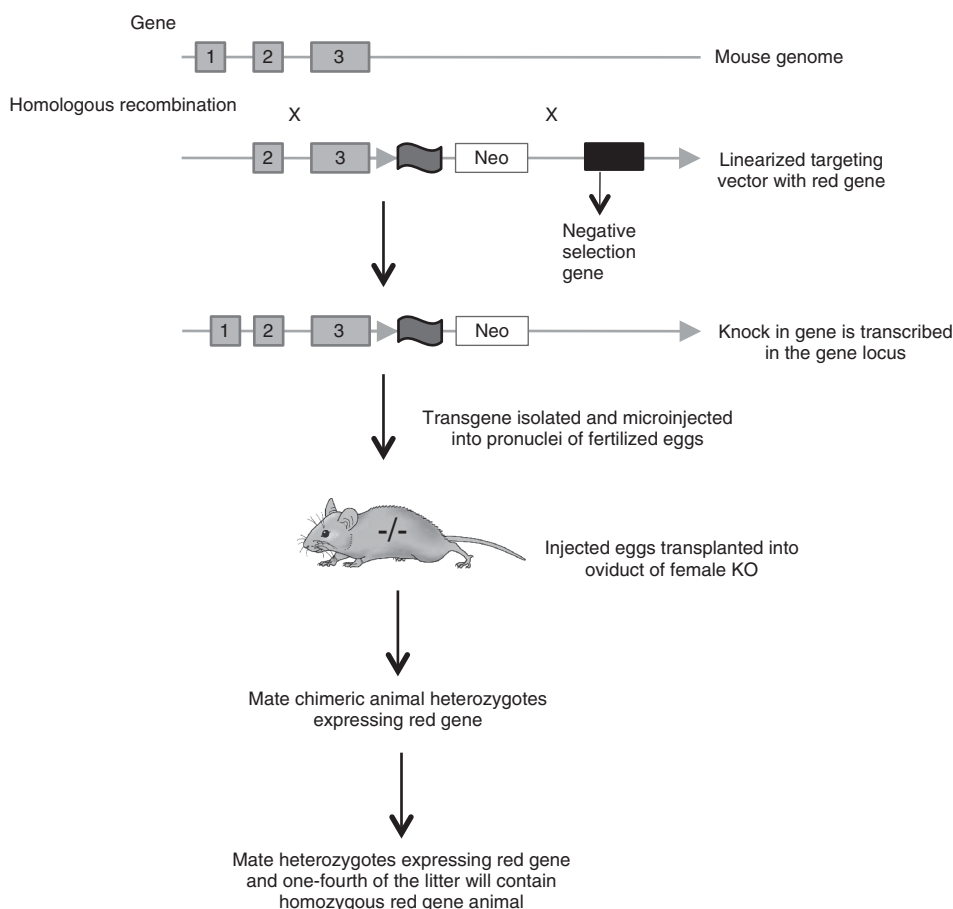


Figure 19.2 Diagram to construct knock-in/knock-out animal model. (See color insert.)

hepatocytes. Repopulation levels were estimated by determining the concentration of human serum albumin (hAlb). Levels were found to reach around 5 mg/mL after 60 days posttransplantation. The presence of human hepatocytes in the chimeric livers was also confirmed by taking liver sections and subjecting them to human-specific cytokeratin 8/18 (hCK8/18), an antibody that reacts specifically with human hepatocytes and bile duct cells, but not with those of the host mice. Given that hepatocytes from various donors and cryopreserved hepatocytes could be used to generate such chimeric-HL mice, interindividual variability in the pharmacokinetics and toxicity of drugs can potentially be evaluated.

19.3.3.2 Chimeric-HL (*Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}) Mouse. Despite engraftment levels of up to 70% having been reported in uPA-expressing animal models, the system has several major disadvantages that have prevented its widespread use. These include poor breeding efficiency, a narrow time window for transplantation, and renal disease in repopulated mice [7]. In order to provide a broadly useful hepatic xenorepopulation system, Azuma *et al.* [8] have recently generated an immunodeficient strain of mice

deficient in the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (*Fah*). *Fah* mutant mice, which develop liver disease only when the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) is withdrawn, have proven to be a reliable model of liver repopulation with transplanted cells [9,10]. The authors showed that their severely immunodeficient mice triply mutant for *Fah* (this gene encodes the last enzyme in the tyrosine catabolism pathway), *Rag2* (the recombination activating gene encodes enzymes that play an important role in the rearrangement and recombination of the genes of immunoglobulin and T-cell receptor molecules), and the common γ -chain of the interleukin receptor (FRG) could, after pretreatment with a urokinase-expressing adenovirus, be highly engrafted (up to 90%) with human hepatocytes from multiple sources, including liver biopsies. Furthermore, human cells could be serially transplanted from primary donors and repopulate the liver for at least four sequential rounds. Using standard drug metabolism assays, the authors reported that the hepatocytes from repopulated FRG livers were indistinguishable from normal human adult hepatocytes.

19.4 CYTOCHROME P450

19.4.1 Cytochrome P450

It has been well documented that approximately two-thirds of the marketed drugs are metabolized by members of the CYP superfamily, in particular the subfamilies: CYP1, CYP2, and CYP3 [11]. Model systems in which genes from cytochrome P450s have been deleted or inactivated have great potential to further our understanding of the function of these enzymes.

Given the complexity of genes that encode DMEs and their overlapping substrate specificities, there is a significant challenge when it comes to analyzing KO experiments, since loss of a single enzyme may be partially or completely compensated by one or more remaining enzymes. Furthermore, the widespread distribution of genes such as those for cytochrome P450s throughout the genome makes their collective obliteration technically unfeasible, at least with current technology. This creates potential problems in the interpretation of phenotypes and in delineation of the function of individual enzymes.

With respect to cytochrome P450 genes, a number of them have been deleted over the past decade or so. These can be divided into two types: (i) those with no overt phenotype, which tend to result from the deletion of P450s involved in xenobiotic metabolism and (ii) those where inactivation of P450s that undertake essential “housekeeping” functions, that is, CYP19 (aromatase) and CYP7A (cholesterol 7 α -hydroxylase) [12,13], results in a phenotypic change, normally embryonic lethality or perinatal morbidity. Henderson *et al.* [14] have recently argued that a more informative approach might be to delete several or all P450s simultaneously, which could be achieved by deletion of cytochrome P450 reductase (CPR), the sole electron donor to microsomal P450 enzymes. However, as indicated above, given the reported lethality following deletion of individual P450s involved in cholesterol or steroid hormone biosynthesis, complete deletion of all P450 function would not be the most pragmatic approach [15]. This embryonic lethality was circumvented via Cre/loxP technology, which selectively deletes liver CPR [14]. Although lacking hepatic CPR and hence completely devoid of liver P450 function, these mice (Hepatic Reductase Null, HRNTM

mice) were both viable and fertile [16]. As such it was argued that this model has the potential to be a valuable tool in drug development, in assessing the role of hepatic CYPs in drug bioavailability and efficacy [17,18]. In contrast, if a CYP responsible for xenobiotic metabolism is deleted, it is not a concern, at least in the absence of an appropriate chemical challenge. The following sections summarize information from the literature on the application of GEMA models in the assessment of the P450 enzymes: CYP1A, CYP2D, CYP3A, and CYP2E.

19.4.2 CYP1A

CYP1A family consists of CYP1A1, CYP1B1, and CYP1A2. CYP1A2 is the major P450, accounting for 10% of hepatic P450s. In contrast, CYP1A1, also known as *aryl hydrocarbon hydroxylase* (ARH), is mainly expressed in lung, placenta, and lymphocytes, albeit in a much lesser abundance. Both CYP1A1 and CYP1A2 are inducible on exposure to xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene (3-MC), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls [19]. Both P450s are primarily involved in the metabolic activation of procarcinogens, such as arylamines, PAHs, and heterocyclic amines [19], the latter being produced during the cooking of meats and fish, with the most abundant being 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Metabolic activation of PhIP is principally mediated by CYP1A2. Increased activity of CYP1A2 in combination with higher *N*-acetyltransferase activity has been associated with an elevated risk for colon cancer in individuals eating well-cooked meats [20]. Species differences in the oxidative metabolism of PhIP between humans and rodents [21] make extrapolation of potential cancer risk from experimental animals to humans difficult. However, by generating humanized mouse lines, suitable models can be provided for human health risk assessment studies of PhIP, as well as other dietary or environmental toxicants. These models have been also been applied by researchers investigating the potential disposition of drugs that are substrates for CYP1A1 or CYP1A2.

19.4.2.1 CYP1A Knock-Out Mouse Model. The *Cyp1a2*^{-/-} mice have been used to demonstrate the role of CYP1A2 in the pharmacokinetics and pharmacodynamics of the muscle relaxant zoxazolamine [22], where a ninefold decrease in enzyme activity was observed in the *Cyp1a2*^{-/-} mice. The wild-type (WT) and *Cyp1a2*^{-/-} mice have also been used to study the pharmacokinetics of caffeine [23]. The half-life of caffeine elimination from blood was about seven times longer in the *Cyp1a2*^{-/-} mice than in the WT mice, while the elimination clearance was eight times slower in the *Cyp1a2*-deficient mice compared to that in the WT mice. Theophylline pharmacokinetics has also been studied in WT and *Cyp1a2*^{-/-} KO mice [24]. Theophylline clearance was estimated as being 10 and 2.5 mL/min/kg for WT and *Cyp1a2*^{-/-} KO mice, respectively, with corresponding half-lives of 40 and 172 min.

Cyp1a2^{-/-} KO mice have also been used to demonstrate the involvement of CYP1A2 in microsomal degradation of bilirubin [25]. Previous studies have shown that liver microsomes from animals administered β -naphthoflavone (BNF) or 3-MC had increased activity for bilirubin degradation compared to microsomes from uninduced animals and that this rate could be increased further when a coplanar halogenated molecule, such as 3,4,39,49-tetrachlorobiphenyl (TCB), was added to the microsomal incubation together with NADPH [26]. It was also reported that CYP1A1 was

responsible for catalyzing bilirubin degradation by induced rat liver microsomes in the presence of TCB. However, Zaccaro *et al.* [25] used Cyp1a2 (2/2) mutant mice to investigate (i) the relative roles of Cyp1a2 and Cyp1a1 in bilirubin degradation and (ii) whether Cyp1a2 and Cyp1a1 require TCB addition for bilirubin-degrading activity. In WT mice, 3-MC treatment caused a threefold increase in the rate of bilirubin degradation. TCB caused a small but significant increase in bilirubin degradation. In Cyp1a2 (2/2) mutant mice, there was no MC-induced increase in microsomal bilirubin degradation in the absence of TCB. However, addition of TCB caused a three- to fivefold increase in bilirubin degradation in these mice. The authors concluded that CYP1A2 was responsible for microsomal bilirubin degradation in the absence of TCB and that TCB was required for bilirubin degradation by CYP1A1. This observation suggests that manipulation of CYP1A2 may be of therapeutic benefit in patients with these diseases of bilirubin conjugation.

19.4.2.2 CYP1A Humanized (Knock-In/Knock-Out) Mouse Model. Pharmacokinetics of theophylline has been evaluated in the WT, Cyp1a2^{-/-}, and humanized hCYP1A1_1A2 Cyp1a2^{-/-} mice [24]. Without the TCDD treatment, theophylline clearance values were 10, 2.8, and 4.5 mL/min/kg for the WT, Cyp1a2^{-/-}, and hCYP1A1_1A2 Cyp1a2^{-/-} mice, respectively. Following TCDD treatment, a 3.5-fold increase in theophylline clearance was observed in the WT mice, compared to 1.8-fold in the humanized CYP1A1_1A2 Cyp1a2^{-/-} mice. However, no change in the clearance was seen in Cyp1a2^{-/-}. These data are consistent with the metabolism of theophylline being mediated by mouse Cyp1a2 as well as human CYP1A2. Toxicokinetics of benzo[a]pyrene (B[a]P) studied in five mouse lines, WT, Cyp1a1^{-/-}, Cyp1a2^{-/-}, Cyp1a1/1a2^{-/-}, and CYP1A1_1A2 Cyp1a1/1a2^{-/-} [27], showed plasma steady-state levels of 1.3, 29, 0.51, 62, and 0.8 ng/mL, respectively. This together with corresponding plasma alanine transaminase (ALT) and aspartate transaminase (AST) suggest that both human CYP1A1 and mouse Cyp1a1 are important in metabolism of oral B[a]P.

Shi *et al.* [28] recently discussed an hCYP1A1_1A2_Cyp1a1/1a2^{-/-}_Ahrd mouse model generated, following the breeding of CYP1A1_1A2_Cyp1a1/1a2^{-/-}_Ahrb1 mice with B6.D2-Ahrd congenic mice. In this humanized model, human CYP1A1 and CYP1A2 are controlled by the endogenous mouse high affinity AHR in the hCYP1A1_1A2_Cyp1a1/1a2^{-/-}_Ahrb1 line and by the endogenous mouse poor affinity AHR in the hCYP1A1_1A2_Cyp1a1/1a2^{-/-}_Ahrd line. The humanized lines were characterized by quantifying human CYP1A messenger RNA (mRNA) levels in liver, lung, and kidney, as a function of four doses of the potent inducer, TCDD. Maximally induced mRNA levels of mouse Cyp1a1 were described as ~10 times higher than those of human CYP1A1; in contrast, maximally induced mRNA levels of mouse Cyp1a2 were less than twofold higher than those of human CYP1A2 in liver. They also reported that maximally induced mRNA levels of human CYP1A2 in liver were ~12 times higher than those of human CYP1A1, whereas maximally induced mRNA levels of mouse Cyp1a2 were ~threefold greater than those of mouse Cyp1a1. It was argued that the newly developed hCYP1A1_1A2_Cyp1a1/1a2^{-/-}_Ahrd line complemented their previously described hCYP1A1_1A2_Cyp1a1/1a2^{-/-}_Ahrb1 line, for use in carrying out pharmacokinetic and human risk assessment studies, involving any drug or environmental toxicant that is an effective substrate for CYP1A1 or CYP1A2.

Uno *et al.* [29] questioned the physiological relevance of these human mRNA levels in the intact mouse and whether these humanized mouse lines actually reflect average *CYP1A1* and *CYP1A2* gene expression that might be expected among individuals in a human population or was the expression too low or high? They compared basal and dioxin-induced CYP1A mRNA copy numbers, protein levels, and enzyme activity from four enzymes (benzo[a]pyrene hydroxylase, ethoxyresorufin-*O*-deethylase, acetanilide-4-hydroxylase, and methoxyresorufin-*O*-demethylase) in livers from hCYP1A1_1A2_Cyp1a1/1a2-/-_Ahr^d and uPA/SCID chimeric mouse line carrying human hepatocytes. In contrast to the findings from Shi *et al.* [28], Uno *et al.* [29] reported a mouse/human-induced CYP1A1 ratio of ~0.03 and a mouse/human-induced CYP1A2 ratio of ~4. Moreover, the mRNA levels of human CYP1A2 in the liver compared to those of human CYP1A1 was only 0.13, while that between maximally induced mRNA levels of mouse CYP1A2 and CYP1A1 was approximately threefold. It was argued that this inconsistency in the calculated ratios reflects the disparity between the “relative values” by Shi *et al.* [28] and their “absolute values” (i.e., copy numbers per microgram total RNA) in this study.

The procarcinogen PhIP is the most abundant heterocyclic amine formed during the cooking of foods. Metabolism of PhIP by CYP1A2 differs substantially between humans and rodents, with more N2-hydroxylation (activation) and less 4'-hydroxylation (detoxication) in humans. Thus, given that the human response to PhIP and potentially other heterocyclic amine exposure may not be accurately reflected in standard laboratory rodents, Cheung *et al.* [30] studied species differences in heterocyclic amine metabolism using two transgenic mouse lines: one expressing the human CYP1A1 CYP1A2 transgene in a mouse Cyp1a1-null background (hCYP1A1) and another expressing human CYP1A1 CYP1A2 in a mouse Cyp1a2-null background (hCYP1A2). Differences in the metabolism of the heterocyclic amine PhIP were observed between WT and hCYP1A2 mice. Moreover, PhIP was preferentially metabolized by N2-hydroxylation in hCYP1A2 mice, whereas in WT mice, 4'-hydroxylation was the predominant pathway. This confirms earlier work [31] that first showed the human CYP1A2 preference for N2-hydroxylation relative to 4 α -hydroxylation (ratio of 97:1) compared with the mouse ratio of 1.7:1 for these pathways. These results highlight the potential effectiveness of using these transgenic, humanized mice as models for determining human health risks to PhIP and other heterocyclic amines instead of WT mice.

19.4.3 CYP2D6

CYP2D isozymes have been identified in many mammalian species and are involved in oxidation of various xenobiotics and drugs. In humans, the only functional gene is *CYP2D6*, while *CYP2D7p* and *CYP2D8p* are two inactive pseudogenes [32]. In contrast, mice have at least nine *CYP2D* genes, *Cyp2d9–2d13*, *Cyp2d22*, *Cyp2d26*, *Cyp2d34*, and *Cyp2d40*, and rats have six *CYP2D* genes: *Cyp2d1–2d5* and *Cyp2d18* [33]. Given this degree of diversity, it is not surprising that marked species differences exist in CYP2D metabolism between humans and rodent. For humans, understanding metabolism via CYP2D6 is particularly important as it has been shown to be involved in the metabolism of more than 20% of prescribed drugs, most of which act in either the heart or central nervous system (CNS) [34]. As important is the fact that it is polymorphically expressed resulting in three distinct human phenotypes, namely poor metabolizers (PMs), who lack the functional enzyme; extensive metabolizers

(EMs); and ultrarapid metabolizers (UMs). The most common phenotyping procedure of CYP2D6 is the determination of the metabolic ratio (MR) of debrisoquine (DEB)/4-hydroxy debrisoquine (as measured in the urine). In PMs, MR values greater than 12.6 are seen, while values less than 12.6 are seen in EMs. Individuals with an MR value much less than 0.1 are referred to as *UMs* [35]. To overcome the species difference in CYP2D metabolism, a number of GEMA have been established and are discussed below.

19.4.3.1 CYP2D6 Humanized (Knock-In) Mouse Model. Corchero *et al.* [36] first established homozygous and heterozygous CYP2D6 humanized mice models. After a single oral dose of DEB (2.5 mg/kg), both CYP2D6 heterozygous and homozygous mice had DEB serum levels significantly lower than in WT. Consistently, 4-hydroxy debrisoquine (4-OH-DEB) levels are highest in CYP2D6 homozygous, intermediate in CYP2D6 heterozygous, and extremely low in the WT mice. In addition, the pharmacokinetic data showed the DEB area under the curve (AUC) to be three- and sixfold higher in WT mice than in heterozygous and homozygous CYP2D6 humanized mice, respectively, reflecting differences in the elimination half-life of DEB, which was 2.1 and 1.4 times shorter in the heterozygous and homozygous CYP2D6 humanized mice than in WT mice. Accordingly, CYP2D6 humanized mice showed a clearance six- and fourfold higher than WT mice. On the basis of the urinary recovery data, the MR was estimated to be 10 for the WT mice and 0.5 for the CYP2D6 humanized mice. Interestingly, the MR of DEB in the WT mice was similar to that in human PMs, while the MR of DEB in humanized CYP2D6 mice was close to that in human EMs.

Work from the same group also looked at the role played by the transcriptional factor, hepatic nuclear factor 4 α (HNF4 α) [36], which had previously been shown to regulate the expression of *CYP2D6* gene *in vitro* [37]. A humanized mouse line carrying the human *CYP2D6* gene and the HNF4 α conditional mutation was created by cross-breeding the humanized CYP2D6 mice with an HNF4 α conditional null mouse line to investigate the effect of this transcriptional factor on the CYP2D6 expression *in vivo*. Disruption of HNF4 α expression resulted in a twofold decrease in the expression of CYP2D6 enzyme. Moreover, the metabolism of DEB was significantly reduced in the humanized CYP2D6 mice lacking HNF4 α gene. The urinary recovery was 7.2% of dose for 4-hydroxylated DEB in the humanized CYP2D6 mice carrying HNF4 α gene. In the absence of HNF4 α gene, the urinary recovery was only 2.9%. On the basis of the urinary recovery data, the MR was estimated to be 2.6 and 3.6, respectively, in the humanized CYP2D6 mice carrying HNF4 α gene and those lacking HNF4 α gene.

This model has been further validated as a model for CYP2D6 [38]. The expression of human CYP2D6 and mouse CYP2D enzymes in these humanized and WT mice were quantified by immunoblotting and detected at the cellular level by immunocytochemistry. In humanized mice, the cell-specific expression of CYP2D6 in liver, kidney, and intestine was similar to those reported in humans. Human CYP2D6 was not detected in the brain of transgenic mice, while mouse CYP2D proteins were present. This has been observed before in a transgenic CYP3A4 mouse model, where this protein is expressed in intestine but not in liver [39], a site of high expression levels in humans. Miksys *et al.* [38] concluded that the transgenic mouse model is useful when investigating CYP2D6-mediated metabolism in liver, kidney, and especially the intestine, where expression patterns demonstrated substantial species differences.

Cytochrome P450 2D6 is known to be absent from the liver and brain for a small percentage (~10%) of the population [40], but its function in the human brain remains elusive even though personality differences have been reported between EMs and PMs, suggesting the potential existence of endogenous substrates for CYP2D6 [41]. To address this, the CYP2D6-humanized mouse model has been further applied to the search for potential endogenous substrates for CYP2D6 [42–44]. *Ex vivo* studies using microsomes from these humanized mice showed that CYP2D6 catalyzed the O-demethylation of a number of psychotropic methoxyindolethylamines derived from serotonin (5-hydroxytryptamine, 5-HT) [42,43,45]. In this regard, Yu *et al.* [43] established that 5-methoxytryptamine (5-MT), a metabolite and precursor of melatonin, was a specific and high turnover endogenous substrate of CYP2D6. After the intravenous (IV) coadministration of 5-MT and pargyline [to protect against monoamine oxidase A (MAO-A) metabolism], serum 5-HT levels were approximately threefold higher in CYP2D6-transgenic mice than WT mice. The authors postulated that this regeneration of 5-HT from 5-MT provided the missing link in the serotonin–melatonin cycle. Interestingly, following coadministration of pargyline and 5-MT, all the mice exhibited tremors, although CYP2D6-transgenic mice appeared less pronounced than the WT. Such tremors were thought to result from dramatically elevated serum 5-MT levels. The authors suggested that caution be taken when using 5-MT (as mood enhancer) with MAO inhibitors in humans, especially in CYP2D6 PMs. More recently, WT and humanized CYP2D6 mice have been used to investigate the metabolism of 5-methoxy-*N,N*-dimethyltryptamine (5-MeO-DMT) [46], a natural psychoactive indolealkylamine drug that has been used for recreational purposes [47]. Previous *in vitro* studies have shown that bufotenine, the major metabolite of 5-MeO-DMT, is produced by CYP2D6 [45]. Following the administration of a high dose of 5-MeO-DMT, humanized CYP2D6 mice showed significantly higher systemic exposure (AUC) to the active metabolite bufotenine than WT mice. As humanized CYP2D6 mice differ from WT mice in the expression of functional CYP2D6 enzyme, these findings have prompted the expectation that subjects with regular or increased CYP2D6 activity would have more complex drug effects induced by bufotenine metabolite.

In light of the fact that 5-MeO-DMT is predominantly inactivated by MAO-A, coabuse with MAO inhibitors such as harmaline (one of the major psychotropic ingredients in recreational beverage *Ayahuasca* [48]) would likely elicit a metabolic drug interactions (DDI) and increase the risk of 5-MeO-DMT intoxication. Studies have shown that pretreatment of harmaline led to a prolonged residual time within the body (referred to by the *mean residence time*, MRT) and increased AUC in humanized CYP2D6 mice [46,49]. Together, their observations in humanized CYP2D6 mice suggested that MAO inhibitors and CYP2D6 status may have significant impact on 5-MeO-DMT metabolism and pharmacokinetics as well as the formation of bufotenine. As such the model allowed novel insights toward biochemical determinants in 5-MeO-DMT pharmacokinetics and harmaline-5-MeO-DMT DDI, and further advanced understanding of risks in 5-MeO-DMT intoxication.

19.4.4 CYP2E1

Cytochrome P450 2E1 is predominately expressed in human liver, with some lower level extrahepatic expression in the kidney, lung, and brain. A number of genetic polymorphisms have been described for the human *CYP2E1* gene, with ethnic differences

demonstrated between European and Japanese populations [50]. CYP2E1 has been found to be involved in the oxidative metabolism of a small range of substrates (mostly small polar molecules). Furthermore, it has also been shown to be a key enzyme in the metabolism and can activate numerous toxicologically important chemicals such as acetone, ethanol, benzene, halothane, and carbon tetrachloride as well as clinically used drugs as acetaminophen (APAP), disulfiram, chlorzoxazone, and carcinogens, such as the low molecular weight nitrosamines [51–54]. Before establishing CYP2E1 GEMAs, the majority of studies on its pharmacological and biochemical actions was conducted in rodents, rabbits, and cultured hepatocytes.

19.4.4.1 CYP2E1 (Knock-Out/Knock-In) Mouse. Lee *et al.* [55] first reported the production of mice homozygous for the disrupted allele, designated *Cyp2e1*^{–/–}. The authors confirmed the complete absence of protein and mRNA expression in the livers of *Cyp2e1*^{–/–} mice. Using this animal model, they went on to show how CYP2E1 influences the toxicity of APAP (also known as *paracetamol*), an over-the-counter analgesic and antipyretic that is commonly used worldwide as a substitute for acetylsalicylic acid (Aspirin®) due to its lack of gastric ulceration and general low toxicity when used within the recommended dose range [56]. Compared to WT mice, *Cyp2e1*^{–/–} mice survived up to doses of 400 mg/kg, whereas this dose was lethal in 50% of WT animals. At all doses, levels of creatinine, bilirubin, and alkaline phosphatase were within the normal range and were not significantly different between the *Cyp2e1*^{–/–} and WT mice. In contrast, liver enzymes aspartate aminotransferase and alanine aminotransferase (considered as measures of liver cell death) were elevated at high doses of APAP but were unchanged at higher doses in the *Cyp2e1*^{–/–} mice. Together these data indicated that liver damage due to formation of a reactive metabolite is involved in mediating the toxicity of APAP. CYP2E1 metabolizes APAP to a highly reactive metabolite, *N*-acetyl-*p*-benzoquinone imine, which can covalently bind to cellular nucleophiles, such as DNA, RNA, and proteins. *N*-Acetyl-*p*-benzoquinone imine is typically inactivated by glutathione-*S*-transferase (GST) [57]; however, in an overdose of APAP, the amount of the reactive intermediate metabolite formed depletes hepatic glutathione (GSH) levels, overwhelming this detoxification pathway.

This model has also been used to investigate the involvement of CYP2E1 in the *in vivo* metabolism of benzene and in the development of benzene-induced toxicity. For the metabolism studies, male *Cyp2e1*^{–/–} and WT control mice were exposed to benzene, along with a radiolabeled tracer dose of [¹⁴C]benzene. Total urinary radioactivity and all individual radiolabeled metabolites were reduced in the urine of *Cyp2e1*^{–/–} mice compared to WT controls. This tissue radioactivity analysis demonstrated that CYP2E1 was the major determinant of *in vivo* benzene metabolism and benzene-induced myelotoxicity in mice. Together, these results suggest that *Cyp2e1*-null mice can be used as a tool to screen for potential CYP2E1-induced hepatotoxicity of drug candidates by comparing with the WT mice. In fact, toxicities associated with carbon tetrachloride [58], chloroform [59], methacrylonitrile [60,61], acrylamide [60,61], and trichloroethylene metabolism [62] have all been shown using this animal model.

The transgenic model reported by Morgan *et al.* [63] (see above) still retained host expression of CYP2E1. To establish a true humanized model, transgenic mice were generated using a bacterial artificial chromosome (BAC) clone containing the entire human *CYP2E1* gene, and these were bred into a mouse *Cyp2e1*-null background, thus creating CYP2E1 humanized mice [64]. Using this mouse model, the functional

differences between human and mouse hCYP2E1 Cyp2E1^{-/-} were directly compared. Human CYP2E1 protein and mRNA expression were expressed at high levels in the liver of CYP2E1-humanized mice, with the human CYP2E1 mRNA additionally detected in some extrahepatic tissues such as kidney, small intestine, and lung, consistent with the distribution pattern reported in humans [65]. Human CYP2E1 activity was demonstrated in the livers of CYP2E1-humanized mice by the increased chlorzoxazone 6-hydroxylation (commonly used to assess human CYP2E1 activity) [50] and increased *p*-nitrocatechol formation compared with the level in Cyp2e1^{-/-} mice. Further studies showed induction of CYP2E1 in the CYP2E1-humanized mice using the known inducer acetone.

Cheng and colleagues [64] went on to investigate the effect of APAP on the WT, Cyp2e1^{-/-}, and CYP2E1-humanized mice. At a dose of 400 mg/kg APAP, liver necrosis was detected in WT but not Cyp2e1^{-/-} mice, consistent with previous studies [55]. At this dose, CYP2E1-humanized mice were found to have moderate-to-severe liver necrosis, whereas in WT mice, moderate liver necrosis was identified. However, the serum ALT levels were elevated to approximately the same levels in both WT and CYP2E1-humanized mice treated with 400 mg/kg APAP, indicating a similar toxic response. It was suggested that the current model could also provide a more conclusive answer to the role that CYP2E1 plays in ethanol toxicity, since the model used by Morgan *et al.* [63] also contained endogenous mouse Cyp2e1.

19.4.5 CYP3A

The human CYP3A subfamily consists of four members: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. Among them, CYP3A4 is the most abundant hepatic and intestinal CYP enzyme in humans, accounting for 30% and 70% of total CYP, respectively, and catalyzes the metabolism of over 50% of drugs that are clinically used [66]. In mice, at least six CYP3A isoforms have been identified: Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41, and Cyp3a44 [33]. Additionally, there are at least six CYP3A isozymes identified in the rat: Cyp3a1, Cyp3a2, Cyp3a9, Cyp3a18, Cyp3a23, and Cyp3a62 [33]. Like that of other P450s, the species differences in the expression pattern of different CYP3A isozymes and their intrinsic enzyme activity between humans and rodents makes the extrapolation of drug metabolism from animals to humans quite difficult. To address this difference, various GEMA models have been established to aid the understanding of drug metabolism by this isozyme.

19.4.5.1 CYP3A Humanized (Knock-In) Mouse. Mice expressing human CYP3A4 (designated hCYP3A4) were first reported by Granvil *et al.* [39]. To generate these mice (hCYP3A4), the BAC *CYP3A4* gene, including the upstream PXR-binding sites, was microinjected into a fertilized mouse egg. IV and oral pharmacokinetics of midazolam have been studied in both the WT and hCYP3A4 mice [39]. The IV data showed no significant differences in half-life and clearance of midazolam between WT and hCYP3A4 mice. In contrast, there were significant differences in the pharmacokinetics of midazolam between the WT and hCYP3A4 mice after oral administration. The high metabolic clearance of midazolam in the hCYP3A4 mice was supported by the fact that the AUC of 1'-OH midazolam (a major metabolite of midazolam) was higher in the hCYP3A4 mice compared with WT mice. Moreover, the potent CYP3A4

inhibitor ketoconazole markedly lowered the rate of midazolam metabolism and elimination when coadministered orally with midazolam, as compared with midazolam mono-treatment. Possible explanations postulated to explain the disparity between these inhibitory effects include greater first-pass metabolism in hCYP3A4 mice, potential differences in the affinity for midazolam and ketoconazole between hCYP3A4 and mouse CYP3a proteins, and differences in tissue expression pattern for CYP3A4 and Cyp3a proteins in the mice. In this regard, Granvil *et al.* [39] measured CYP3A4 expression in hCYP3A4 mice and found it to be highly expressed in the small intestine, but surprisingly, no expression was found in the liver, despite it being the major tissue of CYP3A4 expression. The predominate expression of human CYP3A4 in the intestine but not in liver explains the lack of pharmacokinetic difference between hCYP3A4 mice and WT following IV dosing. In contrast, Cyp3a proteins were highly expressed in the liver of WT mice and to a lesser extent in the small intestine. Subsequent studies using mice generated from a BAC clone, which contains both *CYP3A4* and *CYP3A7* genes [67], revealed constitutive CYP3A4 expression in the livers of immature male and adult female mice, indicating that the transgene is developmentally regulated in a gender-specific manner [67,68]. However, the level of CYP3A4 in the adult female liver was still substantially lower than that in the small intestine. Gender-specific regulation of P450s has been extensively studied in rodents and is suggested to be attributed to differences in growth hormone secretory patterns between males and females [69]. Continuous infusion of growth hormone to mimic female secretory patterns has been shown to convert the male growth hormone secretory pattern to that of the female pattern in hCYP3A4 mice [67].

19.4.5.2 CYP3A Humanized (Knock-In/Knock-Out) Mouse. To address some of the limitations with the aforementioned hCYP3A4 mouse model, which exhibited poor hepatic CYP3A4 levels, van Herwaarden *et al.* generated and characterized transgenic mice with functional expression of human CYP3A4 cDNA in the liver, moderate levels in kidney, but not in small intestine [70]. Using midazolam and cyclosporin A to test functional activity of liver CYP3A4, the authors showed that midazolam plasma AUC concentrations decreased in the transgenic mice compared with WT. Similarly, following IV administration of cyclosporin A, CYP3A4 transgenic mice displayed reduced plasma AUC compared with WT. Yet the major disadvantage of these aforementioned hCYP3A4 mice models is the coexpression of mouse Cyp3a. To address this, the same group generated two strains of Cyp3a KO mice lacking all functional murine *Cyp3a* genes (*Cyp3a*^{-/-}), yet remained viable, fertile, and without marked physiological abnormalities. These models included *Cyp3a*^{-/-}V mice where human CYP3A4 was expressed in high amounts in the duodenum, jejunum, ileum, and colon but not in liver and *Cyp3a*^{-/-}A mice where expression was high in the liver but not in the intestine [71]. *Cyp3a*^{-/-} mice were shown to display a greatly impaired ability to detoxify the cytotoxic drug docetaxel. Expression of transgenic human CYP3A4 in the intestine alone resulted in blockage of parental docetaxel entry from the gut but had very little effect on the systemic clearance of docetaxel. In contrast, liver expression of transgenic CYP3A4 greatly reduced systemic exposure after IV docetaxel administration but had a relatively modest impact on oral administration.

More recently, observations from the same laboratory have shown that CYP2C are upregulated in *Cyp3a*^{-/-} mice and, consequently, contribute to the metabolism of midazolam [72]. Consistent with previous *in vitro* studies [73], their findings showed

that in WT mice, 1'-OH midazolam formation is dependent on not only CYP3A but also CYP2C. Of particular interests were the observations that 1'-OH midazolam formation in Cyp3a^{-/-} mice resulting from CYP2C activity was only slightly lower compared with that in WT mice, and also significant level of 4-OH midazolam formation was seen in Cyp3a^{-/-} mouse liver microsomes, a reaction that was also considered to be CYP3A specific in mice [74]. Midazolam metabolism in small intestinal microsomes from Cyp3a^{-/-} mice was not observed, suggesting that the small intestinal expression of CYP2C enzymes involved in midazolam metabolism is too low to make any significant contribution to the overall clearance. These examples highlight confounding of target metabolism across species, where a probe metabolic pathway in one species may be metabolized by multiple enzymatic pathways in another.

19.4.5.3 CYP3A Chimeric-HL Mouse. A uPA^{+/+}/severe combined immunodeficient transgenic (uPA^{+/+}/SCID) mouse line (see Section 19.3.3) has been established, in which the liver could be replaced by more than 80% with human hepatocytes. Given that the liver is the critical organ involved in the pharmacokinetics of drugs, one could argue that human liver is essential for the development of new drugs. In this regard, to better predict the human drug metabolism and pharmacokinetics, human hepatocytes and liver microsomes are frequently used. Thus, chimeric-HL mice with humanized liver provide an advantage in studies on drug metabolism and pharmacokinetics. Human CYP3A4 mRNA and human CYP3A4 protein can be detected using human albumin as a marker by real-time reverse-transcription polymerase chain reaction (RT-PCR) and western blotting, in a chimeric mouse model [75]. *Ex vivo* studies using hepatocytes from these chimeric-HL mice with near-completely humanized liver were used to investigate the induction of human CYP1A2 and CYP3A4 mRNA by BNF and rifampicin (Rif), respectively. For both P450s, their mRNA was significantly increased compared to fresh human hepatocytes [76]. The same group conducted additional evaluation by evaluating the effects of Rif treatment [intraperitoneal (IP)] on dexamethasone metabolism [77]. The expression levels of human CYP3A4 mRNA and CYP3A4 protein and dexamethasone 6-hydroxylase activity, specific for human CYP3A4, were increased 8- to 22-, 3- to 10-, and 5- to 12-fold, respectively, by treatment with Rif. The authors postulated that because dexamethasone is mainly metabolized to 6-hydroxyldexamethasone in human but to 6-hydroxy-9 α -fluoro-androsta-14-diene-11 β -hydroxy-16 α -methyl-3,17-dione in mice [78], increase in dexamethasone-6-hydroxylase suggested that the human CYP3A4 in the chimeric-HL mice had induction potential. This hypothesis was supported by studies, again from the same group, who investigated CYP3A4 mRNA, protein, and enzyme activity in liver of chimeric-HL mice following exposure to an analogue of Rif, rifabutin which is a specific inducer of human CYP3A4 but not mouse Cyp3a [79]. Each marker was found to be significantly elevated in chimeric mice. In contrast, rifabutin was not shown to increase the murine Cyp3a enzyme activities in the control mice. Together all these studies lend themselves to suggest that, given the environment of P450s in the liver, chimeric-HL mice resembles human *in vivo* more so than they do WT mice, the chimeric-HL mouse line could be a useful animal model to predict CYP3A4 induction in human.

In summary, the previous sections have reviewed the broad applications of chimeric, humanized, as well as KO/knock-in animals for predicting the variation of metabolism by cytochrome P450s, disposition of drugs or drug candidates, *in vivo* drug-drug interactions, and pharmacokinetics and pharmacodynamics for individualized drug therapy

in the human population. The multitude of available models will also permit investigations into the physiological significance of these endogenous substrates. Given the diverse nature of the models available, there is no single model that provides significant advantage over the others. Each will have its advantages and disadvantages, based on the question being asked.

19.5 EPOXIDE HYDROLASES

EHs (formerly epoxide hydratase or epoxide hydrase) were first identified as detoxification enzymes [80]. The two mammalian xenobiotic-metabolizing EHs, microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH), are members of the α/β hydrolase fold family of enzymes [81–83]. The α/β hydrolase fold family contains a catalytic triad that consists of a nucleophile for formation of an acyl or an alkyl enzyme intermediate, a general base, and a charge relay residue [84]. This family is diverse, consisting of a broad variety of enzymes, including esterases (i.e., AChE, EHs, haloalkane dehalogenases, and lipases [85].

EH catalyzes the hydrolysis of epoxides (also called *oxiranes*) to corresponding *trans*-dihydrodiols with water as the cofactor. Epoxides are cyclic ethers with three ring atoms, and due to the short bond angle of their ring system, the ring is highly strained. Depending on the substitution pattern of the epoxide, the epoxide may have significant electrophilic reactivity and thus when formed via metabolism may have the potential to be a reactive metabolite. Reactive electrophiles can potentially react with nucleic acids (DNA) and proteins, leading to mutagenic, toxic, and carcinogenic effects [86,87]. However, some epoxides are relatively stable at physiological pHs (i.e., carbamazepine-10,11-epoxide [88,89]), but they still may be associated with toxicity [90,91]. The addition of water to epoxides by EH to form the corresponding *trans*-dihydrodiols is one way in which the body can eliminate epoxides and is usually considered a detoxification pathway. However, in certain circumstances (i.e., benzo[a]pyrene-4,5-oxide), diol formation maybe an intermediate step for the formation of a second epoxide (i.e., via the cytochrome P450 system [92]) and these paired reactions can result in an epoxide with vastly increased mutagenic and carcinogenic properties [86,93,94].

19.5.1 Microsomal Epoxide Hydrolase

mEH is the major xenobiotic-metabolizing EH with a broad substrate selectivity against a diverse group of epoxides [95]. Substrates for mEH are epoxides, hydrophobic in nature, and mono-1,1-di, or 1,2-*cis*-disubstituted. mEH can accommodate bulky substrates such as benzo[a]pyrene-4,5-oxide as well as less bulky compounds such as octane-1,2-oxide. *trans*-1,2-Disubstituted or trisubstituted epoxides are poorly metabolized if at all. However, *trans*-1,2-substituted epoxides are potential substrates of sEH.

mEH has been found in nearly all evaluated mammalian tissues and it is particularly concentrated in the liver, testes, lungs, kidneys, adrenal gland, and intestines. mEH is located in the smooth endoplasmic reticulum; however, there have been a few reports indicating that it is also located on plasma membranes [96,97]. Within the endoplasmic reticulum, mEH is attached to the membrane with a single N-terminal anchor [98] and is facing toward the cell cytosol [99] similar to the CYP enzymes and not toward the endoplasmic reticulum lumen, like the UGTs. A few endogenous substrates have been

reported for mEH, including androstene oxide and estrooxide [100], suggesting a role in development. Recently, mEH has been linked to neurodegenerative disorders such as Alzheimer's and Parkinson's disease [101,102]. Epoxy fatty acids such as epoxysteric acid are relatively poor substrates for mEH compared with sEH (Section 19.5.2).

19.5.1.1 mEH-Null (Knock-Out) Mouse Model. In a study by Miyata *et al.* [103], mEH-null mice were produced to evaluate the physiological importance of mEH and to evaluate the impact of mEH on 7,12-dimethylbenz[a]anthracene (DMBA) carcinogenesis. To produce mEH-null mice, ES cell clones heterozygous for the disrupted *mEH* gene were prepared and injected into C57BL/6 blastocysts to generate germline chimera mice. Pseudopregnant recipient NIH Swiss females were then impregnated with the injected blastocysts to produce germline chimeras mice. The male were bred with C57BL/6 females to establish heterozygous offspring. Homozygotes were then produced from the heterozygous mice that had germline transmission of the targeted mEH allele. The mEH-null mice produced were found to have no obvious phenotype, indicating that mEH is not essential for reproduction and physiological homeostasis. The mice were then subsequently utilized to evaluate DMBA carcinogenesis.

DMBA is a polycyclic hydrocarbon carcinogen that requires multiple metabolic steps to produce the active carcinogenic metabolite. mEH catalysis is required to produce the diol that undergoes subsequent epoxidation by CYP1B1 [92]. Therefore, it would be predicted that mice lacking mEH would be resistant to DMBA-induced cancer. This hypothesis was tested using the mouse skin cancer bioassay with both an initiation-promotion protocol and the complete carcinogenesis protocol. In the initiation-promotion protocol, DMBA was utilized topically for initiation and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used during the promotion phase. For the complete carcinogenesis study, only DMBA was applied. In the promotion protocol, about 20% of the mEH-null mice developed papillomas at 17 weeks compared to 70% of the WT controls at 15 weeks. In the full carcinogenesis protocol, after 25 weeks of treatment, nearly 80% of the WT mice had papillomas, whereas there were no evident papillomas on the mEH-null mice. Thus, mEH-null mice were found to be resistant to DMBA-skin cancer. However, the fact that tumors were found in mEH-null mice treated with TPA suggests a second activation pathway for DMBA, which is dependent on TPA. These findings could be further investigated using P450-null mice as well as mEH-null mice.

The impact of mEH on DMBA toxicity was further investigated, where DMBA was administered orally [104]. In this study, the mEH-null mice were bred from mEH-null/- mice [103] obtained from the National Institute of Health. mEH-null/- and control mice were orally gavaged with DMBA (0, 17, 50, and 150 mg/kg) once a day for five days. DMBA treatment of mEH/- mice did not produce changes in body weight, spleen weight, or spleen cellularity, which was observed with the WT mice. In contrast to the WT mice, DMBA treatments did not affect the plaque forming cell response, alter natural killer cell activity, or affect B-cell mitogenesis (although T-cell mitogenesis was partially suppressed at the 50 and 150 mg/kg doses) in mEH/- mice. To determine if the difference observed between the two groups of mice could have been due to differences in pharmacokinetics (drug distribution), the biodistribution of DMBA was determined in the WT and the mEH/- mice in the spleen, thymus, and liver after 24 h and 7 days of oral dosing. The concentrations of DMBA were not significantly different between the WT and the mEH/- mice, demonstrating that mEH is crucial for the activation of DMBA *in vivo*.

KO mice have also been utilized to evaluate benzene toxicity [105,106]. Benzene is another example of toxicity, where mEH is involved in the formation of an intermediate that undergoes subsequent metabolism to the final reactive form. It was interesting to note that in a study by Bauer *et al.* [103], benzene-induced toxicity was observed in the WT male but not in the WT female. As in the previous studies, the mEH^{-/-} mice (male and female) did not exhibit toxicity on the administration of benzene. In the study of Recio *et al.* [106], the additional NAD(P)H: quinone oxidoreductase 1 (NQO1)-null mice were also included in the study design to further elucidate the mechanistic pathways.

mEH^{-/-} mice have also been utilized in studies evaluating the impact of mEH on detoxification pathways [107]. In a study evaluating the impact of mEH^{-/-} mice on the toxicity of 1,3-butadiene (BD) and its metabolite [butadienediepoxy (BDO₂)], the mEH^{-/-} and WT mice were exposed to 20 ppm BD (inhalation) or 30 mg/kg BDO₂ (IP injection). The Hprt mutant frequencies (MFs) were significantly higher in the mEH^{-/-} group exposed to BD when compared to controls (12.4-fold increase) and significantly greater than the corresponding WT group (5.4-fold increase). Additionally, mEH^{-/-} mice exposed to BDO₂ exhibited a significant increase in MF over controls (4.5-fold increase) and WT mice (1.7-fold increase). Genomic damage (measured via comet tail moment) of BDO₂ was evaluated in controls, WT, and mEH^{-/-} mice. mEH^{-/-} mice exposed to the high dose of BDO₂ exhibited significantly more DNA damage than all other groups (2.3- to 27.9-fold increase). Thus, the absence of mEH catalytic activity increases the genetic sensitivity of mice exposed to BD and BDO₂, which is consistent with mEH catalysis being a component of the detoxification pathway.

19.5.1.2 mEH and sEH Chimeric-HL Mouse Model. Nishimura *et al.* [108] investigated the hepatic mRNA expression of human DMEs by TaqMan real-time RT-PCR in chimeric-HL mice with almost completely humanized liver (replacement index (RI): 71–89%). The humanized mice were prepared using the method outlined in Section 19.3.3 using (uPA^{+/+}/SCID) mice. The mRNA of 35 phase I enzymes, including the mRNA of mEH (EPHX1) and sEH (EPHX2) were evaluated. The ratio of the target mRNA levels in the chimeric-HL mice when compared to target mRNA levels in human liver were ~0.75 and 0.6 for mEH and sEH, respectively. Chimeric-HL mice with humanized liver represent another transgenic model for evaluation of mEH and sEH.

19.5.2 Soluble Epoxide Hydrolase

sEH does not have the broad xenobiotic substrate selectivity of mEH; however, it complements mEH in that it is capable of hydrolyzing 1,2-*trans*-substituted epoxides [109]. Typical xenobiotics metabolized by sEH are *trans*-stilbene oxide and *trans*-ethylstyrene oxide. Unlike mEH, sEH does not generally metabolize bulky substrates; however, sEH does metabolize a limited number of PAH epoxides to their corresponding diols [110]. The primary substrates for sEH are endogenous epoxides such as arachidonic epoxides (also called *epoxyeicosatrienoic acids* or EETs), linoleic acid epoxides, and other epoxides derived from fatty acids [111–113]. Owing to its importance in lipid metabolism, sEH has recently been identified as a potential target for multiple therapeutic indications including hypertension [114,115], inflammatory disease [116], diabetes [117], and stroke [118].

sEH has been found in nearly all evaluated mammalian tissues, including liver, lungs, kidney, adrenals, brain, and intestines. sEH was first identified in the cell cytosol [119], which is why it was previously known as *cytosolic epoxide hydrolase*; however, in some cells, sEH has also been identified in peroxisomes [120,121], and therefore, the current naming convention has been updated. sEH catalysis also appears to be involved in a toxicity pathway via its formation of a dihydrodiol from the epoxide of an unsaturated fatty acid. The diol of leukotoxin, the epoxide of linolenic acid, has been implicated as the causative agent of the multiple organ failure and adult respiratory distress syndrome after severe body burns previously associated with leukotoxin [111,122,123]. In mice, where sEH is the major leukotoxin-metabolizing enzyme, pre-treatment with a sEH inhibitor decreases the toxic effects of leukotoxin [111].

19.5.2.1 sEH-Null (Knock-Out) Mouse Model. In studies by Sinal *et al.* [124,125], sEH-null mice were produced to evaluate the impact of sEH on blood pressure and renal arachidonic acid (AA) metabolism. To prepare the sEH^{-/-} mice, a fragment containing exon 1 was isolated from mouse sEH genomic clones and subcloned into pUC9. Bacterial phosphoribosyltransferase II gene cassette (TK-Neo) was used to disrupt the gene. ES cell clones prepared using the disrupted *sEH* gene were injected into blastocysts (Genome Systems). The subsequent male chimeric mice were crossed with C57BL6 females to obtain heterozygous mice. The heterozygous mice were then crossed to produce WT, heterozygous mice, and the sEH^{-/-} mice. Systolic blood pressure was then monitored in the mice in both the presence and absence of salt loading. The systolic blood pressure in sEH^{-/-} male mice was significantly lower than the blood pressure in the WT male mice in both the absence and presence of the dietary salt loading. Female mice (WT and sEH 9^{-/-}) had blood pressures significantly lower than the WT male mice.

For *in vitro* evaluation of the impact of sEH^{-/-} mice on AA metabolism, S9 fractions were prepared from the liver and the kidney of WT and sEH^{-/-} mice. AA is metabolized via CYP450s to EETs and then subsequently metabolized to dihydroxyeicosatrienoic acids (DHETs) by sEH. In the liver S9 fractions from WT mice, the level of EETs was extremely small in contrast to the sEH^{-/-} mice. However, the corresponding levels of DHETs were much higher in the WT mice as compared to the sEH^{-/-} mice. It was hypothesized that the small level of DHETs observed in the liver fractions of the sEH^{-/-} mice could be due to mEH metabolism as mEH metabolizes EETs albeit much less efficiently than sEH. The kidney S9 *in vitro* data were consistent with the liver data in that the levels of DHETs observed was greatly reduced in the sEH^{-/-} mice. Striking sex differences were observed in the formation of DHETs from EETs in the kidney fractions with AA metabolites much greater in males than females. The *in vitro* findings were consistent with the *in vivo* findings of the sex differences observed for the impact of sEH^{-/-} mice. These data provide evidence for the role of sEH in blood pressure regulation and thus, sEH as a target that could be used to regulate blood pressure associated with hypertension.

In addition to the examples above, the impact of sEH on EET metabolism has also be evaluated in brain [126,127]. To test the hypothesis that *sEH* gene deletion would be protective against focal cerebral ischemia [126], sEH^{-/-} mice with and without an EET antagonist (14,15-epoxyeicosa-5(Z)-enoic acid) were subjected to a 2-h middle cerebral artery occlusion and the subsequent infarct size was measured and compared to WT controls. sEH detected via immunohistochemistry was detected in WT but not

sEH^{-/-} mice brains. The EET metabolite DHET was abundantly present in the WT mice but virtually absent in the sEH^{-/-} mice, which is consistent with the sEH-null phenotype. However, the levels of EETs in brain tissue between WT and sEH^{-/-} mice were not significant thus suggesting an alternative route of elimination of EETs in mouse brain. The infarct size was significantly smaller with regional cerebral blood flow rates significantly higher in sEH^{-/-} mice when compared to WT mice. It was interesting to note that the presence of the EET antagonist did not alter infarct size.

In a follow-up study [127], sex differences in EET metabolism and infarct size were evaluated in sEH^{-/-} mice. In WT female mice, sEH activity was lower, DHET levels were lower, cerebral blood flow was higher, and infarct size post middle cerebral artery occlusion was lower than in male WT mice. These sex differences were not observed in sEH^{-/-} mice and were negated in female WT mice following ovariectomy. This is consistent with the impact of estrogen on sEH. Thus, it was concluded that sEH activity was an important mechanism underlying sex-linked differences in blood flow and brain damage after cerebral ischemia in mice.

sEH^{-/-} mice are a key tool in evaluating the physiological impact of sEH on disease states and subsequently building evidence of sEH as a potential target for therapeutic intervention. Additional studies have been conducted using sEH^{-/-} mice in regard to blood homeostasis [128], inflammation [128,129], and cholesterol levels [130]. sEH^{-/-} mice have also been utilized to better understand the metabolic formation of toxic metabolites [131]. sEH^{-/-} mice, CYP2E1^{-/-} mice, and *in vitro* studies were used to gain a better understanding of the formation of cyanide from either methacrylonitrile or acrylonitrile, including additional enzyme contributions and understanding the observed sex differences in mice.

19.5.2.2 sEH Humanized (Knock-In) Mouse Model. To evaluate the impact of neuronal overexpression of sEH, Syn1-FLAG-sEH transgenic mice were generated by fusing the rat synapsin 1 promoter to an N-terminally FLAG-tagged cDNA for mouse sEH [132]. The fusion tag consisting of 8 amino acids (FLAG) epitope tag was used to differentiate between endogenous sEH and transgenic sEH. Four transgenic founders were established and two of the founders were successfully bred to create the transgenic lines. Western blots using antibodies against sEH and the FLAG epitope tag showed that the transgenic sEH was limited to the brains and testes of the transgenic mice. A total sEH western blot showed that the overall sEH total brain content was fivefold than that observed in the WT mice (nontransgenic littermates). This corresponded to a threefold increase in sEH activity in the transgenic brain homogenates, which demonstrated that the overexpressed protein was functional.

19.5.2.3 sEH Chimeric-HL Mouse Model. See Section 19.5.1.2 for details.

19.6 OXIDASES

19.6.1 Aldehyde Oxidase

Aldehyde oxidases (AOXs) are molybdoflavoenzymes related to xanthine oxidoreductase (XOR). Rodents have four AOX genes, *Aox1*, *Aoh1*, *Aoh2*, and *Aoh3*, which are located a short distance from each other on chromosome 1c1 in mice and chromosome

9 in rats [133]. Humans have a single functional gene, *AOX1*, and two inactive pseudogenes located on chromosome 2q [133,134]. AOX activity was highest in the livers of all mammals studied, but other organs such as lung, kidney, and small intestine also had activity [135–137]. However, in brain homogenates of preclinical species, AOX activity was very low or absent [138]. The AOX enzymes perform a broad range of metabolic reactions, oxidizing both aliphatic and aromatic aldehydes to their corresponding acids, as well as oxidizing nitrogen-containing heterocycles. A number of species and strain differences have been observed with respect to the AOX activities on a variety of substrates. Some substrates, such as famciclovir and quinoxaline, show metabolic differences between species, while others, such as methotrexate (MTX) and phthalazine, have similar profiles between species. These facts complicate the use of preclinical species for the prediction of human metabolism by AOX.

19.6.1.1 Aldehyde Oxidase Chimeric-HL Mice. To date, the only KO mouse model to be produced is a mouse deficient in *Aoh2*, an enzyme similar to human AOX, but not contained in the human genome [134]. However, the chimeric-HL mouse model reported by Tateno *et al.* [7] (Section 19.3.3), in which mouse livers were repopulated to a high degree with human hepatocytes, has been used in the study of the AOX-catalyzed metabolism of *N*¹-methylnicotinamide (NMN) [139]. In this study, NMN was chosen as a test substrate because of differences observed in the metabolic profiles of humans and normal mice. Nicotinamide is converted to NMN by nicotinamide *N*-methyl transferase, and the subsequent metabolism to *N*¹-methyl-2-pyridone-5-carboxamide (2-PY) and *N*¹-methyl-4-pyridone-3-carboxamide (4-PY) is catalyzed by AOX. The metabolism of NMN is species dependent, with humans forming primarily 2-PY, and mice forming 2-PY and 4-PY in equal abundance [140]. The *in vivo* metabolism of NMN was monitored by measuring NMN, 2-PY, and 4-PY ratios (the RP value) [141] in the urine of chimeric-HL and control mice. The RP value in urine increased as the human hepatocyte RI of the chimeric-HL mice increased. Also, in chimeric-HL mice, the ratio of urinary 2-PY/4-PY increased as RI increased, while in control mice, the amounts of each metabolite were approximately the same. A similar result was also observed *in vitro* by measuring the NMN oxidase activity of liver cytosols produced from chimeric-HL mice, with the 2-PY/4-PY ratio tracking well with the RI. At 80–90% RI, the 2-PY/4-PY ratio in chimeric-HL mice was similar to that observed in pooled human liver cytosol. These results suggest that chimeric-HL mice could be a useful model for AOX-mediated drug metabolism, although the source of the human hepatocytes used for repopulation should be considered due to interindividual variations in human AOX activity [142–144].

19.6.2 Monoamine Oxidases

Amine-containing compounds are ubiquitous in nature, being commonly found in foods and beverages (tryptamine and trimethylamine) [145]; as environmental contaminants (allylamine); in drugs such as sumatriptan and citalopram; in addition to endogenous amines such as the neurotransmitters dopamine (DA), norepinephrine (NE), and serotonin (5-HT). The detoxification and regulation of amines, regardless of source, is critical to maintain normal biological function. In addition to metabolism by cytochrome P450s, amine-containing compounds can also be metabolized by the MAO enzymes. For those compounds that are substrates of MAO, the contribution of these

enzymes to metabolism and clearance can be significant. For example, MAO oxidation represents the primary clearance pathway for some members of the triptan class of antimigraine drugs, such as sumatriptan and rizatriptan. Additionally, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is metabolized primarily by MAO-B to the neurotoxic 1-methyl-4-phenylpyridinium (MPP⁺) species, which destroys nigrostriatal dopaminergic neurons [162–164].

MAO is a flavoenzyme found on the mouse X chromosome [149–151]. In humans, it exists in two forms, MAO-A and MAO-B, comprising 527 and 520 amino acids, respectively, with 70% sequence identity [152]. MAO-A and MAO-B have similar X-ray crystal structures [153,154] but different substrate specificities and kinetic properties. For example, MAO-A has a preference for the substrates NE and 5-HT, and is inhibited by low concentrations of clorgyline, while MAO-B is more selective for β -phenylethylamine (PEA) and can be selectively inhibited by low doses of *l*-deprenyl [155].

Deficiencies in either MAO-A or -B have been linked to several different behavioral and developmental abnormalities in animals and humans. Reduced expression of MAO-A can cause mental retardation and increased aggression, as observed in the males of a Dutch family having a mutation in the *MAO-A* gene [156]. Norrie's disease, with symptoms including impaired hearing and blindness, has been linked to genetic defects in the *MAO-B* gene [157,158]. KO models for MAO-A, MAO-B, and MAO-A/B have all been described in the literature, and numerous studies investigating the behavioral characteristics of genetically altered MAO expression in mice have been reported.

19.6.2.1 MAO-A (Knock-Out) Mouse Models. A line of MAO-A KO mice were inadvertently generated by the insertion of a β -interferon (IFN- β) transgene into the gene encoding MAO-A [159,160] in one-cell C3H/HeJ mouse embryos, producing a Tg(H2-IFN- β)8 (Tg8) transgenic line. Abnormal behaviors, including fearfulness, trembling, aggression, and postural changes, were observed in these mice, and the abnormal behaviors varied with age. Pup brain concentrations of 5-HT increased up to ninefold, while those of NE increased twofold in pup and adult brains. MAO-A activity was found to have been abolished via use of 5-HT as a substrate, but no difference was observed in the activity of MAO-B on PEA. Furthermore, chronic administration of parachlorophenylalanine, a 5-HT synthesis inhibitor, reversed the abnormal behavioral traits of the Tg8 pups, but no change was observed with administration of the catecholamine synthesis inhibitor α -methylparatyrosine.

19.6.2.2 MAO-B (Knock-Out) Models. MAO-B KO mice were produced by insertion of a neomycin resistance gene into exon 6. This generated a stop codon in the *MAO-B* gene, resulting in a truncated and inactive enzyme [161]. Injection of selected ES cells into mouse blastocytes derived from male 129/Sv animals resulted in chimeric animals lacking the WT MAO-B allele. Germline transmission was demonstrated by PCR in 90% of the progeny. The effects of MAO-B deficiency did not yield any statistical difference in brain concentrations of the neurotransmitters 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine, NE, or 3-methoxy-4-hydroxy-phenylglycol between WT and MAO-B-deficient mice. However, urinary and brain concentrations of PEA were approximately eightfold higher in the latter mice. It has been shown previously that the cyclic tertiary amine MPTP is converted to the toxic

metabolite MPP⁺ by MAO-B [162–164], leading to destruction of dopaminergic neurons in the midbrain with symptoms similar to Parkinson's disease. In WT mice, MPTP toxicity produced the depletion of DA and DOPAC, while MAO-B-deficient mice in this study did not suffer similar effects. These observations suggest that this line of MAO-B KO mice may have utility in drug metabolism studies of MAO substrates.

19.6.2.3 MAO-A/B (Double Knock-Out) Models. MAO-A/B double KO mice were produced by a spontaneous point mutation in a colony of MAO-B KO mice [165]. The mutation in exon 8 (A863T) introduced a premature stop codon in the gene for MAO-A in the progenitor mouse, which was propagated by selective breeding. The resulting MAO-A/B double KO mice exhibited a phenotype nontypical of the MAO-B single KO mice, including low body weight and exaggerated starting reactions to normal stimuli. Brain concentrations of 5-HT, NE, DA, and PEA were increased, and 5-HIAA decreased, relative to either MAO-A or MAO-B single KO mice. In a battery of behavioral tests, the characteristics of these mice were also different from those of either MAO-A or MAO-B single KO mice. These differences were principally in the lack of exploratory activity in an unfamiliar open field and in postural attitudes suggestive of fear or anxiety not typical of either MAO-A or B mice. These mice provide an additional tool for the evaluation of drugs for which MAO-A/B metabolism may be important.

19.6.3 Alcohol and Aldehyde Dehydrogenases

Alcohols, most notably ethanol, are metabolized to the corresponding aldehydes by the enzymes alcohol dehydrogenase (ADH), in addition to metabolism by other enzymatic processes. ADH is a family of cytosolic zinc-containing enzymes. These enzymes are dimeric, and each subunit is ~40 kDa. Humans possess five forms of ADH, while mice have only three forms, corresponding to human ADH1, ADH3, and ADH4 [166]. ADH1 and ADH4 both have retinol and ethanol dehydrogenase activity [167–171], while ADH3 is primarily involved in the oxidation of *S*-hydroxymethylglutathione, a product of the reaction of formaldehyde with GSH [147,148].

The aldehydes resulting from ADH oxidation are potentially toxic, and further oxidation to the corresponding carboxylic acids facilitates their detoxification and clearance from the body. The enzymes primarily responsible for this oxidation are the aldehyde dehydrogenase (ALDH), a set of polymorphic enzymes expressed in numerous tissues in the body. The most important of these polymorphs for ethanol metabolism are cytosolic ALDH1 and mitochondrial ALDH2, based on their broad tissue distribution and low K_m values for acetaldehyde of 30 and 1 μM , respectively [146]. Both are tetrameric enzymes, composed of 54-kDa subunits. The precise role of most of the ALDH polymorphs in ethanol metabolism is unknown, but a mutation in ALDH2 in which glutamate is replaced with lysine at position 487, denoted *ALDH2**2 [172,173], has been associated with far higher acetaldehyde levels after consumption of ethanol than in individuals having the normal *ALDH2**1 allele. A large proportion of Japanese, Chinese, and Koreans lack ALDH2 activity due to this mutation, and consumption of alcohol in this population leads to a facial flushing reaction [174,175], which is associated with up to a 10-fold increase in acetaldehyde levels in comparison to individuals who do not exhibit facial flushing [176–178]. An overview of the roles

of ADH and ALDH in ethanol-related pathology has been reported by Crabb *et al.* [179].

19.6.3.1 ADH (Knock-Out) Models. A strain of deer mice (*Peromyscus maniculatus*) naturally deficient in ADH [180] has been used to study various aspects of ethanol metabolism [181,182]. In animals fed a 4% ethanol diet for 60 days, these studies demonstrated increased nonoxidative metabolism of ethanol to fatty acid ethyl esters in ADH^{-/-} versus WT animals, in addition to increased mortality and histological changes to the liver and pancreas.

Other scientists have described a gene KO model in which gene-targeting vectors for murine Adh1, Adh3, and Adh4 were separately introduced into ES cells [183]. Hetero- and homozygous offspring of mice with each mutation developed normally compared with WT mice. Six hours after administration of 3.5 g ethanol/kg body weight, an intoxicating dose, Adh1^{-/-} mice had blood ethanol concentrations of 0.30%, while Adh4^{-/-} mice had concentrations of 0.15%. WT and Adh3^{-/-} mice had blood ethanol concentrations of ~0.03%. Thus, Adh3 was not important for ethanol clearance, while Adh1 and Adh4 played a key role, with the former being more significant. The role of Adh3 in formaldehyde detoxification in these mice was then examined by IP injections of 10% formalin in saline at doses of 0.09–0.22 g/kg. Adh3^{-/-} mice had a formaldehyde LD₅₀ of 0.135 g/kg, where the lethal dose was defined as an amount of formaldehyde resulting in death within 90 min, whereas in WT mice, the LD₅₀ was 0.200 g/kg, a statistically significant difference. For Adh1^{-/-} and Adh4^{-/-} mice, the LD₅₀ values were 0.175 and 0.190 g/kg, respectively, not statistically different from WT mice, suggesting an important role for Adh3 in the clearance of formaldehyde. Mice of each genotype were then administered 100 µg/kg retinol or vehicle control, then retinoic acid (RA) levels were measured after 2 h in kidney homogenates. Vehicle-treated mice had undetectable levels of RA in kidney tissue, while retinol-treated WT, Adh1^{-/-}, and Adh4^{-/-} mice had RA levels of 273 ± 186, 57 ± 15, and 34 ± 16 pmol/g, respectively. Thus, Adh1 and Adh4 played an important role in the ability to metabolize retinol to RA. A second study in this line of Adh1, Adh3, and Adh4 KO mice was used to examine retinol metabolism in more detail [184]. In this study, liver concentrations of RA were measured 2 h after administration of 50 mg/kg all-*trans*-retinol. WT mice produced 2.0 mg/g RA, Adh1^{-/-} mice had a 10-fold reduction in RA, Adh3^{-/-} mice had a 3.8-fold reduction, and Adh4^{-/-} mice had an insignificant reduction in RA levels. These results suggest that Adh1 is mainly responsible for retinol metabolism, with a lesser role for Adh3.

19.6.3.2 ALDH KO Models. A line of Aldh2^{-/-} KO mice was produced by gene-targeting techniques in ES cells [185]. Male chimeras were mated with female C57BL/6 mice. Liver subcellular fractions were generated from these mice to investigate the ability to oxidize methoxyacetaldehyde (MALD) to methoxyacetic acid (MAA). No oxidation was observed in mitochondrial fractions, and very low activity was observed in cytosolic fractions as compared with activities using fractions derived from Aldh2WT mice, suggesting that ALDH2 is the key enzyme involved in the oxidation of MALD. This strain of mice has also been used to investigate the inhalation toxicity of acetaldehyde [186] with the conclusion that toxicity is greater in Aldh2^{-/-} mice and therefore that toxicity may also be greater in humans with low ALDH2 activity. A pathological study of acetaldehyde toxicity has also been

performed using this model [187]. Other investigators have used this strain to study the effects of subacute ethanol treatment on the survival rate and expression of Aldh1, Aldh2, Cyp1a1, Cyp2e1, and Cyp4b1 enzymes [187]. The authors conclude that mortality is higher in the Aldh2^{-/-} mice after ethanol treatment and that Cyp2e1 is inducible in this model after treatment as compared to Aldh2WT mice. Other application of the Aldh2^{-/-} mouse model include (i) the effect of ethanol on DNA and Cyp2e1 expression in liver using this strain [188] and (ii) formation of DNA adducts of *N*2-ethylidene-2'-deoxyguanosine as a product of ethanol exposure [189].

19.6.4 Flavin Monooxygenases

The FMO enzymes are a family of enzymes located in the endoplasmic reticulum, which catalyze the oxidation of nucleophilic heteroatoms such as nitrogen, sulfur, and phosphorus. Their substrate specificity overlaps with other DMEs, such as cytochrome P450s. There are five distinct *FMO* genes and six pseudogenes in humans [190–192], while mice have nine genes [191]. One or more FMO isoforms are expressed in almost all the various mammalian tissues examined. FMOs 1–5 have a primary amino acid sequence which is >50% identical, and the corresponding FMOs across species are more than 80% identical [193]. FMO1, FMO2, and FMO3 are the predominant xenobiotic-metabolizing enzymes among all the species studied. The level of expression of these isoforms is dependent on species, tissue, sex, and stage of development. In adult humans, FMO3 is the most abundant isoform found in liver, along with lesser amounts of FMO5, while in the lung of humans and most other species, FMO2 is dominant. Male rat liver contains abundant amounts of Fmo1, which is absent in adult human liver [194]. Sex differences have been observed in mice, with females producing Fmo1 and Fmo3 in equal amounts, whereas males did not express Fmo3, and Fmo1 was found at less than half the amounts in females. Fmo5 expression was low but equal in male and female mice [195,196].

19.6.4.1 FMO (Knock-Out) Mouse Model. Despite these differences from human FMO expression, a mouse FMO KO model has been reported to investigate the role of this enzyme in drug metabolism. The model was produced by Cre-loxP-mediated deletions of the mouse *Fmo1* gene and portions of the *Fmo2* and *Fmo4* genes [197,198]. It was used to investigate the contribution of FMO1 to interindividual variations in plasma concentrations (greater than 30-fold) [199,200] observed in patients taking the antidepressant drug imipramine. Imipramine is converted to an active metabolite, desipramine, by CYPs 1A2 and 3A4 [201] and is inactivated by hydroxylation via CYP2D6 [202] and N-oxygenation via FMO1 [203,204]. Following a 30 mg/kg IP injection of imipramine, WT mice appeared sedated and had impaired motor coordination within 15 min. The KO mice developed body tremors and spasm within 25 min, unlike the WT mice, suggesting differences in metabolism of the drug. The serum concentrations of imipramine at 20 min postdose were 2.7-fold higher in KO mice than in WT mice ($p < 0.001$). Imipramine-*N*-oxide was present in tissues of WT mice, but were absent in the KO mice, further demonstrating metabolic differences in this model.

19.7 ESTERASES

19.7.1 Cholinesterases

Cholinesterases comprise two sets of enzymes that hydrolyze esters of the neurotransmitter choline. AChE is selective for acetylcholine hydrolysis and functions to terminate nerve impulse conduction in the nerve synapse. BChE, also known as *pseudocholinesterase* or *nonspecific cholinesterase*, is active on a variety of ester substrates, such as succinylcholine and heroin [205], in addition to acetylcholine. AChE and BChE each have nonesterase activity as well, including hydrolysis of substance P [206,207], and the hydrolysis of enkephalins by AChE [208]. The two cholinesterases share ~50% sequence identity [209] and are found in numerous tissues, including muscle, nerve, blood, and heart [210]. In human and mouse brains, AChE activity predominates, in muscle, AChE and BChE activities are similar, but in plasma and other tissues of the body, BChE activity is greater than that of AChE [210,211].

Disruptions in either brain AChE activity or the levels of acetylcholine are observed in neurologic diseases such as Alzheimer's disease (AD) [212–218]. Additionally, acute exposure to organophosphate compounds, such as parathion or the nerve gas sarin, and carbamates such as the pesticide carbaryl, inhibit AChE and can produce seizures and death by respiratory arrest due to the overstimulation of cholinergic neurons [219,220]. Also, naturally occurring BChE deficiencies have been observed in the human population [221,222], contributing to phenomena such as prolonged apnea in patients receiving the muscle relaxant succinylcholine [223–225]. Inhibition of BChE by the bronchodilator bambuterol has been shown to decrease the clearance of another muscle relaxant, mivacurium, prolonging the duration of neuromuscular blockade by three- to fourfold [226]. In all the foregoing examples, AChE and/or BChE KO mice can be used as tools to further investigate the metabolism of compounds known to be substrates or inhibitors of these enzymes.

19.7.1.1 Acetylcholinesterase (Knock-Out) Mice. An AChE KO mouse (AChE^{-/-}) model was produced by gene-targeting techniques in which a targeting vector was introduced into R1 ES cells [227]. Five kilobase of the mouse *AChE* gene on exons 2–5 was deleted by this procedure, removing 93% of the amino acids comprising this gene. Mice produced from cell colonies having the desired gene deletion were mated with 129sv mice to produce offspring having the desired AChE KO. A previous study in *Drosophila* [228] in which AChE was deleted by targeted mutagenesis resulted in the death of the embryos. In the mouse KO, however, the pups survived on average 14 days after birth. In later studies, the average lifespan of the AChE KO mice could be extended to 60–80 days by feeding the dams a high fat diet during the nursing period, and feeding the pups a liquid high fiber diet after weaning. The surviving pups were smaller than their heterozygous AChE^{+/-} or WT littermates and had other phenotypic abnormalities such as lack of a righting response, sealed eyelids, and the development of a fine motor tremor three to four days after birth. The postnatal survival of these mice was proposed to be due to the action of intact BChE on acetylcholine [229], which could possibly compensate for the absence of AChE. Toxicity studies [227] with the nonselective esterase inhibitor diisopropylfluorophosphate (DFP) and the BChE-specific inhibitor

bambuterol demonstrated an increased sensitivity of these KO mice to DFP. A dose of DFP which was sublethal to WT mice killed AChE^{-/-} mice within 3 min and had an intermediate level of toxicity to AChE^{+/-} animals. The administration of bambuterol at 0.3 mg/kg had no effect on WT mice but killed AChE^{-/-} animals within 10 min.

The AChE^{-/-} mouse model was used to investigate the toxicity of the nerve agent *O*-ethyl-*S*-[2-(diisopropylamino)ethyl]methylphosphonothioate (VX) relative to WT mice [230]. VX is the most potent organophosphorus ester known and inhibits both AChE and BChE. It is poorly reactive with carboxylesterase, however, which in rodents is in great excess over the cholinesterases. The AChE^{-/-} mice were observed to be more sensitive to VX than WT mice, with an LD₅₀ of 10–12 µg/kg, approximately one half that of normal mice. These and other observations by the authors led to the conclusion that VX toxicity is due at least in part to the inhibition of targets other than AChE.

In severe AD, AChE activity is greatly reduced, while BChE activity in the brain may remain unchanged or even increase [216,231,232]. One proposed therapeutic approach in AD treatment is to use BChE inhibitors in an effort to increase brain ACh levels. Naik *et al.* [232] have used these AChE KO mice to investigate the effect of the AChE-inhibiting drugs rivastigmine and donepezil on extracellular concentrations of ACh in the hippocampus of AChE^{-/-} mice. ACh levels were 30-fold higher in AChE^{-/-} mice than in WT mice before drug administration. After administration through a microdialysis probe of 1 µM rivastigmine, an inhibitor of both AChE and BChE, ACh levels increased a further twofold within 90–120 min. In contrast, administration of 1 µM donepezil, a selective inhibitor of AChE, did not produce any further increase in hippocampal ACh levels in AChE^{-/-} mice, although in WT mice, it increased ACh levels more than twofold. The authors concluded that inhibition of BChE by rivastigmine led to a significant increase in ACh levels and may be an important observation relevant to the treatment of AD.

19.7.1.2 Butyrylcholinesterase (Knock-Out) Mouse Model. BChE KO mice were produced by gene-targeting techniques in which 891 bp were deleted from the *BCHE* gene [233]. Propagation of the line was effected by breeding BChE^{+/-} mice to produce BChE^{+/+}, ^{+/-}, and ^{-/-} littermates. BChE^{-/-} mice have no differences in appearance, weight gain, reflexes, or fertility relative to WT mice [234]. In this regard, these mice are proposed as a model for human BChE deficiency, since humans with “silent” BChE, caused by various mutations in the *BCHE* gene, appear normal until challenged with a BChE substrate, such as succinylcholine [235–237]. Tolerant to the AChE-specific inhibitors donepezil and (–)-huperzine A was investigated in both AChE-deficient and BChE-deficient mice [230]. A dose of donepezil, which was non-lethal to WT mice, was toxic to BChE^{+/-} and ^{-/-} mice, with the greatest toxicity evident for the ^{-/-} mice. AChE^{-/-} mice, however, did not exhibit any toxic symptoms. Following a nonlethal dose of (–)-huperzine A to WT, ^{+/-}, and ^{-/-} mice, similar results were observed, with toxic symptoms increasing for BChE^{+/-} and ^{-/-} mice, and, as with donepezil, AChE^{-/-} mice did not exhibit any toxicity. These results indicated a role for BChE in the hydrolysis of acetylcholine *in vivo*.

BChE^{-/-} mice were used to investigate the toxicity of cocaine in the absence of this esterase [238]. In mice, cocaine is inactivated by BChE and carboxylesterase, while humans detoxify 90% of the drug via BChE [239]. Human toxicity of cocaine is not well correlated to dose or blood concentrations, and it has been suggested

that low BChE levels due to natural genetic variations may be involved [240]. In this study, cocaine or saline vehicle was administered to BChE $+/+$, $+/-$, and $-/-$ mice at 20 mg/kg for seven days before sacrifice. Liver morphology was then assessed microscopically. Changes were observed to the greatest extent in BChE $-/-$ mice, which displayed lymphocytic infiltration, hepatocellular dropout, and regeneration. Progressively less pathology was observed in the livers of BChE $+/-$ and $+/+$ mice, respectively. No differences in cardiac function were observed between BChE $+/+$ and $-/-$ mice coadministered a single 100 mg/kg dose of cocaine and 2 mg/kg CBDP, a carboxylesterase inhibitor. However, respiration rates were decreased significantly in BChE $-/-$ mice, and remained lower through 12 h postdose, while respiratory rates in WT mice returned to normal after 30 min. All BChE $-/-$ mice also exhibited apneusis (termination of breathing at end-inspiration), while none of the BChE $+/+$ mice presented this symptom. The authors therefore concluded that the observed hepatotoxicity of cocaine and the respiratory effects were due to the absence of BChE in the deficient mice and suggest that natural mutations in BChE may potentiate the effects of cocaine and possibly other drugs, in human beings. The authors suggest the use of this model for the determination of other drug sensitivities in patients with BChE deficiencies.

19.8 UDP-GLUCURONYL TRANSFERASE

UGTs are a family of enzymes that catalyze the glucuronidation of endogenous compounds and xenobiotics utilizing the cofactor UDP-glucuronic acid (UDPGA). UGTs are separated into two major subfamilies based on amino acid sequence similarities [241–243]. Members within a single UGT subfamily exhibit greater than 60% sequence similarity. The differences in the UGT1 family are confined to the N-terminus, with all members of this family having an identical 246 amino acid carboxy terminus. In contrast, the differences in the UGT2 family are observed throughout the protein [241–243].

UGTs are membrane-bound enzymes located in the endoplasmic reticulum [244]. They are found primarily in the liver and are also present in other tissues, including intestine, kidney, skin, lung, and brain [245–247]. UGTs catalyze the formation of a wide variety of glucuronide conjugates, including oxygen, nitrogen, sulfur, and even carbon glucuronides [245]. Glucuronide conjugates are primarily excreted in the urine. However, biliary excretion may become an important route of elimination when the molecular weight of the glucuronide conjugate exceeds 300 Da [248]. Glucuronide conjugates excreted in the bile may be hydrolyzed to their unconjugated forms via β -glucuronidase enzymes present in the intestines. These unconjugated products may subsequently be reabsorbed in the intestines, thus undergoing enterohepatic recirculations [249]. Glucuronidation catalyzed by the UGTs is a major pathway for drug metabolism and elimination in humans. The human UGT enzymes most commonly listed in catalyzing drug glucuronidation are UGT1A1, UGT1A4, and UGT2B7 [250].

19.8.1 UGT1A (Knock-Out) Mouse Model

Genetic polymorphism of some UGT isoforms have been reported, most notably for UGT1A1. Many mutated alleles of UGT1A1 have been identified and some mutations

leading to decreases in UGT1A1 enzymatic activity have been associated with Crigler-Najjar syndrome and Gilbert's syndrome in humans [246]. Crigler-Najjar syndrome and Gilbert's syndrome are the result of unconjugated bilirubin. Crigler-Najjar syndrome type 1 is diagnosed as a complete lack of bilirubin glucuronidation and is often fatal.

In a study by Nguyen *et al.* [251], the physiological importance of the Ugt1 locus in mice was evaluated via the creation of Ugt1 KO (Ugt1^{-/-}) mice. Ugt1^{-/-} mice were developed via an interruption to exon 4 in the Ugt1 locus in C57BL/6 mouse blastocysts. Male chimeras were then backcrossed into C57BL/6 females to generate heterozygous founders that were bred to develop Ugt1^{-/-} mice. In mouse liver, Ugt1a1, Ugt1a5, Ugt1a6, and Ugt1a9 are the predominant forms of Ugt1a [247]. There were no detectable levels of UGT1A RNA or UGT1A proteins in liver microsomes from Ugt1^{-/-} mice. However, cross-reactive UGT2 proteins were detected via immunoblot in the Ugt1^{-/-}, Ugt1^{+/-} heterozygous, and WT mouse livers using a human anti-2B7 antibody. UGT1A enzymatic activity in Ugt1^{-/-} mouse liver microsomes was evaluated using the probe substrates bilirubin and thyroxine. Neither bilirubin nor thyroxine conjugation was observed in Ugt^{-/-} mice microsomes. The thyroxine activity in Ugt^{+/-} mice was 50% that of WT mice in microsomes. In contrast, testosterone metabolism (which was utilized as a UGT2B probe substrate based on activity data in monkey [252] and humans [253]) was similar for the Ugt1^{-/-}, Ugt^{+/-}, and WT mice. These findings suggest the interruption to exon 4 in the Ugt1 locus affected UGT1A but did not affect the formation or enzymatic activity of UGT2B *in vivo*, the newborn Ugt1^{-/-} mice developed serum levels of unconjugated bilirubin that were 40–60 times higher than Ugt 1^{+/-} heterozygous mice or WT mice, respectively. The highly elevated levels of unconjugated bilirubin observed in the Ugt1^{-/-} mice were consistent with the values observed in humans with Crigler-Najjar type 1 syndrome. Extreme diagnostic jaundice due to elevated bilirubin levels was observed in the skin of Ugt1^{-/-} mice within 8 h. However, at five days old, thyroxine levels in the neonates were similar between Ugt^{-/-} and Ugt^{+/-}, indicating more complex mechanisms *in vivo* than simply UGT1A activity for thyroid hormones. Overall, the interruption of the Ugt1 locus was a lethal mutation with all Ugt1^{-/-} mice dying within two weeks of birth.

19.8.2 UGT1A Humanized (Knock-In) Mouse Model

To establish the transgenic UGT1 knock-in mice (Tg-UGT1 mice) [254], a purified BAC encoding the entire human UGT1 locus was microinjected into the pronucleus of CB6F₁ (a F1 hybrid between BALB/c and C57BL/6 N mice) mouse eggs and transplanted into pseudopregnant C57BL/6 N mice. The subsequent offspring were genotyped and five founders containing the entire UGT1 locus were bred into C57BL/6 N mice. The insertion of the DNA did not affect fertility and there were no gross pathological differences from the WT littermates. It should be noted that the addition of the human UGT1 was on a background of mouse UGTs.

Microsomes were prepared from the liver and large and small intestines of the Tg-UGT1 mice. Using antibodies for specific human UGTs, UGT1A1, UGT1A4, and UGT1A6, expression levels were determined for the Tg-UGT1 mice and compared to both human liver microsomes and expressed UGTs prepared from SF-9 insect cells. UGT1A1 was found to be expressed in both the small and large intestines (being more abundant in the small intestines). UGT1A4 was also observed in the small intestines at similar levels to UGT1A1. In contrast to UGT1A1 and UGT1A4, UGT1A6 was

difficult to detect in the small intestines but was observed in the colon. In the liver, there was limited expression of UGT1A1 and UGT1A4 but UGT1A6 was observed at levels similar to human microsomes. There were, however, differences in expression levels across the Tg-UGT1 mice examined. *UGT1A* gene expression was also evaluated across multiple tissues (liver, brain, stomach, small intestines, colon, kidney, and heart). UGT1A10 was found to be exclusively expressed in nonhepatic tissues. UGT1A6 and UGT1A9 were the predominant forms identified in the kidney. Induction patterns were evaluated in the Tg-UGT1 mice (in comparison to the WT mice) with activity evaluated using probe substrates. UGT induction by pregnenolone-16 α -carbonitrile (PCN) and TCDD was observed in the Tg-UGT1 mice and appeared to be dependent on glucocorticoids.

The expression levels of human UGT1 isozymes and the induction profiles suggest that this model will be useful to examine the functional and regulatory properties of human UGT1. This model has subsequently been utilized to understand the impact of a peroxisome-proliferator-activated receptor (PPAR) agonist [255,256], an Ah receptor agonist (chrysin) [257], liver-X-receptor (LXR α) agonists [258], and Nrf2-Keap-dependent UGT1A1 induction by prooxidants [259] in Tg-UGT1 mice. A UGT1A knock-in model has also been developed on a background of UGT1 $-/-$ mice thus eliminating the mouse background UGT1 levels (Section 19.8.3).

19.8.3 UGT1A (Combined Knock-In/Knock-Out) Mouse Models

A transgenic UGT1 knock-in model developed on a background of Ugt1 $-/-$ mice was developed by inserting human BAC encoding either normal (UGT1A1*1) or containing the mutation associated with Gilbert's syndrome (UGT1A1*28) into mice as transgenes [260]. Then, the knock-in transgenic mice were crossed with the heterozygous Ugt1 $+/-$ mice creating mice carrying the UGT transgene in a Ugt1 $+/-$ background. These mice were further interbred to establish Tg(UGT1A1*1)Ugt1 $-/-$ and Tg(UGT1A1*28)Ugt1 $-/-$ mice. The Tg(UGT1A1*28)Ugt1 $-/-$ mice encoded all nine of the UGT1A isozymes (UGTs1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10), while the Tg(UGT1A1*1)Ugt1 $-/-$ mice were missing the *UGT1A8* gene.

The absence of UGT1A1 in the Ugt1 $-/-$ mice results in death within two weeks due to bilirubin toxicity [251]; however, the insertion of the human *UGT1A1* gene into the Ugt1 $-/-$ mice results in survival of the mice. This confirms the importance of UGT1A1 in the early developmental stages of bilirubin metabolism. In the less severe Gilbert's syndrome in humans, the promoter variant linked to the *UGT1A1**28 gene is hypothesized to result in reduced UGT1A1-RNA and protein in the liver [261,262], thus resulting in decreased bilirubin conjugation and mild hyperbilirubinemia. This hypothesized difference was observed in the Tg(UGT1A1*28)Ugt1 $-/-$ mice when compared to the Tg(UGT1A1*1)Ugt1 $-/-$ mice. The RNA levels for the liver-specific expression of UGT1A3, -1A4, -1A6, and -1A9 were similar for both transgenic mice, however, UGT1A1 RNA expression was more than sevenfold higher in the Tg(UGT1A1*1)Ugt1 $-/-$ mice when compared to the Tg(UGT1A1*28)Ugt1 $-/-$ mice. In contrast, expression levels of UGT1A1 in extrahepatic tissues (such as the small intestines) were similar between the Tg(UGT1A1*1)Ugt1 $-/-$ and the Tg(UGT1A1*28)Ugt1 $-/-$ mice showing the promoter polymorphism altered expression of UGT1A1 only in hepatic tissue. It was noted that the drop in total bilirubin during development mirrored the intestinal rather than the hepatic expression levels.

The impact of artificially increased total bilirubin was also evaluated in the transgenic mice utilizing phenylhydrazine to induce hydrolysis and spontaneously increase total bilirubin levels. The differential expression of UGT1A1 in the liver and small intestines of Tg(UGT1A1*28)Ugt1^{-/-} mice emphasizes the importance of understanding which organs are hypothesized to be affected in transgenic mice and to confirm expression levels of the enzyme of interest in multiple tissues of interest as expression levels could differ and lead to confounding results unless it is understood *a priori*.

Tg(UGT1A1*28)Ugt1^{-/-} mice have also been evaluated as a model to improve predictions of human UGT1A-dependent drug clearance in human subjects [263]. Pharmacokinetic and enzyme kinetic parameters were evaluated for three probe substrates in both Tg(UGT1A1*28)Ugt1^{-/-} [with and with phenobarbital (PB) treatment] and WT mice. The substrates evaluated were SN-38 (an exclusive UGT1A1 substrate [264]), ezetimibe (a partial UGT1A1 substrate [265]), and naloxone (a UGT2B7 substrate [266]). The UGT1A1 substrate SN-38 showed the largest difference in both drug exposure and clearance between the Tg(UGT1A1*28)Ugt1^{-/-} mice, Tg(UGT1A1*28)Ugt1^{-/-} mice treated with PB, and WT mice. The clearance of the UGT2B7 substrate (naloxone) was unaltered in the Tg(UGT1A1*28)Ugt1^{-/-} mice, which was as expected. Surprisingly, the pharmacokinetic parameters with ezetimibe showed small to no difference in the Tg(UGT1A1*28)Ugt1^{-/-} mice. The *in vivo* findings for SN-38, naloxone, and ezetimibe were consistent with the enzyme's kinetic parameters determined *in vitro* in WT, Tg(UGT1A1*28)Ugt1^{-/-}, and Tg(UGT1A1*28)Ugt1^{-/-} (treated with PB) mouse liver microsomes. They were also consistent with the enzyme's kinetic parameters determined using human liver microsomes without and homozygous for the UGT1A1*28 polymorphism. These findings suggest that glucuronidation by UGT1A1 may not play a major role in the overall metabolism of ezetimibe.

19.8.4 UGT Chimeric-HL Mouse Model

Chimeric-HL mice were developed using uPA/SCID mice [7], as previously mentioned in Section 19.3.3. These mice have been evaluated for their capacity to metabolize drugs catalyzed by either phase I [75] or phase II [267] enzyme systems. The UGT enzyme family was evaluated along with the other major phase II enzyme systems (SULT, NAT, and GSTs) in the livers of the chimeric-HL mice by mRNA, protein, and enzyme activity using RT-PCR, western blot analysis, and high performance liquid chromatography [267].

The mRNA expression of the evaluated UGT isozymes (UGT1A1, UGT1A9, and UGT2B7) increased in a concentration-dependent manner along with the positive control (human albumin). Further evaluation of the protein content for UGT1A1 and UGT2B7 also showed the specific UGT protein levels increasing in a concentration-dependent manner along with the human albumin. Since there are currently no specific substrates for UGT1A1, the specific enzyme activity of human UGT1A1 could not be measured in the chimeric-HL mice. However, UGT2B7 activity was measured using the probe substrate morphine and monitoring the formation of the 3- and 6-glucuronide metabolites. The ratios of UGT activity toward 3- and 6-glucuronidation in liver microsomes from mice, rats, guinea pigs, and rabbits has been shown to differ across species [268], thus indicating that this metabolic pathway may be of use in defining the human/mice enzymatic activity for UGT2B7 in the chimeric mice. In this

study evaluating the chimeric mice-HL [267], the morphine 6-glucuronidation activity in human liver microsomes was 36-fold higher than that of control mice. The UGT2B7 protein and morphine 6-glucuronidation activity in one chimeric mouse were 2.6- and 3.0-fold higher, respectively, than the corresponding protein content and activity of a human donor.

In addition, chimerical-HL mice developed using immunodeficient Fah-deficient mice repopulated with human hepatocytes have also been developed (Grompe mice) [8]. After pretreatment with a urokinase-expressing adenovirus, these mice were engrafted (up to 90%) with human hepatocytes. RT-PCR was performed on mRNA obtained from the chimeric-HL mouse livers and human UGT1A1 was evaluated and found to be abundantly expressed.

These results indicate the potential for chimeric-HL mice to be used for the study of human glucuronidation (with the most detailed information available for UGT2B7). However, additional characterization work using the chimeric-HL mice of interest should be conducted before specific use.

19.8.5 Other Mouse Models Affecting UGT (Brief Overview)

Transgenic animals developed to evaluate systems in addition to direct UGT models (UGT-null and knock-in models) have been shown to affect UGT activity. Specifically transgenic animals affect transcription factor-mediated UGT regulation such as nuclear hormone receptors, the AHR, and nuclear factor erythroid 2-related factor 2 (Nrf2) [259]. The nuclear hormone receptors and AhR models are covered in Section 19.10. This interconnectivity of receptors and enzyme systems bring forward an important point with regard to transgenic animals. Understanding downstream impacts of transgenic modifications and the impact on other systems is key to fully understanding the results obtained from transgenic animals.

19.9 SULFOTRANSFERASE

Sulfation is a conjugation reaction that serves to increase the polarity of molecules, enhancing the body's ability to excrete them. It is generally associated with the detoxification of xenobiotics, but it is also an important mechanism for the detoxification, regulation, or activation of many endogenous compounds, such as thyroid hormones and steroids [269]. Some compounds, such as morphine and minoxidil, exhibit enhanced pharmacological activity after sulfation [270–272], and it can also be a pathway to the bioactivation of some xenobiotics [273–279]. The enzymatic sulfation of -OH and -NH₂ groups of endogenous compounds or xenobiotics is accomplished by the action of cytosolic sulfotransferase (SULT) enzymes, a large gene family containing at least 11 subfamilies in humans [280]. All the SULTs require 5'-phosphoadenosine-3'-phosphosulfate (PAPS) as a cofactor supplying the sulfonyl moiety. The different SULT enzymes are expressed to varying levels in numerous tissues of the body. In humans, the most important SULTs for drug metabolism are SULT1A1, SULT1A3/4, SULT1B1, SULT1E1, and SULT2A1 [281]. SULT1A1 and 1B1 are active on a range of different substrates, but 1B1 has a higher K_m for most substrates [282,283]. SULT1A3 has a high selectivity for catecholamine substrates in primates [284,285]. SULT1E1,

also known as *estrogen-specific sulfotransferase* (EST), and SULT2A1 are selective for steroids, but also act on other xenobiotic compounds [286–288].

19.9.1 Sulfotransferase (Knock-Out) Mouse Model

SULT1E1 is important in the regulation of estrogen (17 β -estradiol), having an affinity for the substrate in the low nanomolar range. Sulfation of estrogen inhibits its binding to the estrogen receptor (ER), thus inactivating it. A line of SULT1E1 KO mice (SULT1E1 $-/-$) was generated by gene-targeting techniques [289]. Heterozygous chimeric mice from transfected ES cells were cross-bred to produce homozygous SULT1E1 $-/-$ -deficient mice. The absence of SULT1E1 activity was verified by northern and western blot and estrogen sulfotransferase activity assays. This line was used to investigate the reduced fertility observed in SULT1E1 $-/-$ mice [290]. The authors were able to demonstrate that the abnormally high estrogen levels associated with the absence of SULT1E1 not only impaired ovulation but also reduced the expression of COX-2. Administration of human chorionic gonadotropin improved the expression of COX-2, stimulating normal ovulation.

19.9.2 Natural Sulfotransferase-Deficient Animal Model

Brachymorphic mice are genetically deficient in the ability to synthesize PAPS due to reduced activity of ATP kinase [291]. These mice have been used to investigate the bioactivation of safrole in mouse liver [292]. An initial breeding stock of male brachymorphic mice was bred to B6C3F₁ females. Successive generations of mice were bred similarly, and bm/bm progeny were phenotyped by their shortened stature and dome-shaped skulls. After administration of [³H]-1'-hydroxysafrole to brachymorphic and normal mice, brachymorphic mice had 8–15% of the amount of liver DNA- and RNA-bound adducts as compared to their normal littermates. On the basis of the reduced incidence of hepatocarcinomas in the brachymorphic mice, the authors concluded that 1'-sulfooxysafrole is the ultimate electrophilic metabolite of 1'-hydroxysafrole. Other researchers have used this mouse model to investigate the activation of *N*-nitrosomethyl(2-hydroxyethyl)amine to a DNA alkylating agent [293], concluding that a sulfate intermediate is at least partially responsible for the observed carcinogenicity.

19.10 NUCLEAR HORMONE RECEPTOR (PXR, CAR, FXR, AND PPAR)

PXR, CAR, PPAR, and FXR were identified as novel orphan nuclear receptors of the nuclear receptor superfamily of ligand-activated transcription factors. These receptors function by forming a heterodimer with the retinoid X receptor α (RXR α , NR2B1). The nuclear receptor/RXR/ligand subsequently binds to a transcriptional response elements in the 5'-flanking region of the targeted gene resulting in the transcriptional activation [294–298]. The transcriptional factor/RXR/ligand complex works in concert to regulate the expression of specific genes that control the differentiation, development, homeostasis, and metabolism of the organism [295].

Various transcriptional factors regulate phase I and phase II metabolic enzymes and transporters that play a role in ADME of xenobiotics and endobiotics [299]. The development of GEMA can provide an *in vivo* tool to investigate the role of transcriptional

factors in the regulation of DMEs and transporters. Also, the GEMAs can facilitate the elucidation of receptor-independent/dependent pathways of drug-induced metabolism, toxicities, and role in mammalian development.

19.10.1 Pregnane X Receptor (PXR) (Knock-Out) Mouse Model

Two laboratories have generated mice lacking functional PXR [227,300] by disrupting the mouse *PXR* gene. The PXR KO/null mouse (*PXR*^{-/-}) developed by Staudinger *et al.* [300] replaced the first coding exon (amino acids 1–63) with a neo resistance cassette by homologous recombination, which resulted in a *PXR* transcript ~200 bp shorter than the native transcript. This resulted in a nonfunctional *PXR* gene response. The laboratory of Xie *et al.* [227] developed a *PXR*^{-/-} mouse by deleting two exons (amino acid residues from 63 to 170 of the DNA-binding domain), generating a nonfunctional *PXR* gene. The *PXR*^{-/-} mice generated by these two methods were viable and fertile with no overt biochemical alterations in levels of cholesterol, steroid hormones, triglycerides, albumin, free bilirubin, and bile acids. More recently, it was found that PXR activity is required for normal femoral bone mineral density development as its absence manifests in a phenotype known as *hypophosphatemia* [301], based on studies conducted in *PXR*^{-/-} mouse. In addition, as discussed briefly above, the *PXR*^{-/-} animal model has been utilized to confirm the role of PXR in the expression of DMEs such as cytochrome P450s, UDPGA glucuronyl transferases (UDPGTs), sulfotransferases, PAPS, and drug transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), organic anion polypeptide transporter (Oatp2) [300,302–306]. Additionally, it was assumed that PCN induced the activity of Cyp3a11 via PXR activation; however, CYP3a11 mRNA was increased ~fourfold in the *PXR*^{-/-} mice compared to WT mice suggesting that PXR may actually play a repressive role [300]. However, the *PXR*^{-/-} mouse generated by Xie *et al.* [227] exhibited a different response phenotype in which it did not respond to PCN and the basal levels of CYP3a11 were not higher than that in the WT mice. Thus, slight changes in the removal of PXR amino acid sequences appear to lead to varying gene regulations in response to inducers.

The effects of PCN via PXR activation do extend beyond xenobiotic-metabolizing enzymes in which CYPs involved in cholesterol and bile acid formation are modulated such as CYP7a1 [300]. It has been shown that PCN exposure can reduce the activity of Cyp7a1, the enzyme involved in the metabolism of cholesterol to bile acids. The role of PXR in regulating Cyp7a1 has been elucidated via studies conducted in the *PXR*^{-/-} mice in which KO animals showed a twofold less basal activity and were refractory to PCN treatment compared to WT animals [300]. In addition to modulating enzyme activity, PXR can activate the transporter Oatp2, which transports bile acids, and is inducible by PCN. Interestingly, Staudinger *et al.* [300] have shown that bile acids can directly upregulate PXR, specifically. PXR is activated by lithocholic acid (LCA), a toxic secondary bile acid associated with cholestasis. LCA via PXR can stimulate the expression of Cyp3a11 and Oatp2, which then work in concert to rid excess bile salts from the body [227,300]. In these studies, PXR is required for the expression of CYPs (Cyp7a1 cited as an example) and can provide a protective mechanism to modulate LCA-induced toxicities.

PXR as a drug target has been proposed to enhance circulating levels of the anti-cancer drug paclitaxel [307]. Paclitaxel, a microtubule-binding drug, can activate both mouse and human PXR, resulting in the activation of DMEs and drug transporters

at the transcriptional level to accelerate its own metabolism following chronic dosing [308,309]. In the WT mouse, chronic dosing of paclitaxel resulted in a 31% and 59% decrease in AUC and $C_{\max}$, respectively, on day 5 compared to day 1 consistent with PXR-mediated induction [310]. In contrast, paclitaxel administered to PXR $-/-$ mouse showed an increase in drug exposure with chronic dosing. In humans, ketoconazole has been shown to modulate paclitaxel biotransformation by inhibiting CYP3A4 and CYP2C8 activity as well as by inhibiting MDR1-related transport [307]. More recently, it has been shown that ketoconazole can inhibit PXR activation [308]. It has been proposed that ketoconazole binds to the outside of the PXR ligand-binding pocket based on mutagenesis studies conducted in the AF-2 region of PXR. Consequently, ketoconazole appears to repress the activity of PXR, which in turn can alter the metabolic enzyme and transport activities dependent on this transcriptional factor.

19.10.1.1 PXR Humanized (Knock-In/Knock-Out) Mouse Models. Species differences in CYP induction in response to various inducing agents have been well documented. Comparison of PXR sequences from different species (mouse, rat, rabbit, and human) shows ~95% similarity in their DNA-binding domains; however, it shares only 80% amino acid similarity in the ligand-binding domain (LBD) [311]. In human and rabbits, PXR is sensitive to Rif pretreatment but is insensitive in mouse and rat, while PCN can induce CYP3A activity in rat and rabbit and modestly in mouse but has no effect in humans [311]. The utility of WT and PXR $-/-$ mouse models have provided insight toward the potential of ligands to induce PXR, however, extrapolation to humans is limited due to species differences in response to drugs such as Rif, clotrimazole, PB, and troglitazone [311]. Because of the species differences in response to PXR ligands, a humanized PXR mouse model provides a more appropriate *in vivo* model to identify and evaluate potential human hPXR Pxr $-/-$ inducers. Several humanized PXR mouse models have been reported. The two models are Alb-hPXR and BAC-hPXR [227,312] in which human PXR gene is expressed in the absence of the mouse Pxr. The activity of these humanized PXR animal models were confirmed by treatment with Rif and PCN. The humanized animal model responded to Rif pretreatment and did not respond to PCN. The humanize PXR model provides a tool to anticipate potential drug interactions involving PXR, minimizing untoward reduction in drug efficacy as a result of enhanced metabolic or transport clearance of the drug. Also, such animal models can identify ligands that may repress PXR activity resulting in increased toxicity risks due to elevated drug levels [313]. In summary, the humanized PXR mouse models provide researchers with an *in vivo* tool to elucidate the role of PXR in many disease states and modulation of xenobiotic DMEs and transporters.

19.10.2 CAR

The nuclear CAR has been shown to increase and decrease the regulation of genes sensitive to PB [314–316]. Similar to PXR, CAR plays a role in the regulation of xenobiotic drug metabolism enzymes and transporters. In response to PB exposure, CAR translocates to the nucleus, forms a heterodimer with the RXR, and activates the 51-bp PB-responsive enhancer module conserved in the mouse, rat, and human CYP2B genes [314,317–320]. Drug-metabolizing and transporter proteins modulated by PB are CYP1A2, CYP2B1, CYP3A, CYP4A10, CYP2E1, UGT1A1, GST, MRP2, BCRP, and OATP1B1, and suppresses CYP2E1 [315,321–323].

19.10.2.1 CAR (Knock-Out) Mouse Model. On the basis of cDNA microarray analysis, CAR appears to play both an activating and repressing role in the regulation of DMEs. For example, Cyp2b1 was induced by PB in the WT mice and induction was absent in CAR-null mice [316]. However, Ueda *et al.* [316] found that CYP4a10 is induced by PB in the null CAR mouse compared to WT suggesting that the presence of CAR represses protein expression in the presence of an inducer. In addition to elucidating the role of CAR in drug clearance, the CAR-null mice model has helped identify proteins involved in drug-induced hepatotoxicities. For example, APAP (250 mg/kg)-related hepatotoxicity was observed in WT mice pretreated with 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), a CAR agonist, while CAR-null mice showed no such hepatotoxicities [323]. It was found that TCPOBOP treatment modulated proteins involved in APAP-induced toxicity such as Cyp2e1, Cyp1a2, Cyp3a11, and GST. Specifically, TCPOBOP modestly suppressed Cyp2e1 mRNA levels but induced Cyp1a2, Cyp3a11, and GST mRNAs in WT animals, while these genes were not altered in the CAR-null mice.

Additionally, the CAR-null mice have helped elucidate enzymes involved in physiological regulation of bilirubin homeostasis. Five enzymes have been identified to mediate the *in vivo* levels of bilirubin [324]. In systemic circulation, bilirubin is noncovalently bound to hAlb. It has been postulated that only free bilirubin enters hepatocytes by a facilitated diffusion mechanism likely involving hepatic uptake transporters as organic anion polypeptide transporter 1B1. Once bilirubin is inside the hepatocyte, it readily binds to GST (A1 or A2), which reduces its efflux from the hepatocyte to the blood [324]. In hepatocytes, bilirubin is cleared from the body by UGT1A1. Bilirubin diglucuronide is formed in the liver and is secreted across the bile canalicular membrane by an active transporter, multidrug-resistance-associated protein 2 (MRP2, ABCC2). Complete loss of UGT1A1 function leads to severe hyperbilirubinemia, a condition known as the *type 1 form of Crigler-Najjar syndrome*, a fatal disease rescued only by liver transplantation [325], and a defect in MRP2 results in the accumulation of the conjugated bilirubin, a disease known as *Dubin-Johnson syndrome* [326]. In humans, PB treatment has been known to increase bilirubin conjugation activity and decrease elevated bilirubin levels [327]. Because PB induction is regulated by CAR, it has been associated to play a role in bilirubin clearance. Huang *et al.* [324] showed that WT mice pretreatment with TCPOBOP showed an increase in hepatocyte expression of OATP1B1, UGT1A1, GSTA1, GSTA2, and MRP2, the five enzymes involved in bilirubin elimination, while induction was absent in CAR-null mice.

It has been reported that PXR, like CAR, can also regulate the expressions of bilirubin-detoxifying enzymes and transporters [324,328]. However, in cell cultures that did not contain PXR ligands, PXR was shown to suppress the ability of CAR to induce MRP2 [329]. A double null mouse line (2XENKO) lacking CAR and PXR showed that both receptors contribute to the protective response to the hydrophobic bile acid LCA [330], protecting against LCA toxicity. Furthermore, the double KO mice model has shown that CAR and PXR function coordinated to regulate both xenobiotic and bile acid metabolism [331].

As for transporters, CAR regulates the expression of several transport proteins such as P-gp, BCRP, MRP2, MRP4, OATP1B1, and bile salt export pump (BSEP) [332,333]. Among these transporters, the CAR-null mouse has been applied to understand P-gp, MRP2, and MRP4 [334,335]. In the presence of dexamethasone and PCN, CAR ligand and activators, MRP2 expression was not present in hepatocytes isolated from null

CAR mouse compared to WT mice [335]. It has been shown that Mrp4 can transport sulfated bile acids. Under normal conditions, hepatic levels of sulfated bile acids are low but are increased under cholestatic conditions. Assem *et al.* [334] hypothesized that the upregulation of Mrp4 during cholestasis is a compensatory mechanism to protect the liver from accumulation of bile acids. Additionally, Sult2a1, an enzyme that conjugates a sulfate to hydroxyl bile acids and steroids, is also involved in the protection of the liver from bile acid accumulation. It was shown that CAR is required to coordinately upregulate hepatic Mrp4 and Sult2a1. It was shown that Sult2a1 is down-regulated in Mrp4-null mice, which highlighted relation between *Mrp4* and *Sult2a1* gene expression.

19.10.2.2 Humanized (Knock-In/Knock-Out) CAR Mouse Model. Species differences in response to ligands activating CAR have been observed. A transgenic mouse specifically expressing hCAR but not mouse CAR in the liver has been constructed [323]. The utility of a “humanized” CAR animal model provides an *in vivo* tool to identify ligands sensitive to human CAR activity. The humanized CAR model was validated with pretreatment to PB as increased sensitivity to APAP toxicities was observed based on elevated mRNA levels of *Cyp1a2*, *Cyp3a11*, and *Gstpi* [323]. Meclizine, an antiemetic agent, is an example to highlight species differences in CAR response [336]. In WT mice, meclizine was shown to induce the expression of *Cyp2b10*, *Cyp3a11*, and *Cyp1a2*, target genes of CAR. In contrast, in humanized CAR animals, meclizine treatment in primary hepatocytes isolated from the liver of humanized CAR mice showed suppression of *Cyp2b10*, *Cyp3a11*, and *Cyp1a2* gene expression. Moreover, it was shown that meclizine can suppress APAP-induced hepatotoxicity as the CYP responsible for forming the reactive metabolite of APAP is not expressed. In summary, the humanize CAR animal models provide insight toward the disposition of compounds when species differences in substrate selectivity and response may occur. The utility of the humanized model provides an *in vivo* system to evaluate the induction potential of new drug entities involving human CAR.

19.10.3 Peroxisome-Proliferator-Activated Receptors

PPARs play a role in the control of xenobiotic metabolism, lipid metabolism, glucose homeostasis, adipocyte differentiation, and inflammation [337–341]. As mentioned above, there are three different PPAR isoforms: PPAR α , PPAR β/σ , and PPAR γ . PPARs regulate gene expression by binding, as heterodimers with RXRs to specific PPAR response elements in the promoter regions of specific target genes. In the absence of a ligand, high affinity complexes are formed between the PPAR–RXR heterodimer [342]. These three receptors are present in most tissues in humans and rodents; however, protein levels are highly variable. PPAR α has been associated with fatty acid metabolism and gluconeogenesis [343–346]. It has been shown that tissue-specific expression profile of PPAR α in humans is very different from that in rat and mouse. Specifically, human liver contains lower levels of PPAR α in contrast to the rodents, in which potent peroxisomal proliferators can cause liver tumors due to activation of the gene [347–349]. In human livers that express PPAR α , levels are approximately one order of magnitude lower than mouse liver. Human cDNA for PPAR α does not encode a functional protein because it lacks exon 6 due to alternate RNA splicing, this was observed in all 10 human liver samples examined [350]. In animals, PPAR α

is associated with the upregulation of CYP4A6 (fatty acid omega hydroxylase) [351]. PPAR α is the receptor for the fibrate drugs that lower triglycerides and raise high density lipoprotein cholesterol levels in the treatment and prevention of coronary artery disease [352,353].

PPAR γ plays a critical role in adipocyte differentiation and is the receptor for the glitazone class of insulin-sensitizing drugs used in the treatment of type 2 diabetes and management of dyslipidemia [354]. PPAR γ activates genes that increase glucose and lipid uptake, enhances glucose oxidation, and lowers free fatty acid levels and insulin resistance [355]. It is highly expressed in fat, but is low in the liver, and has trace levels in the muscle tissues [356]. The thiazolidinediones (TZD) are synthetic ligands of PPAR γ . In patients with type 2 diabetes, TZD had been shown to decrease mean glycated hemoglobin [HbA(1c)] by 1.5% and lower HbA(1c) to less than 7% in 30% of patients [356]. Other indications for PPAR γ include cell cycle control by inhibiting growth and/or inducing differentiation of several cancer types, such as prostate, breast, pancreatic, and colon [357] and in neurodegenerative disorders such as AD [358,359].

PPAR β/σ is involved in fatty acid oxidation in muscle tissues, inflammation, and oxidative stress [356]. Persistent low level of inflammation is believed to contribute to several metabolic diseases such as obesity, type 2 diabetes mellitus, and atherosclerosis [360]. By activating PPAR β/δ genes, this appears to reduce the production of inflammatory cytokines by adipocytes and prevents fatty acid-induced inflammation in skeletal muscle cells. Also, gene activation appears to minimize the development of cardiac hypertrophy and suppresses macrophage-derived inflammation in atherosclerosis [360].

19.10.3.1 PPAR (Knock-Out) Mouse Model. The ability to oxidize fatty acids is necessary for the normal response to fasting as demonstrated by severe hypoglycemia in which premature death was observed in fasted PPAR α -deficient (PPAR $-/-$) mice. PPAR $-/-$ mice have a defect in fatty acid metabolism but have normal reproduction capabilities. Gemfibrozil, a lipid-lowering agent, can activate PPAR α . Studies conducted in PPAR $-/-$ mouse model have shown that this receptor plays a key role in lipid homeostasis and is important in the regulation of fatty acid oxidation enzymes. CYP4A isoforms are under the control of PPAR α , in which starvation and diabetes can triggers its upregulation [361]. PPAR α is required for the fasting-activated expression of target genes involved in mitochondrial and extramitochondrial fatty acid oxidation in liver and heart, tissues known to increase fatty acid oxidation rates during short-term starvation. Studies in the PPAR $-/-$ mice have shown that CYP4A contributes to the cellular metabolic response to fasting. In the fasting state, endogenous lipids activate PPAR α that induces several genes involved in fatty acid oxidation, due to either increase fatty acid uptake and/or utilization. *trans*-4-Hydroxy-2-nonenal (HNE) is a potent cytotoxic and genotoxic compound originating from the peroxidation of n-6 polyunsaturated fatty acids [362]. Studies conducted in WT mice and PPAR α -null mice have confirmed the role of CYP4A in the metabolism of HNE. In the presence of an inducer, clofibrate, WT mice were able to form the two metabolites derived from HNE; however, PPAR α - $-/-$ mice did not form them [362].

PPAR γ , in both rodents and humans, is highly expressed in adipose tissue, where it is a major regulator of adipogenesis, and lower expression levels are found in skeletal muscle and liver [363]. KO mice engineered with a deletion of muscle PPAR γ have been utilized to elucidate the physiologic role of muscle PPAR γ and elucidate the mechanism of the diabetic drugs known as TZDs in response to insulin [364]. Norris

et al. [364] found that these mice develop excess adiposity as well as whole-body insulin resistance. Tissue-specific insulin sensitivity in these mice was normal in skeletal muscle but impaired in the liver and possibly in adipose tissue. Unlike PPAR α that is activated by fibrates to upregulate the activity of CYP4A family, not as much is known for PPAR γ . However, studies utilizing DMBA, a tumor initiator, suggests that PPAR γ plays a protective role in carcinogenesis. This was exemplified in studies conducted with heterozygous PPAR γ mouse models, as a null model does not exist, since this gene is necessary for normal development and null mice die *in utero* around gestational day 10 [365]. In the heterozygous PPAR γ mice, an increase in DMBA-mediated skin tumors was observed compared to the WT animals. Studies with CYP1B1 $-/-$ mice have shown that this enzyme appears to have a protective role in skin tumor formation compared to WT mice [366]. Taken together, PPAR γ may regulate CYP1B1-mediated bioactivation of DMBA to its carcinogenic metabolites [367].

19.10.3.2 Humanize PPAR (Knock-In/Knock-Out) Mouse Model. When species difference in activity exists, humanized animal models are ideal to evaluate gene response in the presences of ligands. As previously discussed, PPAR α exhibits species differences and it has been shown that humans retain the capacity to encode PPAR α but a portion of the gene lacks exon 6 producing nonfunctional receptor and the mRNA levels are 10 times lower than the levels present in rodents [368,369]. The humanized PPAR α mouse that was generated by introducing human PPAR α transgene into a PPAR α $-/-$ mouse did not manifest hepatocellular proliferation when exposed to fibrates unlike that observed in WT mouse. The utility of humanized PPAR α mice would aid in the understanding of the response differences and enable better management of drug development risks in moving a PPAR α agonist forward [370].

19.10.4 Farnesoid X-Activated Receptor

The FXR, also known as the *bile acid receptor*, plays a role in the regulation of bile acids, lipid, and glucose homeostasis [371,372]. The receptor is expressed primarily in the liver, kidney, intestine, and adrenal gland [373,374]. As mentioned above, the FXR heterodimerizes with RXR and binds to farnesoid X-activated receptor response elements (FXREs) within the regulatory regions of target genes [374]. Bile acids function as ligand activators for both rodent and human FXR [375]. Chenodeoxycholic acid (CDCA) and cholic acid, primary bile acids, are synthesized in the liver from either cholesterol or oxysterols and then transported across the basement membrane of the hepatocyte into the bile canaliculi and stored in the gallbladder [376]. Bile acids excreted into the intestinal lumen can be further metabolized by bacteria into secondary [deoxycholic acid (DCA) or LCA] or tertiary bile acids before their resorption in the distal ileum. Bile acid concentrations are controlled by a feedback regulatory pathway in which the activation of the FXR represses transcription of both the *CYP7A1* gene, which encodes the rate-limiting enzyme in the synthesis pathway, and the *CYP8B1* gene required for synthesis of cholic acid [377]. Various diseases are attributed to defects in this cycle. Examples are cholestasis (impaired bile flow) that leads to hepatic accumulation of toxic bile acids such as LCA and 3-keto-LCA as well as toxins that would normally be excreted in the bile [378]. FXR is activated by various bile acids such as CDCA, DCA, LCA, and cholic acid [379]. In response to bile acids, FXR regulates several transport proteins and metabolizing enzymes involved in bile

acid homeostasis such as Ntcp, the hepatic basolateral sodium taurocholate cotransporter protein [380], Bsep, the hepatic canalicular BSEP transporter [381], and CYP7a, a cholesterol 7 α -hydroxylase [382].

19.10.4.1 FXR (Knock-Out) Mouse Model. In evaluating the mechanistic role of FXR in bile acid regulation, the development of FXR KO mouse (FXR $-/-$) model has provided an *in vivo* tool to assess its role. A FXR $-/-$ mouse has been engineered by Sinal *et al.* [124], in which the animals were visibly normal, exhibited normal physiological developmental characteristics, and were indistinguishable from WT littermates. However, FXR $-/-$ animals could not control bile acid synthesis and excretion. These animals have been utilized to study the effects of dietary bile acid, namely, 1% cholic acid (added to animals food), and have confirmed that FXR is essential in regulating bile acid homeostasis as high systemic levels can lead to severe wasting, dyslipidemia, and hypothermia and even death (30% of the null mice by day 7) [124]. Organ-specific FXR $-/-$ mouse models have been generated in which activity in the liver or intestine has been disrupted [383]. Kim *et al.* [383] showed that FXR intestinal deletion or liver deletion models provide a mechanistic tool to understand organ-specific FXR-mediated regulation and whether there are potential interplay between intestinal and liver FXR activity in the regulation of bile acid homeostasis. It was shown that treatment with a FXR-selective agonist GW4064 significantly repressed CYP7A1 levels in FXR liver-deficient mice but had no effect in the FXR intestinal deficient mice. In contrast, it was shown that FXR-mediated repression of CYP8B1 was dependent on liver FXR and less dependent on intestinal FXR. Thus, the different organ-specific expression of FXR provided a valuable tool to demonstrate that FXR-related repression of bile acid synthesis requires the complementary actions of FXR in both liver and intestine and enabled the mechanistic understanding of the role of CYP7A1 and CYP8B1 in maintaining bile acid homeostasis [383].

19.10.5 Multiple Nuclear Receptor GEMA

As discussed above, PXR and CAR play critical role in the transcriptional regulation of phase I and II enzymes as well as transporters. There are unique substrates as well as overlapping substrates with different affinities to PXR and CAR that contribute to the induction of a wide array of genes involving cross talk between the nuclear receptors and transcriptional cofactors [384]. Enzymes and transporters such as CYP3A, CYP2B, UGT, SULT, GST, P-gp, MRPs, and OATPs, which contain the responsive element (PXRREs) and PB-responsive element (PBREMs) in the promoter region can be regulated by both PXR and CAR [385]. To evaluate the complex mechanisms governing the cross talk between PXR and CAR, Scheer *et al.* [385] generated a variety of possible combinations of PXR and CAR mouse lines. Mouse models generated were (i) humanized PXR and CAR KO, (ii) PXR KO and humanized CAR, (iii) PXR KO and CAR KO, and (iv) humanized PXR and CAR. The utility of these mouse models provide a tool to discriminate between the PXR- and CAR-mediated effects of these two receptors. For example, PB is reported to activate PXR *in vitro* and studies with humanized PXR and CAR animals suggest that the PB-mediated activation of Cyp3a11 and Cyp2b10 *in vivo* is CAR dependent [385]. This example highlights a disconnect between *in vitro* and *in vivo* responsiveness to PXR and CAR. Moreover, PB did not induce the expression of mouse Cyp3a1 and Cyp2b10 in humanized PXR and CAR

KO mice, which demonstrates that human PXR is not able to compensate for the loss of CAR activity [385]. The above examples demonstrate how the use of combination animal models of CAR and PXR can elucidate the dependence or lack of dependence of these transcription factors in the regulation of genes that may affect drug disposition and toxicity in humans.

19.10.6 Aryl Hydrocarbon Receptor (AHR)

The AHR has been studied for its pathological activity in response to man-made environmental toxins/pollutants such as halogenated PAHs. AHR is distinct from the nuclear receptor superfamily in that it is a ligand-activated transcriptional factor. It is found in the cytosol and is normally present in the inactive form associated with a chaperone complex consisting of two molecules of heat shock protein-90, the immunophilin-like X-associated protein 2 (XAP2), and p23. AHR is highly conserved and expressed in various cell types. AHR is a member of the basic-helix-loop-helix (bHLH) superfamily of DNA-binding proteins [386–388]. In the cytosol, AHR is activated on ligand binding resulting in the dissociation from its inactive complex formation and translocates to the nucleus where it dimerizes with the Ah receptor nuclear translocator protein (ARNT) resulting in gene transcription. The genes transcriptionally regulated are *CYP1A1*, *CYP1A2*, *CYP1B1*, xanthine oxidase, xanthine dehydrogenase, *GST-Ya*, and *UDPGT* [387,389–391]. A variety of lipophilic toxins such as TCDD, B[a]P, and naphthalene have been shown to mediate the toxic response through the AHR regulation of various CYP enzymes [389,392–396].

19.10.6.1 AHR (Knock-Out) Mouse Model. AHR-deficient mouse (*Ahr*^{−/−}) are typically generated by homologous recombination in ES cells [397]. *Ahr*^{−/−} animals from different labs where exon 1 or 2 has been deleted have shown that the receptor is critical in embryonic and physiological development, as abnormal development of the liver, heart, spleen, thymus, and kidney occurs in these animals [390,397–399]. The lack of AHR receptor resulted in age-related organ lesions that appear nine months postbirth. Other effects observed were cardiomyopathy (CMP; 100%) with hypertrophy, focal fibrosis, and T-cell deficiency only in their spleens, while other lymphoid organs were not immune compromised [400]. In humans, the expression of the AHR in the left ventricle specimens obtained from explanted hearts of patients with CMP based on immunohistological analysis revealed significantly increased AhR levels in the diseased heart. Weak to intermediate staining of anti-AhR was observed in control hearts (hearts from healthy accident victims) but weak to intense staining in CMP hearts [401].

Ahr^{−/−} mice failed to induce drug-metabolizing genes that encode enzymes that catalyze the metabolism of foreign compounds. This is exemplified by studies with TCDD and B[a]P in which *AhR*^{−/−} mice were resistant to TCDD-induced immune suppression and showed reduced tumor formation in the presence of B[a]P, due to low expression levels of cytochrome P450 1A1 (*CYP1A1*) required to activate these carcinogens to reactive metabolites [395,402–404]. The *Ahr*^{−/−} mice has been utilized to understand the role of Ahr in the immune system as phenotypic differences exist among the different KO mice lines. For example, the *Ahr*^{−/−} mice [397] are more susceptible to infections with *Helicobacter hepaticus*, an opportunistic infection associated with immunodeficiency [405]. It has been speculated that *CYP1B1* may metabolize B[a]P to benzo[α]pyrene-4,5-epoxide, the resultant mutagen in mice resulting in B[a]P-induced

carcinogenesis. Moreover, unlike the *Ahr*^{-/-} mice, the expression of CYP1A1 and 1B1 mRNAs in liver and lung were highly induced in WT mice by several PAHs such as 7,12-dimethylbenz[a]anthracene, dibenz[a,l]pyrene, 3-methylcholanthrene, 1,2,5,6-dibenzanthracene, benzo[b]fluoranthene, and benzo[a]anthracene [393]. The role of CYP1B1 in B[a]P-induced carcinogenesis was elucidated in which *Ahr*^{-/-} mice had lower levels of this enzyme compared to WT animals.

19.10.6.2 Humanized AhR (Knock-In/Knock-Out) Mouse Model. The large inter- and intraspecies differences in the susceptibility to dioxin-induced toxicities are due to the species differences in AhR receptor [406]. The humanized AhR mouse model was generated by knock-in strategy via homologous recombination in which the human *AHR* gene is transcribed under the same regulatory mechanism as the replaced murine gene [406]. The humanized AHR mouse model affords an *in vivo* model to evaluate human AHR-induced gene responses in the presence of toxins. The affinity of human AHR to TCDD is 10-fold lower compared to mouse AhR [393]. The humanized mouse studies have provided evidence that impaired receptor ligand binding is associated with *in vivo* toxin resistance due to the lower affinity of human AHR [406]. Since ligand affinity differs in rodents and humans, the humanized AHR model ensures that strong ligands for human AHR are anticipated.

19.10.6.3 AHR Chimeric Mouse Model. A transgenic C57BL/6J mice containing Han/Wistar *Ahr* gene (endogenous mouse *Ahr* gene was eliminated) provides an animal model to further evaluate the molecular mechanisms governing aryl hydrocarbon enabled toxicities [407]. The Han/Wistar rats are resistant to TCDD toxicity due to a mutation in the *Ahr* gene, in which altered splicing of the *Ahr* mRNA results in two *Ahr* proteins. The two proteins consist of a deletion (DEL) and insertion (INS) variants, with the latter dominating in expression. The laboratory of Pohjanvirta [407] showed that the transgenic INS mouse were phenotypically normal except for increase in testes weight. For drug metabolism properties, the INS mouse responded to TCDD induction similar to WT mice; however, sex differences in response to TCDD were not observed as both sexes showed resistance to TCDD toxicities. It has been observed that the *Ahr* is capable of associating to the ER, resulting in modulation of ER trans-activation function. Specifically, the development of reproductive organ tumors such as breast cancer often depends on the action of sex hormones. Further, PAHs can be activated the *Ahr*/Arnt heterodimer, which directly associates with the ER subtypes “a” and “b.” This association results in recruitment of unliganded ER and coactivator p300 to estrogen-responsive gene promoters, leading to activation of transcription and estrogenic effects in the absence of estrogen [408]. This INS transgenic mouse model provides tools to evaluate PAH-related toxicities related to body weight, testicular function, and hormone-dependent diseases.

19.11 DRUG TRANSPORTERS IN DISPOSITION

19.11.1 Multidrug Resistance

The ATP-binding cassette (ABC) transporter P-gp protein was discovered in the mid-1970s for its ability to confer multidrug resistance in tumor cells [409]. It is an integral

membrane transporter in humans encoded by the *MDR1* (*ABCB1*) gene [410]. *MDR1* gene is expressed in various human tissues and the protein serves as a permeation barrier in the gastrointestinal tract and brain, while contributing to the elimination of drugs in the liver and kidneys. It is localized to the apical surface of intestinal epithelial cells, bile canaliculi, renal tubular cells, and placenta and on the luminal surface of capillary endothelial cells in the brain and testes [411]. Humans carry a single *MDR1* gene, while rodents contain two genes specifically *mdr1a* and *mdr1b* [412–415]. In rodents, the two *mdr* genes are expressed together in liver, kidney, heart, lung, and spleen and independently in different organs where mouse *mdr1a* is expressed in the intestinal epithelium, blood–brain barrier (BBB), and blood–testis barrier, while *mdr1b* is expressed in the adrenal gland and ovaries [416].

19.11.1.1 P-gp (Knock-Out) Mouse Model. To better understand the role of P-gp in barrier tissues such as the gastrointestinal tract and the BBB, *MDR1* knock-out/null animal models were generated to understand the role of this transporter. Mice deficient in either *mdr1a* or *mdr1b* (*Mdr1a/b*–/–) have been generated. The *mdr1a* KO (*Mdr1a*–/–) mouse was generated first in which the *mdr1a* gene was disrupted in which fragments of exon 6 and 7 were replaced by hygromycin phosphotransferase cassette. For the *Mdr1b* knock (*Mdr1b*–/–) mouse, fragments from exon 3 and 4 were deleted. In both mice models, both showed that disruption of the *mdr1a* or *mdr1b* gene did not affect animal viability and these animals exhibit normal development and were fertile giving rise to normal size litters [410,417]. In *Mdr1a*–/– mouse, as discussed previously, this protein is highly expressed at the BBB. Studies with *Mdr1a*–/– mice showed that ivermectin and vinblastine brain levels were 87- and 22-fold greater than their WT counterparts following a 0.2-mg/kg oral dose of ivermectin and a 1-mg/mg IV dose of vinblastine, respectively [410]. Similarly, studies conducted with nelfinavir demonstrated that *mdr1a* in the BBB can limit drug access in which brain levels were elevated 36-fold in the *mdr1a*–/– model, while plasma levels increased only 2.5-fold in WT [418]. The *Mdr1b*–/– mice showed slightly elevated [³H]digoxin levels in the adrenal glands (632 vs 320 ng/g tissue) and ovaries (708 vs 288 ng/g tissue), while all other tissues were no different in [³H]digoxin exposure compared to WT mice [419]. A double KO mice of *mdr1a*–/– and *mdr1b*–/– has also been produced. These animals were developmentally normal with no overt abnormalities [419]. To understand the pharmacokinetic ramifications of having both *mdr1a* and *mdr1b* genes absent, studies were conducted in which double KO animals were intravenously administered 1-mg/kg [³H]digoxin. Tissue exposure in the double KO mice resemble the *mdr1a*–/– mice with increased levels observed in the brain and testis (male animals) after 4 h postdose, which suggested that the *mdr1a* is the dominant phenotype [419].

After oral administration, plasma concentrations were elevated two- to fivefold in *mdr1a*–/– mice and with IV administration, brain concentrations were elevated 7- to 36-fold. These data demonstrate that P-gp limits the oral bioavailability and penetration of these agents into the brain. The double KO mouse provides a tool to understand the mechanistic role of P-gp in barrier tissues as well as in excretory organs such as the liver and kidney. Studies with *mdr1a/b*–/– double KO animals showed that compounds that are not known to cross the BBB exhibit tremendous increase on exposure in the brain such as asimadoline [420]. Also, the *mdr1a/b*–/– mice and WT animals have been utilized to elucidate the sedating and nonsedating effects of H1 antagonists. For example, the first-generation H1 antagonists such as diphenhydramine, triprolidine,

and hydroxyzine are known as *sedating* and cause somnolence, while the second-generation H1 antagonists such as cetirizine, loratadine, and fexofenadine produce very little somnolence or CNS effects [421]. Using the *mdr1a/b*^{-/-} double KO mice, Chen *et al.* [421] were able to demonstrate that the nonsedating antihistamines were substrates for P-gp with brain to plasma ratios between 2 and >14 compared to brain to plasma ratio observed in WT mice. In contrast, the nonsedating antihistamines exhibited brain to plasma ratios ~1.

Mdr1a/b^{-/-} animals are a useful tool to identify P-gp as a factor of brain uptake and to assess whether comedices may limit CNS exposure; however, extrapolation of brain exposure from animal data to humans is poor. For example, the brain to plasma concentration ratio of nelfinavir increased 7- to 36-fold, paclitaxel increased 6- to 28-fold, and verapamil increased 8.5-fold in *mdr1a/b*^{-/-} mice compared to WT [422]. In rat studies, where cyclosporine (CsA) is coadministered as a P-gp inhibitor, the brain to plasma ratio of verapamil increased 24.1-fold [423]. Studies in humans utilizing noninvasive positron emission topography (PET) imaging techniques have provided a tool to measure P-gp activity based on drug exposure in the brain. In human healthy volunteer study, [¹¹C]verapamil, 0.2 mCi/kg, was intravenously infused to patients for 1 min before and after a 1-h infusion of CsA (2.5 mg/kg/h) [424]. The brain to plasma ratio of [¹¹C] radioactivity increased by 79% at a measured blood concentration of 2.8 μ M CsA, which is much less than the brain exposures observed in rodent models. Hsiao *et al.* [425] conducted a P-gp drug interaction study with verapamil and CsA, where CsA doses were administered to achieve a range of systemic concentrations ranging from 0 to 12 μ M. The authors showed that drug levels achieved in rodent brain far exceed the levels achieved in human brain. However, when CsA was dosed in rodents to achieve brain concentrations similar to the levels observed in humans, similar brain to blood exposure of [³H]-verapamil was observed in the rodents of ~75% [425].

19.11.1.2 Dog *MDR1* Model. The mouse *Mdr1* KO model is derived from experimental manipulations of the *MDR1* gene. As an alternative model, a dog collie model exists in which a mutation in the *MDR1* gene leads to inactive P-gp activity. Specifically, a frame shift deletion of 4 bp results in a premature stop codon leading to nonfunctional P-gp protein in which the dogs show sensitivity toward ivermectin-induced neurotoxicity [426]. Studies demonstrated that the *Mdr1*-deficient dog models are comparable to the mouse *Mdr1a/b* models in the distribution patterns of loperamide and ivermectin, thus providing a nonrodent model to investigate *Mdr1* gene [426,427].

19.11.2 Breast Cancer Resistant Protein

BCRP is a member of the ABC family of drug transporters. It can actively transport various anticancer drugs such as doxorubicin, mitoxantrone, camptothecin, and topotecan from cells, causing multidrug resistance [428–431]. Like P-gp, BCRP is localized on the apical membrane and found in barrier tissues such as the gastrointestinal tract, blood placenta and brain barriers, and in eliminating organs such as the liver and very low levels in the kidney [432].

19.11.2.1 *Bcrp* (Knock-Out) Mouse Model. The *Bcrp* KO (*Bcrp*^{-/-}) mouse was developed by deleting a large portion of the ATP-binding domain in exons 3–6 and

replaced with a 1.8-kb pgk-hygro cassette in reverse-transcriptional orientation. The Bcrp^{-/-} mice were developmentally normal and fertile, with similar lifespan as the WT animals [433]. The first application of Bcrp^{-/-} mice was in the evaluation of skin photosensitivity to porphyrins, namely, pheophorbide a and protoporphyrin. Pheophorbide a is a phototoxic porphyrin catabolite of chlorophyll. Animals ingest a vast amount of food products containing chlorophyll and Bcrp is known to transport pheophorbide a. Bcrp is known to reduce the bioavailability of pheophorbide a from the diet by preventing its uptake from the intestine and possibly effluxing it via liver and kidney [433]. Phototoxicity has not been observed in WT mice but bcrp^{-/-} mice show extreme hypersensitivity ~100-fold more sensitive toward pheophorbide a compared to WT mice.

Since BCRP is present at the BBB, it is believed to limit CNS exposure of many drugs. Of interest are the human immunodeficiency virus 1 (HIV) nucleoside reverse-transcriptase inhibitors (NRTIs) that exhibit low CNS penetration due to active efflux at the BBB. Zidovudine (AZT) and abacavir (ABA) are *in vitro* substrates for BCRP [434]. Utilizing the Bcrp^{-/-} KO animals has helped identify the role of this transporter in plasma exposure and brain penetration. Studies were conducted with AZT and ABA in WT and Bcrp^{-/-} KO mice in which no difference in either area under the concentration–time profiles for plasma (AUC_{plasma}) or brain (AUC_{brain}) were observed for AZT between the WT and Bcrp^{-/-} mice. However, for ABA, brain exposure in the Bcrp^{-/-} mice was 1.5-fold greater than WT mice [435]. These results indicate that BCRP may not play a role in CNS exposure for AZT but appears to contribute for ABA.

19.11.3 Multidrug Resistance Protein

The MRP transporter family consists of nine members. Among these transporters, MRP1, MRP2, MRP3, and MRP4 have been widely investigated and associated with the transport of xenobiotics and endogenous substances [436]. Typical substrates for MRPs are organic anions, for example, anticancer and antiviral drugs conjugated to GSH, glucuronate, or sulfate and endogenous substrates as bile salts, bilirubin glucuronides, prostaglandins, and leukotrienes [437–452]. These MRPs are associated with drug resistance in which the mechanism of action requires a constant supply of GSH to form GSH conjugate in the cell so that conjugated drug is then transported out of the tumor by these transporters [439]. Tissue distribution of the MRPs are broad, where MRP1 is ubiquitous except low levels in liver; MRP2 is predominately expressed in the liver, kidney, and gastrointestinal tract; MRP3 is predominately found in the liver, kidney, adrenals, and gastrointestinal tract; and MRP4 is most abundantly expressed in the kidney, brain, and prostate [439,449,451,453]. In humans, MRP2 deficiency leads to a mild liver disease known as the *Dubin-Johnson syndrome*.

19.11.3.1 *Mrp1 and Mrp2 (Knock-Out) Mouse Model.* MRP1 KO (Mrp1^{-/-}) animal models have been constructed by Lorico *et al.* [454] in which the mouse *Mrp* gene in W9.5 ES cells was disrupted by replacing a small segment of the ATP-binding domain with a neomycin resistance gene cassette. The Mrp1^{-/-} mice subsequently generated from this modification were viable and fertile and appeared healthy without any developmental alterations. After western blot analysis, the *Mrp* gene was absent in the colon, lung, and muscle in the Mrp1^{-/-} mice, tissues that normally express

high levels. Also, plasma levels and tissue levels of GSH in the *Mrp1*^{-/-} mice were elevated, where tissue levels increased by 25–90% and most pronounced in tissues that express Mrps in the WT state [454]. The utility of the *Mrp1*^{-/-} mouse model has been used to explore the role of *Mrp1* in anticancer-mediated cytotoxicity and drug resistance. Several investigators have shown that the *Mrp* gene affords protection against the toxic effects of agents such as etoposide, vincristine methotrexate, and arsenite in which compounds exhibited toxicities twofold or greater in *Mrp1*^{-/-} mouse compared to WT mouse based on LD₅₀ values, bone marrow toxicity as a result of depletion of nucleated cells, and poor leukocyte count recovery [439,454,455]. Moreover, dose tolerance to toxicity for etoposide was lower in the *Mrp1*^{-/-} mice in which maximum tolerated dose was 125 mg/kg compared to 200 mg/kg in WT mice [455].

MRP2 is localized in the apical membrane of epithelial cells in hepatocytes, renal proximal tubules, and enterocytes [456]. *Mrp2*-deficient mouse models have been described by several laboratories in which *Mrp2* gene has been disrupted by the insertion of a Lac-Neo cassette [457] or insertion of a 2-kb pgk-hygro cassette to delete exons 4–6 [458]. The *Mrp2*^{-/-} mice showed normal development, was fertile, and exhibited a lifespan similar to WT mice [457]. However, Chu *et al.* [457] reported that total bilirubin levels were fivefold higher and conjugated bilirubin was 60% of total bilirubin (conjugated and unconjugated) in the *Mrp2*^{-/-} mice compared to WT animals. Vlaming *et al.* [458] observed a 20–25% increase in liver weight in adult *Mrp2*^{-/-} mice compared to WT mice [457,458]. It was also observed that the suppression of *Mrp2* resulted in the upregulation of liver *Mrp4* levels by six- to sevenfold in liver and 2.5-fold in kidney and *Oat1* levels in the kidney by 2.5-fold [457]. The utility of the *Mrp2*^{-/-} mouse has elucidated the role of *Mrp2* in various tissues. *Mrp2*^{-/-} mice exhibit reduced intestinal secretion, biliary secretion, and renal elimination. This was demonstrated by a significant reduction in the excretion of GSH and bilirubin glucuronides in *Mrp2*^{-/-} mice compared to WT [457]. In another example, mycophenolate mofetil (MMF), the prodrug of mycophenolic acid (MPA), is widely used to prevent allograft rejection in solid organ transplantation and as an immunomodulator in the treatment of autoimmune diseases [459]. In humans, MPA is taken up by the liver, undergoes glucuronidation to MPA-glucuronide, is excreted into the bile, and is deconjugated by gut bacteria to MPA. The coadministration of CsA in patients taking MPA resulted in less biliary secretion of MPA-glucuronide and reduced systemic exposure of MPA compared to patients coadministered tacrolimus and MPA. Unlike CsA, tacrolimus is not an inhibitor of MRP2 and therefore did not alter MPA disposition [460,461]. To clarify the role of P-gp and *Mrp2* in MPA pharmacokinetics, the evaluation of MPA and its glucuronide in *Mdr1a/b*^{-/-} and *Mrp2*^{-/-} animals provided insights toward CsA-mediated drug interaction. Following oral MMF administration, the coadministration of CsA resulted in the expected changes in MFA levels in WT, *Mdr1a/b*^{-/-}, and *Mrp2*^{-/-} mice but MFA-glucuronide increased in both KO animals indicating that P-gp and *Mrp2* cannot account completely for the effect of CsA [462]. In the clinical setting, MMF is monitored for potential drug transporter interactions.

19.11.3.2 Rat MRP2 Model. Two natural mutant rat strains for *Mrp2* are the TR and Eisai hyperbilirubinemic rat (EHBR), these are spontaneous mutants of the Wistar and Sprague-Dawley strains, respectively [463,464]. The main differences between the normal rodent versus the mutant *Mrp2* rodent is decreased bile flow, increased plasma levels of bilirubin glucuronides, decreased biliary secretion of reduced GSH,

and decreased elimination of GSH, glucuronide, sulfate conjugates, and unconjugated compounds [465]. These rat strains lacking functional Mrp2 have been utilized to examine the role of this transporter in the efflux of conjugated bilirubin; however, it has been noted that Mrp3 and UGT1a are upregulated in these Mrp2-deficient animals [457].

19.11.3.3 Mrp3 (Knock-Out) Mouse Model. MRP3 is localized in the basolateral membrane of epithelial cells in hepatocytes and enterocytes [456]. Several Mrp3-deficient (Mrp3^{-/-}) mice have been constructed by disrupting a segment in exons 2–8 of the *Mrp3* gene with a hygromycin resistance gene [466] or deletion of 2.1-kb fragment in exons 6–8 of the *Mrp3* gene [467]. The Mrp3^{-/-} mice showed normal development, were fertile, and exhibited a lifespan similar to WT mice. The transport of taurocholate in Mrp3^{-/-} and WT mice were similar. However, it does appear that Mrp3^{-/-} mice exhibit lower plasma bilirubin glucuronide levels. Because Mrp3 preferentially transports glucuronide conjugates, the role of Mrp3 in drug disposition has been investigated for drugs such as APAP, morphine, and etoposide [466–468]. The disposition of APAP is predominately mediated by glucuronidation and sulfation with minor contribution by cytochrome P450 to form the reactive metabolite that is detoxified by conjugation to GSH [469]. Following an IV dose of APAP, no differences were observed in the formation of the APAP-GSH and APAP-sulfate conjugates but large differences were observed in APAP-glucuronide conjugate in Mrp3^{-/-} and WT mice. A 20-fold higher accumulation of APAP-glucuronide was found in the liver of Mrp3^{-/-} mice and 10-fold reduction in biliary secretion of this conjugate [470]. Moreover, Manautou *et al.* [470] reported that Mrp3^{-/-} mice are more resistant to APAP hepatotoxicity than WT mice, which is most likely a result of the faster repletion of hepatic GSH, as the Mrp3^{-/-} mice had approximately threefold greater GSH. Thus, results obtained from the Mrp3^{-/-} mice model provided direct evidence that Mrp3 appears to be the only basolateral transporter that can efficiently secrete APAP-glucuronide into the blood from hepatocytes [470]. Another utility of the Mrp3^{-/-} mice model provided direct information of the sensitivity of *Mrp3* gene to protect normal tissues from the cytotoxicities of anticancer agents such as etoposide. Further, etoposide drug resistance occurs in human cancer patients so studies conducted in Mrp3^{-/-} provide mechanistic insight toward drug resistance [447]. The Mrp3^{-/-} mice did not show sensitivity toward etoposide toxicity as no difference in LD₅₀ was observed among Mrp3-deficient and WT animals. Unlike the Mrp1^{-/-} mouse model that did show sensitivity to cytotoxicity and lower dose toleration, Mrp3^{-/-} did not show this effect, suggesting that etoposide sensitivity and drug resistance may be more attributed to Mrp1 function.

19.11.3.4 Mrp4 (Knock-Out) Mouse Model. The tissue localization of MRP4 is not uniform as it is found at the apical membrane of the proximal tubules [451], the basolateral membrane of hepatocytes [471], the basolateral membrane of the choroid plexus epithelium, and the apical membrane of the endothelial cells of the brain capillaries [472]. Mrp4-deficient (Mrp4^{-/-}) mice have been generated in which exon 27 was disrupted in murine ES cells by replacing it with a neomycin resistance cassette via homologous recombination [472]. The generated Mrp4^{-/-}-deficient mice showed normal development and fertility and exhibited a lifespan similar to WT mice. The role of MRP4 in drug disposition has been evaluated with several anticancer and antiviral agents. Specifically, Mrp4 has been shown to have functional involvement in the renal

elimination of adefovir, tenofovir, and cidofovir [473]. These compounds were administered by constant IV infusion. Plasma levels of adefovir was higher in Mrp4^{-/-} mice compared to WT; however, kidney concentration of adefovir and tenofovir was almost twofold greater in Mrp4^{-/-} mice, which reflects an inability of the proximal tubules that efflux the drug from the proximal tubules to the urine. No significant differences were observed for cidofovir between Mrp4^{-/-} and WT animals, indicating that its pharmacokinetics are not dependent on Mrp4 activity. In another example with topotecan, the pharmacokinetics were compared between Mrp4^{-/-} and WT mice following IV administration [472]. The pharmacokinetic concentration–time profile was similar in the two animal models, exhibiting a biphasic elimination; however, at 6 h postdose, topotecan plasma levels were 50% higher in the Mrp4^{-/-} animals. Other observations were (i) 36% lower topotecan excretion in the urine and (ii) brain concentrations were sixfold higher in Mrp4^{-/-} mice compared to WT. Topotecan was shown to be a substrate for Mrp4 and localization of Mrp4 at the choroid epithelium appears to play a role in limiting drug penetration into the cerebral spinal fluid (CSF). The Mrp4^{-/-} mice models provide the *in vivo* evidence that Mrp4 plays a role in protecting the brain from cytotoxins [472].

19.11.4 Organic Anion Transporting Polypeptides

OATP transporters consist of a superfamily of proteins [474]. The OATPs are sodium-independent uptake transporters involved in the transport of anionic drugs in clinical use as well as endogenous substrates such as bile acids, hormones, and steroids [436,475]. OATPs are expressed in several organs involved in drug disposition such as intestine, liver, kidney, and brain [476]. The OATPs are highly expressed in the sinusoidal membrane of hepatocytes or in the apical membrane of enterocytes, where they may affect liver penetration or intestinal uptake, respectively [474]. Several OATPs are of interest in specific organs such as OATP1A2 in the intestine and OATP1B1, OATP1B3, and OATP2B1 in the liver. Inhibition of the hepatic uptake transporters may lead to significant drug interactions, as observed for several statins such as pravastatin and fluvastatin [477,478].

19.11.4.1 *Oatp* (Knock-Out) Mouse Model. OATP1B1 and OATP1B3 can play the role of a rate-limiting step to the hepatic uptake of many drugs. In rodents, there is only one member of the Slco1b family, known as *Slco1b2* (*Oatp1b2*) in both rats and mouse that corresponds to human OATP1B1 and 1B3 [479–481]. Several laboratories have generated *Slco1b2* KO (*Oatp1b2*^{-/-}) mice models in which one model was produced by deletion of exons 10–12 of the mouse *Oatp1b2* gene sequence and insertion of a neomycin resistance cassette [482] and the second one by disruption in intron 1 through part of intron 3 [483]. Both methods revealed that the absence of these genes did not alter normal development and fertility and no apparent phenotypic abnormalities were observed. In all cases, the expression levels of *Oatp1b2* mRNA in the liver were markedly lower or absent in comparison to WT mice. Zaher *et al.* [482] also reported sex differences in expression of other *Oatps* in the *Oatp1b2*^{-/-} animals compared to WT mice. In male *Oatp1b2*^{-/-} mice, *Oatp1a4* and *Oatp2b1* were unaltered compared to WT, although *Oatp1a1* was greater in the KO animal. In female *Oatp1b2*^{-/-}, no difference was observed for *Oatp1a1*, but KO mice exhibited greater expression of *Oatp1a4* and *Oatp2b1* [482]. Additionally, Lu *et al.* [483] also reported

similar alterations in the Oatps as Zaher *et al.* [482] and also noted that KO mice exhibited moderate conjugated hyperbilirubinemia. In general, the Oatp1b2^{-/-} KO mice lack expression of Oatp1b2 but expressions of non-Oatp transporters and DMEs were comparable to WT mice [484].

The Oatp1b2^{-/-} animal model provides a tool to gain insight toward the *in vivo* relevance of human OATP1B1 and OATP1B3 to the hepatic uptake of substrates. Lu *et al.* [483] showed that the organic anionic dye dibromosulfophthalein disulfonate, a compound that is actively taken up by Oatps in the liver and largely biliary excreted, is eliminated more slowly in the Oatp1b2^{-/-} mice compared to WT mice. In studies evaluating Rif and lovastatin acid, significantly higher liver to plasma partition ratio ($K_{p,liver}$) or liver uptake were observed in WT compared to the Oatp1b2^{-/-} mice consistent with reduced expression of the liver uptake transporter in the KO animals [484]. Consistently, Zaher *et al.* [482] conducted a single IV study with rifampin (1 mg/kg) and observed a 1.7-fold increase in plasma AUC in the Oatp1b2^{-/-} mice and the $K_{p,liver}$ was reduced by fivefold, and nearly eightfold when assessed at steady-state conditions after 24 h of continuous subcutaneous infusion compared to WT mice. Also pharmacokinetic studies with pravastatin showed fourfold lower $K_{p,liver}$ in the Oatp1b2^{-/-} mice compared to WT mice [482]. Thus, the pharmacokinetic results observed in the Oatp1b2^{-/-} mice were in line with expectations of an animal model lacking a critical hepatic uptake observed for compounds that are actively taken up by the liver.

19.11.4.2 Oatp (Multiple Knock-Out) Mouse Model. It is widely known that for the OATP there are no ortholog genes between rodents and humans. In some cases, there appears to be substrate overlap among rodents and humans despite the lack of orthologs. For example, humans express only a single OATP1A member (OATP1A2), while rats and mice express several members (Oatp1a1, 1a4, 1a5, and 1a6), believed to have risen by gene duplication events. In contrast, humans express OATP1B1 and OATP1B3, while rodents express only Oatp1b2 [485,486]. The generation of a double KO animal lacking *Slco1a* (*Oatp1a*) and *Slco1b* (*Oatp1b*) genes (Oatp1a/1b^{-/-} mice) would enable the further understanding of these Oatps in drug disposition. van de Steeget *al.* [486] generated this double KO mice model by insertion of loxP sites at both ends of the *Slco1a/1b* gene cluster (~620 kb), followed by Cre-mediated deletion. The double KO mice were developmentally normal and fertile and exhibited a normal lifespan comparable to WT animals. However, both male and female Slco1a/1b^{-/-} mice had significantly increased body weights, and by 85 weeks, they exhibited hyperbilirubinemia compared with WT mice.

van de Steeget *al.* [486] examined the pharmacokinetics of MTX in the double KO animals (Slco1a/1b^{-/-}) to understand the role of these two Oatps in the *in vivo* disposition of MTX, a known substrate for OATP1A/1B family. Plasma MTX levels were 4.8- and 3.8-fold elevated in the double KO mice compared to WT, respectively, following IV and oral doses of MTX (10 mg/kg). Additionally, the accumulation of MTX in WT animals was 50% of the MTX dose, while the Slco1a/1b^{-/-} mice showed only 2% accumulation of the dose. Similarly, the metabolite of MTX, 7-hydroxy MTX, was reduced in Slco1a/1b^{-/-} mice compared to WT, which highlights that lower uptake of drug in the liver results in less metabolism. These studies with MTX clearly highlight the contribution of Oatps toward the elimination of drugs.

19.11.4.3 Humanize OATP (Knock-In/Knock-Out) Mouse Models. As discussed above, orthologous *OATP* genes between rodents and humans do not exist. For example, nafcillin, a β -lactam antibiotic, is a substrate for rat *Oatp1a4*; however, in humans, both OATP1B1 and OATP1B3 contribute to liver uptake. Studies conducted with human *in vitro* OATP1B1 and OATP1B3 transfected cell lines have shown that nafcillin is a substrate for both transporters, but OATP1B3 is the primary uptake transporter [487]. The utility of a mouse KO model enables pharmacokinetic evaluation to assess the role of *Oatps* in the disposition of a compound. It is important to note that the lack of orthologous genes among rodent to human can make translation or predictions from animals to humans difficult. Thus, the creation of OATP1B1 humanized mouse models provide improved tools to explore the pharmacokinetic contribution of human transporters toward a compound's disposition [488]. From immunohistochemical staining, OATP1B1 was localized in the liver with no expression found in the intestine or kidney. van de Steeg *et al.* [488] used these humanized OATP1B1 mice (*Oatp1a/1b* were KO) to explore the *in vivo* role of human OATP1B1 in the disposition of the anticancer drug MTX. Following IV administration of MTX, the AUC was 1.5-fold less in the humanized OATP1B1 mice, but liver concentrations were twofold higher compared to WT mice. Thus, the humanized OATP1B1 mice model affords an *in vivo* tool to assess the role of human OATPs toward the disposition of a new chemical entity as both a substrate and an inhibitor.

19.11.5 Organic Cation Transporters

Organic cation transporters (OCTs) mediate the transport of hydrophilic, low molecular weight organic cations in organs such as the intestines, liver, kidney, and brain. OCT mediate the uptake of both endogenous (e.g., DA and choline) and exogenous (e.g., cimetidine, metformin, cisplatin, ranitidine, and procainamide) substrates that are positively charged at physiological pH [436]. There are three members of the OCT family (OCT1, OCT2, and OCT3). OCT1 is broadly distributed with highest expression in the liver and less expression in small intestine, kidney, and lung. OCT2 is highly expressed in the kidney and less expressed in small intestine, lung, and brain. OCT3 is broadly distributed in several tissues such as skeletal muscle, kidney, placenta, and heart and to a lesser extent in the liver [489]. Among these OCTs, OCT1 and OCT2 have been well characterized for substrates and inhibitors. The OCTs are facilitative diffusion transporters that in principle can transport cations in both directions and operate independently of H^+ and Na^+ gradients. The driving force determining the direction of transport is provided by the concentration gradient of the transported substrate and by the membrane potential [490]. The clinical relevance of the OCTs has been identified in which (i) OCT1 contributes to hepatic uptake affecting drug disposition and efficacy; (ii) OCT2 contributes to renal uptake, which may affect renal clearance and potential drug interactions; and (iii) both transporters are associated with genetic polymorphisms [491].

19.11.5.1 Oct (Knock-Out) Mouse Model. Several OCT KO animal models have been constructed. OCT1 is localized at the basolateral membrane of epithelial cells in the liver, kidney, and intestine in the rodent and is primarily expressed in the liver of humans [492]. This transporter facilitates the uptake of hydrophilic cationic

compounds into the liver. The OCT1 KO mice model was constructed by disrupting the mouse *Oct1* gene by replacing exon 7 [493]. The Oct1 KO mice (Oct1^{-/-}) were developmentally normal and fertile. These animals have been utilized to investigate the pharmacologic and physiologic role of Oct1 in the liver, intestine, and kidney by comparing the pharmacokinetics of tetraethyl ammonium ion (TEA) following an IV administration in the Oct1^{-/-} and WT animals. In rodents, Oct1 is highly expressed in the above organs. Consistent with the uptake role of Oct1, Oct1^{-/-} mice accumulated four- to sixfold less TEA in liver, twofold less direct intestinal excretion of TEA, but 1.5-fold greater excretion of TEA in the urine compared to WT mice [493]. The authors hypothesized that the increase in urinary excretion of TEA in the Oct1^{-/-} mice was likely due to a secondary effect of limited liver uptake as 20% of the administered dose accumulates in the liver of WT animals compared to only 3% of the dose in the Oct1^{-/-} animals.

19.11.5.2 *Oct1 and Oct2 (Double Knock-Out) Mouse Model.* Other OCT KO animals have been generated such as Oct2 KO (Oct2^{-/-}) and double KO of Oct1 and Oct2 (Oct1/2^{-/-}) [494]. For the Oct2^{-/-} mice, the model was generated by disrupting exon 1 with a replacement with an inverted *pgk-neo* cassette via homologous recombination in ES cells. To generate the double KO animals, Jonker *et al.* [494] started with Oct1^{-/-} ES cells in which *Oct2* gene was disrupted by removing exon 1. By western blot, excised liver and kidney showed no detectable levels of Oct1 and Oct2 in the double KO animals and Oct2^{-/-} animals lacked Oct2 in the kidney. Oct2^{-/-} and WT mice were dosed intravenously with TEA, a substrate for Oct2, but no difference in compound accumulation in kidney, liver, and plasma were observed, indicating that the absence of Oct2 plays a minor role in TEA disposition. However, in the double KO animals, TEA plasma levels were ~fourfold greater and the accumulation of TEA in the kidney and liver were approximately fourfold lower than in WT animals. Thus, the advantage of the double KO animals provides evidence that both genes contribute to the distribution and excretion of the compound.

19.11.6 Organic Anion Transporters

Organic anion transporters (OATs; SLC22A family) consist of 10 members but most clinical interest lies with OAT1 and OAT3. The OATs mediate the transport of hydrophilic, low molecular weight organic anions in kidney (OAT1 and OAT3) and brain (OAT3) [436]. OATs mediate the tissue distribution of a wide variety of small and hydrophilic organic anions of endogenous (e.g., bile salts, taurocholate, and cortisol) and exogenous origins (e.g., cimetidine, adefovir, AZT, ciprofloxacin, and furosemide) that are negatively charged at physiological pH [436,491]. OAT1/3 functions as organic anion exchanger. The OATs require an energy source that is several steps upstream, which is derived from a Na⁺/K⁺-ATPase pump that creates an Na⁺ gradient across the plasma membrane. Then an Na⁺/dicarboxylate cotransporter protein moves dicarboxylic acids (such as glutarate and ketoglutarate) against its electrochemical gradient, which creates an outwardly directed gradient that enables the uptake of an organic anion into the cell. Therefore, when one molecule of an organic anion is transported into a cell by an OAT exchanger, one molecule of an endogenous dicarboxylic acid is simultaneously transported out of the cell [495]. The clinical relevance of the OATs has been linked to nephrotoxicities with therapeutic

agents such as adefovir and cidofovir [496]. To minimize nephrotoxicity, probenecid, an OAT inhibitor, has been coadministered with cidofovi, which inhibits the tubular accumulation [497].

19.11.6.1 Oat (Knock-Out) Mouse Model. Several OAT KO animal models have been constructed. OAT1 is localized at the basolateral membrane of epithelial cells in the kidney, choroid plexus, and olfactory mucosa [498,499]. A 52-base segment within the coding region of exon 1 of *OAT* gene was disrupted by homologous recombination with a cassette encoding β -galactosidase and a neomycin-selectable marker (LacZ-Neo), which results in the inability to transcribe or translate the gene [500]. The *Oat1* KO (*Oat1*^{-/-}) mice of both sexes were viable and fertile, and they appeared healthy without any developmental alterations. In WT animals, expression levels of *Oat1* mRNA and protein levels are higher in male animals compared to female animals and greater renal transporter activity is observed in the male animals [500]. Pharmacokinetic studies were conducted to elucidate the role of *Oat1* in the disposition of furosemide. Following an IV dose of furosemide, the renal excretion of furosemide was diminished in the *Oat*^{-/-} animals compared to WT confirming that the renal secretion of furosemide is mediated by *Oat1* activity.

An *Oat3* KO (*Oat3*^{-/-}) mouse model has been generated by replacing exon 3 of the murine *Oat3* gene, with an inverted neomycin cassette via homologous recombination in CJ-7 ES cells [499]. The *Oat3*^{-/-} of both sexes were viable and fertile, and they appeared healthy without any developmental alterations and appear to live longer than WT animals [499]. The utility of *Oat3*^{-/-} mouse has helped clarify its role in the uptake of PAH, which was presumed to be transported by *Oat1*. In a pharmacokinetic study, low levels of PAH were excreted in the urine of *Oat3*^{-/-} animals compared to *Oat1*^{-/-} and WT animals [499,500].

19.11.7 Bile Salt Export Pump

The BSEP is considered to be a sister of P-gp [501], a member of the ABC superfamily of transporters. This transporter is considered a major transporter involved in the biliary excretion of bile salts. BSEP is expressed in the apical membrane of hepatocytes and responsible for bile salt-dependent bile flow. The clinical significance of BSEP is associated with the finding that mutations in this gene result in a form of progressive familial intrahepatic cholestasis (PFIC), also known as *PFIC type II* [325].

19.11.7.1 Bsep (Knock-Out) Mouse Model. A KO mouse model for *Bsep* has been constructed by disruption of the gene. A 1.4-kb fragment of the ATP-binding domain was replaced with neomycin-resistant cassette. The subsequent *Bsep*-deficient mouse (*Bsep*^{-/-}) was viable and fertile but displayed growth retardation [502]. Other physiological observations with the *Bsep*^{-/-} mice were 20% lower body weight than WT mice. The utility of the *Bsep*^{-/-} mice model provides an *in vivo* model to confirm the role of *Bsep* in the biliary excretion of bile salts in which its accumulation in the liver is one of several mechanisms for cholestasis. [¹⁴C]-Taurocholate was infused in *Bsep*^{-/-} and WT mice, and impaired secretion of radioactivity was observed 30 min following dosing in the KO animals (5% compared to 66% for WT) and accumulation in the liver was 70% compared to 2% for WT [502]. Additionally, total concentration of bile salts in the *Bsep*^{-/-} animals was reduced by fourfold and total bile salts in the liver and

plasma were elevated 4- and 5.8-fold, respectively, compared to WT animals. Also, the secretion of hydrophobic bile salts such as deoxycholate and chenodeoxycholate were statistically different between Bsep^{-/-} and WT animals but not for hydrophilic bile salts such as ursodeoxycholate, muricholate- ω , and muricholate- β . Taking this together, Bsep is the main transporter of hydrophobic bile salts and secretion of the hydrophilic bile salts appears to be mediated by a compensatory transporter.

Because Bsep does not efflux all the bile salts, Bsep^{-/-} mice exhibits mild cholestasis [502] unlike humans, where a mutation in BSEP can lead to fatal cholestatic disease [503]. Wang *et al.* [504] observed elevation of Mdr1a and 1b expression in Bsep^{-/-} mice. A triple KO mice model lacking Mdr1a, Mdr1b, and Bsep were generated by Wang *et al.* [504]. The triple KO animals showed histological changes typical of cholestatic stress, such as jaundice, limited bile flow in early adulthood, and signs of hepatic inflammation. More specifically, bile flow was reduced by 73% and bile salts accumulated by 50% in the liver of the triple KO animals but not in the WT animals. The studies conducted with the triple KO clearly demonstrate that the mild cholestasis observed in Bsep^{-/-} was the result of upregulation of murine Mdr1a and 1b, which compensated for the reduction in biliary clearance of hydrophobic bile salts as the triple KO mice demonstrated a severe cholestatic phenotype. As a note of caution, Bsep^{-/-} animal models exemplify the danger in utilizing KO animals to gain further insight toward a mechanism, as upregulation of compensatory proteins may occur, which may obscure the true role of a protein of interest. One of the key points is the observation that Bsep^{-/-} mice did not exhibit the severe form of cholestasis as that observed in humans.

19.12 CONCLUSION

GEMA have provided a means to gain new insights and validate mechanisms, disposition, and consequences of exposure to drugs or toxins. Specifically, the role of DMEs, transcriptional factors, and transporters have been further elucidated to understand activation and detoxification mechanisms that govern the toxicities of a molecule. The examples given in section 19.5 illustrate the importance of the enzyme in forming intermediates that can be further metabolized to toxic products which otherwise is thought of as a detoxification step. Many of the examples, however, illustrate the complexity of drug disposition processes in which most enzymes and transporters are relatively nonselective and promiscuous. Moreover, feedback mechanisms are present to rebalance homeostasis, which may upregulate parallel processes that make it difficult to achieve unequivocal conclusions. GEMAs provide a mechanistic tool to gain an overall assessment of the potential role of DMEs, transcriptional factors, and transporters as well as evaluate the potential interplay of these systems; however, species differences poses a challenge to truly predict potential outcomes in humans. While it is unlikely that GEMAs will become a routine tool in preclinical drug development, they do provide a tool to answer some specific questions and provide bespoke solutions (such as in section 19.11.1 and ATP-binding cassette transporter and intestinal and brain permeability). Overall, the animal models in research and drug development have provided enormous insight and validated mechanisms to provide greater understanding of the role of DMEs, transcriptional factors, and transporters in the absorption, disposition, metabolism, and drug-related toxicities.

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1 Introduction: An Overview of the Role of Metabolism in Drug Toxicity

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Up until the 1960s, the metabolism of drugs and other chemicals was primarily associated with detoxication processes. In fact, the first major monograph on the metabolism of drugs and other chemicals was written by R. Tecwyn Williams and appeared in 1947 under the title *Detoxication Mechanisms—The Metabolism of Drugs and Allied Organic Chemicals* [1]. This landmark book in the field of drug metabolism was followed by others that were widely used as texts and reference guides, which highlighted the basic enzyme systems involved in drug metabolism and the chemical structural groups affected. *Fundamentals of Drug Metabolism and Drug Disposition* by La Du *et al.* [2] and a two-part series entitled *Drug Metabolism: Chemical and Biochemical Aspects* and *Concepts in Drug Metabolism, Part B* by Bernard Testa and Peter Jenner are now classics in the field [3,4].

Since the 1980s, essentially every monograph in the area of drug metabolism has incorporated concepts of reactive metabolites and drug toxicity, and drug interactions and drug toxicity [5–8]. Some monographs have been primarily devoted to these concepts because in several instances, metabolism-based toxicities have caused human morbidity and mortality and limit the development and use of drugs and other chemicals that otherwise may be very beneficial [9]. Some of the earliest work on the characterization of reactive metabolites as mediators of toxicity were carried out in the 1950s during the investigations on mechanisms of chemical carcinogenesis by the Millers (see Ref. 10 for a review of this early work). Meanwhile, in approximately the same time period, the first case reports of drug interactions that caused drug toxicities appeared (see Ref. 11 for a proceedings report of these early cases).

Reactive metabolite-based drug toxicities came to the forefront with the now classic series of papers published in the late 1970s by researchers led by Jerry Mitchell, Jim Gillette, and B. B. Brodie in the Laboratory of Chemical Pharmacology of the National Heart, Lung, and Blood Institute at the NIH on the bioactivation of the widely used analgesic/antipyretic drug, acetaminophen, to a reactive metabolite that caused hepatotoxicity [12–15]. Thousands of research papers, many book chapters and reviews, and even a book have since been published on the mechanism of liver injury caused by this drug (for more recent reviews, see Refs 16–19). Several other examples of reactive metabolite formation from drugs leading to toxic effects in animals

and humans soon followed, and biological reactive intermediates have been the subject of numerous symposia and symposia proceedings. One of the most recent of these provided data showing that drug toxicity is now responsible for approximately one-third of the terminations of drug candidates in development and that over 70% of those cases were related to drug metabolism [20].

Although reports of drug interactions leading to toxicities have appeared since the early 1950s [21], drug interactions did not receive serious attention until the publication of *Drug Interactions: Clinical Significance of Drug Interactions and Drug Effects on Clinical Laboratory Results* by Hansten in 1971 [22]. The public did not really take note until the discovery in the early 1990s that inhibition of cytochrome P450 3A4 caused cardiac toxicity and death in patients taking the antihistamine, terfenadine [23–25]. This resulted in the issuance of new guidelines in 1997 by the FDA and other regulatory agencies worldwide, stipulating the need for *in vitro* drug interaction studies as part of the safety assessment of new drugs. Since then, books have been published on mechanisms of drug interactions [7].

The chapters in this volume of this encyclopedia represent an update on the multiplicity of chemical and biological factors that are involved in both reactive metabolites and their role in drug toxicities and in drug interactions. Box 1.1 is a brief synopsis of some of the major factors that are discussed. Drug structure plays a significant role, in that it determines the structures of metabolites that can be formed by a cadre of enzymes that can catalyze a wide variety of reactions on organic chemicals. The chapters titled *Bioactivation I: Bioactivation by Cytochrome P450s*, *Bioactivation II: Phase I (Non-P450)*, *Bioactivation by Phase-II-Enzyme-Catalyzed Conjugation of Xenobiotics*, *Safety Testing of Drug Metabolites: A Practical Approach for the Implementation of the MIST Guidance in PKDM*, *In Vitro Toxicity Screening in Early Drug Discovery: Importance of Metabolism and Reactive Metabolites* describe the mechanisms of formation of drug metabolites with a focus on bioactivation mechanisms and the various enzymes that catalyze the reactions. The chapter titled *Stereochemical Elements Associated with Drug Metabolism and Toxicity* describes specific examples where the stereochemistry of a drug and its metabolites play a significant role in drug toxicities and drug interactions. It is important for the reader to understand that all drug-metabolizing enzymes can form products that can cause toxicities depending on the drug structure, which when coupled to the particular reaction catalyzed, defines the structure of the metabolite.

BOX 1.1 SEVERAL IMPORTANT DETERMINANTS OF DRUG TOXICITIES.

Chemical structure of a drug and its metabolites

Dose of the drug

Rates of toxic metabolite formation vs. detoxification

(Dependent on concentrations of enzymes and cofactors, which in turn are affected by genetics, gut microflora, diet, and other drugs as inducers, inhibitors, etc.)

Immune recognition of macromolecular adducts of a drug or its metabolites

Efficiency of macromolecular repair mechanisms

BOX 1.2 A LIST OF SUBSTRUCTURES FOUND IN DRUGS THAT HAVE BEEN MOST COMMONLY ASSOCIATED WITH THE FORMATION OF REACTIVE METABOLITES THAT HAVE CAUSED TOXICITIES.

Drugs that contain or can be metabolized to the following structures may cause toxic effects via the formation of reactive metabolites:

- Hydrazines and hydrazides
- Arylacetic or aryl-propionic acids
- Thiophene, furan, or pyrrole
- Anilines or anilides
- Quinone and quinone imines
- Medium chain fatty acids
- Halogenated hydrocarbons and some halogenated aromatics
- Nitroaromatics
- Moieties that can form reactive α,β -unsaturated enal-like structures
- Thiols, thiono compounds, and thiazolidinediones

The major chemical substructures found in drugs that have caused overt tissue injury in the past are listed in Box 1.2. For the most part, these structures are metabolized to reactive electrophiles that interact with tissue nucleophiles (primarily proteins) to form covalent adducts, a topic of considerable interest at present that is discussed in detail in the chapter titled *Covalent Drug–Protein Adducts: An Exploration of Their Role in Drug-Induced Liver and General Organ Toxicity*. In some cases, these electrophilic species can also lead to oxidation of tissue macromolecules, and in a few cases, radical metabolites can be formed that can react with oxygen to form toxic oxygen products (Fig. 1.1).

It is well known that there are a host of factors that influence not only the enzymes involved in the metabolism of drugs but also the disposition of these metabolites and their ultimate effect on our biological system. Genetics and gene regulation play a very significant role in the rates of reactive metabolite formation, in their disposition as described in the chapter on *Genetics of Drug Disposition*, and in the response of the cell/tissue/organism to the effects of the metabolites. The first work in the area of pharmacogenetics was published over 50 years ago [26], and now, genome-wide association studies are helping to define the role of particular genes involved in drug toxicities (for discussions, see Refs 27 and 28). The advancing field of toxicogenomics is discussed in the chapter titled *The Application of Preclinical Toxicogenomics for Predicting and Understanding Drug-Induced Toxicity and Metabolism*, and the influence of genetics and gene regulation on drug interactions and drug metabolism is the topic of the chapters titled *Clinical Implications of CYP Induction-Mediated Drug–Drug Interactions*, and *Regulation of Drug Metabolizing Enzymes and Transporters in Infection, Inflammation, and Cancer*. Additional work in proteomics and metabolomics/metabonomics is extending our understanding of the effects of drugs and

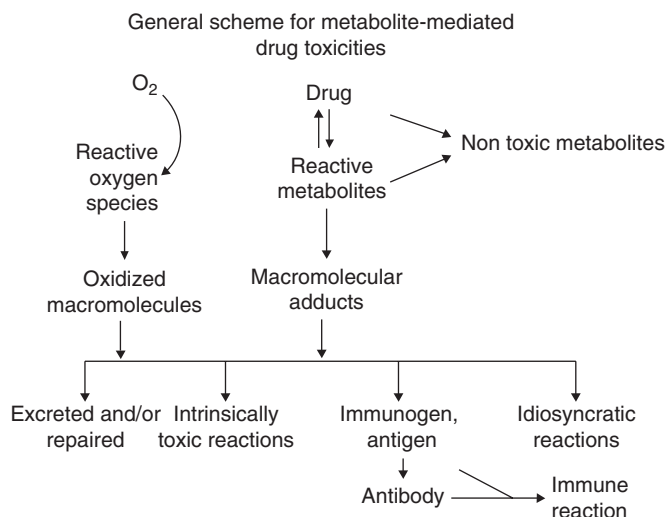


Figure 1.1 A simplified scheme for general mechanisms of drug metabolite-mediated drug toxicities.

drug metabolites at the cellular and whole body levels, thereby providing additional insights on phenotypic variations in drug metabolism, drug toxicities, and drug interactions. Proteomics in drug toxicities is the subject of the chapter titled *Proteomics in Drug Discovery and Development*, and metabolomics/metabonomics is the subject of the chapter titled *Metabonomics in Understanding Drug Metabolism and Toxicology*.

Finally, there are a host of other factors that can affect either the metabolism of drugs or the response of our biological system to the metabolites formed (Box 1.1). The dose of a drug (or any chemical substance) was recognized as a very important factor in as early as the 1500s when Paracelsus stated, “All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.” Thus, the lower the dose of a drug, the lower the amounts of toxic metabolites that can be generated. In most instances, this translates to a decreased risk for toxicity. However, many toxic effects of drugs occur in only a small percentage of individuals who take the drug. These idiosyncratic drug reactions are not yet very predictable, and several factors, such as inflammatory episodes, may leave an individual at more risk to an idiosyncratic drug reaction. This is the subject of the chapters titled *Idiosyncratic Drug Reactions* and *Animal Models of Idiosyncratic, Drug-Induced Liver Injury: Emphasis on the Inflammatory Stress Hypothesis*. Other factors such as drugs acting as inhibitors of drug metabolizing enzymes (see chapter titled *Drug–Drug Interactions 1: Inhibition*) age and diet (see chapters titled *Age-Dependent Expression of Human Drug-Metabolizing Enzymes* and *Phytochemical Modulators of Human Drug Metabolism: Drug Interactions with Fruits, Vegetables, and Botanical Dietary Supplements*) can also play a significant role in drug metabolism-related drug toxicities.

In summary, our knowledge of drug-mediated toxicities and the role that drug metabolites play in those toxicities has increased dramatically over the last 50 years. Advances in systems biology approaches to better define the most important factors in drug-metabolism-based toxicities should identify new biomarkers that can provide early signals of potential drug toxicities and lead to improvements in drug safety.

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2 Bioactivation I: Bioactivation by Cytochrome P450s

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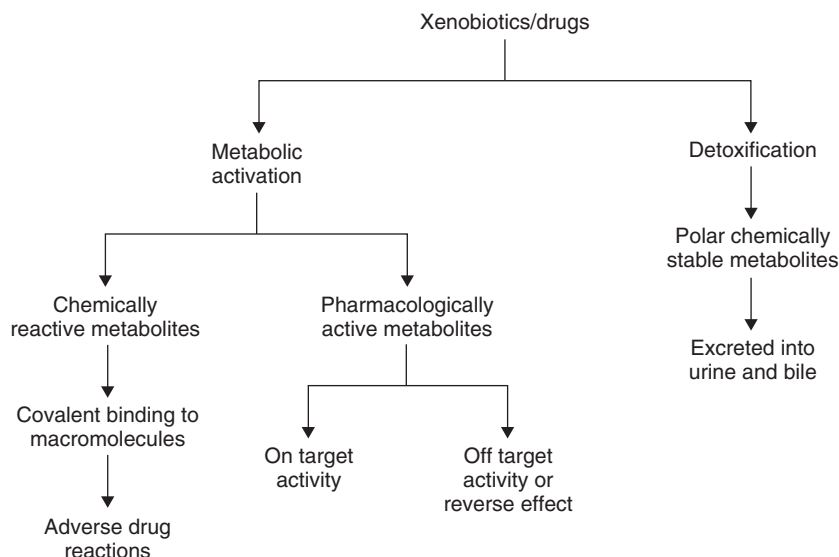
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2.1	Introduction	1
2.2	Bioactivation to pharmacologically active metabolites	2
2.3	Bioactivation chemically reactive metabolites	19
2.4	Summary	42
	References	42

2.1 INTRODUCTION

Metabolism is a process that converts nonpolar, lipid-soluble substances to polar more water-soluble products that facilitates their excretion from the body, renally or via the biliary route. It is the body's major line of defense against potentially toxic xenobiotic substances, including drugs and environmental chemicals. Consequently, it is the major clearance mechanism for most drugs and reduces the total body burden of these compounds by transforming them into one or multiple products. Although the conversion of compounds can occur chemically, most metabolic reactions are catalyzed by an array of enzyme systems in the body. These reactions are typically categorized as phase 1 or functionalization and phase 2 or conjugative reaction types. In functionalization reactions, xenobiotics are metabolized via oxidative, reductive, and/or hydrolytic pathways, with P450-mediated oxidation reactions predominating [1,2]. Conjugative reactions, on the other hand, result in conversion of the substituents introduced during functionalization reactions, or those already present in the xenobiotic, by derivatization to polar metabolites such as glucuronide and sulfate conjugates.

Metabolism reactions generally result in detoxification of xenobiotics and are accompanied by the formation of chemically stable metabolites, which are devoid of pharmacological or toxicological activities. However, in some cases, these reactions can convert drugs to products that are either chemically reactive or pharmacologically active, a process that is commonly referred to as *metabolic activation* or *bioactivation*



Scheme 2.1 Bioactivation of drugs.

(Scheme 2.1) [2,3]. The chemically reactive metabolites that are formed may interact with cells in numerous ways, such as covalently binding to cellular constituents or by stimulating peroxidation and decomposition of cellular lipids. In contrast, pharmacologically active metabolites are chemically stable. These metabolites can influence the therapeutic effect of a drug (on-target activity) or they could have off-target activities unrelated to the action of the parent molecule. In some instances, they might even reverse the pharmacological action of the parent drug.

Most, if not all, functionalization and conjugation reactions can result in bioactivation. The production of an ultimate toxin or a pharmacologically active metabolites involves either a single enzymatic reaction or, in some cases, several enzymatic and/or chemical reactions. Even though all enzymes have the propensity to catalyze the metabolic activation of compounds, cytochrome P450 (P450) enzymes, which generally dominate metabolism, play a major role in the metabolic activation of drugs [4–10]. The primary focus of this treatise is to provide an overview of the bioactivation by P450 to pharmacologically active and chemically reactive metabolites.

2.2 BIOACTIVATION TO PHARMACOLOGICALLY ACTIVE METABOLITES

Compounds transforming into active metabolites fall into two classes. One category includes conversion of active parent molecules to active metabolic products [11–16]. Alternatively, a biologically inactive compound can also undergo enzymatic conversion to metabolites that are pharmacologically active. Prodrugs are compounds that fall in this category [17–21].

2.2.1 Active Metabolites Produced from Bioactivation of Pharmacologically Active Drugs

Approximately 20% of the drugs marketed in the United States have been shown to form active metabolites [11]. Several classes of drugs produce active metabolites via P450 catalysis. Although all P450-mediated reactions can convert drugs to active metabolites, hydroxylation of aliphatic, alicyclic, or aromatic rings and N- or O-dealkylation reactions commonly result in metabolites that are equipotent or more potent than parent compound. Other biotransformation reactions such as epoxidation of an unsaturated system or heteroatom oxidation can also form active metabolites. In addition to improved pharmacodynamics due to increased potency, the metabolite may sometimes possess improved pharmacokinetic properties such as a longer half-life, lower probability of drug–drug interactions (DDIs) and lesser variability. Alternatively, the metabolites may have improved overall safety profile and better solubility than the parent drug. Active metabolites possessing one or more of the above attributes have been developed and marketed as drugs. Discussed below are examples of P450-catalyzed transformation reactions that convert the parent drug to pharmacologically active metabolites, some of which have been introduced as commercial products.

2.2.1.1 Aromatic and Aliphatic Hydroxylation. Two classic examples where aromatic oxidation of drug molecules leads to activation are atorvastatin (Lipitor) and tamoxifen. Atorvastatin undergoes extensive CYP3A4-catalyzed metabolism in the liver to two active hydroxy metabolites, *ortho*-hydroxy-atorvastatin (*o*-OH-atorvastatin) and *para*-hydroxy-atorvastatin (*p*-OH-atorvastatin) (Fig. 2.1). Both metabolites have

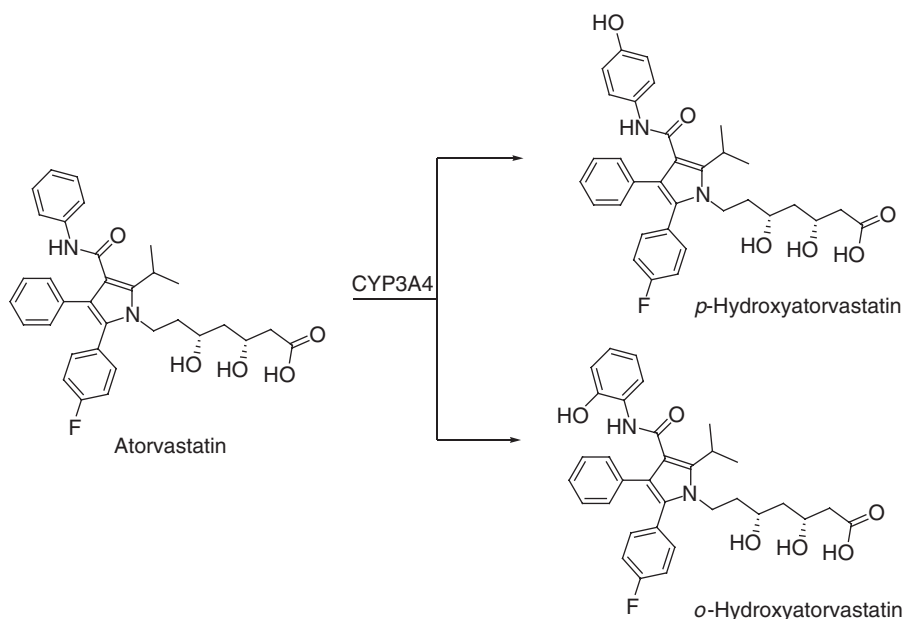


Figure 2.1 CYP3A4-catalyzed conversion of atorvastatin to *para*- and *ortho*-hydroxy-atorvastatin.

potency that is similar to that of the parent drug *in vitro* and it is speculated that at least 70% or more of the HMG CoA reductase inhibitory activity of atorvastatin is attributed to these two metabolites [22].

Likewise, tamoxifen, an estrogen receptor antagonist, which is extensively used for endocrine treatment of breast cancer is primarily converted to two metabolites that contribute to the antiestrogenic activity of the parent drug. These metabolic transformations are the CYP2D6-mediated 4-hydroxytamoxifen, and a second-generation metabolite, *N*-desmethyl-4-hydroxytamoxifen (endoxifen) (Fig. 2.2). The latter is probably formed via CYP2D6-catalyzed aromatic hydroxylation of *N*-desmethyltamoxifen, which is a major circulating metabolite in humans. Although both metabolites are more potent than the parent drug in terms of binding affinity and in suppressing estrogen-dependent cell growth, endoxifen is said to be the main contributor to the therapeutic activity of tamoxifen in patients [23,24].

Pharmacological activation of flutamide, a potent antiandrogen used for the treatment of prostatic cancer, by CYP1A2 to 2-hydroxyflutamide is a good example of instances in which the oxidation of an aliphatic side chain results in the formation of an active metabolite (Fig. 2.3) [25]. After a 250 mg dose of flutamide, the plasma concentrations of 2-hydroxyflutamide are much higher than those of the parent drug and contribute to the efficacy [26].

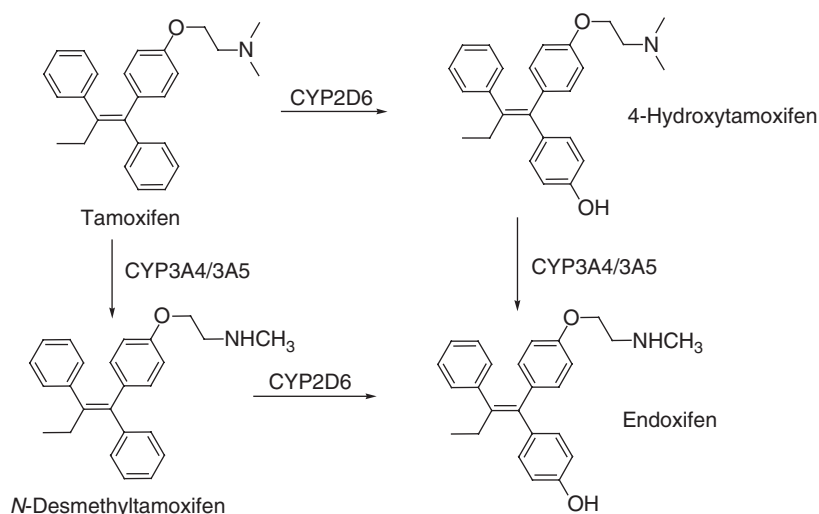


Figure 2.2 Metabolism of tamoxifen to its active metabolites, hydroxytamoxifen and endoxifen.

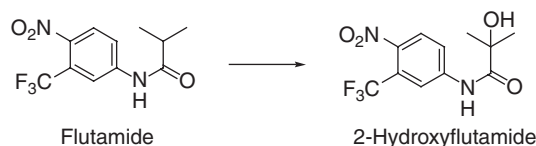


Figure 2.3 Metabolism of flutamide to its active metabolite 2-hydroxyflutamide by CYP1A2.

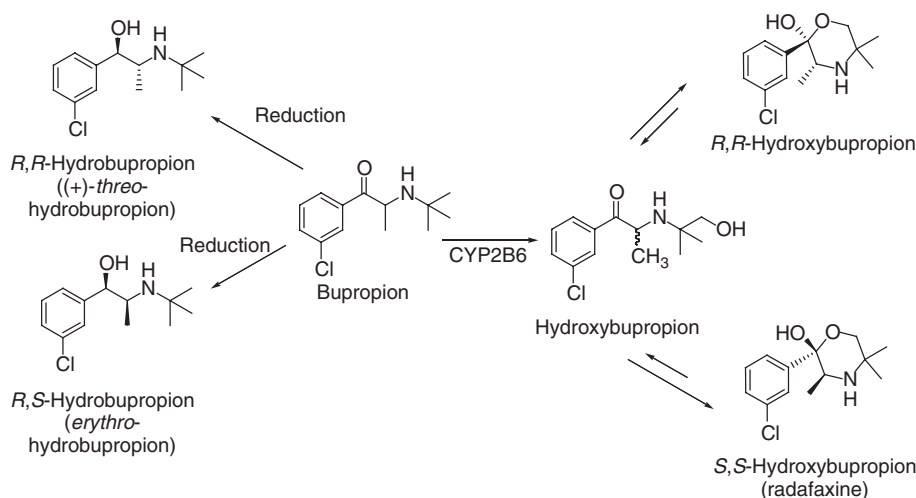


Figure 2.4 Metabolism of bupropion to active metabolites, *R,R*-hydroxybupropion, *S,S*-hydroxybupropion (radafaxine), *threo*-hydrobupropion, and *erythro*-hydrobupropion.

Similarly, clinical data have indicated that the biological activity of bupropion, an atypical antidepressant and smoking cessation aid, has been attributed to its active metabolites, *R,R*-hydroxybupropion, (*S,S*)-hydroxybupropion (radafaxine), *threo*-hydrobupropion, and *erythro*-hydrobupropion [27–30] (Fig. 2.4). The pharmacological activity appears to be primarily due to radafaxine, as plasma levels of these metabolites greatly exceed those of the parent drug. Although the *threo*-hydrobupropion and *erythro*-bupropion are probably formed via reduction of the carbonyl group, the formation of (*R,R*)-hydroxybupropion and radafaxine is mediated by CYP2B6 via hydroxylation of the *t*-butyl group [31]. The hydroxyated metabolites are generally in equilibrium with their respective morphinol derivatives that are formed via intramolecular cyclization [29].

Some drugs (e.g., terfenadine and losartan) are bioactivated to pharmacologically active metabolites following multiple P450-catalyzed oxidations. Terfenadine, an antihistamine used for the treatment of allergic conditions is converted to the carboxylic acid metabolite fexofenadine in two steps (Fig. 2.5) [32,33]. The first step in the formation of fexofenadine involves hydroxylation of the methyl group which is subsequently converted to fexofenadine. Most antihistaminic activity of terfenadine is attributed to fexofenadine formation *in vivo* [34,35]. Terfenadine was shown to promote the inhibition of cardiac ion channels (potassium ion currents) leading to prolongation of the QTc interval and ventricular arrhythmia. This risk of cardiac arrhythmia led to the withdrawal of terfenadine in the late 1990s. Fexofenadine lacks this property and is devoid of antiarrhythmic side effects of terfenadine [36,37]. Additionally, fexofenadine causes less drowsiness than first-generation histamine-receptor antagonists as a result of its poor permeability across the blood–brain barrier because of its disposition by efflux transporters such as Pgp [38].

Losartan, the first selective angiotensin II (AT1-subtype) receptor antagonist to be used in the treatment of hypertension and congestive heart failure, is oxidized by CYP2C9 to an active carboxylic acid metabolite (Fig. 2.6) [39]. Its formation involves

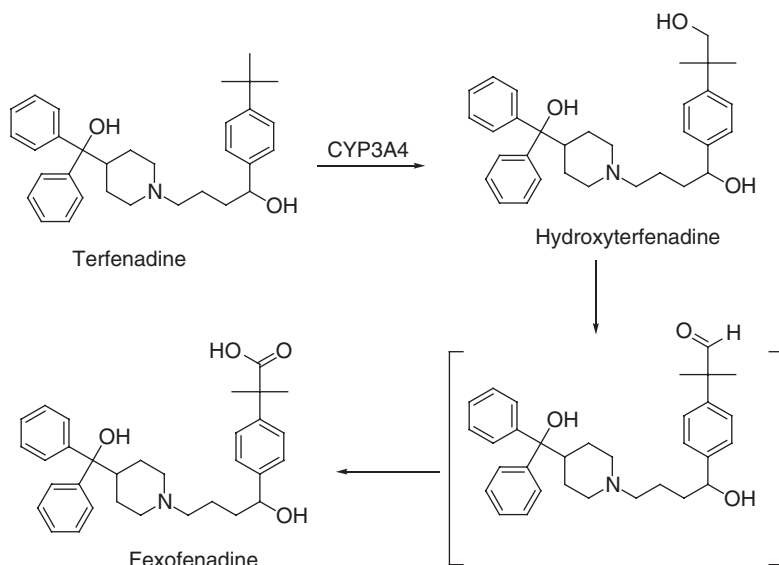


Figure 2.5 Metabolism of terfenadine to fexofenadine by CYP3A4.

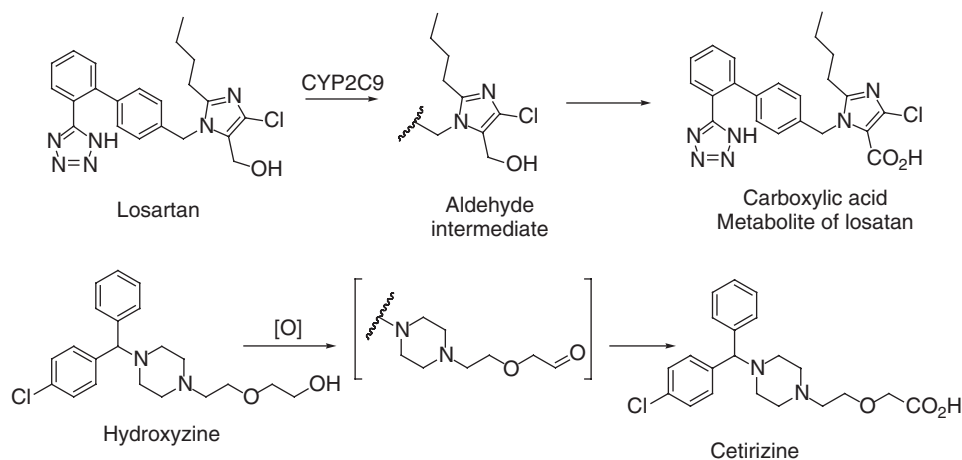


Figure 2.6 Metabolism of losartan and hydroxyzine to its carboxylic acid metabolites.

oxidation of the hydroxymethyl group to the corresponding aldehyde intermediate, which is consequently converted to the corresponding carboxylic acid metabolite (Fig. 2.6). This metabolite is a long-acting (6–8 h), noncompetitive antagonist at the AT₁ receptor and is 10–40 times more potent than the parent drug [40].

An example where the conversion of a hydroxymethyl group to an active carboxylic acid metabolite has resulted in commercialization of an active metabolite is that of cetirizine. Cetirizine (Zyrtec) is a second-generation antihistamine that is selective against H₁-receptor antagonist and is widely prescribed for seasonal and perennial allergic

rhinitis, and for dermatological conditions such as urticaria and pruritus. It is an acid metabolite of hydroxyzine (Atarax) and is formed via oxidation of the primary alcohol (Fig. 2.6). Approximately 45–60% of orally administered hydroxyzine undergoes oxidative metabolism to a carboxylic acid metabolite that possesses activity at H_1 histamine receptors and has a relatively small volume of distribution [41]. Although the enzymes for this biotransformation are not known, it can be speculated that this reaction is at least partially catalyzed by P450 enzymes. Not only does cetirizine have greater affinity for the H_1 receptor compared to hydroxyzine, but is also a long-acting nonsedating agent. This is partially attributed to its lack of distribution into brain tissue as a result of its strong Pgp substrate properties and poor membrane permeability [42].

2.2.1.2 Dealkylation Reactions. N/O-Dealkylation is one of the most common biotransformation reactions that can result in active metabolites. A classic example where O-dealkylated metabolite has replaced the parent drug in the market is that of acetaminophen. Acetaminophen is an active metabolite of phenacetin, which was once widely used as an analgesic (Fig. 2.7). Phenacetin was withdrawn because of its carcinogenic and kidney-damaging properties. Acetaminophen, on the other hand, is devoid of any carcinogenicity risks at therapeutic doses and quickly replaced phenacetin. It is currently one of the most popular analgesics in the market [43,44]. Likewise, desvenlafaxine (Pristiq), which was developed as a serotonin norepinephrine reuptake inhibitor (SNRIs) in the treatment of depression, is a major active O-dealkylated metabolite of venlafaxine (Effexor) (Fig. 2.7). The CYP2D6 catalyzed O-dealkylation leads to approximately 10-fold increase in potency at inhibiting serotonin uptake than norepinephrine uptake compared to venlafaxine [45]. Desvenlafaxine as a drug has several advantages over venlafaxine in that the former has a better safety and pharmacokinetic profile relative to the latter.

A P450-catalyzed O-dealkylation, which led to the development of a second-generation antihistaminic agent, is that of desloratadine (Clarinx). Desloratadine is an active metabolite of loratadine (Claritin) and approximately 10-fold more potent than loratadine [46]. Desloratadine is formed via CYP3A4-mediated oxidative decarbamylation of loratadine (Fig. 2.8) [47]. It is more potent and also has better pharmacokinetic properties such as lower oral clearance with greater systemic

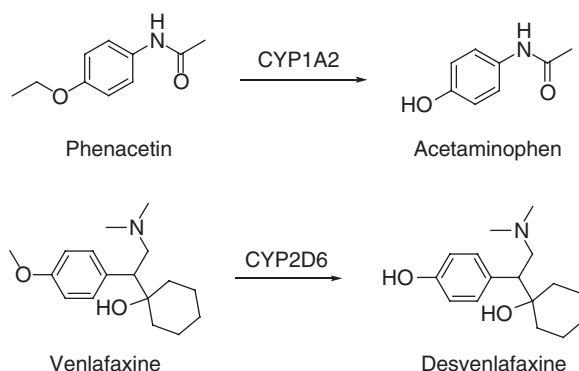


Figure 2.7 O-Dealkylation of phenacetin and venlafaxine to their active metabolites, acetaminophen and desvenlafaxine.

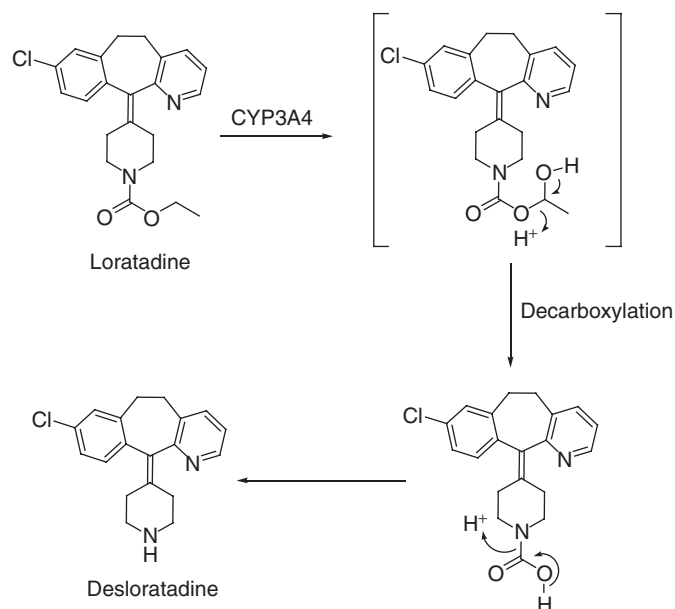


Figure 2.8 Metabolism of loratadine to desloratadine.

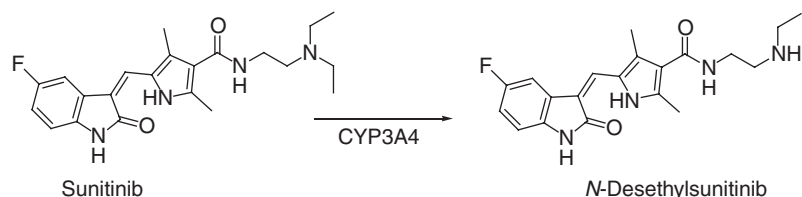


Figure 2.9 CYP3A4-catalyzed metabolism of sunitinib to *N*-desethylsunitinib, an active metabolite.

concentrations at comparable oral doses and a longer plasma elimination half-life and shows less pharmacokinetic variability in comparison to loratadine [46]. Further desloratadine does not cause drowsiness because it does not readily enter the central nervous system. Owing to its superior pharmacokinetics and pharmacodynamic and safety profiles, the standard human dose is half as much as that of loratadine.

Active metabolites that have been either marketed as drugs, or have to be monitored *in vivo* as a result of N-dealkylation, are quite common. For instance, the CYP3A4-catalyzed N-dealkylated metabolite of sunitinib (Sutent) is equipotent and has a longer half-life than the parent drug (Fig. 2.9) [48–50].

Many psychotropic drugs are converted into active metabolites via the N-dealkylation pathway [51–53]. Benzodiazepines are one such class of psychoactive agents that undergo N-dealkylation or hydroxylation and usually yield pharmacologically active metabolites. The formation of active metabolites is one of the factors that have been actually used in their classification as long-acting or short-acting agents. Those compounds that are transformed into an active metabolite which has a long

elimination half-life are classified as long-acting agents. Examples of benzodiazepines that fall into this class are diazepam (Fig. 2.10) and flurazepam (Fig. 2.11). On the other hand, temazepam, oxazepam (both of which are active metabolites of diazepam), and lorazepam (Fig. 2.11) are classified as short-acting agents as extensive metabolism of these agents results in rapid elimination of the active principle.

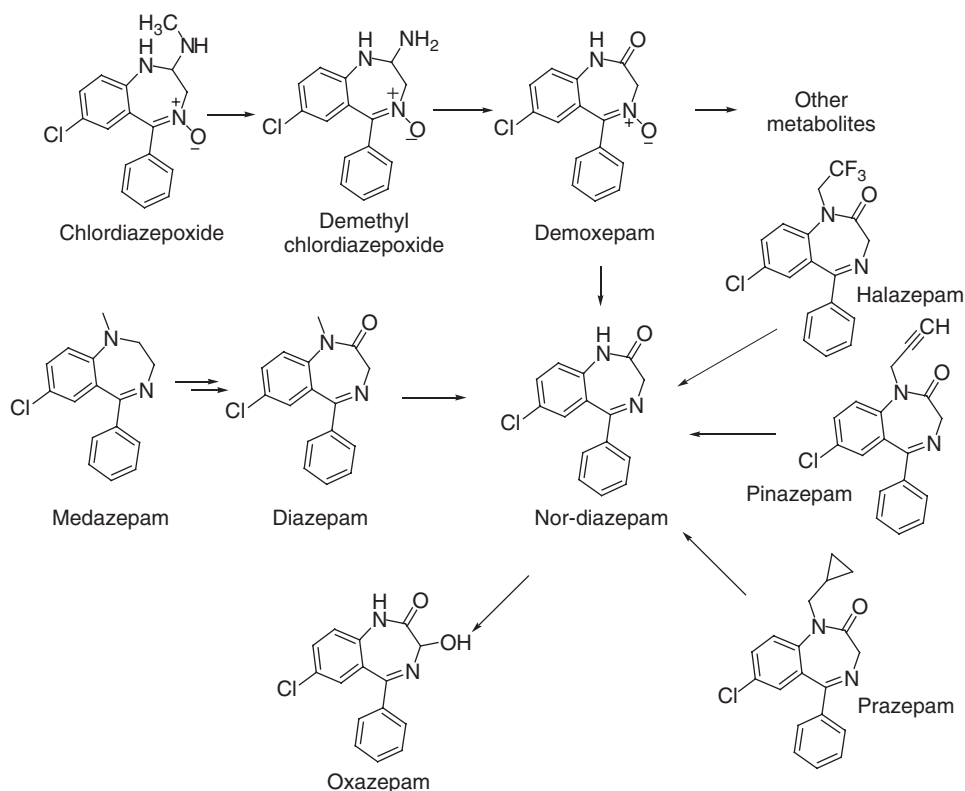


Figure 2.10 Metabolism of various benzodiazepines to active metabolite, nordiazepam.

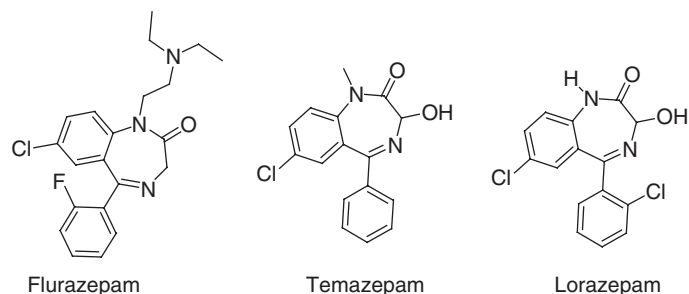


Figure 2.11 Structures of other benzodiazepines, flurazepam, temazepam, and lorazepam.

Chlordiazepoxide (Fig. 2.10) was the first benzodiazepine to be synthesized and marketed for clinical use as an antianxiety agent in the 1960s. The compound is extensively metabolized and most of its activity is attributed to nordiazepam, which is formed via a sequence of reactions (Fig. 2.10). Since nordiazepam has a long half-life due to very slow biotransformation, and it can potentially accumulate in the body after few days of treatment following repeated administration. Nordiazepam has also been detected as a major metabolite in the metabolism of other benzodiazepines including pinazepam, diazepam, medazepam, prazepam, and halazepam (Fig. 2.10). Hydroxylation of nordiazepam at position 3 yields another active metabolite oxazepam (Fig. 2.10), which has been marketed as a short-acting agent for the treatment of anxiety and insomnia and in the control of symptoms of alcohol withdrawal.

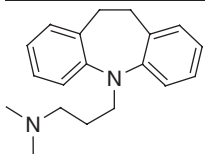
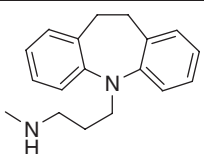
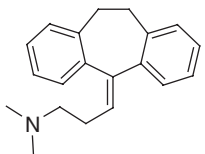
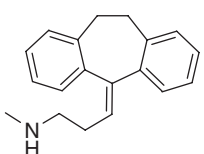
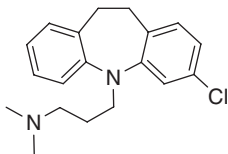
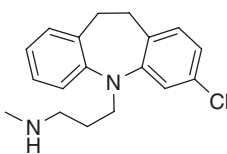
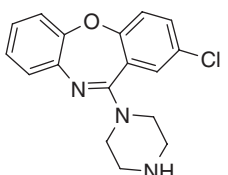
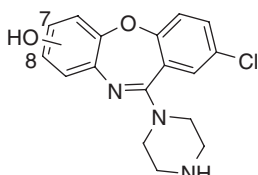
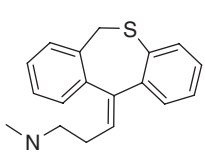
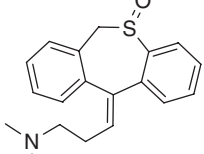
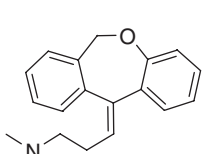
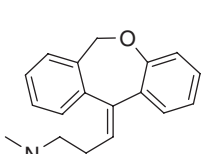
Like the benzodiazepines, several tricyclic antidepressants (TCAs) are also converted to active metabolites via N-dealkylation. Some of these demethylated metabolites have better pharmacokinetic properties and have been marketed as drugs in their own right. For example, amitriptyline and imipramine, used for the treatment of major depression in the United States, are primarily converted to its respective N-demethylated metabolites, nortriptyline and desipramine (Table 2.1), which have been marketed as second-generation antidepressant agents. The incidence of side effects in these second-generation antidepressants is lower than their predecessors. Table 2.1 also shows examples of other TCAs that are transformed to active metabolites via N-dealkylation.

Even though drug activation to a pharmacologically active metabolite is a minor pathway for some drugs, metabolism of the parent drug directly in the target organ can sometimes lead to pharmacological modulation at the site of action. For example, antianxiety agent alprazolam is metabolized to two hydroxylated metabolites by CYP3A enzymes, a major, 4-hydroxy alprazolam metabolite that is pharmacologically less active and a minor α -hydroxy alprazolam (α -OHALP) metabolite which is (pharmacologically more active) in humans (Fig. 2.12) [54–56]. It is speculated that the formation of large amounts of α -OHALP at the site of action potentially impacts the pharmacological activity of this antianxiety drug [57,58].

2.2.1.3 Other Reactions Leading to Active Metabolites. As mentioned previously, other P450 catalyzed can also result in conversion of drugs to active metabolites. For instance carbamazepine-10,11-epoxide, derived from carbamazepine, has anticonvulsant activity comparable with that of its progenitor (Fig. 2.13) [59]. Similarly, CYP2D6- and 2J2-catalyzed S-oxidation of the methylthio group in thioridazine yields two active metabolites, the sulfoxide, mesoridazine, and the sulfone, sulforidazine [60,61]. Mesoridazine is twice as active as the parent drug and as a result it was introduced into clinical use as antipsychotic agent (Fig. 2.13) [62].

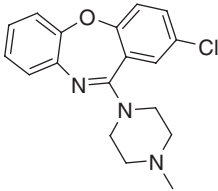
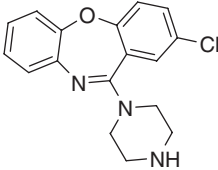
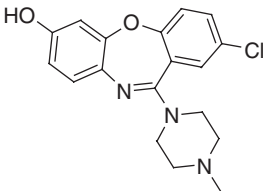
Leflunomide (Arawa) represents another interesting example which involves P450-mediated reductive bioactivation to an active metabolite. This drug is marketed as an orally active disease-modifying anti-inflammatory agent for the treatment of advanced rheumatoid arthritis [63]. The principal biotransformation pathway of leflunomide in humans involves an N–O bond cleavage of its isoxazole ring leading to the formation of α -cyanoenol metabolite (A771726) which is the active entity (Fig. 2.14) [63,64]. *In vitro* studies have shown that this ring cleavage is catalyzed by CYP1A2 [65]. All activity of leflunomide is ascribed to this ring-opened metabolite which is found

TABLE 2.1 Tricyclic Antidepressants that Are Activated to Pharmacologically Active Metabolites^a

Parent	Active Metabolite
 Imipramine	 Desipramine
 Amitriptyline	 Nortriptyline
 Clomipramine	 Nor-clomipramine
 Amoxapine	 8 and 7 hydroxyamoxapine
 Dothiepin	 Dothiepinsulfoxide
 Doxepine	 Nordoxepine

(continued overleaf)

TABLE 2.1 (continued)

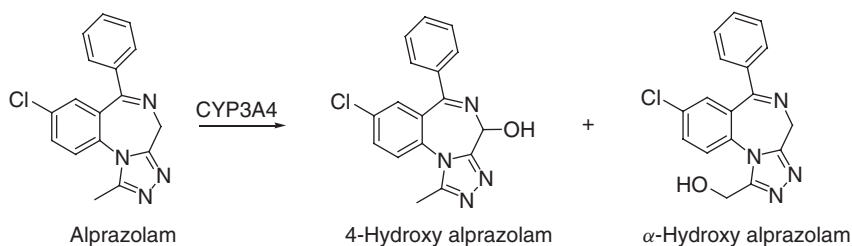
Parent	Active Metabolite
 Loxapine	 Amoxapine
	 7-hydroxyloxapine

^aRef. 51.

circulating in humans since the circulating levels of the parent drug are below the limit of detection.

There are instances where the biotransformation could result in metabolites with activities that might even reverse the pharmacological action of the parent drug. For instance nefazodone is metabolized by humans into three pharmacologically active metabolites, hydroxynefazodone, triazolodione, and N-dealkylation of nefazodone to *m*-chloro phenylpiperazine (mCPP) by CYP3A4 (Fig. 2.15) [66–69]. Although, triazolodione and hydroxynefazodone may contribute significantly to the antidepressant effects of nefazodone, mCPP acts an agonist of 5-HT_{2c} receptor, an action which might be expected to produce an anxiogenic effect [70,71].

Similarly, the truncated phenolic metabolite of tamoxifen (metabolite E) formed by the cleavage of dimethylaminoethyl side chain (Fig. 2.16) is predominantly estrogenic

**Figure 2.12** Hydroxylation of alprazolam to its hydroxylated metabolites.

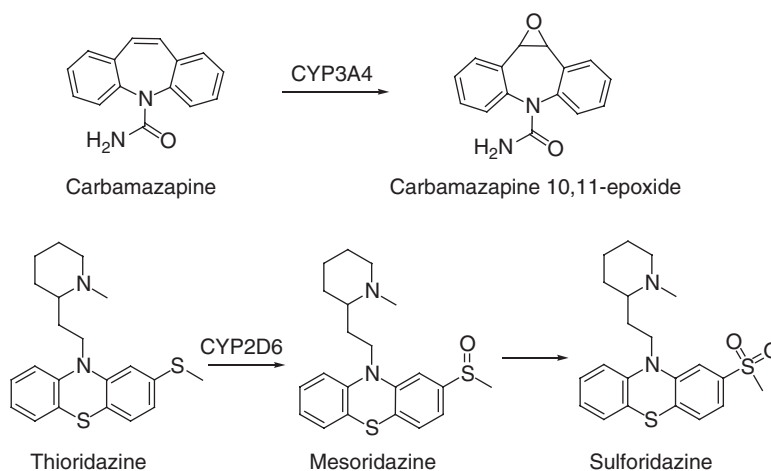


Figure 2.13 Metabolism of carbamazepine and thioridazine to its active metabolites.

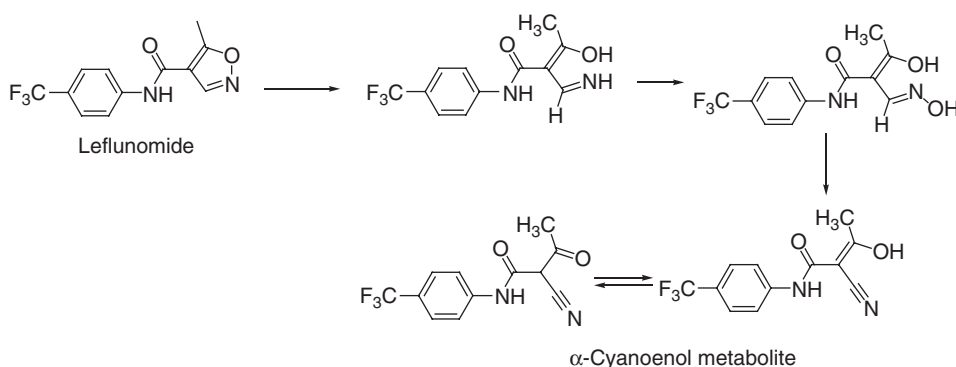


Figure 2.14 Ring opening of the isoxazole ring of leflunomide to an active metabolite.

in its activity in contrast to tamoxifen and its two metabolites, hydroxytamoxifen and endoxifen (Fig. 2.2), which are antiestrogenic [72–74].

It is important to note that active metabolites which contribute to the efficacy can also elicit side effects that are observed after administration of the parent drug. For instance, mephenytoin is associated with increased incidence of serious toxic events including rash, agranulocytosis, aplastic anemia, and hepatitis [59]. Reports indicate that the *N*-desmethyl metabolite of *R*-mephenytoin (Methoin) (Fig. 2.17), which was once marketed under the name of nirvanol, is responsible for the bone marrow toxicity observed following mephenytoin administration [59]. Similarly serious adverse effects such as neurotoxicity and cardiotoxicity arising from meperidine (also referred to as *Demerol*) and propoxyphene (Fig. 2.17) are primarily attributable to their metabolites, normeperidine and norpropoxyphene, respectively [75]. Active metabolites themselves could induce or inhibit drug-metabolizing enzymes and as a result could also affect the pharmacokinetic profile of other drugs. For example, norfluoxetine, an active metabolite

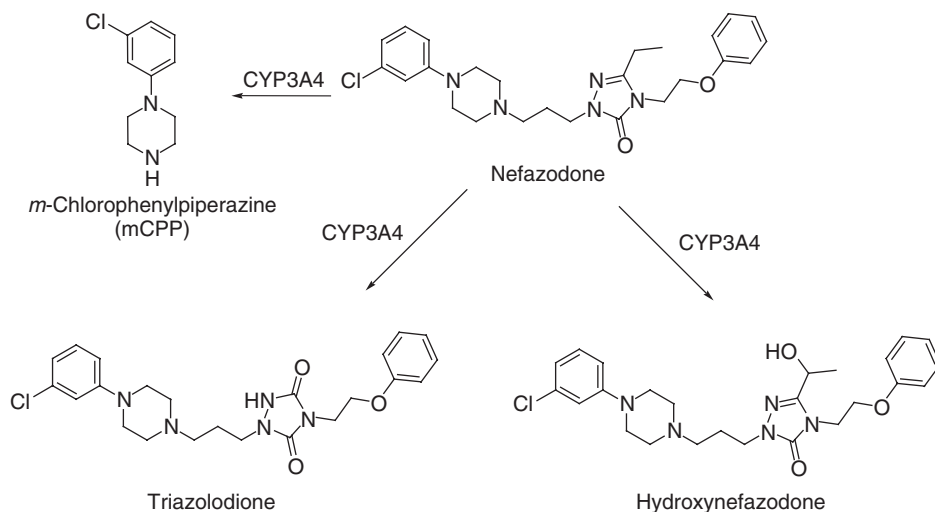


Figure 2.15 Metabolism of nefazodone.

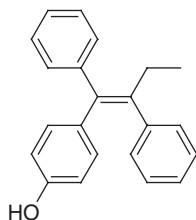


Figure 2.16 Structure of metabolite E of tamoxifen.

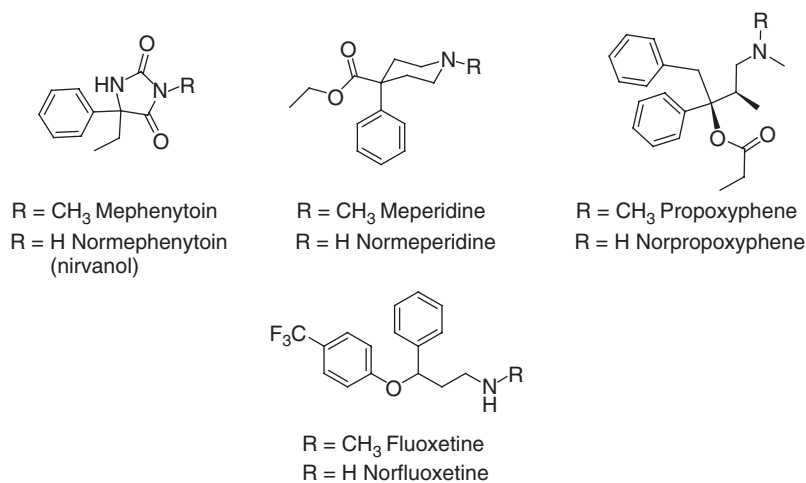


Figure 2.17 Structures of mephenytoin, meperidine, propoxyphene fluoxetine, and its N-demethylated metabolites.

of fluoxetine (Fig. 2.17) is a potent inhibitor of CYP2D6. As a result of the long half-life (up to two weeks) of norfluoxetine, the inhibitory effect persists for weeks after administration of fluoxetine [76]. This has led to the discontinuation of this drug.

2.2.2 Bioactivation of Inactive Compounds (Prodrugs)

As noted earlier, prodrugs are compounds with little or no pharmacological activity, which undergo biotransformation to a therapeutically active metabolite. Enzyme-mediated bioactivation of pharmacologically inactive compounds to active metabolites has been commonly used as a part of drug design when the drugs possess poor absorption properties or are unstable in the acidic pH of the GI tract following oral administration. Common reactions that usually catalyze this conversion are mediated by a broad class of hydrolytic enzymes, such as esterases, amidases, and phosphatases. However, P450-mediated oxidation reactions have also been employed to bioactivate molecules to moieties that can elicit their therapeutic action once formed *in vivo*. Some examples of where inactive compounds are activated via CYP-mediated biotransformation include encainide, cyclophosphamide, ticlopidine, and clopidogrel.

Encainide is a class IC antiarrhythmic agent. Indirect evidence has strongly suggested that the slowing of intracardiac conduction (prolongation of QRS) by encainide is mediated by its metabolites [77]. In humans, encainide is primarily metabolized to *O*-demethyl encainide and 3-methoxy-*O*-demethyl encainide. A third metabolite, *N*-demethyl encainide, also has been identified in serum from some patients [78]. Assessment of the activity of these metabolites revealed that *O*-demethyl encainide was nine times as potent as the parent drug and 3-methoxy-*O*-demethyl encainide equipotent to the parent drug. Some studies show that metabolic transformation of encainide to its *O*-demethyl derivative (Fig. 2.18) is a prerequisite for its antiarrhythmic activity as patients with reduced formation of this CYP2D6-catalyzed product show poor response to the drug [79,80]. Thus, in its own right, encainide is a prodrug of desmethylencaïnide that is activated by its CYP2D6-mediated *O*-demethylation. Similarly, some reports indicate that tamoxifen and leflunomide can also be classified as prodrugs because both these compounds are converted to metabolites that are

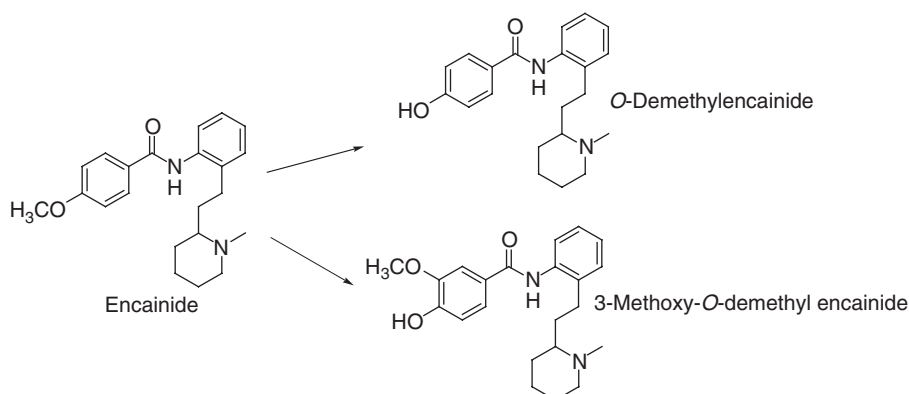


Figure 2.18 Metabolism of encainide to its active metabolites.

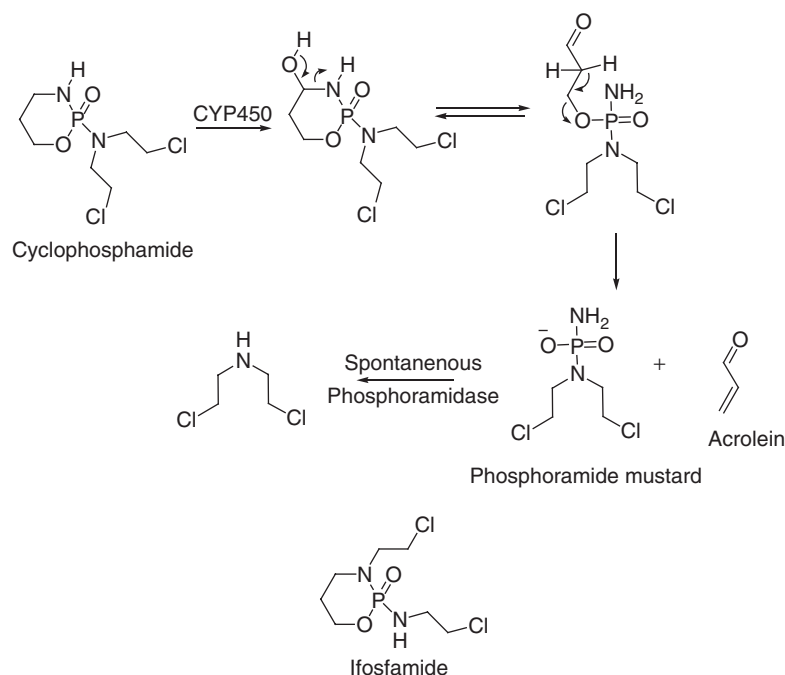


Figure 2.19 Bioactivation of cyclophosphamide by P450 and structure of ifosfamide.

even more potent than the parent and almost all the effects observed are due to these metabolites.

The oxazaphosphorines, cyclophosphamide and ifosfamide (Fig. 2.19), are alkylating agents that require bioactivation via P450 isoenzymes including CYP2C9 and 2B6 enzymes [81–83]. Cyclophosphamide has emerged as an important drug for the treatment of a wide variety of malignant diseases. The parent compound as such is inactive in the tissue culture. However, preincubation of the drug with liver homogenates activates the molecule suggesting that cyclophosphamide is a prodrug that requires oxidative metabolism to generate the active principle. The activation mechanism is depicted in Fig. 2.19. The initial step entails CYP-mediated oxidation of the ring on the carbon adjacent to the nitrogen of the phosphoramidate moiety. The hydroxylated metabolite is in equilibrium with the ring-opened aldehyde which undergoes spontaneous decomposition to the corresponding phosphoramidate mustard and acrolein. It is not clear if the phosphoramidate mustard or the parent nitrogen mustard (that is formed on hydrolysis, chemically or by phosphoramidase) is responsible for the therapeutic action [84,85].

Another interesting example which entails P450-mediated mechanism for activation to an active moiety is exemplified by drugs such as clopidogrel and ticlopidine (Fig. 2.20) [86]. These tetrahydrothienopyridine derivatives were developed as antithrombotic agents that inhibited platelet aggregation. Ticlopidine (Ticlid) was the first compound of this class, introduced to the market in 1979 for the prevention of thrombotic stroke. Clopidogrel (Plavix/Iscover) was later launched in the United States and Europe in the late 1990s for the reduction of atherosclerotic events in patients with

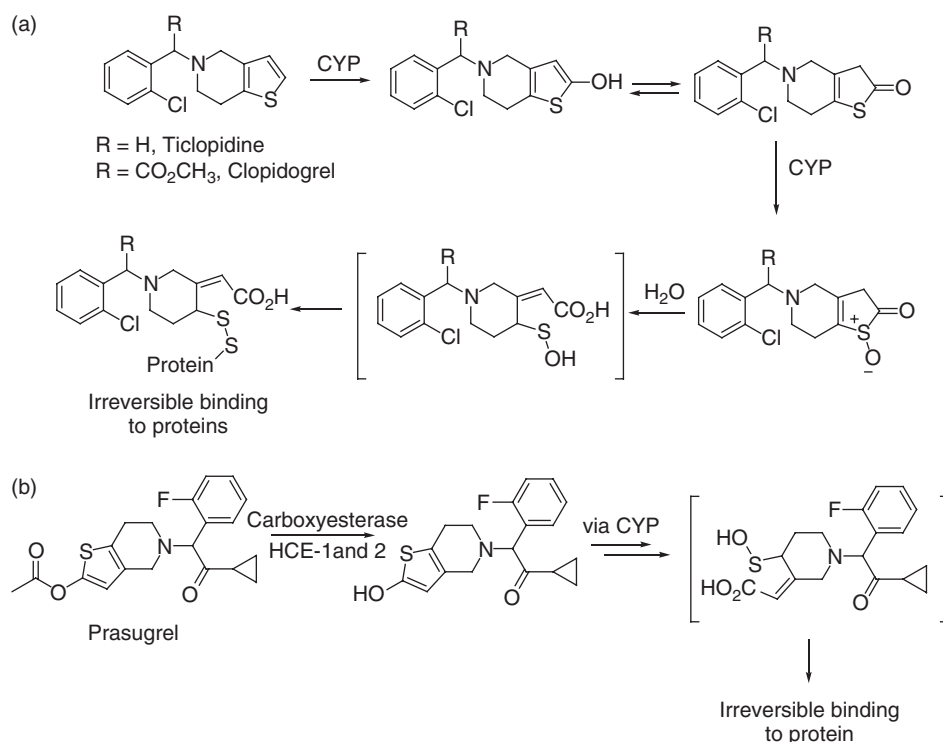


Figure 2.20 Metabolic activation of (a) clopidogrel and ticlopidine and (b) prasugrel.

stroke, myocardial infarction (MI), or peripheral arterial disease. Both ticlopidine and clopidogrel must be metabolized *in vivo* into a pharmacologically active derivative, in order to exert their activity as antagonists of the platelet ADP receptor P2Y (Fig. 2.20). Savi and coworkers [87] have demonstrated the importance of hepatic metabolism in eliciting clopidogrel's effect on platelet aggregation and that the active metabolite was formed through an intermediate, 2-oxo-clopidogrel. The metabolic activation occurs in two steps. The first step is a classical CYP2C19-catalyzed monooxygenation of the thiophene ring that leads to the thiolactone metabolites, 2-oxoticlopidine and 2-oxoclopidogrel. The second step involves sulfoxidation of the thiolactone, which undergoes spontaneous cleavage to the corresponding sulfenic acid intermediate. This highly reactive intermediate forms a covalent S–S bridge with a thiol group in platelet ADP receptors, thereby causing their long-lasting inactivation.

Prasugrel (Effient/Efient) (Fig. 2.20) is a newest member of this class of drugs [86]. Like ticlopidine and clopidogrel, it is extensively metabolized and also undergoes activation in the same way as clopidogrel and ticlopidine. The key difference between prasugrel and its predecessors, however, is the presence of the acetoxycarbonyl group at position 2 of the thiophene ring in prasugrel. Hence, the first step in the activation of prasugrel involves hCE2 ester hydrolysis to the thiolactone in contrast to the CYP-catalyzed oxidation to the thiolactone for clopidogrel or ticlopidine. The thiolactone is then activated to sulfenic acid via a CYP-mediated pathway that is similar to that described for clopidogrel and ticlopidine (Fig. 2.20).

2.2.3 Consequences of Formation of Pharmacologically Active Metabolites

Contribution of an active metabolite(s) to the overall pharmacological action of drugs depends on the disposition characteristics of the metabolites. Therefore, just the knowledge that a particular compound forms an active metabolite and has specific pharmacological properties is not sufficient. The free concentration of this metabolite should be sufficient enough at the target site to produce a significant effect. Further, most active metabolites have longer plasma elimination half-lives than parent drugs. This can lead to increased accumulation of the metabolites upon chronic dosing, even when they are formed as minor metabolites and their systemic concentrations upon acute dosing appear to be very low. Thus, if an active metabolite has significant potency and a good pharmacokinetic profile, and is formed in sufficient concentrations, it is very likely that there is a greater and/or longer duration of pharmacological action following administration of a parent drug. The result is a complex dose–response relationship, especially if the metabolite contributes significantly to the therapeutic effect of the drug and accumulates in tissues. This might necessitate dose adjustment. It is therefore necessary to identify active metabolites early on and monitor their plasma concentrations, in addition to the parent, in order to assess its concentration–response relationship and guide therapy [88,89].

Secondly, it is important to assess the factors affecting the formation of active metabolites. All factors that affect the metabolism of a principle drug will influence the formation of active metabolites. Some important factors include genetics, hepatic and renal impairment, and age. Additionally, other disease states, interactions with other drugs (inhibition or induction of enzymes) that can alter the levels of enzymes and therefore affect the amount of active metabolites formed. Among all these, genetic polymorphism can have a significant impact on efficacy/safety if active metabolites that are formed by polymorphic enzymes contribute to the therapeutic effect of the drug. For instance, codeine is primarily metabolized by CYP2D6 and undergoes O-demethylation to morphine. Owing to genetic variability in the CYP2D6 enzyme, patients receiving codeine can be poor, intermediate, or fast metabolizers of codeine into its active metabolite, morphine. Given the same dose, poor metabolizers may receive minimal relief, whereas fast metabolizers may experience a morphine overdose [75]. Likewise coadministration of codeine with quinidine a 2D6 inhibitor results in increased levels of the drug, lesser efficacy, and increased codeine-induced toxicity [90]. On a similar note, as CYP2D6 is the rate-limiting enzyme catalyzing the conversion of tamoxifen into metabolites with significantly greater affinity for the estrogen receptor and greater ability to inhibit cell proliferation, not all women benefit from tamoxifen treatment. Also, the side-effect profiles vary considerably from patient to patient. Thus, both genetic and environmental (drug-induced) factors that alter CYP2D6-enzyme activity directly affect the concentrations of the active tamoxifen metabolites and the outcomes of patients receiving adjuvant tamoxifen. An *a priori* knowledge of the pharmacogenetic variation in CYP2D6 enzyme activity may therefore be useful in individualizing the hormonal therapy of breast cancer [91]. Further, coadministration of tamoxifen with paroxetine, a CYP2D6 inhibitor, has been shown to alter the efficacy of tamoxifen because of the decrease in the plasma concentrations of 4-hydroxytamoxifen and endoxifen [92].

More recently, a lack of clopidogrel effect has been reported in some patients treated with this drug [93]. As mentioned earlier, the polymorphic enzyme CYP2C19 is the

primary enzyme responsible in the bioactivation of clopidogrel. Thus, compared to the wild-type individuals, CYP2C19 variant allele carriers exhibit a significantly lower capability to metabolize clopidogrel to its active metabolite and inhibit platelet activation. These patients are at a higher risk of adverse cardiovascular events. Investigations to understand this have demonstrated that genotyping of patients can readily predict this nonresponsiveness and poor inhibition of platelet activation. Consequently, the regulatory agencies have modified the prescribing information of this drug to highlight the impact of CYP2C19 polymorphism on efficacy. Other examples where genetic variation can impact the therapeutic efficacy of a drug include losartan and cycloguanil [11]. Overall, these examples emphasize the potential importance of pharmacogenetic considerations in the assessment of active metabolites in the development of drugs.

2.3 BIOACTIVATION CHEMICALLY REACTIVE METABOLITES

The concept of metabolic conversion of chemicals to reactive products that covalently bind critical protein components stems back to the pioneering work by Miller and coworkers who demonstrated the carcinogenic and hepatotoxic activity of polycyclic aromatic hydrocarbons and aminoazo dyes to arise from their P450-mediated bioactivation [94–96]. Since then several reviews on bioactivation of drugs to chemically reactive intermediates have appeared in the past few decades [5–8,97–99]. A brief overview of P450-mediated reactions that result in chemically reactive intermediates and examples of drugs that undergo bioactivation via these mechanisms is described here.

All major isoforms of P450 can catalyze metabolic activation of drugs to reactive intermediates [4–6,98,100]. Many organic functional groups are particularly susceptible toward P450-mediated bioactivation to reactive intermediates. Details on these functional groups and their proposed bioactivation pathways catalyzed by P450 enzymes have been recently reviewed [101]. Although all oxidation reactions are capable of generating a reactive metabolite, some of the common reactions by which the P450 transform drugs to reactive metabolites are described below.

2.3.1 *N*-Oxidation of Aromatic Amines

A compound containing an aniline group is generally associated with serious adverse side effects such as hypersensitivity reactions, lupus, and carcinogenesis [102]. Most of these side effects are attributed to P450-catalyzed metabolic activation of the primary amine via *N*-oxygenation to hydroxylamine derivative. The resulting *N*-hydroxylamine products are acylated to produce highly reactive ester derivatives such as the *N*-acetoxy, *N*-sulfonyloxy, or *N*-phosphatyl esters, which can undergo heterocyclic cleavage to yield reactive aryl nitrenium ion species that covalently bind to DNA (Fig. 2.21) [103]. Several arylamines including 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ), or 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) bind to DNA to form adducts via the nitrenium ion. An alternative mechanism has also been proposed in which the hydroxylamine is oxidized to the electrophilic nitroso derivative, that can covalently bind to macromolecules via reaction with sulfhydryl groups yielding the corresponding

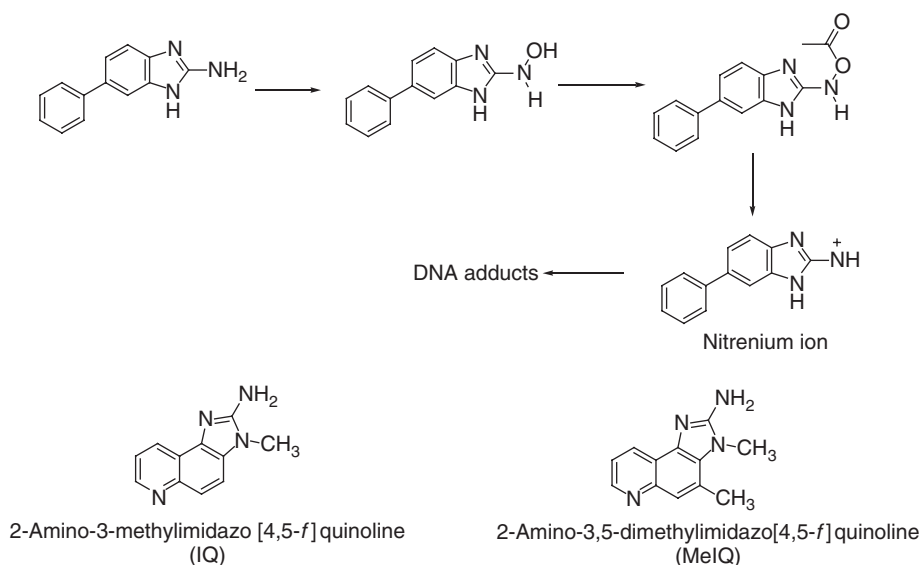


Figure 2.21 Metabolic activation of arylamines by P450 via the reactive ester pathway.

sulfonamide adducts (Fig. 2.22) [104,105]. This mode of bioactivation has been demonstrated for several very popular drugs such as sulfamethoxazole, dapsone, and procainamide (Fig. 2.22) [106–109].

Like arylamines, most drugs containing a nitroaromatic ring also generate a nitroso intermediate. However, the process of bioactivation of nitroaromatics to the nitroso intermediate involves a two-electron reduction as opposed to N-oxidation and is a part of the six-electron reduction to the aniline moiety (Fig. 2.23). Chloramphenicol is a good example where the nitro group is reduced to form a reactive intermediate [110]. Other examples containing a nitro group include nimesulide, dantrolene, and nilutamide (Fig. 2.23). The toxicity of all these compounds is ascribed to the reduction of the nitro group [111]. P450 and/or CYP-reductase is the primary enzyme that catalyzes this bioreduction of the nitro group. However, other enzymes including xanthine oxidase, aldehyde oxidase, and quinone reductase can also play a role [111].

2.3.2 Epoxidation of Arenes and Olefins

P450-catalyzed epoxide formation is an important metabolic transformation of drugs and xenobiotics containing an aromatic or an olefin moiety. Most often, the resulting arene or olefin oxides undergo detoxification via spontaneous rearrangement or subsequent epoxide hydrolase-mediated hydrolysis to the more stable arenols or dihydrodiols. However, in some instances, epoxide intermediates can react with nucleophilic biomolecules (e.g., DNA or proteins) to form macromolecular adducts that can manifest carcinogenicity or hepatotoxicity [5].

Bioactivation of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrenes is a good example where arene oxides have been implicated in carcinogenesis [112]. Numerous metabolic intermediates of benzo[*a*]pyrene have been postulated to be chemically reactive and capable of binding to nucleic acids. However, the key metabolite that efficiently

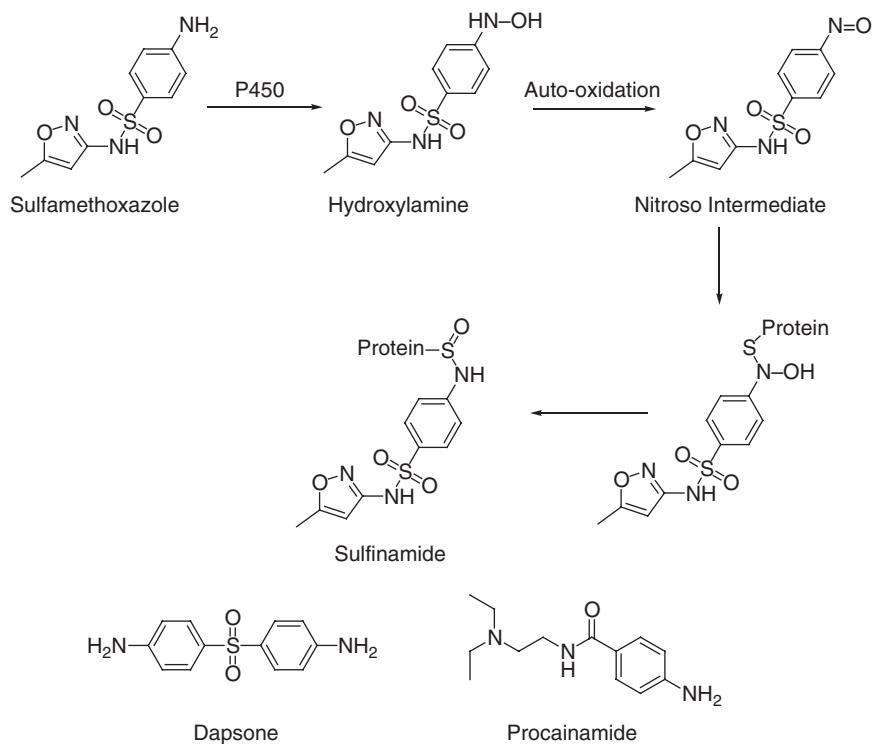


Figure 2.22 Metabolic activation of sulfamethoxazole via the nitroso intermediate.

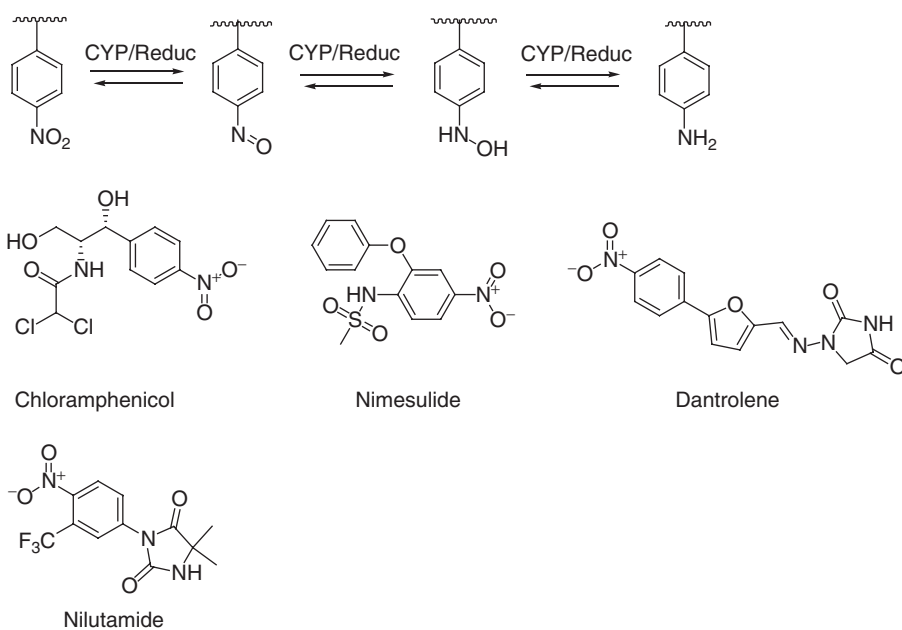


Figure 2.23 Bioactivation of compounds containing nitroaromatic rings.

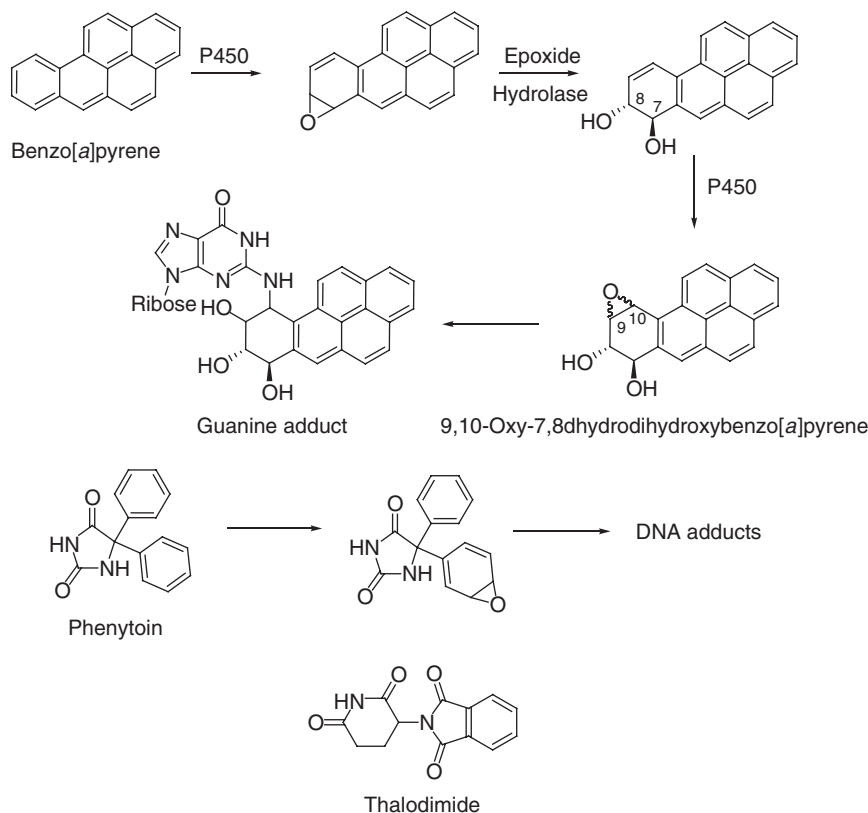


Figure 2.24 Metabolic activation of compounds via the arene oxide pathway.

alkylates nucleic acids is 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Fig. 2.24). The first step involves the oxidation of benzo[*a*]pyrene to 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by P450 and epoxide hydrolase. Subsequent stereoselective epoxidation of the 9,10-double bond of this diol yields *R*9, *R*10 and *S*9, *S*10-epoxides [113]. Both these epimers are highly reactive and react with nucleic acids to form a covalent adduct between the C-2 amino group of guanosine and the C-10 position of the hydrocarbon [114,115]. Similarly, arene oxides have been implicated in cytotoxic and tetratogenic effects that are manifested by phenytoin and thalidomide [116,117] (Fig. 2.24).

The formation of an epoxide intermediate is also a common pathway in the bioactivation of heteroaromatic rings [118]. For example, the chloro-imidazopyridine ring in alpidem is subject to P450 bioactivation, thus leading to the formation of a reactive epoxide that reacts with GSH to yield sulfhydryl-linked conjugates, which have been detected in humans (Fig. 2.24) [119]. Similarly, epoxidation also plays role in the hepatotoxicity of five-membered heteroaromatic rings. For example, the first step in the bioactivation of furan derivatives involves a P450-catalyzed oxidation to a heteroarene oxide. This can directly react as hypothesized in the case of furosemide (Fig. 2.25) [120], or most often it undergoes spontaneous ring opening to the enedialdehyde that reacts with sulfhydryl groups in proteins (Fig. 2.26) [121]. Oxidative ring opening

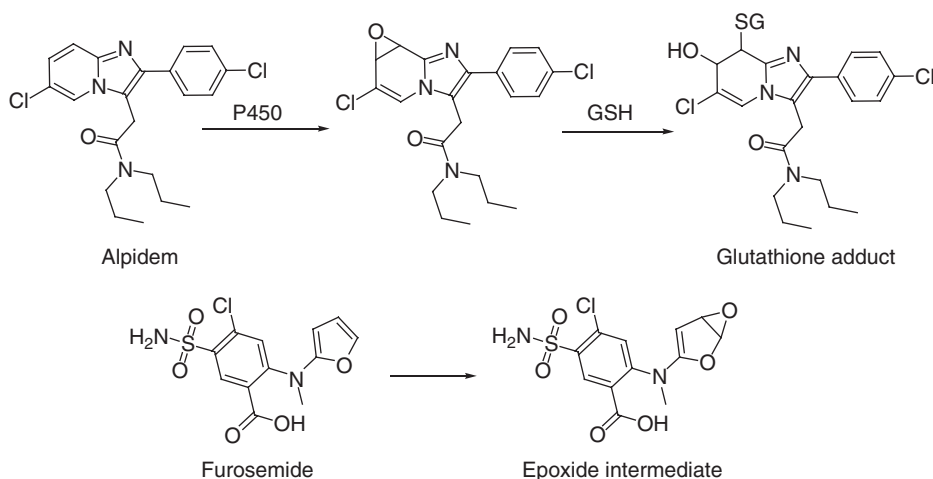


Figure 2.25 Metabolic activation of heteroaromatic rings by epoxidation.

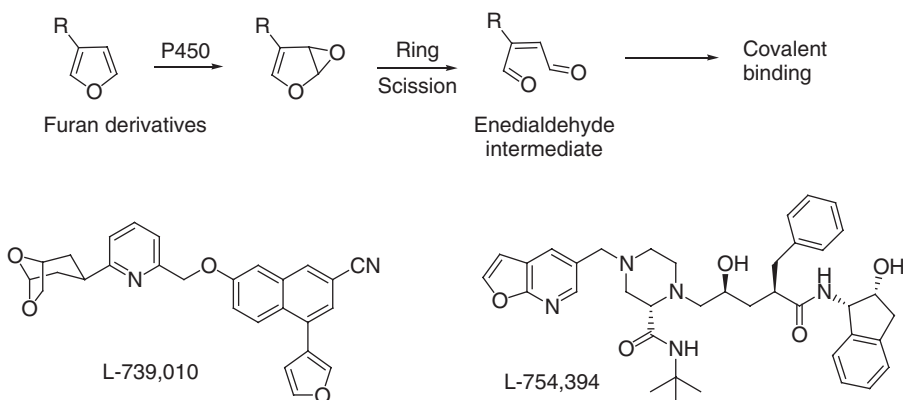


Figure 2.26 Ring opening of furan rings.

of furan and irreversible protein binding of the corresponding substituted ϵ -ketoenals has also been reported during metabolism studies with the lipooxygenase inhibitor L-739,010 and an experimental anti-HIV drug, L-754,394 (Fig. 2.26) [122,123].

The toxicity of thiazole rings is also attributed to their P450-catalyzed oxidative ring scission to the corresponding thioamide metabolites (Fig. 2.27) [124]. This is exemplified by studies on metabolism and toxicity of thiabendazole, a compound that is widely used as an agricultural fungicide and an anthelmintic [125]. As shown in Fig. 2.27, thiabendazole is oxidatively metabolized to thioformamide and the benzimidazol-2-ylglyoxal metabolites in preclinical species and humans. Although the initial step is epoxidation of the thiazole moiety, it subsequently undergoes ring scission to the corresponding thioformamide, which is believed to be the proximate toxicant of the compound. Likewise, the clinical hepatotoxic effects of sudoxicam

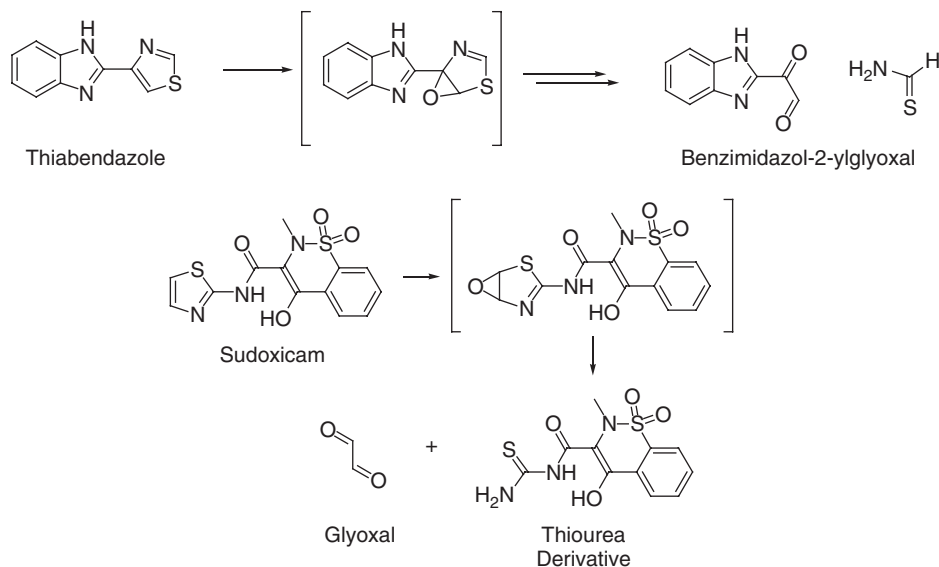


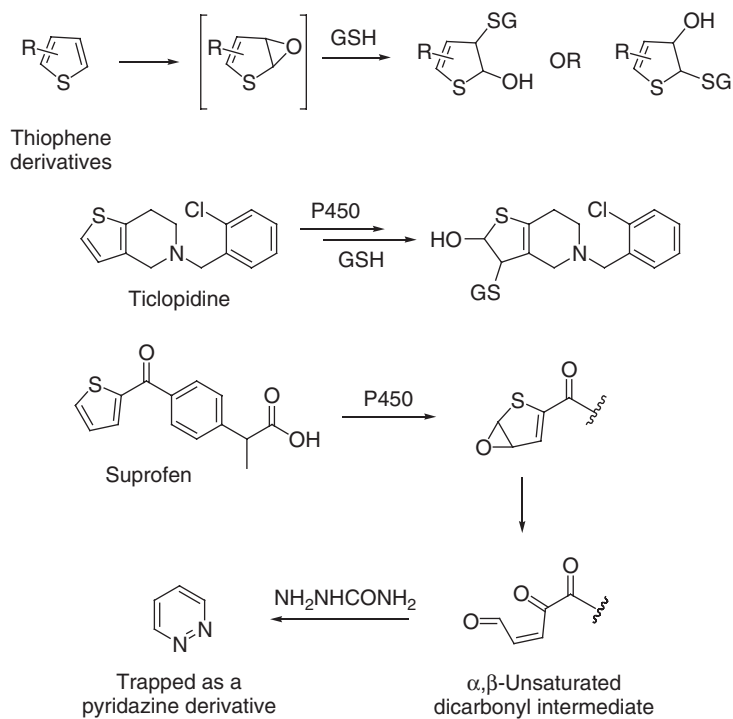
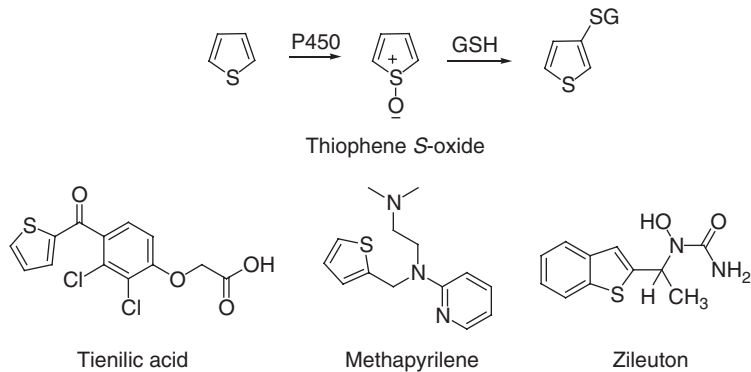
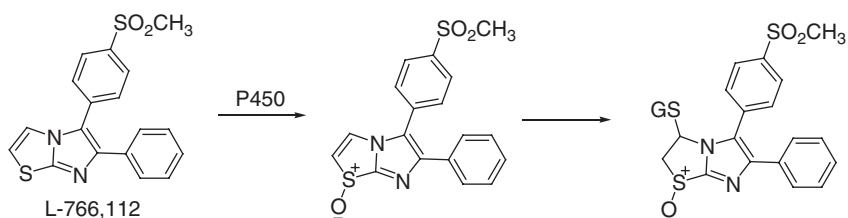
Figure 2.27 Bioactivation of compounds containing a thiazole ring.

have been circumstantially linked to the bioactivation of its thiazole ring to reactive thioureido and thiourea metabolites [126].

The situation is more complex in the case of thiophene compounds, as these compounds form the thiophene epoxides and thiophene-*S*-oxides upon oxidative metabolism [118,127]. Earlier studies by Bray and coworkers [128] provided evidence that thiophene-2,3-epoxide is the obligatory intermediate leading to glutathione adducts. Likewise, the formation of an epoxide in the bioactivation tienilic acid has not been ruled out [129]. More recently, a glutathione adduct of 2-hydroxy-2,3-dihydrothienopyridine which is formed via attack of the glutathione molecule on the epoxide intermediate has been detected and characterized (Fig. 2.28) [130]. In rare cases, the thiophene epoxides have been shown to undergo a ring opening to enedialdehydes as reactive intermediates as demonstrated in the bioactivation of suprofen [131].

Several reports have provided evidence that formation of a thiophene-*S*-oxide intermediate is the primary activation pathway in the oxidative metabolism of thiophene compounds [132–136]. Some drugs that undergo bioactivation via this pathway include tienilic acid, methapyrilene, or zileuton (Fig. 2.29) [134,136–138]. Other heteroaromatic rings containing a sulfur atom in the ring also undergo metabolic activation via the *S*-oxide intermediate in addition to epoxidation. For instance, incubation of L-766,112, a potent and selective inhibitor of human COX-2, with rat and rhesus monkey hepatic microsomal fractions under oxidative conditions generates the electrophilic thiazole-*S*-oxide metabolite (Fig. 2.30) [139]. The corresponding *S*-oxide has been trapped with GSH to give the GSH conjugate. A similar reaction has also been observed with compounds containing a thiazole ring as exemplified in the metabolism of the nonpeptidyl thrombopoietin receptor agonist [140].

Like the arenes, the alkene containing compounds are bioactivated to their corresponding alkene oxide intermediates [98,141–143]. Olefin bioactivation via an epoxide

**Figure 2.28** Bioactivation of thiophene derivatives.**Figure 2.29** Metabolic activation of thiophenes to thiophene-S-oxides.**Figure 2.30** Metabolic activation of L-766,112 by P450.

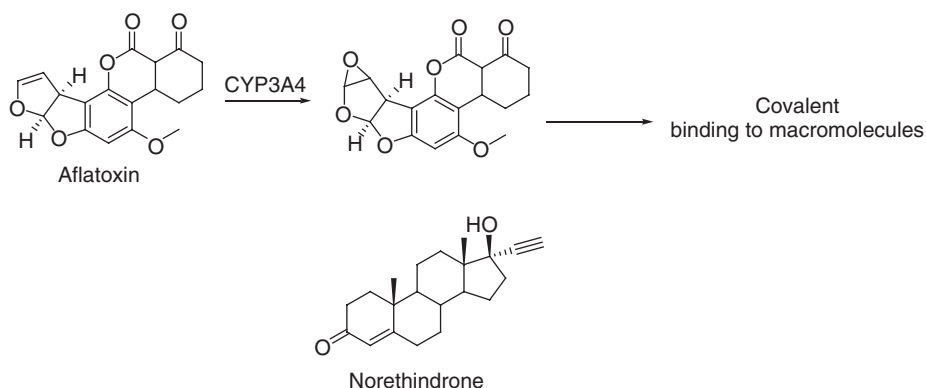


Figure 2.31 Metabolic activation of aflatoxin and structure of norethindrone.

intermediate is well illustrated by metabolism studies of aflatoxin (Fig. 2.31) [144]. A CYP3A4-catalyzed epoxidation of the 2,3-double bond in aflatoxin B1 results in a very reactive epoxide intermediate which binds to RNA rendering the compound extremely toxic. Similarly, the hepatic side effects observed following administration of the contraceptive agent norethindrone have been attributed to the formation of 4,5-epoxide metabolite [145–147].

2.3.3 Two-Electron Oxidation of Electron Rich Compounds

Many drugs containing a nitrogen, oxygen, or carbon atoms ortho or para to one another in an aromatic ring are susceptible to a two-electron oxidation that yields quinone imine, quinones, or quinone methide intermediates. These intermediates are quite reactive (depending on the substituents on the aromatic ring) and the reactivity is ascribed to the presence of α,β -unsaturated carbonyl or imine moieties, that can react with biologically important nucleophiles via a 1,4-Michael addition reaction [148–153].

2.3.3.1 Quinone Imines. Prime examples of drugs undergoing metabolic activation to quinone imines are acetaminophen and amodiaquine. Acetaminophen contains a *para*-hydroxyacetanilide core, which undergoes a CYP3A4-catalyzed two-electron oxidation to form the quinoneimine N-acetyl *para*-benzoquinone imine (NAPQI) species that can form covalent adducts to protein (Fig. 2.32) [154–157]. Similarly, amodiaquine, an antimalarial agent, which is effective against chloroquine resistant isolates of *Plasmodium falciparum* undergoes metabolic activation of the 4-hydroxyphenylamino moiety in the molecule to the corresponding quinone imine and arylates sulfhydryl nucleophiles in a macromolecule (Fig. 2.32) [158–160]. Metabolism studies of amscarine, an antileukemic agent, in rats have also led to the detection of glutathione conjugates as the main biliary metabolite (Fig. 2.32) [161]. Amascarine contains a *para*-aminosulfonamide moiety that is oxidatively transformed to a quinone dimine intermediate which is analogous to a quinone imine observed in the case of amodiaquine (Fig. 2.32) [162]. This reacts with the sulfhydryl group of glutathione resulting in GSH adducts observed in the rats or with other sulfhydryl-containing biological molecules.

Quinone imine intermediates are also observed in drugs that are hydroxylated on the aromatic ring containing a “masked aniline.” For instance, nonsteroidal

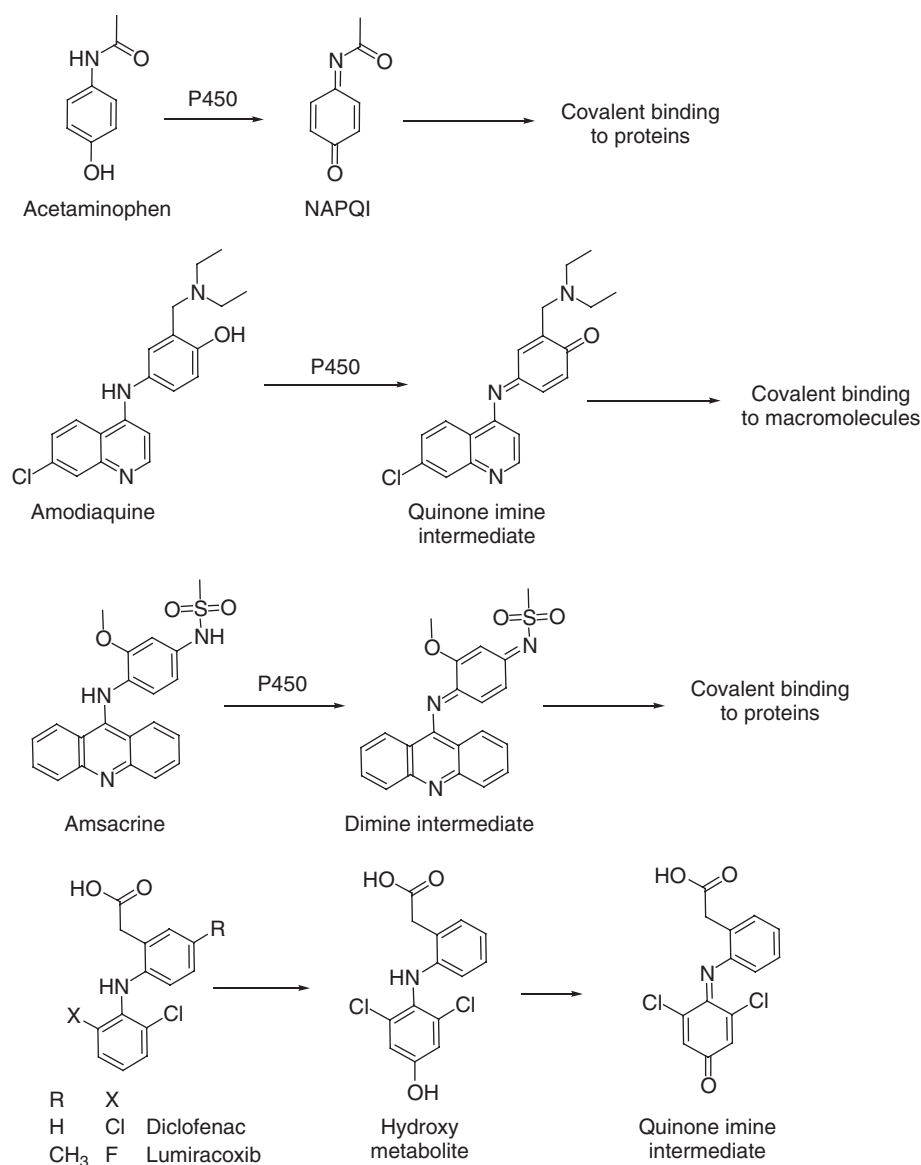


Figure 2.32 P450-catalyzed oxidation of aminophenol derivatives to quinone imines.

anti-inflammatory drugs, diclofenac and lumiracoxib, undergo a CYP3A4- and 2C9-catalyzed aromatic hydroxylation, respectively, to the corresponding aminophenol derivative (Fig. 2.32) [163,164]. These metabolites are known to undergo further P450- or myeloperoxidase (MPO)-catalyzed two-electron oxidation to the corresponding electrophilic quinone imine intermediates, and this event is thought to contribute to the hepatotoxicity associated with these two drugs [165,166].

Other drugs that are postulated to elicit their toxicity by their transformation to the corresponding hydroxyphenyl metabolites and subsequent oxidation to the quinone

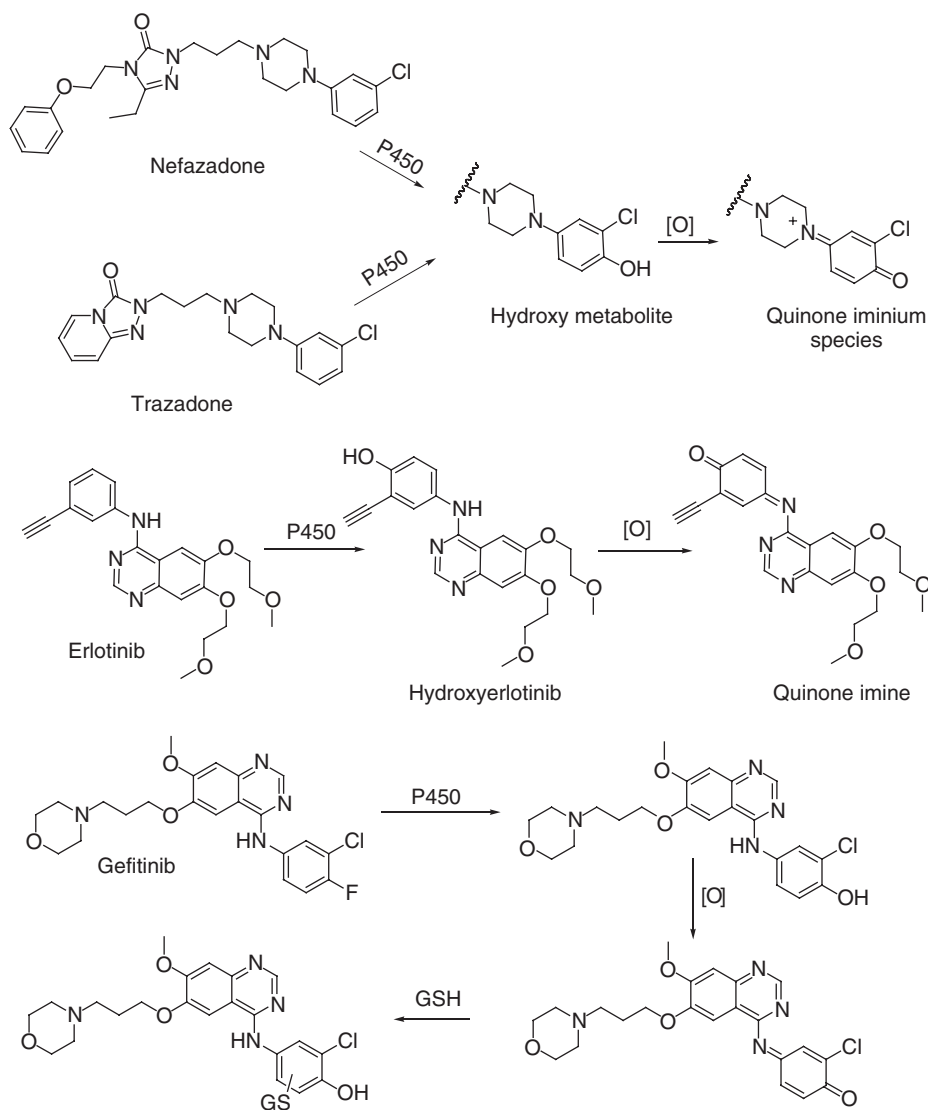


Figure 2.33 Metabolic activation of nefazadone, trazadone, erlotinib, and gefitinib.

imine intermediate are nefazadone, trazadone, and erlotinib (Fig. 2.33) [167–169]. Generally, halogen atoms are added to aromatic rings in order to block the positions that are prone to hydroxylation. However, in rare instances, these halogenated analogs can undergo oxidative dehalogenation by P450 to yield aminophenol derivatives that are further bioactivated to the quinone imines. For instance, gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, is bioactivated to a quinone imine via oxidative defluorination followed by oxidation of the corresponding hydroxyaniline metabolite (Fig. 2.33) [170].

Nitroaromatic compounds can also undergo bioactivation to quinone imines. However, this process involves two pathways. The first step is the six-electron reduction of

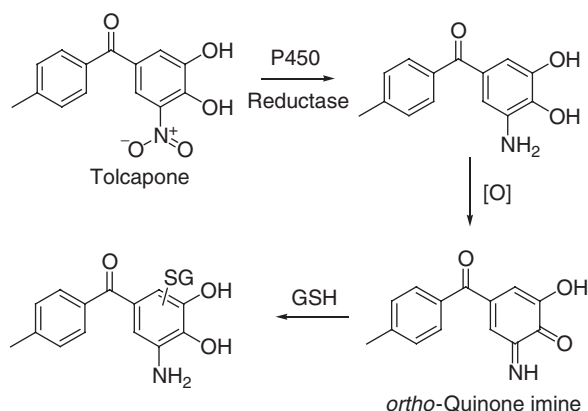


Figure 2.34 Metabolic activation of tolcapone.

the nitro group to the corresponding amine. The corresponding aromatic amine is then oxidized to an *ortho*-quinone imine intermediate either by peroxidases or P450. An example involving CYP-catalyzed bioactivation of a compound containing a nitroaromatic ring is depicted in studies on metabolism of tolcapone (Fig. 2.34) [171].

2.3.3.2 Quinones. Quinones are toxicophores that are produced via a two-electron oxidation of phenolic compounds [148,149,172]. Like the quinone imines, these intermediates are Michael acceptors, which generally react with sulfhydryl nucleophiles on a macromolecule. Interestingly, the presence of an electron rich sulfhydryl group can make the reoxidation of the corresponding hydroquinone back to the quinone intermediate. This in turn can covalently bind with second sulfhydryl-containing residue in the macromolecule and therefore lead to cross-linking of the macromolecule. Because of this, the initial nucleophilic attack of protein-SH to quinones does not generally represent a true detoxication reaction [173,174]. Examples of drugs that bioactivated to the quinones by P450 are raloxifene and ethinylestradiol. Raloxifene is a selective estrogen receptor modulator (SERM), which is effective in the treatment of osteoporosis in postmenopausal women. Glutathione adducts of raloxifene were identified in incubations with human liver microsomes that were supplemented by NADPH and GSH [175]. These adducts are postulated to derive by nucleophilic attack of GSH on the extended quinone intermediate, which is then trapped by GSH conjugation (Fig. 2.35). Likewise, the 2-hydroxyethinylestradiol metabolite formed by P450-catalyzed oxidation of the oral contraceptive, ethinylestradiol, is converted to the corresponding *ortho*-quinone intermediate by P450 (Fig. 2.35) [176].

2.3.3.3 Quinone Methide. Quinone methides differ structurally from quinones, in that one of the carbonyl oxygen atoms is replaced by a methylene or substituted methylene group. These reactive metabolites are generally formed following the oxidation of 4-alkylphenol moiety which may be present in a drug itself or one that is formed following hydroxylation of aromatic rings in a drug. Like all reactive intermediates, the selectivity of a quinone methide depends on its rate of formation as well as its reactivity. The presence of a carbon instead of an oxygen or nitrogen atom in a quinone methide makes this intermediate much more reactive than its analogs (quinone or the

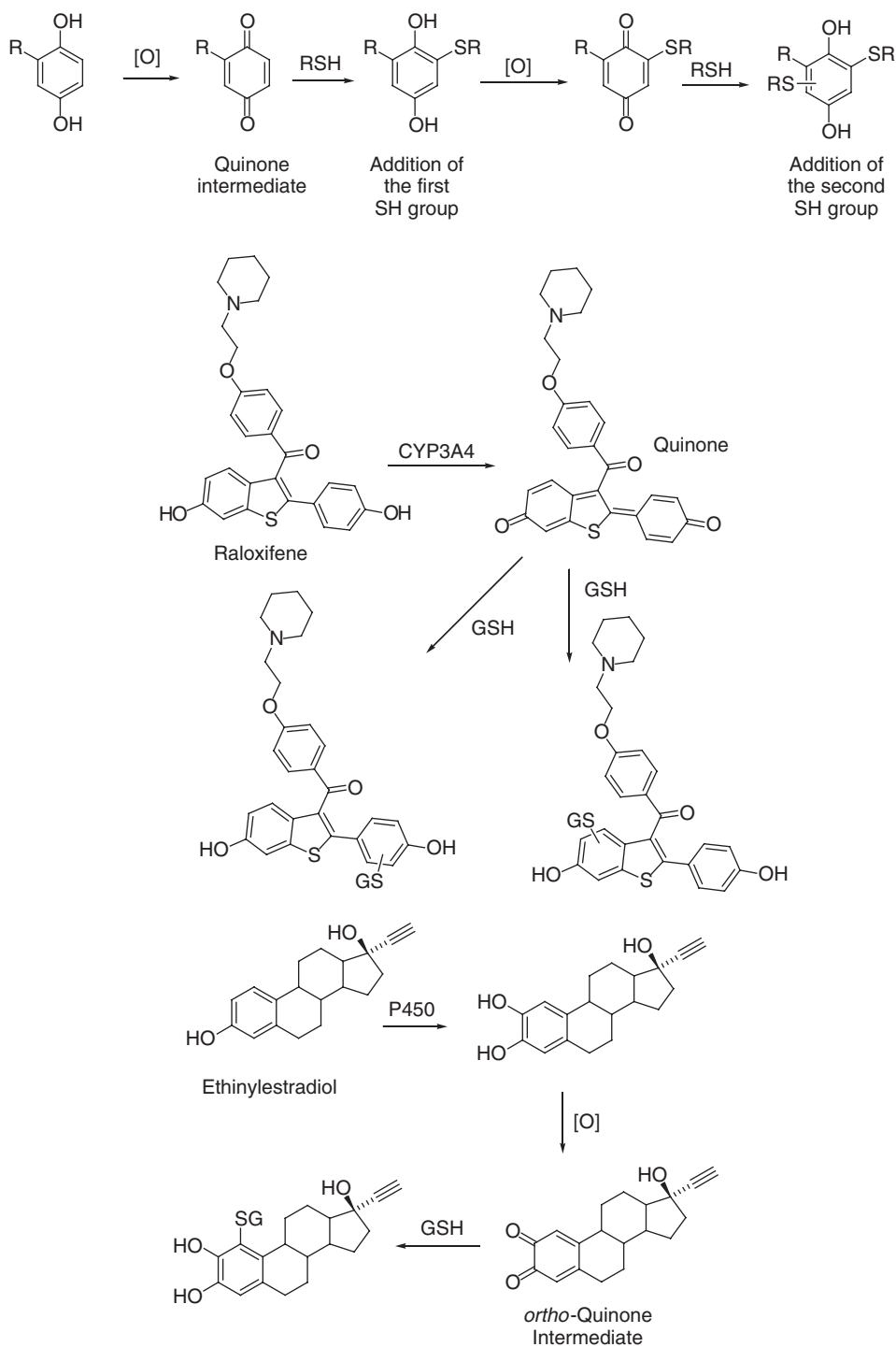


Figure 2.35 Oxidation of phenolic derivatives to quinones.

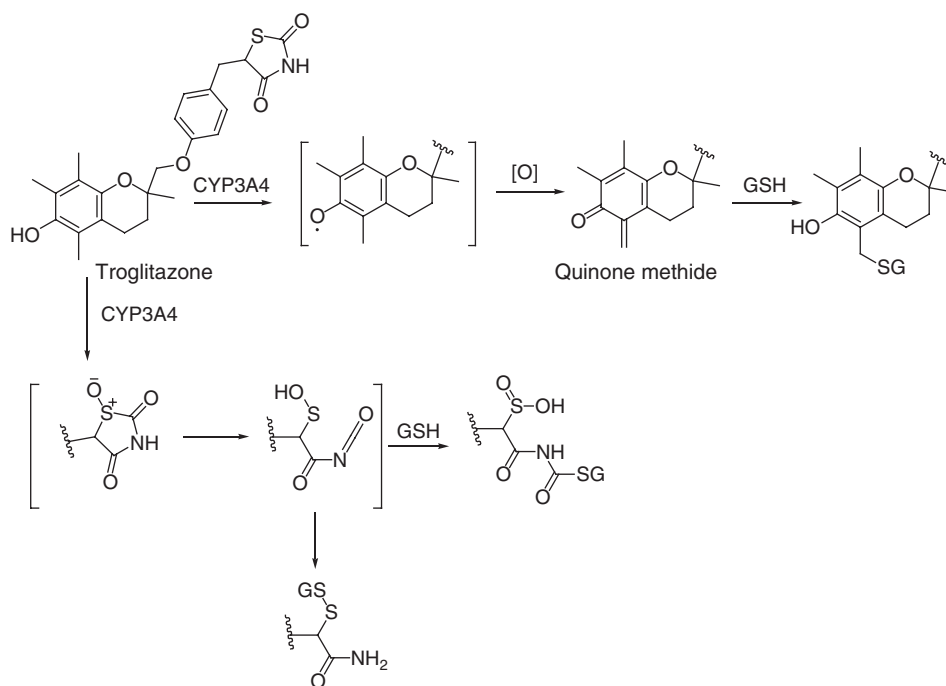


Figure 2.36 Bioactivation of troglitazone.

quinoneimine) with a reduced capacity for redox cycling. Therefore, quinone methides generally react in biological systems via nonenzymatic Michael additions. Most SERMs that undergo conversion to extended quinones are also bioactivated to quinone methides [177]. Troglitazone, the first glitazone used for the treatment of type II diabetes mellitus and withdrawn from the market for liver toxicity, also forms the quinone methide via oxidation of the substituted chromane ring system (Fig. 2.36) [178]. However, an alternative mechanism, which involves a novel oxidative cleavage of the thiazolidine-dione ring, potentially generating highly electrophilic *R*-ketoisocyanate and sulfenic acid intermediate, has also been proposed in the bioactivation of troglitazone [178]. Both the bioactivation processes are CYP3A4 mediated as incubations in the presence of ketoconazole inhibits the formation of the GSH conjugates.

2.3.3.4 Imino Methide. Imino methides are analogous to quinone methides in that a nitrogen atom replaces an oxygen atom. Generally, compounds containing a 3-methylindole motif are bioactivated to imino methides. A prime example for this class is that of 3-methylindole. This is a potent pneumotoxicant, found in tobacco smoke and intestinal or ruminant contents. Reports indicate that its toxic effects are a result of bioactivation via P450-mediated two-electron oxidation to an iminomethide (Fig. 2.37) [179–181]. Some compounds that are bioactivated via this pathway include zafirlukast and L-745,870 (Fig. 2.37) [182,183]. Trimethoprim, a bacteriostatic antibiotic, which is mainly used in the prophylaxis and treatment of urinary tract infections, also undergoes bioactivation to an imino methide intermediate that is formed by P450 or peroxidase-catalyzed oxidation of the diaminopyrimidine moiety (Fig. 2.37) [184].

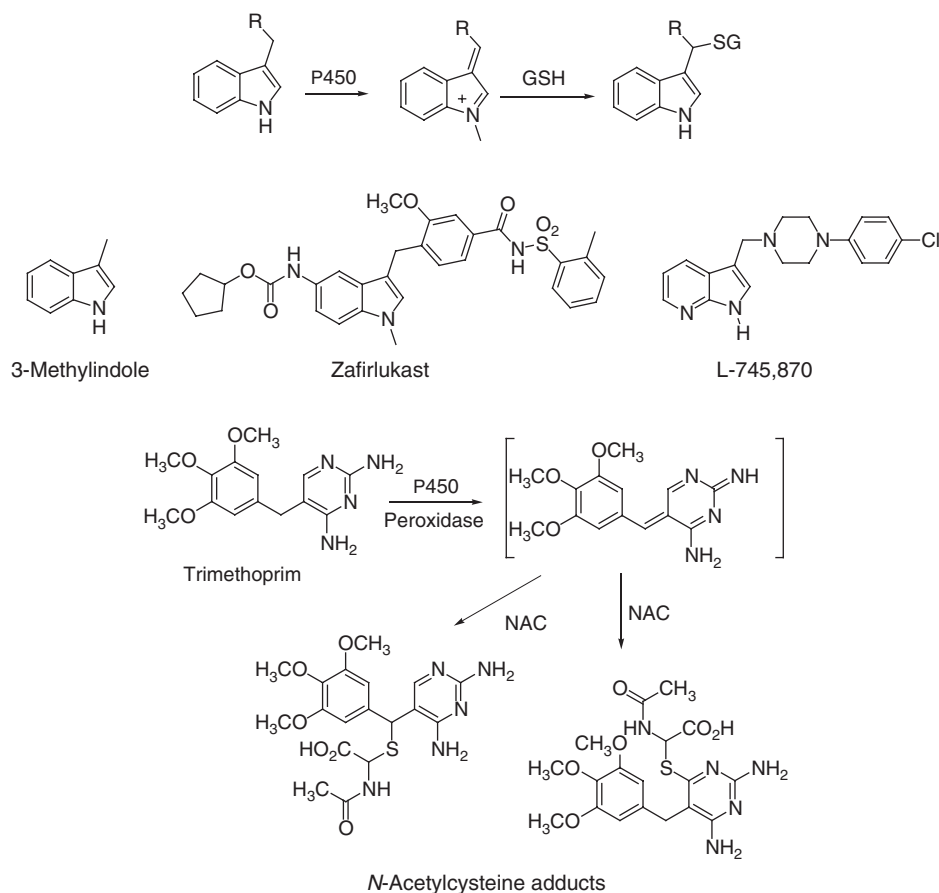


Figure 2.37 Formation imino methide intermediates via bioactivation of 3-alkylindoles and trimethoprim.

2.3.3.5 Iminium Ions. Cyclic tertiary amines can undergo a two-electron oxidation to form iminium ions as intermediates that can serve as electrophilic centers for reactions with macromolecules [185]. As a part of the detoxification pathway, these intermediary iminium ions are often oxidized to the biologically less active lactams in a reaction that is catalyzed by the liver cytosolic enzyme aldehyde oxidase (Fig. 2.38) [186]. The most generally accepted catalytic pathway for this reaction assumes an initial single electron transfer (SET) step from the amine substrate nitrogen lone pair to generate an aminyl radical cation which, following loss of a proton, is converted to a highly reactive carbon-centered radical (Fig. 2.38). A second single-electron oxidation of the carbon-centered radical gives, in the case of the P450-catalyzed reaction, the carbinolamine, which is in equilibrium with the iminium ion product [185]. The same reaction is catalyzed by monoamine oxidase (MAO). However, the electron acceptor in the case of CYP450 is the perferryl oxo species (Fe(V)O) species, while the electron acceptor for the MAO-catalyzed reaction is the oxidized flavin moiety (FAD) [185]. A variety of cyclic tertiary amines undergo P450-catalyzed oxidative metabolism to form chemically reactive iminium metabolites. Phencyclidine undergoes α -carbon oxidation

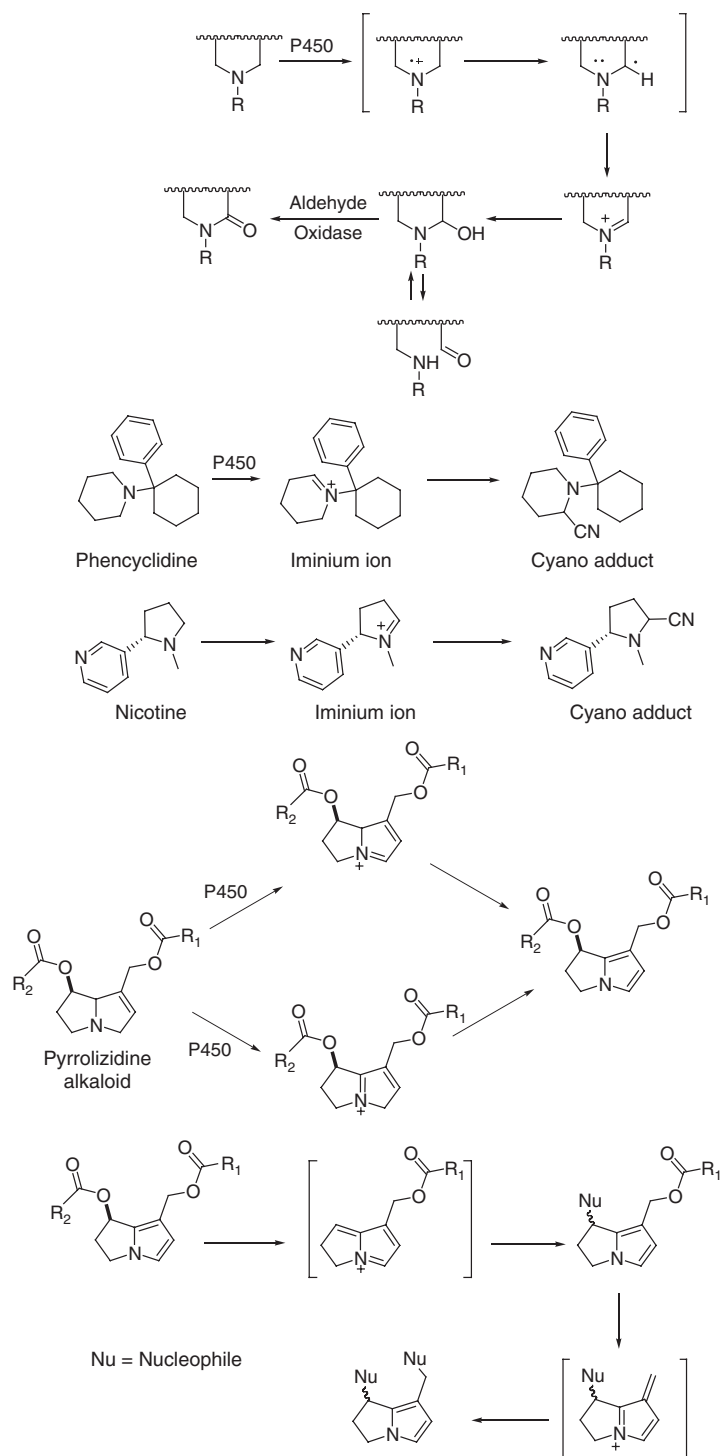


Figure 2.38 Bioactivation of cyclic amines to iminium ion intermediates.

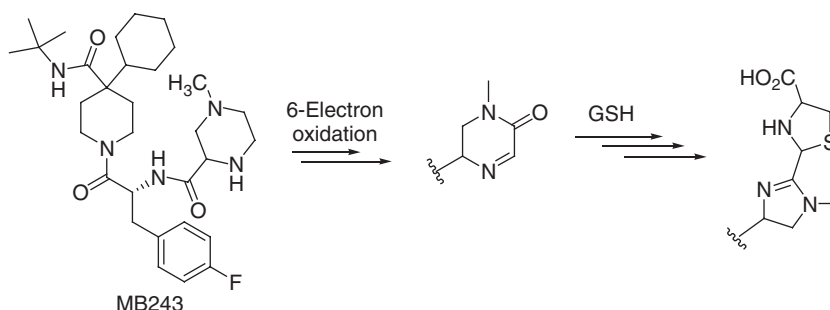


Figure 2.39 Metabolic activation and a ring contraction of a piperazine ring via an iminium intermediate.

to form the chemically reactive iminium intermediate, a pathway which is associated with covalent binding to macromolecules (Fig. 2.38) [187,188]. A similar bioactivation of the tobacco alkaloid (*S*)-nicotine to an iminium ion intermediate (Fig. 2.38) that forms covalent bonds to biomacromolecules has been reported [189].

The pyrrolizidine alkaloids (PAs) constitute a large class of natural products many of which are carcinogenic, genotoxic, neurotoxic, and hepatotoxic [190]. This toxicity is attributed to their conversion to pyrrolyl metabolites via the P450-mediated α -carbon oxidation pathway (Fig. 2.38) [185,191,192].

Doss and coworkers in metabolism studies of a selective melanocortin receptor subtype-4 agonist have demonstrated a novel metabolic activation and a ring contraction of a piperazine ring via an iminium intermediate. The transformation is a consequence of a nucleophilic attack of a GSH molecule on the iminium ion intermediate (Fig. 2.39) [193].

Another interesting case of a P450-catalyzed bioactivation sequence involving an iminium ion as a reactive intermediate has been documented with 6,7-dimethyl-2,4-di-1-pyrrolidinyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (U-89843) (Fig. 2.40) [194]. U-89843 is a novel antioxidant with prophylactic activity in animal models of lung inflammation whose clinical development was suspended because of its genotoxicity. Incubation of [14 C]-U-89843 with liver microsomes resulted in NADPH-dependent covalent binding of radioactive material to microsomal protein. Characterization of the corresponding methanol, GSH, and *N*-acetylcysteinyl adducts of U-89843 in NADPH-supplemented rat liver microsomes supported a bioactivation pathway involving the formation of a resonance-stabilized iminium intermediate formed via dehydration of the alcohol metabolite, in a manner similar to that observed with 3-methylindole. The highly reactive iminium species is suspected to react with nucleophilic sites within proteins resulting in adducts.

2.3.4 Mechanism-Based Inactivators

Generally, P450s that bioactivate compounds are often targets for modification by reactive intermediates that are generated from them. This can lead to irreversible binding of a molecule to the enzyme and eventually inactivation of the enzyme. The compounds that fall into this category are often referred to as “*mechanism-based*,” “*suicide*,” “*time-dependent*,” or “*catalysis-dependent*” inactivators. Two types of

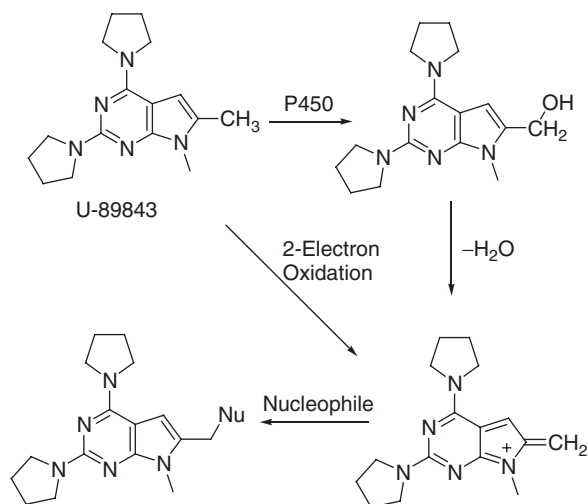


Figure 2.40 Bioactivation of U-89843.

mechanism-based P450 inactivators can result from the bioactivation of compounds, namely, quasi-irreversible and irreversible inhibitors. In the case of quasi-irreversible inhibitors, these reactive intermediates coordinate with the heme prosthetic group leading to the formation of a catalytically inactive metabolite–inhibitor (MI) complex of the P450 enzyme(s). Alternatively, reactive intermediates generated from the metabolism of irreversible inactivators can covalently react with an active site amino acid residue(s) within the apoprotein following their bioactivation and/or cause direct alkylation/arylation of the heme prosthetic group resulting in heme destruction [195–199]. Whether the reactive metabolite formed by a P450 enzyme can covalently bind to the enzyme that generates it depends in part on the reactivity and longevity of the intermediate itself. Additionally, the proximity of amino acid residues in the active site of P450 to the electrophile also influences the potential of a compound to act as an inactivator. If the metabolite is “very reactive” it may not be able to diffuse out of the active site of the enzyme into cell milieu where it can react with cellular constituents and/or reduced glutathione. Instead, it may alkylate the heme moiety, which can lead to heme destruction. Alternatively, the intermediate formed can covalently bind with a nucleophilic amino acid residue within the apoprotein, which is in the vicinity of the active site where the intermediate was formed. In general, heme alkylation invariably inactivates P450, whereas amino acid alkylation may result in loss of catalytic activity.

2.3.4.1 Quasi-Irreversible Inactivation. This type of time-dependent inactivation of P450 is common and involves a metabolite inhibitor complexation (MI complex). Once the compound is metabolized, the initial products of oxidation can tightly coordinate with the P450-Fe(II) enzyme resulting in the formation of MI complex and renders the enzyme inactive. The MI complex exhibits an absorbance maximum in the Soret region between 448 and 456 nm when the heme iron is in the Fe(II) state [200]. Aliphatic or aromatic amines are a class of compounds that can cause enzyme inhibition via this mode of bioactivation [195]. A primary amine is generally required for a compound to

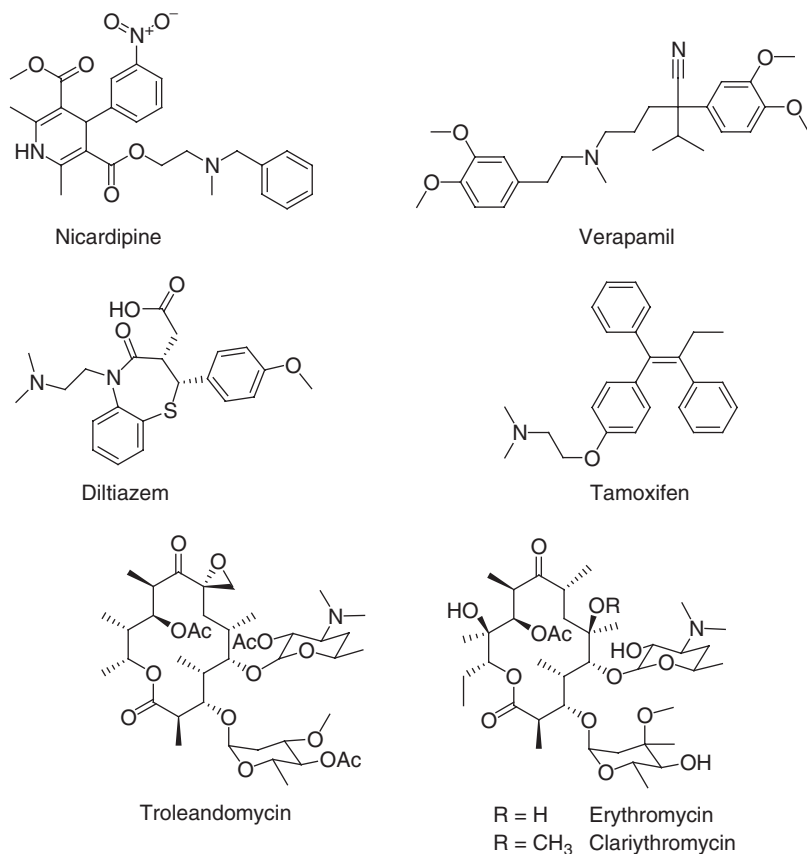


Figure 2.41 Structures of quasi-irreversible inhibitors.

cause a quasi-irreversible inhibition [201,202]. Some noteworthy examples that have shown to undergo MI complexes include amine-based calcium channel blockers that are widely used for the treatment of hypertension, angina pectoris, and other cardiovascular diseases [203]. For instance, 1,4-dihydropyridines such as nicardipine, verapamil, and diltiazem have been classified as quasi-irreversible inhibitors of CYP3A4 (Fig. 2.41). Excluding nicardipine, both verapamil and diltiazem undergo N-demethylation to the corresponding primary or secondary amines which can complex with Fe(II) [204–206]. The irreversible mechanism-based inactivation of P450 3A4 by the estrogen receptor antagonist, tamoxifen and its N-demethylated metabolite is also accompanied by MI complex formation with the ferrous form of the enzyme [207]. Several members of the macrolide class of antibacterial agents that contain a tertiary amine group are also known to form MI complexes with P450–Fe(II) following their metabolism to the nitroso intermediate. For instance, troleandomycin, erythromycin, and clarithromycin can mediate its CYP3A4 inhibitory effects by converting to nitrosoalkanes and the consequent formation of inactive MI complexes of P450 (Fig. 2.41) [105]. The reactive species is believed to be a nitrosoalkane obtained by enzymatic oxidation of the amine through the intermediate hydroxylamine metabolite. Although the primary amine is

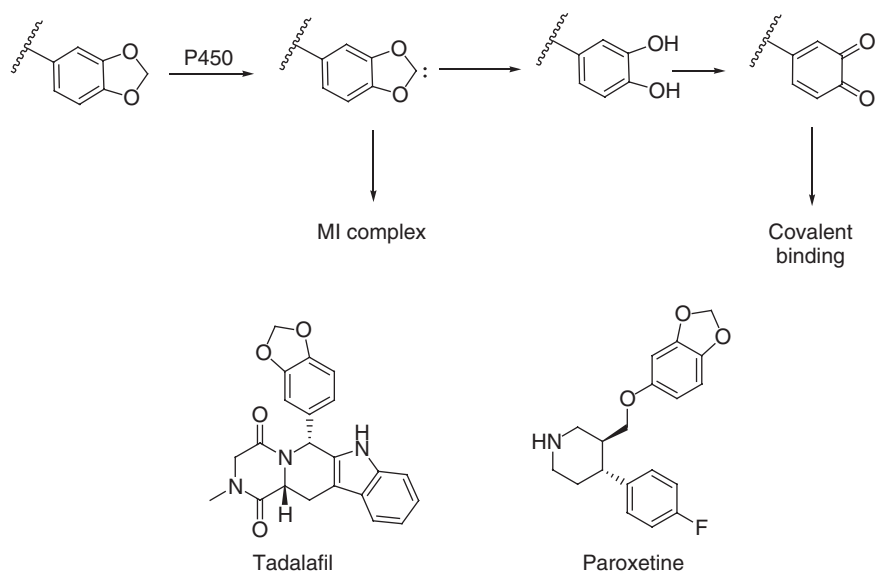


Figure 2.42 Bioactivation of 1,3-benzodioxole groups.

the required moiety for binding, secondary and tertiary amines have also been shown to inactivate the enzyme and can serve as suitable precursors for MI complexation provided they are N-dealkylated to primary amines.

Compounds that contain the methylenedioxyphenyl or 1,3-benzodioxole motif also inactivate P450 enzymes via MI complex formation with the enzyme following bioactivation [208–210]. The phosphodiesterase-5 inhibitor tadalafil and the selective serotonin reuptake inhibitor paroxetine are examples of drugs that contain the 1,3-benzodioxole group that form MI complexes with human P450 3A4 and P450 2D6, respectively, resulting in the mechanism-based inactivation of these enzymes (Fig. 2.42) [211,212]. Although speculative, the evidence points toward a reactive carbene intermediate obtained by hydrogen atom abstraction from the methylene carbon or by the elimination of water from a hydroxymethylene intermediate. An alternate pathway to inactivation of P450 by methylenedioxy group involves metabolism of the dioxole moiety to an *ortho*-quinone via a catechol, which can react with amino residues in the enzyme as exemplified by methylenedioxymethamphetamine (ecstasy) and safrole (Fig. 2.42) [213,214].

2.3.4.2 Mechanism-Based Inactivation of P450s by Covalent Modification. Most functionalities that are bioactivated (described above) and lead to macromolecular adduct formation have the potential to covalently modify the enzyme active site and therefore inactivate it. Hence, it is not surprising that most of the compounds that possess these functionalities are inactivators of P450. For example, the extended quinone formed via bioactivation of raloxifene is responsible in inactivation of CYP3A4 [215]. More recently, Baer and coworkers [216] have demonstrated that a single equivalent of raloxifene is bound to the cysteine residue in the P450 apoprotein. Previous reports have also shown that 3-alkylindoles, that are bioactivated via imino methide intermediates, are potential suicide inactivators of CYP enzymes [182,217–220]. However, more

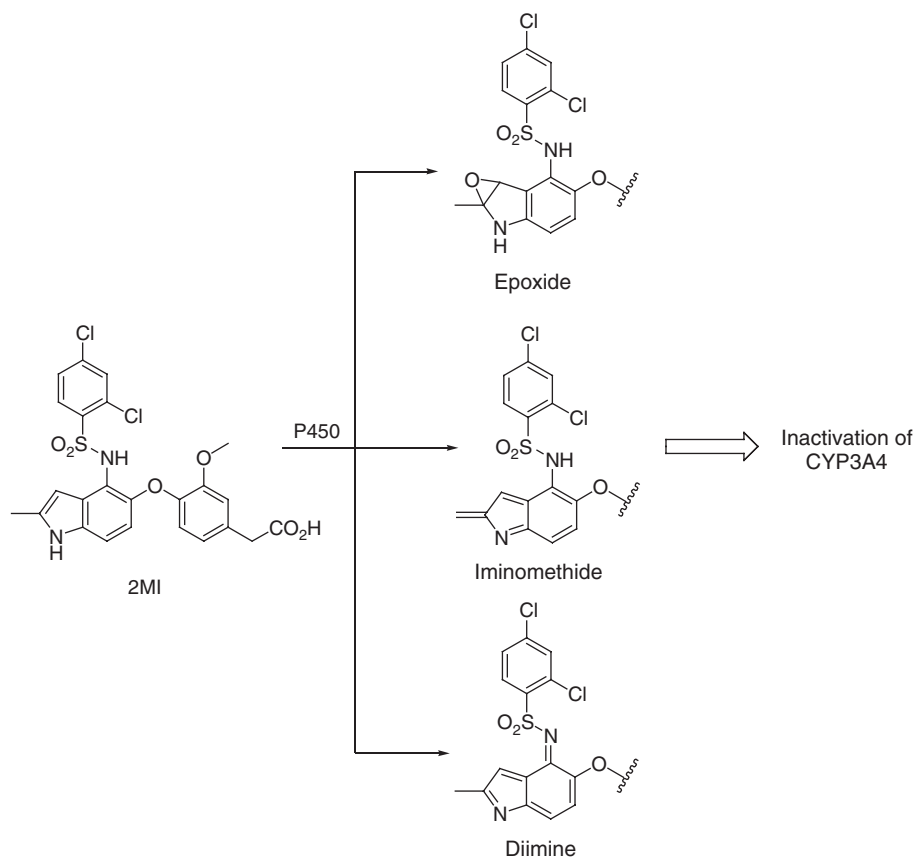


Figure 2.43 Bioactivation and mechanism-based inactivation by a 2-methyl substituted indole derivative.

recently, a report showing bioactivation of 2-methyl-substituted indole (Fig. 2.43), which is a potent dual inhibitor of chemoattractant receptor-homologous molecule expressed on T-helper type-2 cells and D-prostanoid receptor, has been published [221]. During evaluation as a potential treatment for asthma and allergic rhinitis, 2MI was identified as a mechanism-based inactivator of CYP3A4 *in vitro* as well. The NADPH-dependent increase in 2MI covalent binding to a 55- to 60-kDa microsomal protein was consistent with irreversible binding to CYP3A4.

Several compounds containing a furan moiety are inactivators of CYP enzymes. Because of extensive mechanistic and Structure activity relationship (SAR) studies with furan analogs, it has been proposed that P450-mediated furan ring scission to reactive intermediates (discussed above) leads to mechanism-based inactivation of P450. Evidence for P450 inactivation by reactive intermediate(s) derived from furan ring scission has also been presented for the experimental HIV protease inhibitor L-745,394 that inactivates P450s [222]. An interesting example of a furan containing compound where metabolism of the furan moiety is not involved in the inactivation is that of furafylline (Fig. 2.44). This compound is a mechanism-based inactivator of CYP1A2. Several studies have been conducted to understand the mechanism of inactivation of

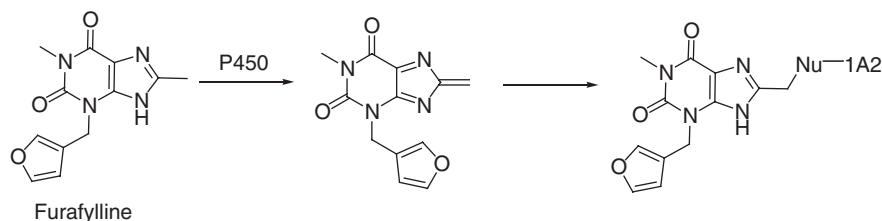


Figure 2.44 Bioactivation and mechanism-based inactivation of CYP1A2 by furafylline.

this compound. SAR and deuterium-isotope studies indicate that inactivation arises via bioactivation involving the methyl group attached to the C-8 carbon of the xanthine nucleus and no metabolism of furan ring to reactive intermediates is discernible [223,224]. Metabolism studies using H_2^{18}O indicate that the inactivation occurs as a result of P450 1A2-catalyzed two-electron oxidation of the 8-methylxanthine to the corresponding imidazomethide intermediate that is covalently bound to the enzyme.

As described above, thiophene containing compounds are bioactivated to thiophene-*S*-oxides and formation of these intermediates have been implicated as the rate-limiting step in the etiology of adverse drug reactions. In addition, these *S*-oxide intermediates can also inactivate 450s as exemplified by studies on metabolism of clopidogrel and ticlopidine. Both these compounds are potent suicide inactivators of CYP2C19 and 2B6 [225–228]. Similarly, *S*-oxidation of the thiophene ring in tienilic acid results in inactivation of CYP2C9 that forms it [229]. The anti-inflammatory agent suprofen also inactivates CYP2C9 and like tienilic acid, oxidation of the thiophene ring in the molecule is implicated in its inactivation process [131].

Drugs containing alkenes or alkynes (especially a terminal alkyne) as a functional group have been associated with mechanism-based inactivation of P450 enzymes [230–234]. Two mechanisms for the inactivation process have been described with alkynes (Fig. 2.45). The first mechanism involves initial oxidation of the triple bond on the terminal carbon followed by a 1,2-hydrogen shift of the terminal hydrogen to the vicinal carbon that is thought to generate the reactive ketene intermediate. This intermediate can have two possible fates: it is hydrolyzed to the carboxylic acid product or it can acylate the apoprotein [234,235]. An alternative mechanism involves the formation of oxirene (especially with internal alkynes), which then leads to heme- or apoprotein alkylation [234]. Some examples of alkyne-containing compounds that are potent mechanism-based inactivators of P450s are 17α -ethinylestradiol and mifepristone (Fig. 2.45) [236,237]. Both are inactivators of CYP3A4. Ethinylestradiol inactivates the enzyme via metabolism of the terminal acetylenic moiety to a reactive ketene intermediate that leads to heme- and apoprotein alkylation. On the other hand, mifepristone has an internal alkyne that alkylates the apoprotein but not the heme prosthetic group.

Hydrazine or hydrazide group containing drugs are also commonly associated with inactivation of major CYP enzymes. For instance, the antihypertensive drug, dihydralazine or the antituberculosis drug, isoniazid displays mechanism-based inactivation of human P450 isoforms (Fig. 2.46) [238,239]. Inactivation by dihydralazine is ascribed to the formation of a free carbon radical via the diazene, which results in alkylation of the heme. Consistent with these findings, P450 1A2 autoantibodies have been found

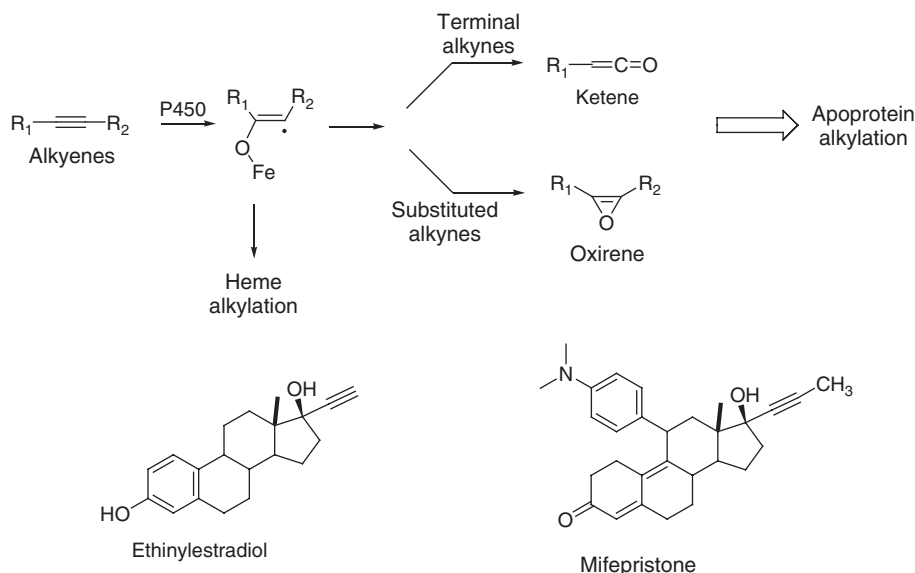


Figure 2.45 Mechanism-based inactivation by alkynes.

in the serum of patients suffering from dihydralazine-mediated hepatitis, suggesting a key role for P450 in the overall toxicological process. A nonselective mechanism-based inactivation of multiple mammalian P450 enzymes by the hydrazine analog 1-aminobenzotriazole (ABT) proceeds via alkylation of the heme prosthetic group as demonstrated by the characterization of an N,N' -bridged phenylene-protoporphyrin IX adduct from livers of rats treated with ABT [240,241]. The proposed mechanism, which accounts for the formation of the phenylene-protoporphyrin IX, involves the putative formation of the electrophilic benzyne intermediate via initial oxidation of the primary amine functionality in ABT (Fig. 2.46).

A number of other functionalities have been implicated in mechanism-based inactivation of CYP enzymes, including halogenated compounds (chloramphenicol), dihydropyridines, dihydroquinolines, various organosulfur compounds, including thiols, thioamides, dithiocarbamates and isothiocyanates, and cyclopropylamines. The mechanism of inactivation and examples of drugs containing these functionalities has been extensively reviewed [195,198,199,242].

2.3.5 Consequence of Bioactivation of Drugs to Reactive Metabolites

Circumstantial evidence suggests that bioactivation of relatively inert functional groups to reactive metabolites may contribute toward certain drug-induced adverse reactions. Adverse drug reactions pose a significant health problem as they contribute to patient morbidity and mortality and commonly compromise good patient care and represent one of the most common causes for pharmaceutical product recalls and black box warning labels. Life-threatening idiosyncratic adverse drug reactions that are usually unpredictable and rare such as hepatotoxicity, severe cutaneous reactions, anaphylaxis, and blood dyscrasias are a major cause of drug withdrawal after the drug has

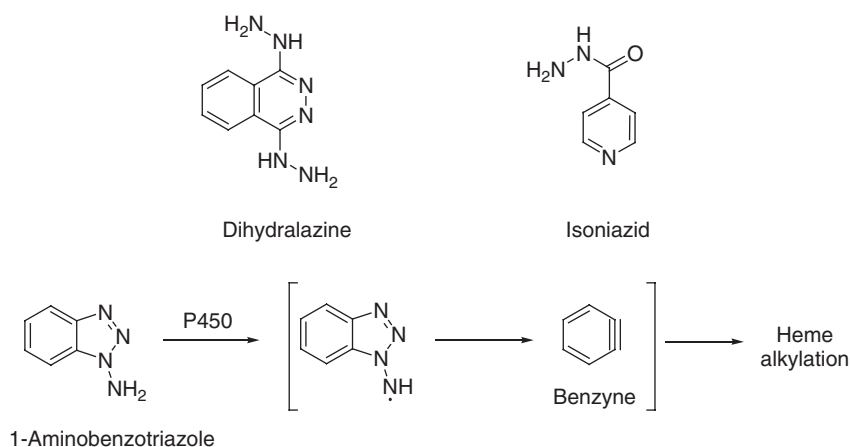


Figure 2.46 Structures of hydrazines derivatives and mechanism-based inactivation by aminobenzotriazole ABT.

been launched. Mechanism-based inactivation, on the other hand, can translate into clinical DDIs. Some of these can be potentially deleterious, and can also lead to withdrawal of the perpetrator drug. Inactivation of enzymes has a greater potential to lead to DDIs when compared to reversible inhibition. This is because a CYP modified by a mechanism-based inactivator is irreversibly modified and has to be replaced by newly synthesized enzyme in order for activity to be regained. Several pharmaceutical companies have therefore implemented definitive screens to address the bioactivation potential of new drug candidates. Mechanism-based inactivation of major human CYP enzymes by new compounds is also routinely assessed in a drug-discovery paradigm. In an event that the new candidate shows a potential to form a reactive intermediate or exhibits time-dependent inhibition of a CYP, an attempt to replace the offending group is made.

Like in the case of formation of active metabolites, genetic polymorphism in addition to other factors can significantly affect the formation of reactive metabolites and protein adducts, leading to altered drug toxicity [243–246]. It can also result in differential inactivation of P450s. Information on whether common genetic polymorphisms affect P450 inactivation and may thereby affect the frequency of DDIs is therefore required. Additionally, drug-associated factors, kinetics of drug–protein adduct formation and degradation, affected proteins and organs, and genetic and pathological conditions of the patients are also important factors that need to be considered to when managing the risks related to adduct formation as a result of bioactivation to reactive metabolites. The potential for DDI and/or ensuing immune-mediated toxicity of a new drug candidate due to bioactivation also depends on the overall disposition (extent of metabolism leading to P450 inactivation relative to latent metabolic or nonmetabolic fate), daily dose, therapeutic regimen (acute vs chronic), and the intended target population.

Despite the risks associated due to covalent binding to enzymes, mechanism-based inactivators have also been used to our advantage and exploited to provide therapeutic benefits. For instance, the coformulation of ritonavir, a potent inhibitor of CYP3A4, with lopinavir, a novel protease inhibitor with relatively low bioavailability, has been shown to significantly improve the pharmacokinetic properties and hence the activity

of lopinavir against HIV-1 protease [247]. Mechanism-based inactivators have also been used as powerful molecular probes for delineating the catalytic specificities of particular P450s and for obtaining important information regarding P450 structure and function.

2.4 SUMMARY

This chapter summarizes the bioactivation of drugs by P450. Even though all enzymes can catalyze metabolic activation of compounds, P450 enzymes, which generally dominate metabolism, play a major role in the metabolic activation of drugs. Bioactivation by P450 enzymes can result in pharmacologically active metabolites that can influence the overall pharmacological response of the drug. Although the major advantage of the formation of active metabolites is the prolonged pharmacological effect of the drug, it is important to note that it results in a complex dose–response relationship that results in the monitoring of the parent drug as well as all its metabolites and necessitates the adjustment of the dose of the drug. Alternatively, metabolic activation of drugs can result in reactive intermediates that have the propensity to covalently bind with the nucleophilic centers in biomolecules and elicit drug-induced toxicities and DDIs depending on the target protein. Overall, bioactivation of compounds clearly has its advantages and disadvantages. It is therefore important to evaluate the potential of metabolic activation of a candidate drug early on in discovery and development so that it can allow one to mitigate the risks associated with it.

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3 Bioactivation II: Phase I (Non-P450)

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3.1 Introduction	1
3.2 Flavin-containing monooxygenases (FMO)	2
3.3 Amine oxidases	9
3.4 Alcohol dehydrogenase	13
3.5 Peroxidases	17
3.6 Aldo-ketoreductases (AKR)	24
3.7 Reductive bioactivation	24
3.8 Conclusions and perspectives	32
References	34

3.1 INTRODUCTION

The potential for drug-induced adverse reactions with new drug candidates entering clinical trials is a principal concern for the drug development industry, due largely to the unfortunate frequency of untoward outcomes reported and/or black-box warnings associated with many drugs [1]. In addition, postmarketing incidences of hepatotoxicity and other severe adverse events have been noted with a number of otherwise useful medications, such as troglitazone (withdrawn in 2000), and most recently, lumbiracoxib (withdrawn in 2008). These increasingly observed outcomes have prompted the FDA to draft a guidance on the topic of drug-induced liver injury (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM174090.pdf>), which accounts for up to 50% of liver failure [2]. The debate is ongoing about the ultimate cause of adverse drug reactions (ADRs), and, in particular, idiosyncratic adverse drug reactions (IADRs), which currently cannot be predicted in preclinical animal models. One prevailing hypothesis is that reactive electrophilic metabolites generated by metabolic activation (e.g., bioactivation) may covalently modify critical biomacromolecules such as protein and DNA. These modifications of protein lead to “haptization” and the potential for an autoimmune response and/or various organ toxicities [3]. Considering that the majority of drug molecules are metabolized to some degree by the cytochrome P450 enzyme system, which are predominantly expressed in the liver, their purported role in the bioactivation

of drug molecules is not unexpected. In fact, in recent years, a multitude of reports and reviews describing *in vitro* evidence of reactive metabolite formation via cytochrome P450-mediated metabolism and the functional groups (e.g., structural alerts) that are particularly prone to bioactivation have been published [4,5]. As a result, *in vitro* assays with human liver fractions such as microsomes, where new chemical entities are incubated in the presence of various nucleophilic trapping agents (e.g., glutathione (GSH), cyanide, methoxylamine), are commonplace within drug metabolism departments in pharmaceutical industries as a strategy to identify the potential risk of reactive metabolite formation [6,7].

While the cytochrome P450 enzymes are generally the primary focus of drug metabolism scientists, there are many other drug-metabolizing enzymes that may contribute to the biotransformation of xenobiotics that are perhaps underappreciated, particularly when considering generation of reactive metabolites in extrahepatic target tissues. Non-cytochrome P450 drug-metabolizing enzymes such as flavin-containing monooxygenase (FMO), monoamine oxidase (MAO), alcohol dehydrogenase (ADH), and peroxidases such as prostaglandin H synthase (PGHS) and myeloperoxidase (MPO), have all been reported to contribute to the metabolism of xenobiotics [8,9]. Thus, their potential role in bioactivation of drug molecules also deserves increased attention.

The focus of this chapter is on specific examples of non-P450 drug-metabolizing enzymes and the potential role they may play in bioactivation and toxicity of xenobiotics. Table 3.1 highlights the enzymes discussed in this chapter, including the predominant tissues in which they are expressed and subcellular location. It is the hope of the authors that this compilation of selected examples will lead to an increased knowledge and awareness regarding reactive metabolite formation by metabolic enzymes outside the cytochrome P450 family.

3.2 FLAVIN-CONTAINING MONOOXYGENASES (FMO)

FMOs are a family of microsomal enzymes involved in the oxidation of nitrogen-, sulfur-, phosphorous-, selenium-, and other nucleophilic heteroatom-containing small molecules. Although these enzymes share a similar subcellular location, substrate overlap, coenzyme, and a common role in xenobiotic detoxification with cytochrome P450 enzymes, FMOs are distinct in a number of ways. The rates of xenobiotic turnover by FMOs are generally two to three times higher than that of P450 enzymes [10]. Additionally, the pH optimum for FMO-catalyzed transformations is 9–10. These enzymes also display a thermal instability in the absence of NADPH [11]. These three characteristics have often been exploited in determining the role of FMO in xenobiotic metabolism.

In humans, there are five functional FMO enzymes (FMO1–FMO5) as well as six pseudogenes. Human FMO1 (expressed in kidney) and FMO3 (expressed in liver) appear to play a dominant role in xenobiotic metabolism, while the role of FMO2, FMO4, and FMO5 in xenobiotic metabolism is currently not well established [12]. FMO isoforms have different substrate selectivity, and have distinct age-, gender-, species-, and tissue-dependent expression. This must be kept in mind when comparing FMO-mediated metabolism in different species because the expression of FMOs may differ [13]. The major steps in the mechanism of FMO-mediated oxidation are presented in Fig. 3.1. Typically, products from this mechanism include *N*-oxides and sulfoxide

TABLE 3.1 Expression and Subcellular Localization of the Non-P450 Metabolic Enzymes Described within this Chapter

Enzyme (Isoform)		Tissue(s)	Subcellular Fraction
Flavin-containing monooxygenase (FMO)	FMO1	Kidney	Microsomes
	FMO3	Liver	Microsomes
	FMO5	Liver	Microsomes
Monoamine oxidase (MAO)		Liver	Mitochondria
		Brain	
		GI tract	
		Placenta	
		Heart	
		Blood vessels	
		Kidney	
		Platelets	
Semicarbazide-sensitive amine oxidase (SSAO)		Blood vessels	Membrane-bound
		Plasma	
Alcohol dehydrogenase (ADH)	ADH1	Liver	Cytosol
	ADH2	Liver	Cytosol
	ADH3	Liver	Cytosol
	ADH4	GI tract	Cytosol
		Liver	
Prostaglandin H synthase (PGHS)		Kidney medulla	Microsomes Nuclei
		Platelets	
		GI tract	
		Brain	
		Lung	
		Bladder	
Myeloperoxidase (MPO)		Blood	Leukocytes
Aldo-ketoreductase (AKR)		Liver	Cytosol
		Brain	
NAD(P)H dehydrogenase [quinone] 1 (NQO1)		Stomach	Cytosol
		Kidney	
		Lung	
		Liver	
		GI tract	
NADPH cytochrome P450 reductase (CPR)		Liver	Microsomes
Aldehyde oxidase (AO)		Liver	Cytosol
Xanthine oxidase/xanthine dehydrogenase (XO/XD)		Liver	Cytosol

metabolites that are generally nonreactive, but a few examples exist where a reactive species is generated, which are discussed.

3.2.1 Thiocarbonyls

The conversion of thiocarbonyl compounds to reactive species by FMO enzymes is mechanistically well understood with several examples of compounds showing toxicity resulting from this bioactivation pathway. Thiourea, phenylthiourea, α -naphthylthiourea, ethionamide, thioacetamide, and thiobenzamide (Fig. 3.2) are a few examples

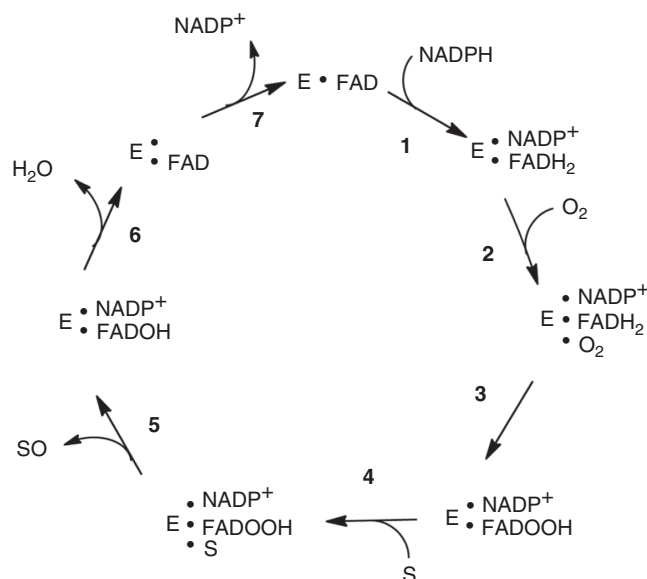


Figure 3.1 Catalytic cycle of FMO.

of thiocarbonyl compounds which have been shown to cause toxic outcomes [14,15]. The most common toxicity associated with thiocarbonyl compounds is pneumotoxicity and hepatotoxicity in rats. Additionally, the tuberculosis drug ethionamide has been associated with hepatotoxicity and dermal hypersensitivity in humans [16].

A generic example of the mechanism of bioactivation of a thiocarbonyl compound is depicted in Fig. 3.2. This bioactivation pathway proceeds via initial oxidation of sulfur generating a thioamide *S*-oxide. Further oxidation of the thioamide *S*-oxide results in formation of the reactive *S*, *S*-dioxide. Tautomerization of the *S*, *S*-dioxide to the iminosulfinic acid generates what is thought to be the reactive species, reported to react with nucleophiles as an acylating species giving rise to covalently modified proteins and lipids [16]. This observation may provide some mechanistic connection between the proposed bioactivation pathways by FMO and toxic outcomes that have been observed with thiocarbonyl-containing compounds, particularly within the lung [14].

3.2.2 Aryl Piperazines

Compared to the thiocarbonyls, less data has been presented to associate any toxicity of aryl piperazine-containing molecules with a particular biotransformation pathway and reactive metabolite. Despite these shortcomings, the examples presented in this section will provide additional instances of FMO-mediated bioactivation of substrates to reactive metabolite(s).

Rodriguez *et al.* have proposed that reactive metabolites of ketoconazole produced by an FMO-mediated pathway are a possible explanation for the observed hepatotoxicity [17]. Studies using rat hepatocytes with ketoconazole and its primary metabolite, *N*-deacetylketoconazole, indicate greater hepatotoxicity for the metabolite than for the

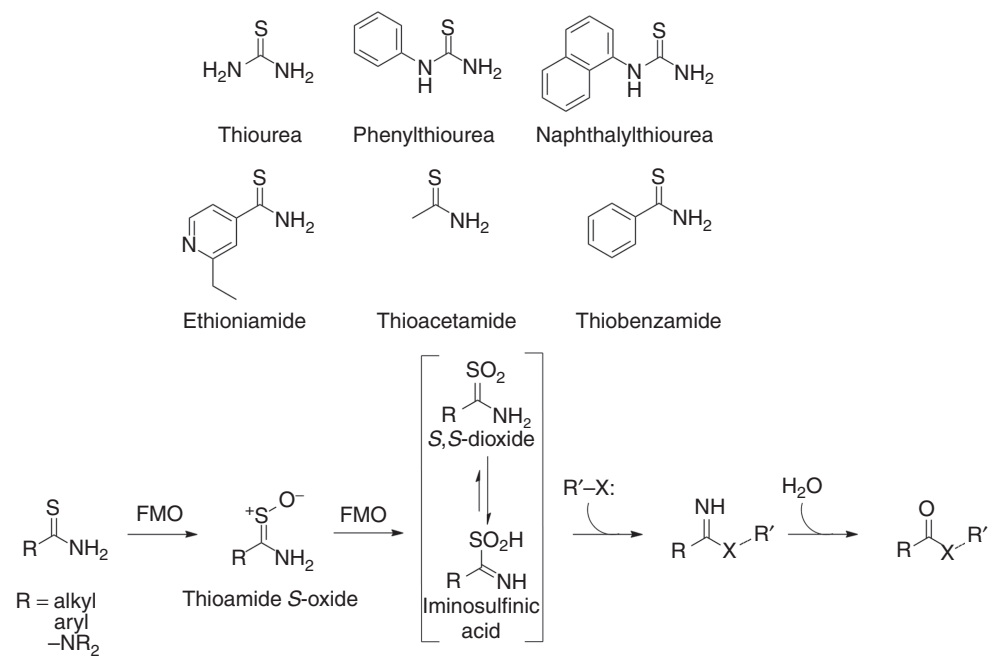


Figure 3.2 Examples of thiocarbonyl-containing compounds and bioactivation mechanism catalyzed by FMO.

parent drug. Additional reports have also shown that binding of radiolabeled ketoconazole or its metabolites to microsomal proteins is abrogated by heat inactivation of FMO in microsomes. Mechanistically, FMO-mediated metabolism of the secondary nitrogen of *N*-deacetyl ketoconazole results in the formation of the secondary hydroxylamine metabolite [18] as shown in Fig. 3.3. Further oxidation of this intermediate has been proposed to result in production of the potentially reactive nitron and/or aldehyde metabolites, although direct evidence supporting the formation or reactivity of these intermediates has not yet been presented.

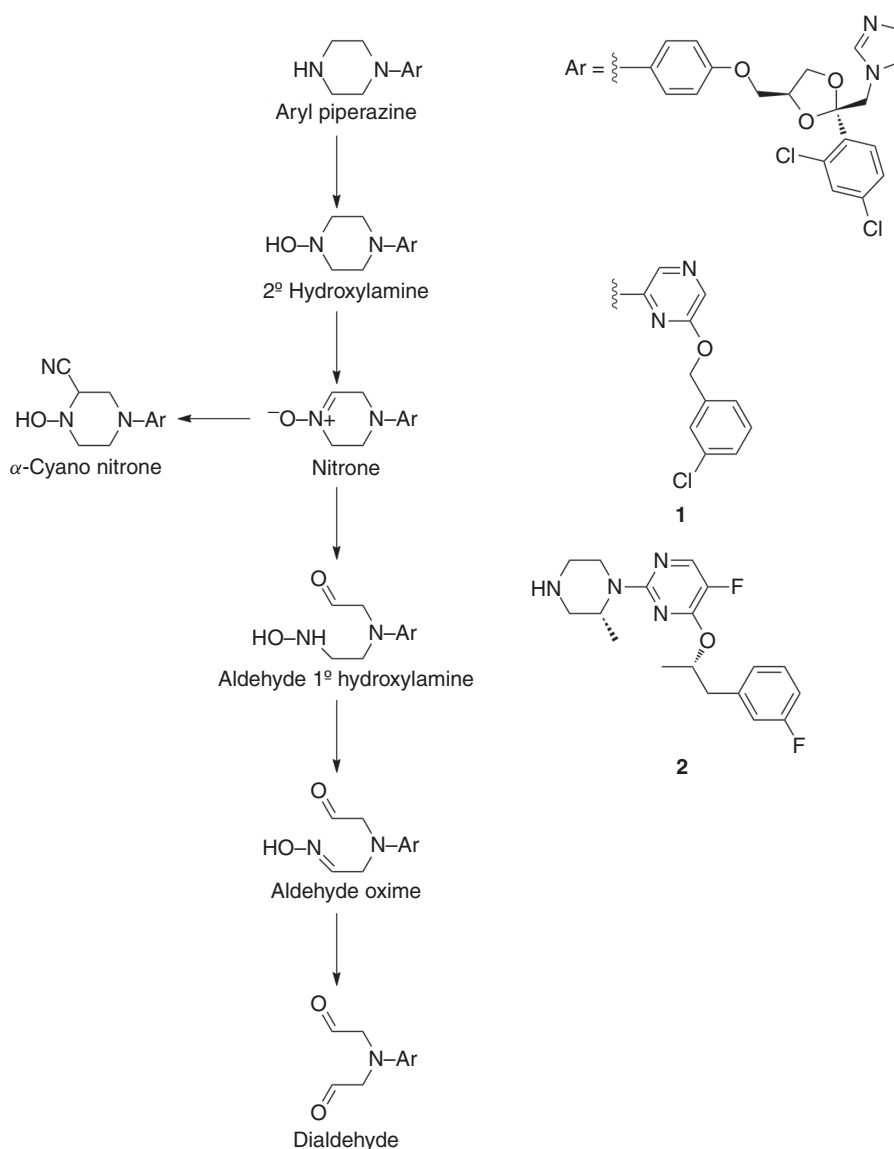


Figure 3.3 Bioactivation of *N*-aryl piperazine moiety by FMO.

Work by Kalgutkar *et al.* involving 5-HT_{2c} agonists has likewise uncovered formation of reactive metabolites associated with the *N*-aryl piperazine moiety [19]. During safety evaluation, exposure of *Salmonella* cells with **1** (shown in Fig. 3.3) in the presence of S9 and NADPH resulted in a dose-dependent increase in reverse mutations. Additionally, incubation of [¹⁴C]-**1** with S9 fortified with NADPH resulted in a concentration-dependent increase in covalent binding of radioactivity to calf thymus DNA. Further examination of the metabolism of **1** in the presence of the trapping agents, GSH, cyanide, or methoxylamine was also undertaken. In addition to a GSH adduct and two methoxylamine trapped metabolites, an α -cyano hydroxylamine metabolite was also observed (Fig. 3.3), indicating the formation of an initial secondary hydroxylamine followed by a reactive nitron intermediate. While the involvement of FMO in the bioactivation of **1** has not been confirmed, the observed and proposed metabolites of **1** are strikingly similar to those of *N*-deacetylketonazole, which suggests that a rat FMO-mediated bioactivation pathway may be involved. Interestingly, in subsequent work with analog **2** in which the 3-chlorobenzyloxy moiety has been removed, no metabolism-dependent mutagenicity was observed [20] despite the finding of both a methoxylamine and cyanide conjugate. The authors propose that differences in the extent or nature of the metabolism of **2** compared to **1** explain the lack of mutagenicity of this compound.

3.2.2.1 4-Fluoro-*N*-Methylanilines. Numerous examples of hydroxylation of anilines or aniline containing structures at the para position have been shown to result in the formation of electrophilic quinoneimine species, which may be reactive with protein nucleophiles resulting in toxicity. A rational approach to mitigating the formation of this reactive species would be to substitute a fluorine atom para to the aniline nitrogen, decreasing the electron density of the aromatic ring and potentially blocking para hydroxylation. Despite attempts to block metabolic activation in this manner, several examples of oxidative dehalogenation resulting in the formation of *para*-hydroxyanilines have been documented. For most of these examples, a reaction mechanism involving cytochrome P450 has been invoked [21]. Interestingly, in addition to the involvement of cytochromes P450, FMO enzymes have also been shown to be proficient at catalyzing this bioactivation pathway.

A thorough characterization of 4-fluoro-*N*-methylaniline metabolism *in vitro* and *in vivo* was undertaken by Boersma *et al.* Formation of the oxidatively defluorinated metabolite, *N*-methyl-4-hydroxyaniline, was identified and its formation reported to be catalyzed by purified rat FMO [22]. Subsequent work demonstrated that recombinant human FMO1 (rFMO1) was also capable of generating this metabolite [23], with a mechanism involving electrophilic aromatic substitution being invoked to explain the product formation (Fig. 3.4). Delocalization of the aniline lone pair would direct the carbon at the para position to attack the distal oxygen of the peroxyflavin of FMO. The resulting tetrahedral intermediate could then eliminate HF to generate a reactive quinoneimine. Subsequent two-electron reduction would generate the final phenolic product. Coincubation of *N*-acetylcysteine (NAC) with 4-fluoro-*N*-methylaniline, rFMO1, and NADPH resulted in the formation of two NAC conjugates. This, along with the observation that the phenolic oxygen atom originates from molecular oxygen, provided evidence in support of the proposed mechanism. Not only does this example demonstrate a novel FMO-mediated biotransformation pathway, but it also highlights

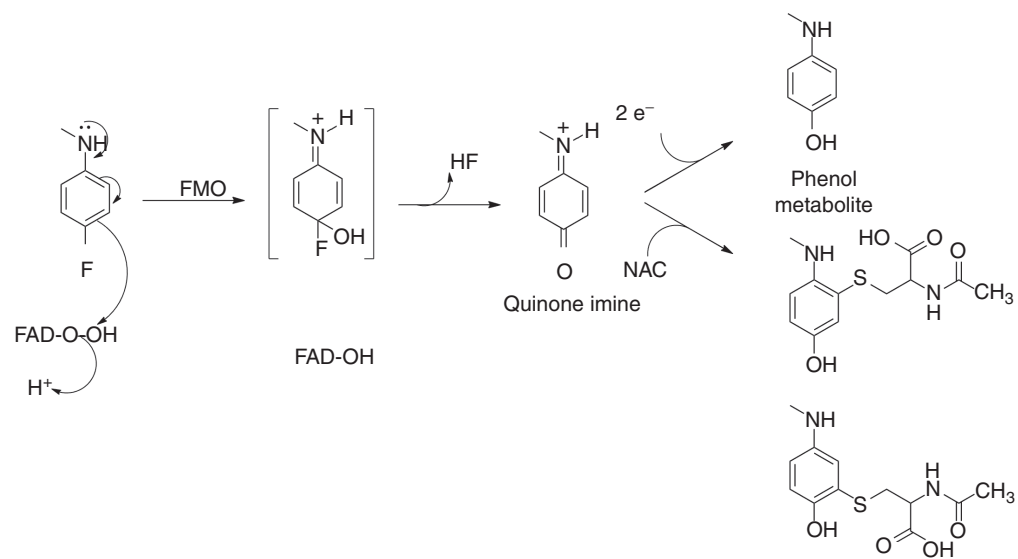


Figure 3.4 Bioactivation of 4-fluoro-*N*-methylaniline by FMO to a reactive quinoneimine intermediate.

an FMO-mediated bioactivation pathway that, until this report, was essentially reserved for the cytochrome P450 enzymes.

3.3 AMINE OXIDASES

3.3.1 Monoamine Oxidase (MAO)

The MAO enzymes are integral membrane proteins localized in the mitochondrial outer membrane of most cell types and are dependent upon the cofactor flavin-adenine dinucleotide (FAD). These amine oxidases typically catalyze the deamination of endogenous primary biogenic amines such as dopamine (DA), norepinephrine, serotonin, and tyramine, and are widely distributed throughout the body, particularly the liver, brain, heart, blood vessels, and kidney [24,25]. The expression of MAO in the brain has made this a popular pharmacological target in the treatment of depression and Parkinson's disease [26]. However, side effects such as hypertensive crisis due to the inhibition of metabolism of tyramine (e.g., "cheese-effect") have lead to reduced interest in this therapeutic approach. MAO exists in two different forms, MAO-A and MAO-B (encoded by distinct genes; ~70% sequence similarity), and can be differentiated by substrate and inhibitor specificities. For example, MAO-A preferentially oxidizes serotonin and norepinephrine, and is selectively inhibited by clorgyline, whereas MAO-B oxidizes phenethylamine and benzylamine, and is inhibited by low concentrations of (*R*)-deprenyl [27]. Despite the predominant role of MAO in oxidizing endogenous amines, it is also involved in oxidizing a number of exogenous primary, secondary, and tertiary amines such as the triptan antimigraine drug sumatriptan [28], and the selective serotonin reuptake inhibitor (SSRI) antidepressant citalopram [29]. The observation of increased xenobiotic metabolism by MAO suggests that more emphasis should be placed on characterizing non-P450-mediated metabolism, particularly for amine-containing drug molecules.

MAO distinguishes itself from cytochrome P450 enzymes in multiple ways, including its subcellular location, which is the mitochondria. Interestingly, however, it has been demonstrated that liver microsomes, when prepared from frozen tissue, may contain MAO activity, as mitochondrial membranes apparently may end up included in the microsomal fraction as a contaminant [30]. The catalytic mechanism of MAO may be broken down into two main reactions: oxidative deamination and cofactor recycling. Steps a–e in Fig. 3.5 represent the "deamination" reaction, while step f represents the FAD-recycling step. MAO incorporates oxygen from water into the substrate to yield an aldehyde and an amine, and upon reoxidation of FADH₂ with molecular oxygen (O₂), produces hydrogen peroxide (H₂O₂) as a by-product. Figure 3.5 depicts the detailed catalytic mechanism by which MAO operates, including an example of MAO metabolism of a cyclic tertiary amine to a ring-opened aldehyde. Alternatively, the iminium intermediate may be oxidized by an enzyme called *aldehyde oxidase* (AO) to form the nonreactive lactam.

3.3.1.1 Tetrahydropyridines. The finding that prompted specific interest in MAO as it relates to bioactivation was the observation that MAO-B metabolically converts 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into a potent neurotoxin (1-methyl-4-phenylpyridinium, MPP⁺) that can destroy dopaminergic neurons, leading to Parkinson-like symptoms in humans [31–33]. The Parkinson-like motor deficits

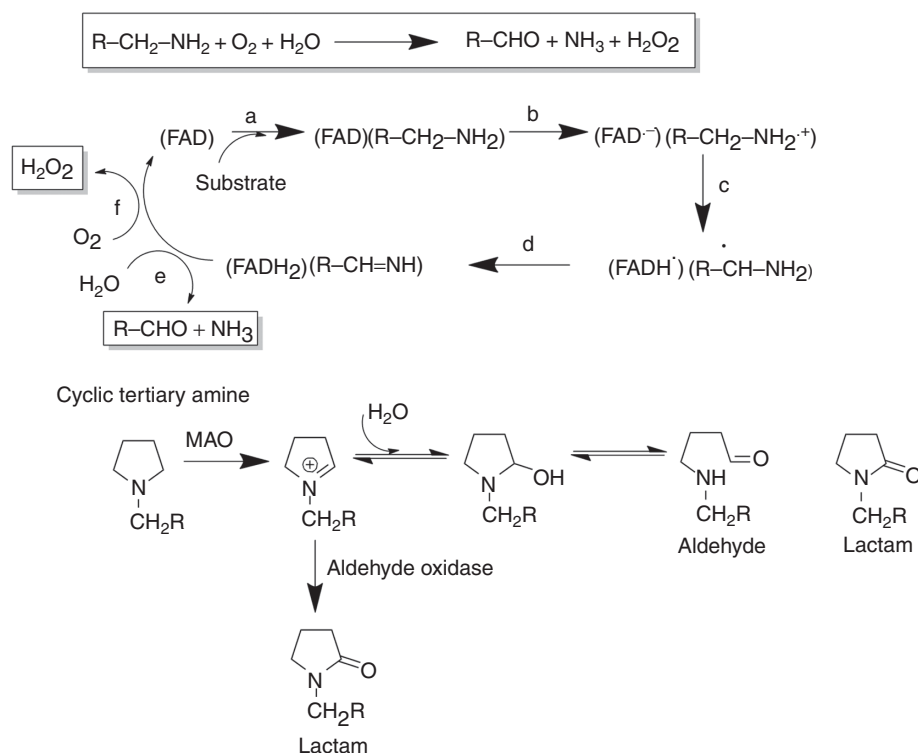


Figure 3.5 Catalytic mechanism of MAO.

were initially observed in drug addicts after self-administration of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), an analog of meperidine that was contaminated with MPTP. MPTP undergoes oxidation to the iminium species 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) by MAO-B. However, instead of undergoing hydrolysis to produce the corresponding aldehyde and amine, is further oxidized to the neurotoxic MPP⁺ species within the brain (Fig. 3.6), which in turn is actively transported into dopaminergic nerve terminals by the DA uptake transport system [34] where it accumulates and interferes with mitochondrial respiration. Solid evidence supporting the role of MAO-B in this pathway was gathered when selective MAO-B inhibitors were shown to protect against MPTP neurotoxicity in mice and primates [35,36]. Also interesting is that while the iminium MPDP⁺ may be converted to the nontoxic lactam product by AO in tissues such as liver, this alternate detoxifying pathway is apparently not present in the brain, resulting in the accumulation of MPP⁺ and subsequent neurotoxicity [37]. Thus, the MPTP story represents a classic example of bioactivation by a non-P450 enzyme, followed by a perfect storm of events, where the lack of a detoxifying pathway in a specific tissue (e.g., brain) and a transport mechanism leads to a localized neurotoxic event in the nigrostriatum.

3.3.1.2 Dopamine. DA is a critical endogenous neurotransmitter in the brain with numerous physiological functions. It undergoes catabolism via multiple pathways, including MAO to form 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL may

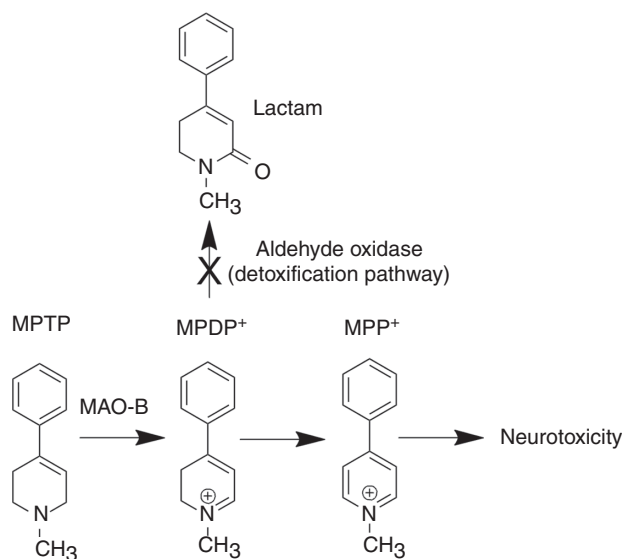


Figure 3.6 Bioactivation of MPTP by MAO-B in brain.

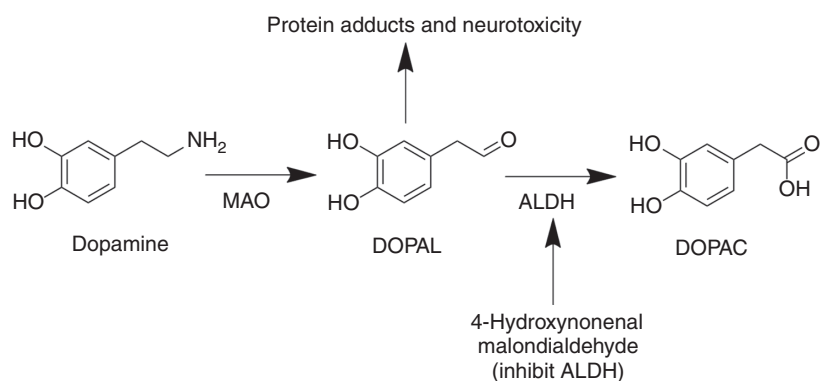


Figure 3.7 Bioactivation of dopamine to a reactive aldehyde by MAO.

then undergo further catabolism to 3,4-dihydroxyphenylacetic acid (DOPAC) via aldehyde dehydrogenase (ALDH) (Fig. 3.7). Despite the critical physiological role that DA plays, it has been implicated as a neurotoxin, with a potential role in Parkinson's disease pathogenesis. However, several publications have pointed to the DOPAL metabolite of DA (generated by MAO) as the actual toxin [38,39]. For example, when both DOPAL and DA were directly injected into rat substantia nigra, DOPAL was toxic to DA neurons in a dose-dependent manner, with tissue necrosis observed at the highest dose, whereas DA itself showed no evidence of neurotoxicity at much higher doses [38]. Additional evidence for the role of DOPAL in neurotoxicity was elegantly demonstrated by showing that the lipid peroxidation products, 4-hydroxynonenal (4HNE), and malondialdehyde (MDA), inhibit further oxidation of DOPAL by the ALDH pathway, resulting in the accumulation of DOPAL and a subsequent increase in protein

modification [40,41]. These data have provided a unique mechanistic link between oxidative stress/lipid peroxidation and degeneration of DA neurons by a reactive aldehyde metabolite generated by MAO. Lastly, it has also been reported that 3,4-dihydroxyphenylglycoaldehyde (DOPEGAL), the MAO metabolite of norepinephrine, display neuronal selective toxicity [39], suggesting that bioactivation of endogenous molecules may be more common than expected. All of these findings confirm what was hypothesized over 50 years ago, which was that aldehyde metabolites of amines would be directly toxic to cells where they are formed [42].

3.3.2 Semicarbazide-Sensitive Amine Oxidase (SSAO)

Semicarbazide-sensitive amine oxidase (SSAO) oxidatively deaminates primary amines such as methylamine and benzylamine [43]. The name of this amine oxidase is derived from the fact that semicarbazide covalently interacts with and inhibits enzyme activity. SSAO is expressed in most mammalian tissues, and exists both as tissue-bound, as well as a soluble form in plasma [44]. The main tissue-bound form is in blood vessel smooth muscle layers, including cardiac tissue.

3.3.2.1 Allylamine. Allylamine is an aliphatic amine that was discovered to cause cardiovascular toxicity (e.g., lesions) in experimental animals [45]. Specifically, when rats were dosed with allylamine, myocardial necrosis and fibrosis resulted, which interestingly, resemble atherosclerotic disease [46]. However, when allylamine was codosed with semicarbazide, the severity of disease was dramatically reduced, which lead to the hypothesis that allylamine may be converted to acrolein by tissue-specific amine oxidases such as SSAO. In fact, the production of acrolein was confirmed in homogenates of rat aorta and heart [45]. Bioactivation of allylamine by SSAO to acrolein, a highly reactive aldehyde, is shown in Fig. 3.8. Providing a link between preclinical species and humans regarding the toxicity of acrolein, one human fatality following ingestion of allyl alcohol has been reported [47]. High concentrations of acrolein ($\sim 128 \mu\text{M}$)

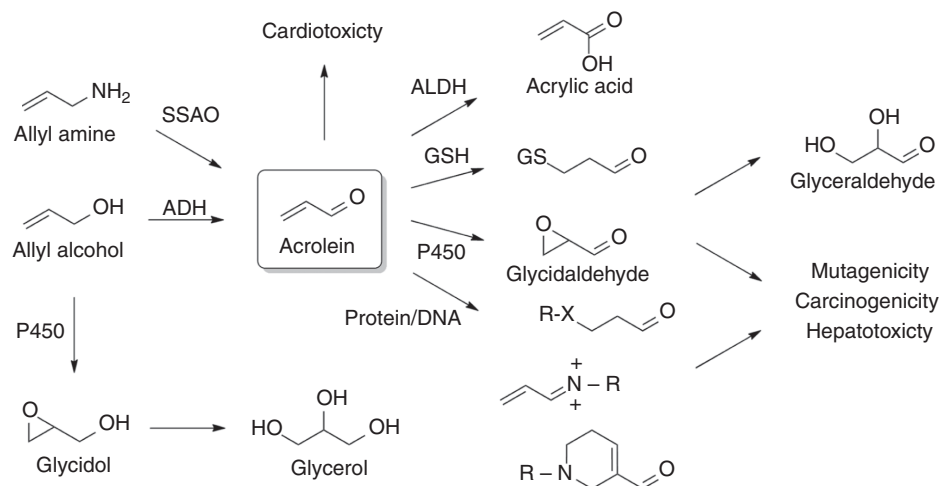


Figure 3.8 Metabolic pathways and formation of the toxic aldehyde metabolite acrolein.

were found in the blood of this individual upon autopsy, and it was concluded that the cause of death was cardiotoxicity caused by acrolein. Interestingly, formation of acrolein in this case is likely catalyzed by another non-cytochrome P450 metabolic enzyme, namely, ADH, which is further discussed in the next section.

3.4 ALCOHOL DEHYDROGENASE

ADH is a member of the medium-chain dehydrogenase/reductase (MDR) superfamily. Other members of the MDR superfamily include quinonereductases and leukotriene B4 dehydrogenase [48]. These enzymes exist as dimers folded into two domains. Each monomeric subunit contains two zinc atoms, one structural and one catalytic, with the catalytic zinc anchored between the two domains. There are five classes of human ADHs designated ADH1–ADH5. Three isozymes of the ADH1 family exist, specifically ADH1A, ADH1B, and ADH1C [49]. Generally, all human isoforms are expressed in the liver, except ADH5, which was only identified at the gene and mRNA level. Additionally, the expression of ADH4 is higher in extrahepatic tissues, specifically the gastrointestinal tract [49].

ADH is involved in the NAD(H)-catalyzed oxidative metabolism of primary and secondary alcohols to aldehydes and ketones, respectively. Additionally, ADH may also catalyze the reduction of aldehydes back to alcohols producing NADH [50]. The general sequence of events in the mechanism of ADH-catalyzed oxidations is as follows [51,52]: (i) NAD^+ binding, (ii) alcohol binding via coordination to the catalytic zinc, (iii) deprotonation of the alcohol generating a zinc-bound alkoxide ion, (iv) hydride transfer from the alkoxide ion to NAD^+ thereby producing a zinc-bound aldehyde or ketone, (v) product release, and (vi) dissociation of NADH. The products of ADH-catalyzed reactions are aldehydes or ketones. Of these two species, aldehydes have been shown to be involved in reactions with macromolecules such as DNA and/or proteins. Despite known detoxifying enzymatic pathways for aldehydes, several examples exist for ADH-mediated bioactivation of molecules resulting in toxic outcomes.

3.4.1 Allyl Alcohol

Allyl alcohol is a widely used industrial chemical and synthetic intermediate. Extensive periportal necrosis has been observed in rats following administration of allyl alcohol [53]. Subsequent studies have shown that allyl alcohol is converted to the well-known toxic substance acrolein, which is present in tobacco smoke and fried foods, and is the toxic metabolite of the cancer drug cyclophosphamide. The metabolic bioactivation of allyl alcohol occurs in rat liver and lung S9 and cytosol, and this biotransformation is NAD^+ -dependent. Neither NADPH- nor NADP^+ -fortified incubations result in conversion of allyl alcohol to acrolein [54]. Additionally, this conversion is significantly inhibited by pyrazole [55]. Together these pieces of evidence support the involvement of ADH in the metabolism of allyl alcohol to acrolein (Fig. 3.8). Upon formation of acrolein, several possible pathways have been proposed which could explain covalent binding of acrolein to DNA or protein, subsequently resulting in potential toxicity. Michael addition of sulfhydryl groups occurs rapidly, resulting in a protective mechanism, such as in the case of GSH, or alkylated proteins, which may cause toxicity. Similarly, the imidazole of histidine has also been shown to be involved

in Michael addition to acrolein [56]. Formation of propenediamines with the nitrogen of lysine is another mechanism of protein adduction which has been shown to occur more slowly than that of Michael addition of sulfhydryls [53]. Uchida *et al.* have also identified an acrolein-lysine adduct, *N*^ε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine), formed by the reaction of lysine with two molecules of acrolein [56].

As shown in Fig. 3.8, there are also several other metabolic pathways that may be protective or may lead to other reactive species. Acrolein is converted by ALDH to acrylic acid which, despite being an α,β -unsaturated acid, is less reactive than acrolein. Addition of an ALDH inhibitor results in greater toxicity in animals administered acrolein, which suggests that this is a protective pathway [53]. In addition, two cytochrome P450-catalyzed pathways result in conversion of allyl alcohol to glycidol and acrolein to glycidaldehyde. Adding complexity to the nature of the toxicity is the fact that glycidaldehyde has been identified as both a mutagen and a carcinogen [53]. Both glycidol and glycidaldehyde are then hydrated to form the benign metabolites glycerol and glyceraldehyde, respectively. This example represents one of the more complex bioactivation pathways known, with multiple drug-metabolizing enzymes playing a role in either generation of reactive metabolite, or as protective mechanisms.

3.4.2 Felbamate

Felbamate (2-phenyl-1,3-propanediol dicarbamate) was approved in 1993 as an antiepileptic drug. In its first year on the market, felbamate was administered to ~120,000 patients [57]. A total of 34 cases of aplastic anemia resulting in 13 deaths and 23 cases of hepatic failure resulting in 5 deaths have been reported [58–60]. Studies of the *in vivo* metabolism of ¹⁴C-felbamate in rat, rabbit, dog, and human have resulted in the identification of two mono-hydroxylated metabolites (*p*-OHF and 2-OHF), a monocarbamate (MCF), and the carbamoyl propionic acid (CPPA) (Fig. 3.9). Of particular interest was the fact that CPPA was the major human

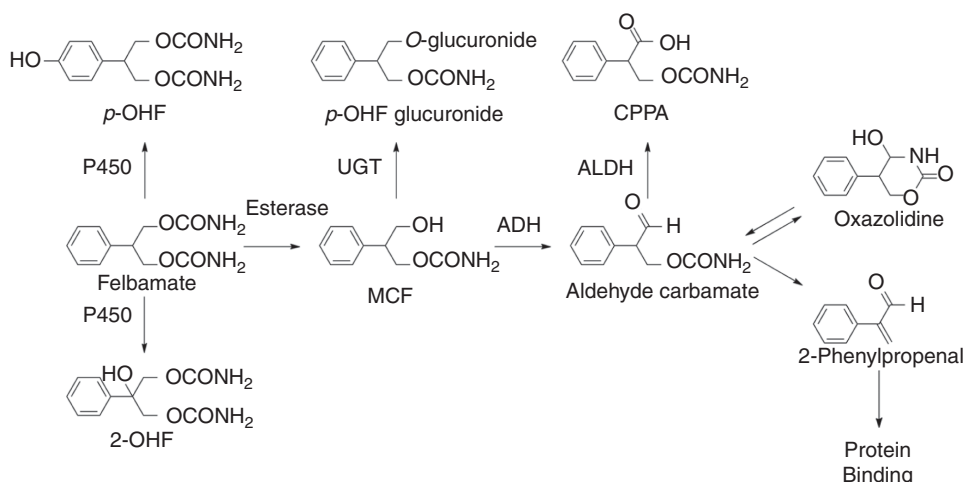


Figure 3.9 Bioactivation mechanism of felbamate to 2-phenylpropenal.

metabolite at ~12%, and the only other species in which this metabolite was observed was the dog [61]. On the basis of the observation of CPPA and the likely intermediacy of an aldehyde carbamate metabolite, the MacDonald group at the University of Virginia hypothesized that once formed, the aldehyde carbamate may undergo β -elimination to give 2-phenylpropenal as the reactive electrophile responsible for the observed toxicities of felbamate [62].

To support the hypothesis of toxicity mediated by 2-phenylpropenal, 2-phenylpropenal and its proposed precursor, the carbamoyl aldehyde, were synthetically prepared. Toxicity of these two compounds, as well as felbamate, MCF, CPPA, and two known toxic compounds, acrolein and 4-HNE was tested in a cytotoxicity assay. In this assay, 2-phenylpropenal showed similar toxicity to acrolein and 4-HNE, although the aldehyde carbamate was shown to be 10–100 times less potent, while MCF and CPPA were not toxic [61]. Additional studies of the reaction of GSH with 2-phenylpropenal in phosphate buffer indicated that the reaction was fast, with an observed half-life of ~40 s [63].

With data to support toxicity and reactivity of a likely intermediate in the metabolism of felbamate, attention turned toward the mechanism and rate of conversion of the aldehyde carbamate to 2-phenylpropenal. These studies showed the aldehyde carbamate to be chemically reactive, generating the expected 2-phenylpropenal and a cyclized oxazolidine product. Under phosphate buffered conditions at physiological pH, formation of the oxazolidine was favored but longer incubation times resulted in 2-phenylpropenal [61]. The observation of the oxazolidine species also leads to the proposal that this species acts as a latent form of 2-hydroxypropenal, able to escape the site of formation and travel to sites distal from the liver, where it may exert its effects [62].

The aforementioned metabolic conversions of felbamate to both nontoxic and toxic metabolites are demonstrated in Fig. 3.9. The bioactivation pathway leading to the aplastic anemia and hepatotoxicity observed for felbamate involves several non-P450-mediated biotransformations. Initially, one of the carbamate functionalities of felbamate is cleaved via an esterase, amidase, or hydrolase to give MCF, although neither the exact enzyme nor its subcellular location is known [64]. Oxidation of the primary alcohol to the aldehyde carbamate occurs via an NAD^+ -catalyzed ADH-mediated reaction. The aldehyde carbamate and the oxazolidine species are in equilibrium with decomposition of the aldehyde carbamate leading to the formation of 2-phenylpropenal. ALDH may oxidize the aldehyde carbamate to give CPPA [62]. Interestingly, species differences in the enzymes involved in the metabolism of felbamate are thought to have contributed to the lack of observed toxicity in preclinical studies. For example, in rats, 2-phenylpropenal formation is minor because of the significant P450-mediated metabolism of felbamate, the relatively poor esterase activity in rats, and the high ALDH activity in this species. The metabolic and chemical transformation uncovered in the numerous studies investigating felbamate toxicity present a fascinatingly complex example of non-P450 bioactivation.

3.4.3 Abacavir

Abacavir is a nucleoside reverse transcriptase inhibitor approved in 1999 for the treatment of HIV-1. Upon release onto the market, hypersensitivity reactions in ~4% of the patient population were reported. The severity of these reactions ranged from fever and rash to a small number of deaths [65]. In order to obtain a better understanding of the

potential for bioactivation pathways being responsible for this idiosyncratic reaction, characterization of the metabolism of abacavir was undertaken. The major metabolic routes for abacavir in humans were found to be O-glucuronidation and oxidation of the primary alcohol of abacavir to the carboxylic acid (Fig. 3.10). The latter conversion likely occurs via a two-step, four-electron oxidation to an aldehyde intermediate. However, this aldehyde was never directly observed in incubations and attempted synthesis failed, most likely the result of its instability/reactivity. On the basis of the likely intermediacy of this metabolite and the known reactivity of aldehyde metabolites, Walsh *et al.* hypothesized that either the aldehyde intermediate or an isomeric species where the cyclopentene double bond had tautomerized to give an α,β -unsaturated aldehyde were likely to blame for the hypersensitivity reactions [66].

In vitro metabolism studies were undertaken to provide evidence for the involvement of one or both of these pathways. These studies subsequently showed that the abacavir acid was only observed in human liver cytosol incubations and that the conversion, as well as covalent binding, was NAD^+ -dependent, suggesting the involvement of ADH [66]. Similar ADH-mediated four-electron oxidations of alcohols to acids previously have been reported [50].

Screening of purified ADH isoforms showed that only ADH1A and ADH1C were capable of forming carboxylic acid metabolites, as well as covalent binding. The observation of multiple isobaric species in the liquid chromatography/mass spectrometry (LC/MS) indicated that tautomerization of the cyclopentene double bond and/or racemization of one or more of the stereocenters likely had occurred. Trapping experiments with methoxylamine, resulting in the formation of an oxime species, provided support for the proposed aldehyde intermediate. Walsh *et al.* concluded that the mechanism of bioactivation for abacavir occurs via initial ADH-mediated oxidation to an unstable aldehyde metabolite. The double bond of the cyclopentene ring is then proposed to tautomerize into conjugation, giving an α,β -unsaturated aldehyde, which is likely to undergo Michael addition of protein nucleophiles. Studies using an analog of abacavir with the double bond of the cyclopentene reduced yielded similar metabolic turnover, but significantly limited covalent binding to protein. This result suggested that the unconjugated aldehyde alone had limited involvement in adduct formation via Schiff

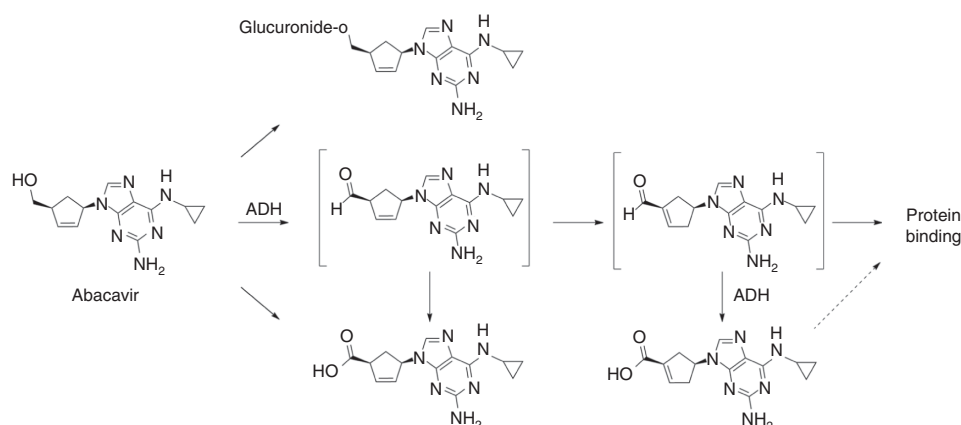


Figure 3.10 Bioactivation of abacavir by ADH to α,β -unsaturated aldehyde.

base formation [66]. However, exploration of the reactivity of the α,β -unsaturated acid (which would likewise be a Michael acceptor) was not explored. Although these studies do not draw a conclusive link between reactive metabolites and toxicity, they do offer possible bioactivation pathways that could result in the observed idiosyncratic hypersensitivity of abacavir.

3.5 PEROXIDASES

Peroxidase enzymes (MPO, lactoperoxidase, and PGHS) are expressed in many tissues such as kidney medulla, platelets, GI tract, brain, lung, urinary bladder (PGHS), mammary gland epithelium (lactoperoxidase), and polymorphonuclear leukocytes (MPO). Thus, bioactivation of xenobiotics by PGHS appears to be more prevalent in these extra-hepatic tissues where various toxicities have been noted [67,68]. These enzymes are similar to cytochrome P450 enzymes in that they are hemoproteins and contain bound protoporphyrin IX, yet they do not require cofactors NADPH or NADH. Instead, peroxidases couple the reduction of hydrogen peroxide (H_2O_2) or lipid hydroperoxides to the oxidation of substrate (e.g., co-oxidation) [69]. Many xenobiotics have been shown to be oxidized by peroxidase enzymes, often to reactive metabolites. Typical classes of compounds that undergo peroxidase-mediated one-electron oxidation to potentially reactive intermediates include aromatic amines, phenols, hydroquinones, and polycyclic hydrocarbons. This section focuses mainly on PGHS and MPO.

3.5.1 Prostaglandin H Synthase (PGHS)

PGHS, most commonly referred to as *cyclooxygenase* (COX), exists in two forms: the constitutive enzyme (PGHS-1) that maintains normal housekeeping functions, and the inducible PGHS-2, which is involved in inflammatory processes, and as such, is the primary target for nonsteroidal anti-inflammatory drugs (NSAIDs). The physiological role of PGHS includes the initial oxidation of arachidonic acid to PGG₂ (e.g., *cyclooxygenase* activity), followed by a *peroxidase* activity, where the hydroperoxide group of PGG₂ acts as an oxygen donor to reduce PGG₂ to PGH₂ (Fig. 3.11). In parallel with the second peroxidase reaction, xenobiotics may undergo what is termed a co-oxidation reaction. It is this co-oxidation mechanism and the role it plays in bioactivation of xenobiotics that is of specific interest here. Three principle mechanisms of PGHS-mediated bioactivation have been characterized, as highlighted in Fig. 3.11, including (A) direct oxidation of the xenobiotic cosubstrate (typically phenols or amines) by the peroxidase, forming a radical and (B) direct oxidation by the peroxy radical (ROO•) that is generated during the cyclooxygenase reaction to form an oxygenated product. A third possible mechanism is a combination of mechanisms A and B, where the formation of a highly reactive radical species (formed during mechanism A) combines with oxygen (O_2) to form a peroxy radical which subsequently acts to bioactivate a secondary xenobiotic, as occurs in mechanism B (e.g., indirect bioactivation) [67]. Many chemicals, including aromatic amines, polyaromatic hydrocarbons (PAHs), aflatoxins, heterocyclic amines, and phenols, are capable of serving as electron donors during the peroxidase reaction. Mutagenicity assays are often used to assess the potential for the formation of mutagenic products, which may occur during PGHS-dependent oxidation of certain xenobiotics. This may be performed by incubating test compounds with *Salmonella*

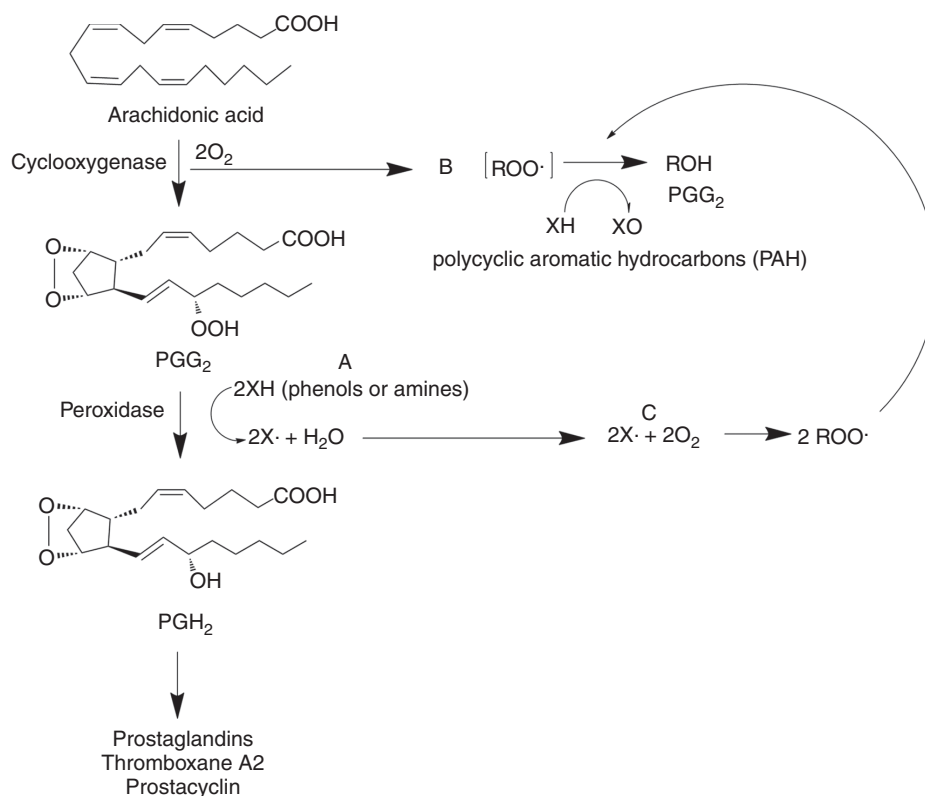


Figure 3.11 Mechanism of PGHS-mediated bioactivation of xenobiotics.

bacterial tester strains, which contain ram seminal vesicle microsomes, a source of PGHS activity, in addition to arachidonic acid or hydrogen peroxide [67]. While specific examples of PGHS-mediated bioactivation are described in the following sections, a more comprehensive review of PGHS and its role in xenobiotic bioactivation has been published [69].

3.5.1.1 Acetaminophen (APAP). Metabolic activation of APAP by the cytochrome P450 enzymes to its reactive quinoneimine metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) is well documented in the literature, and serves as the gold standard example of bioactivation of drug molecules. Formation of NAPQI in the liver is known to cause centrilobular necrosis upon overdose and depletion of cellular pools of detoxifying GSH [70]. In addition, and perhaps less recognized is that chronic exposure to APAP can also lead to covalent binding and damage in the kidney, a tissue that contains low levels of cytochrome P450 [71]. Ironically, it was noticed before fully understanding the mechanisms of bioactivation, that following pretreatment of mice and rats with the P450 inducer 3-methylcholanthrene, the induced changes were greater in the liver when compared to the kidney, suggesting that the formation of reactive metabolite in kidney was by a mechanism distinct from cytochrome P450 [72]. Subsequently, it was proposed that one-electron co-oxidation of APAP by PGHS in the renal medulla produces either the phenoxyl radical or the benzosemiquinoneimine radical (Fig. 3.12),

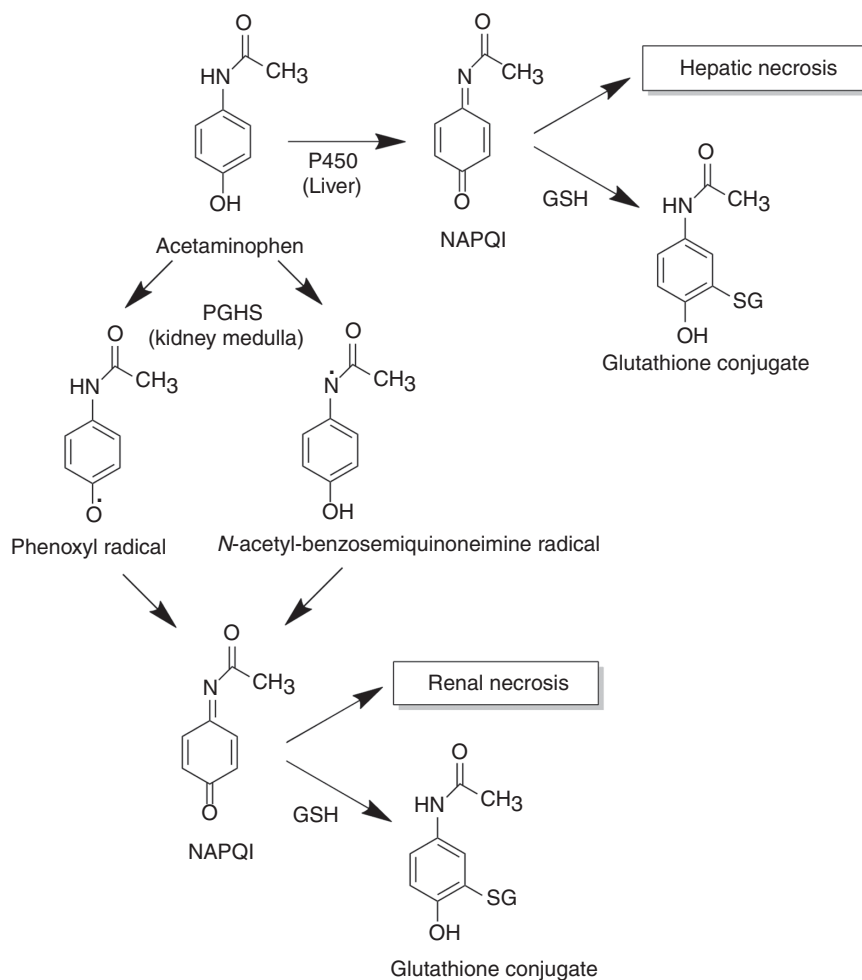


Figure 3.12 Bioactivation of acetaminophen to NAPQI in kidney medulla by PGHS.

leading to the eventual formation of NAPQI and regional injury in this tissue [73]. This serves as an excellent example of a tissue-specific toxicity due to bioactivation by a non-P450 enzyme highly expressed in that particular *microenvironment*, in this case, the kidney.

3.5.1.2 Aromatic Amines. Aromatic amines are ubiquitous in the environment, and are known as *human carcinogens* due to their oxidation to electrophilic intermediates and subsequent covalent modification of DNA. While the liver is often the main focus when studying bioactivation of aromatic amines, extrahepatic tissues such as urinary bladder contains low expression levels of cytochrome P450, while PGHS levels are quite high in both humans and dogs [74]. Aromatic amines such as benzidine, 4-aminobiphenyl, and 2-aminonaphthalene are reported to be bioactivated by PGHS to mutagenic reactive metabolites, such as diimines (Fig. 3.13) that can interact with DNA. This is presumed to be the mechanism by which aromatic amines lead to carcinogenesis

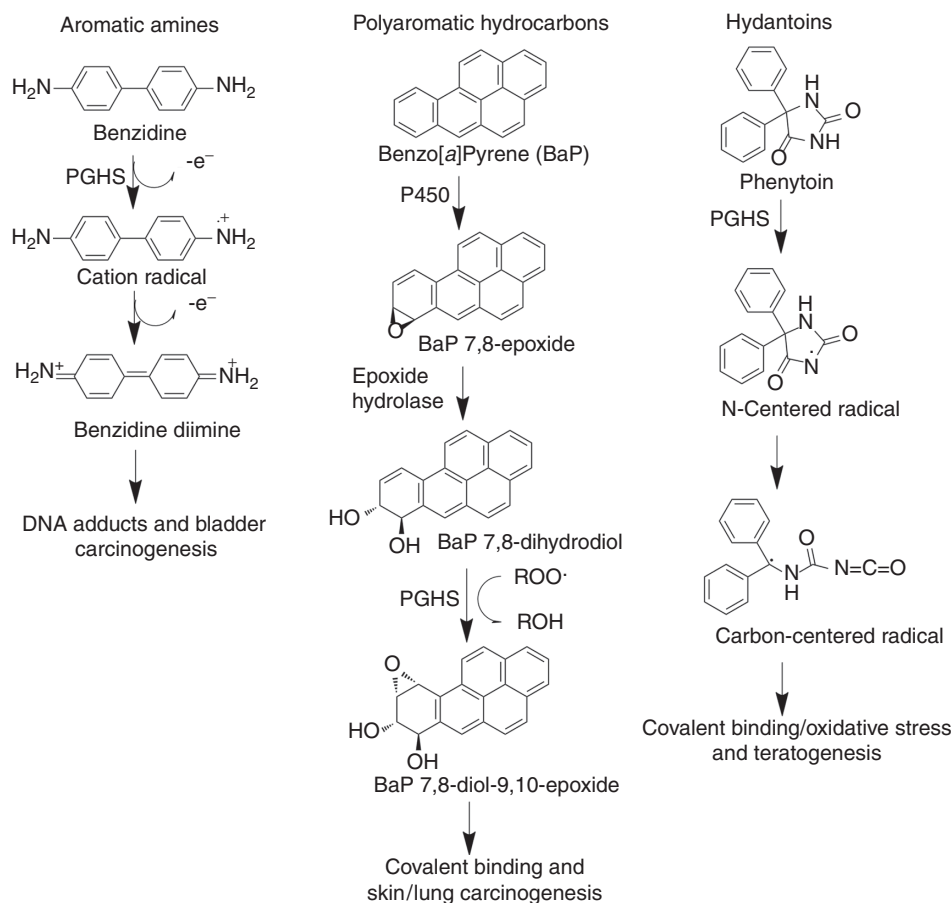


Figure 3.13 Bioactivation of aromatic amine benzidine, polyaromatic hydrocarbon BaP, and hydantoin phenytoin by PGHS leading to various forms of carcinogenesis/teratogenesis.

in the bladder and other tissues [68,75,76]. Specifically, the bioactivation of radiolabeled benzidine to detectable DNA-bound products in various extrahepatic tissues has been investigated. For example, covalent binding to DNA was observed in microsomal preparations from urinary bladder epithelium, prostatic epithelium, colonic mucosa, and peripheral lung tissue [74]. The role of PGHS in the bioactivation of benzidine was supported by the data showing that binding was dependent on the presence of arachidonic acid, and was also inhibited substantially when coincubated with indomethacin, a known inhibitor of PGHS. This bioactivation pathway likely has played a role in the high frequency of bladder cancer in workers who have been continuously exposed to aromatic amines in the chemical and rubber industries, as well as in smokers [77].

3.5.1.3 Polyaromatic Hydrocarbons (PAHs). PAHs are ubiquitous in the environment, found in oil and as by-products of fuel burning. These molecules are of significant concern because of their highly carcinogenic nature and essentially unavoidable exposure by the general population. Formation of the active carcinogenic form generally requires metabolic oxidation of the PAH by the cytochrome P450 enzymes, the most

well characterized of which is benzo[*a*]pyrene (BaP). As shown in Fig. 3.13, BaP is oxidized by the cytochrome P450 enzyme system to the 7,8-epoxide, which is then subsequently converted by epoxide hydrolase to the 7,8-dihydrodiol. It is the 7,8-dihydrodiol of BaP that may then be co-oxidized by PGHS via the peroxy radical mechanism (mechanism B) highlighted in Fig. 3.11 to form the ultimate carcinogen, the bay region 7,8-diol-9,10-epoxide. Interestingly, only dihydrodiols with double bonds adjacent to the bay region are mutagenic with PGHS, demonstrated with multiple PAHs including BaP, benzo[*a*]anthracene, and chrysene [78]. In addition, PGHS-mediated epoxidation of BaP-7,8-diol is reported to be catalyzed by microsomes generated from several known target organs of BaP-induced tumorigenesis, including mouse skin and rat and human lung [79], again highlighting the common link between the region of bioactivation and observed toxicity. Another relevant example of bioactivation of an environmental procarcinogen is aflatoxin B1, which is converted to the mutagenic 8,9-epoxide by PGHS (and lipoxygenases) in the human lung [80].

3.5.1.4 Hydantoins. Phenytoin is a commonly used anticonvulsant that happens to also be teratogenic in animals, as well as humans [81]. For years, the molecular mechanism of teratogenesis by phenytoin, as well as the structurally related thalidomide, has remained elusive. What has been discovered in recent years is that both phenytoin and thalidomide may be bioactivated by embryonic PGHS (highly active in embryo during organogenesis) to free radical intermediates. Specifically, phenytoin and its analogs mephenytoin and trimethadione may be bioactivated to an imidyl nitrogen-centered radical that can undergo ring opening to a carbon-centered radical with an isocyanate moiety (Fig. 3.13). These radicals may either bind to critical embryonic DNA and/or proteins or initiate embryonic reactive oxygen species (ROS) formation that can result in oxidative stress [82]. Evidence supporting this biochemical mechanism includes characterization of free radical spin adducts that were absent in control incubations, as well as when a PGHS inhibitor was included in the incubation. Similarly, for the well-characterized teratogen thalidomide, pretreatment of rabbits with the spin trapping agent α -phenyl-*N*-*t*-butylnitrone (PBN) essentially blocked both thalidomide-mediated embryonic DNA oxidation and teratogenicity *in vivo* [83], which provides strong evidence for a free radical intermediate generated by a non-cytochrome P450 enzyme as a relevant molecular mechanism of teratogenicity.

3.5.2 Myeloperoxidase (MPO)

A major oxidation system in leukocytes (e.g., neutrophils and monocytes) is the combination of NADPH oxidase and MPO. In this complex system, summarized in Fig. 3.14, hydrogen peroxide (H_2O_2), which is generated following dismutation of NADPH oxidase-mediated superoxide ($\text{O}_2^{\cdot-}$) oxidizes the MPO enzyme to a form referred to as *Compound I*. Compound I can in turn oxidize chloride ion (Cl^-) to hypochlorous acid (HOCl), the primary oxidant in neutrophils, which can oxidize or chlorinate drug molecules. In general, the MPO enzyme system is capable of oxidizing xenobiotics with easily oxidizable functional groups [84,85], including aromatic amines, hydrazines, and sulfur compounds, in many cases leading to the formation of reactive chlorinated intermediates. This enzyme system has thus been reported to play a dominant role in the formation of reactive metabolites of drugs that cause certain blood disorders such as agranulocytosis, where neutrophils are the target of toxicity, and

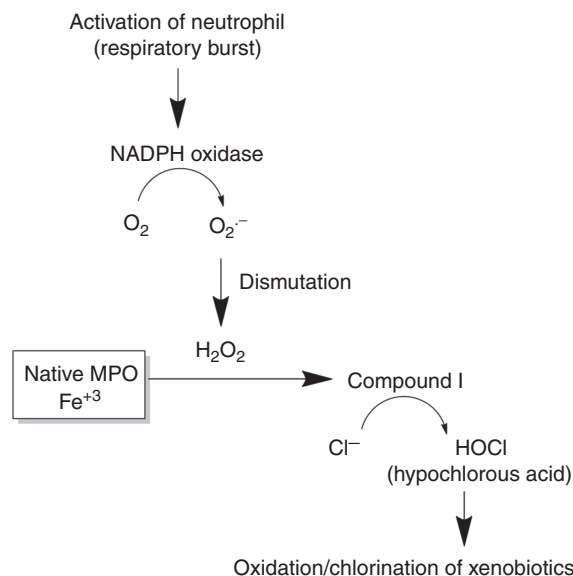


Figure 3.14 Mechanism of MPO-mediated chlorination of xenobiotics in neutrophils.

drug-induced lupus [85], both of which may occur in an idiosyncratic manner. While we concentrate only on selected examples of MPO-mediated bioactivation of xenobiotics, reports of this mechanism in the literature are numerous, and thus, the reader is directed to more focused reviews on this particular enzyme system present in leukocytes [84–86].

3.5.2.1 Diclofenac. Diclofenac is a widely prescribed NSAID that has been associated with cases of severe hepatotoxicity [87]. In addition, rare cases of neutropenia and thrombocytopenia have been observed [88,89], which has triggered interest in the ability of diclofenac to be bioactivated to reactive intermediates in neutrophils, the target of observed toxicities. In an *in vitro* system including MPO/H₂O₂/Cl⁻, as well as activated neutrophils, Miyamoto *et al.* found evidence for the formation of a reactive quinoneimine (Fig. 3.15), proposed to be formed following N-chlorination and oxidation to 5-hydroxydiclofenac [90]. It is thus hypothesized that this reactive metabolite generated in neutrophils may be involved in the idiosyncratic reactions reported with the use of diclofenac. Interestingly, the structurally related COX-2 inhibitor lumiracoxib, which was recently withdrawn from the market in many countries because of severe liver toxicity, has also been reported to undergo similar mechanisms of bioactivation to quinone-imines, both by cytochrome P450, as well as peroxidases MPO and PGHS [91]. Other drugs associated with various toxicities that appear to form quinoneimine intermediates via peroxidase mechanisms include amodiaquine and APAP [86].

3.5.2.2 Ticlopidine. Ticlopidine is an antiplatelet drug that has essentially been supplanted by clopidogrel (Plavix®) due to the significant incidences of agranulocytosis [92]. Ticlopidine contains a thiophene ring, a known structural alert for bioactivation (e.g., tienilic acid and suprofen), and in addition to cytochrome P450-mediated

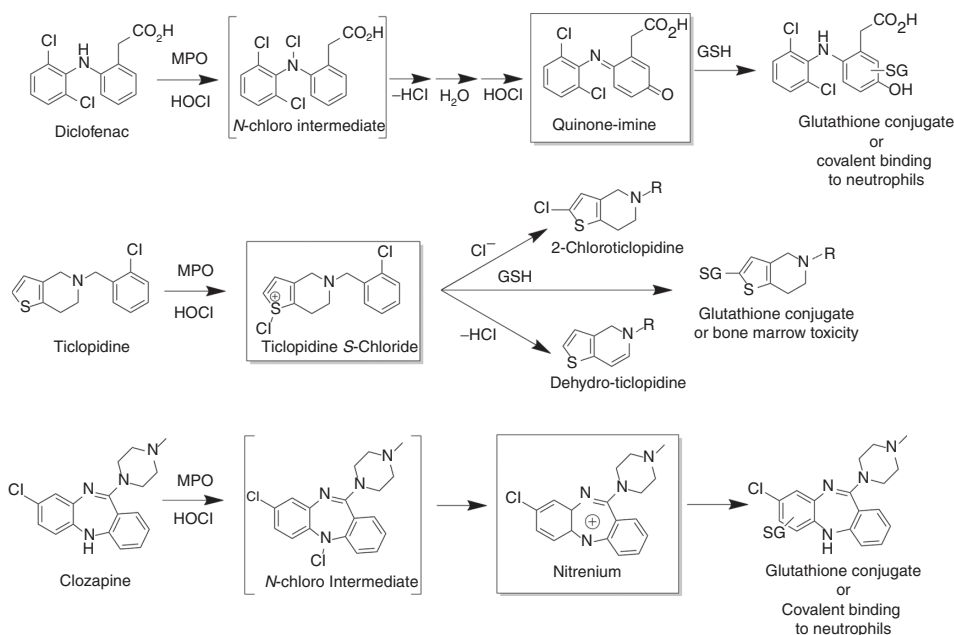


Figure 3.15 Bioactivation of diclofenac, ticlopidine, and clozapine by MPO.

reactive metabolite formation, it has been shown that ticlopidine may be metabolized by MPO and in activated neutrophils to a reactive *S*-chloride metabolite [93]. As Fig. 3.15 illustrates, the *S*-chloride of ticlopidine is reported to likely undergo several subsequent reactions, including with the nucleophilic chloride ion (Cl⁻) to form the proposed 2-chloroticlopidine, loss of HCl to form the predominantly observed dehydro-ticlopidine metabolite, and with GSH to form the proposed conjugate with GSH at the 2-position [93].

3.5.2.3 Clozapine. Clozapine is an antipsychotic agent that has restricted use because of a high incidence (1%) of agranulocytosis [94]. Similar to the previously mentioned examples, clozapine was found to be bioactivated to a reactive species in both the MPO/H₂O₂/Cl⁻ system, as well as in activated neutrophils [95]. In addition, covalent binding to neutrophils was observed following incubation with radiolabeled clozapine. The reactive species was proposed to be a delocalized nitrenium ion (Fig. 3.15), formed through a purported *N*-chloro intermediate (not observed directly), which reacted with GSH to form two GSH conjugates. Structure-activity characterization of clozapine and some close analogs has also been reported [96]. Replacement of the nitrogen that connects the two aryl rings on clozapine with oxygen or sulfur resulted in analogs that did not undergo peroxidase-mediated bioactivation to the reactive iminium species in neutrophils, which supports the proposed mechanism. The benefit to this chemical intervention is apparent in the drugs quetiapine (sulfur instead of bridging nitrogen) and loxapine (oxygen instead of bridging nitrogen), both of which have been in clinical use without a high incidence of adverse events.

3.6 ALDO-KETOREDUCTASES (AKR)

The aldo-ketoreductase (AKR) enzymes are a superfamily of NADPH-dependent cytosolic enzymes that catalyze the reduction of aldehydes and ketones to primary and secondary alcohols, respectively. These enzymes may also utilize NADP⁺ in the oxidation of certain xenobiotics, particularly of alcohols. This is a complex family of enzymes, as there are roughly 114 proteins in the AKR superfamily that are distributed over 14 families (AKR1–AKR14), and expressed widely in liver and brain of prokaryotes and eukaryotes [97]. AKR enzymes are generally thought to be involved in detoxification of xenobiotics, but from a chemical toxicology point of view, some members of the AKR family have been implicated in the oxidation of xenobiotics to carcinogenic metabolites, one of which is dihydrodiol dehydrogenase (DD), a member of the AKR1C subfamily.

3.6.1 Polyaromatic Hydrocarbons (PAHs)

As already described in Section 3.5 of this chapter, PAHs such as BaP are primary environmental pollutants, with the bioactivation pathways of BaP to the proposed carcinogen, BaP 7,8-diol-9,10 epoxide, being catalyzed by cytochrome P450 and peroxidases such as PGHS. An often overlooked alternate bioactivation pathway in this cascade is the NADP⁺-dependent *oxidation* of the 7,8-diol of BaP by DD, which produces the unstable catechol of BaP (Fig. 3.16). This catechol can then undergo auto-oxidation with superoxide to yield hydrogen peroxide (H₂O₂) and the *o*-semiquinone anion radical, with eventual production of the reactive *o*-quinone, BPQ [98], a Michael acceptor that can form adducts with cellular nucleophiles such as DNA [99]. In addition, BPQ can undergo a two-electron reduction back to the catechol using cellular reducing equivalents (NADPH), which may initiate a futile redox cycle, triggering multiple rounds of ROS formation and leading to further DNA damage and tumor initiation [100]. Additional studies where cDNAs were isolated from HepG2 cells have shown that there are four DD isoforms in the liver (DD1, DD2, DD4, and DDX) that are capable of contributing to PAH bioactivation to reactive *o*-quinones [101]. Other PAHs that have also been studied in this context include 1,2-dihydronaphthalene-1,2-diol and benz[*a*]anthracenedihydrodiols [102,103]. Convincing evidence for the role of AKR enzymes in human lung carcinogenesis includes the finding that AKR1C enzymes are expressed in the human lung carcinoma cell line A549, and 7,12-dimethylbenz[*a*]anthracene-3,4-diol (DMBA-3,4-diol) was found to convert to the *o*-quinoneDMBA-dione within A549 cells, trapped as the mono- and bis-thioether conjugates [104]. Thus, the human AKR enzymes appear to be an important family of drug-metabolizing enzymes contributing to the metabolic activation of various PAHs to carcinogenic metabolites.

3.7 REDUCTIVE BIOACTIVATION

Bioactivation of drugs may also proceed via reductive metabolic pathways, particularly reduction of nitroaromatic-containing drugs, which typically involves a six-electron reduction of the nitro group to the aniline, which often results in the formation of mutagenic metabolites [105]. Reductive metabolism of nitroaromatics can be catalyzed

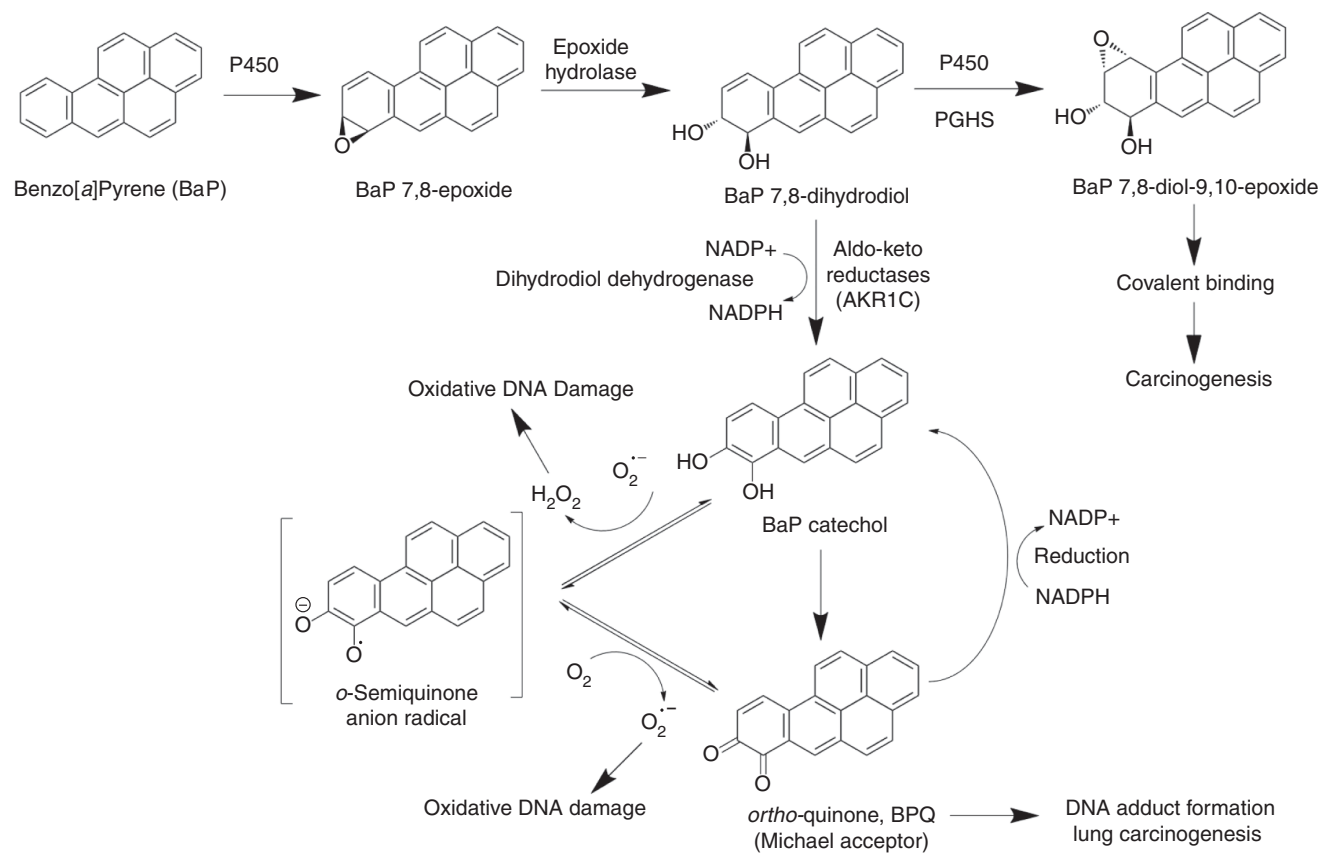


Figure 3.16 Bioactivation of BaP 7,8-dihydrodiol to catechol by the AKR1C enzyme dihydrodiol dehydrogenase, leading to futile cycling and ROS generation.

not only by cytochrome P450, but also by NADPH cytochrome P450 reductase (CPR), xanthine oxidase (XO), AO, nitric oxide synthase (NOS), and quinonereductases such as NQO1 [106]. Given the broad enzyme overlap in the substrate specificity for many of the reductive pathways, we highlight a couple of specific enzymes and make mention of others if a role has been observed.

3.7.1 NADPH-Quinoneoxidoreductase (NQO1)

NADPH-quinoneoxidoreductase (NQO) is a cytosolic flavoprotein that is also commonly referred to as *DT-diaphorase* (DTD) [107]. It is a dimer of two equal subunits, each containing FAD, and is highly expressed in the stomach and kidney, but also in the lung, liver, and intestinal tract [108]. Humans appear to express four forms of DTD, the fourth being the predominant form, the inducible NQO1 [107]. The role of this enzyme is typically in the two-electron reduction of quinones to the relatively stable hydroquinone, a competing detoxification pathway leading to cytoprotection against potentially toxic semiquinones that may be formed via one-electron reduction by enzymes such as NADPH-CPR. The two potential reductive pathways are depicted with menadione in Fig. 3.17. Despite a predominant role in detoxification, there are examples where NQO1 bioactivates drug molecules to reactive metabolites, which ironically, in some cases may be of potential therapeutic benefit, as described in the latter part of this section.

3.7.1.1 Aristolochic Acid. Aristolochic acid (AA) is a plant extract and a component of many Chinese herbs [109] that has been associated with the development of nephrotoxicity in humans, often referred to as *aristolochic acid nephropathy* (AAN), including

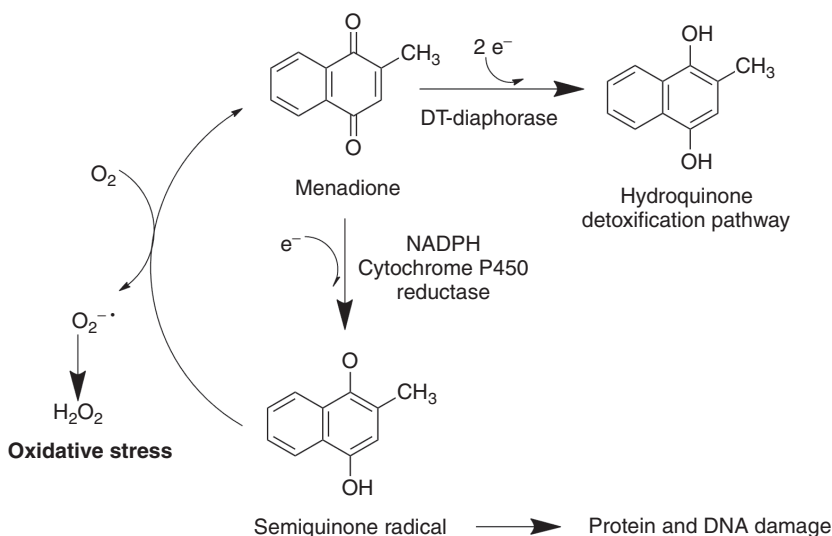


Figure 3.17 Reductive pathways for menadione, including the one-electron reduction to the semiquinone radical by CPR and two-electron reduction to the inert hydroquinone by DT-diaphorase.

urothelial cancer [110]. The two main components of AA are 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAI) and 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAII), as shown in Fig. 3.18. AA has been reported to form covalent DNA adducts, which have been observed in patients with AAN [111,112]. Consistent with the structures of the major DNA adducts identified [113], the initial step in the bioactivation pathway of these naturally occurring nitroarenes to their carcinogenic species appears to be a multistep nitro reduction to an eventual lactam intermediate, followed by nitrenium ion formation (Fig. 3.18). It is unclear what the actual reactive precursor is that forms the lactam. Characterized adducts supporting this pathway include 7-(deoxyadenosin-*N*6-yl)aristolactam I and II (dA-AAI and dA-AAII) and 7-(deoxyguanosin-*N*2-yl)aristolactam I (dG-AAI), which have been used as standards to evaluate which human renal and hepatic cytosolic enzymes are involved in the bioactivation (e.g., nitro reduction) of AA to form these specific adducts. Reported data supports a dominant role for NQO1 in the formation of these DNA adducts, as incubations carried out in the absence and presence of dicoumarol, a potent inhibitor of NQO1, resulted in an 85% decrease in the levels of AAI-DNA adducts in hepatic cytosol, and 75% decrease in renal cytosol [114]. Interestingly,

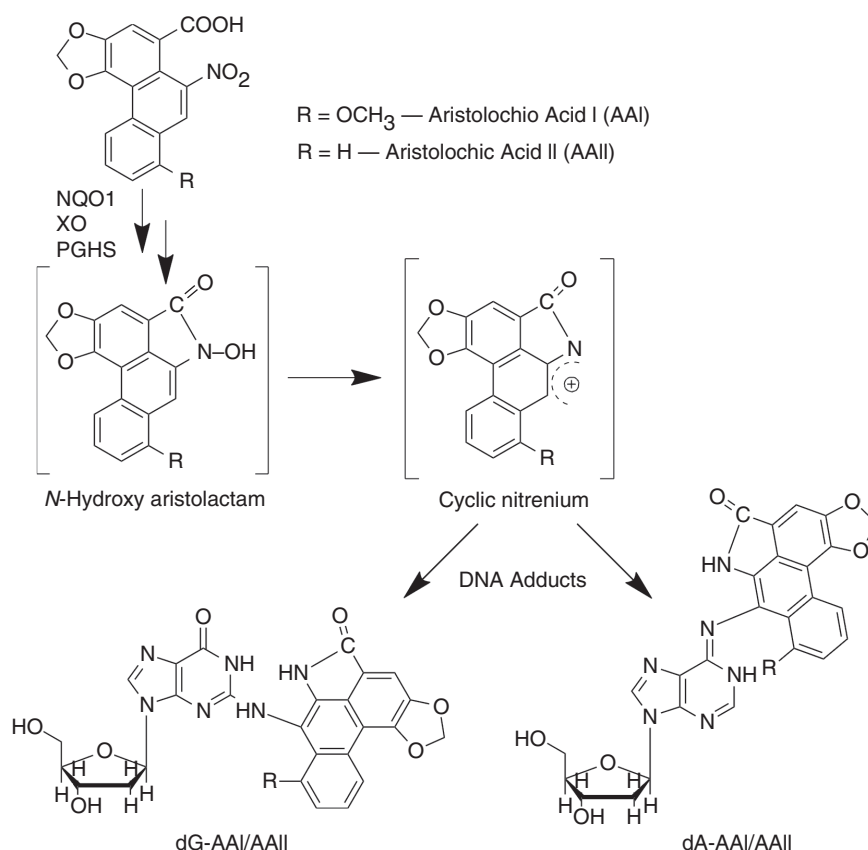


Figure 3.18 Reductive bioactivation of aristolochic acid by NQO1 to the cyclic nitrenium intermediate.

AA has also been reported to be bioactivated by other reductive enzymes, such as XO, as well as peroxidases such as PGHS [115,116], while the expected cytochrome P450-mediated bioactivation of the methylenedioxyphenyl moiety to either a carbene or catechol has not been reported.

3.7.2 NADPH Cytochrome P450 Reductase (CPR)

NADPH-CPR is also known as *NADPH:ferrihemoprotein oxidoreductase*, NADPH: hemoprotein oxidoreductase, NADPH:P450 oxidoreductase, or P450 reductase. CPR is a membrane-anchored enzyme found in the endoplasmic reticulum of cells and is widely expressed throughout the body, although it is most abundant in the liver [117]. Initial establishment of the involvement of CPR for P450 activity came from the work of Lu *et al.* [118,119], who performed the first resolution of P450, CPR, and lipid from rabbit liver microsomes. Subsequently, CPR was found to be required for the transfer of electrons to hemeoxygenase, cytochrome *b*₅, squalenemonooxygenase, and 7-hydroxycholesterol reductase [120]. Additionally, CPR can itself catalyze the metabolism of drugs [121,122].

3.7.2.1 Paraquat. Paraquat (PQ) is a commonly used, fast-acting herbicide. Since its introduction in 1962, numerous accidental or voluntary ingestions have occurred resulting in numerous fatalities and even more cases of “paraquat lung.” In addition to the major site of toxicity in the lung; the kidney, the liver, and the thymus also are affected by PQ [123].

Upon ingestion, PQ is rapidly absorbed and accumulated via a polyamine uptake system into the lung. In the lung, PQ undergoes an alternating process of reduction and oxidation, also known as *redox cycling* [106]. The major reductive enzymes involved in PQ reduction to the cation radical PQ^{•+} are CPR, NADH:ubiquinone oxidoreductase, XO, and NOS [124]. The rapid reoxidation of PQ^{•+} by O₂ in the lung occurs because of the high aveolar pressure, resulting in generation of O₂^{•-}. Subsequently, available NADPH is utilized to reduce O₂^{•-}. The end result of these events include generation of ROS (e.g., peroxide, superoxide, and hydroxyl radical), oxidation of cellular thiols, depletion of GSH stores, and oxidative damage to lipids, proteins, and DNA [124].

3.7.2.2 Flutamide. Flutamide is a nonsteroidal antiandrogen used for the treatment of prostate cancer. Reports of idiosyncratic hepatotoxicity range between 1% and almost 10% of patients treated with flutamide [125,126]. Symptoms of liver dysfunction include elevation of serum aminotransaminase (ALT) and bilirubin, hepatic necrosis, and cholestasis [125]. The major routes of metabolism for flutamide include hydroxylation, hydrolysis, N-acetylation, and nitro reduction (Fig. 3.19). The prominence of nitro-reduction steps in the metabolism of flutamide as well as the established toxicity/reactivity of nitro-reduction intermediates lead Wen *et al.* to hypothesize that reduction of the nitro group was involved in the toxicity of flutamide [127]. On the basis of this hypothesis, flutamide and an analog where the nitro group was replaced with a cyano group, were examined in a hepatocyte toxicity assay [128]. These studies demonstrated time- and concentration-dependent toxicity which was approximately twofold greater for flutamide than its cyano analog. Subsequent to these toxicity studies, Wen *et al.* thoroughly profiled the metabolites of flutamide and its cyano analog in human liver microsomes and human hepatocytes. Similar oxidative GSH adducts were

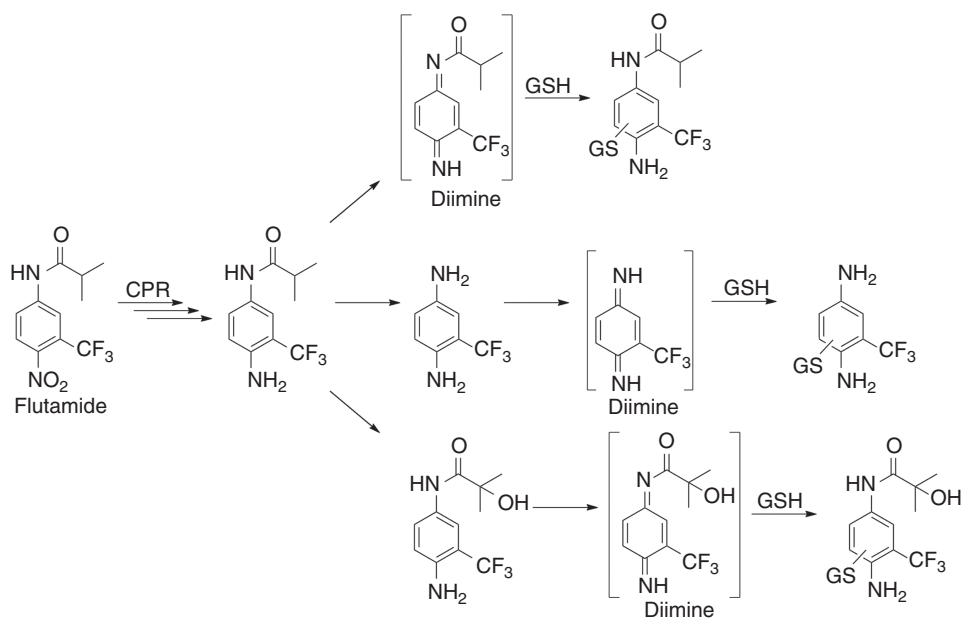


Figure 3.19 Nitro-reductive bioactivation of flutamide by CPR.

detected for both compounds. However, several GSH adducts resulting from reduction of the nitro group were observed only for flutamide. Incubation of the *p*-aniline compound, which would result from nitro reduction, gave these same GSH adducts suggesting the possibility of a reactive diamine species. Additionally, the observed reduction of the nitro group may also implicate toxicity via redox cycling [106].

An interesting finding by Wen *et al.* is that the nitro reduction was solely carried out by CPR under aerobic and anaerobic conditions. Furthermore, a dramatic increase in the rate of the formation of the aniline metabolite was observed under anaerobic conditions using both microsomes and expressed CPR. The involvement of P450s in the reduction of the nitro group was ruled out using specific inhibitors of P450 and expressed enzymes. Likewise, cytochrome *b₅* reductase (another known *nitroreductase*) was shown not to reduce the nitro group of flutamide in microsomal incubations supported by NADH. Further confirmation of CPR involvement comes from the >90% inhibition of the formation of the nitro-reduced metabolite in microsomes, coincubated with α -lipoic acid, a selective and reversible CPR inhibitor. Although these studies supply convincing evidence for the involvement of CPR, the involvement of other cytosolic or mitochondrial “nitroreductases” could not be ruled out.

3.7.3 Bioreductive Antitumor Prodrugs

Contrasting the multitude of examples of bioactivation described to this point, reductive bioactivation to cytotoxic intermediates may actually be used as a therapeutic strategy. Some chemotherapeutic agents such as mitomycin C, tirapazamine (TPZ) (SR4233), and CB 1954 are reductively bioactivated to cytotoxic metabolites that can cause DNA strand breaks, a mechanism referred to broadly as *bioreductive activation* [129]. These

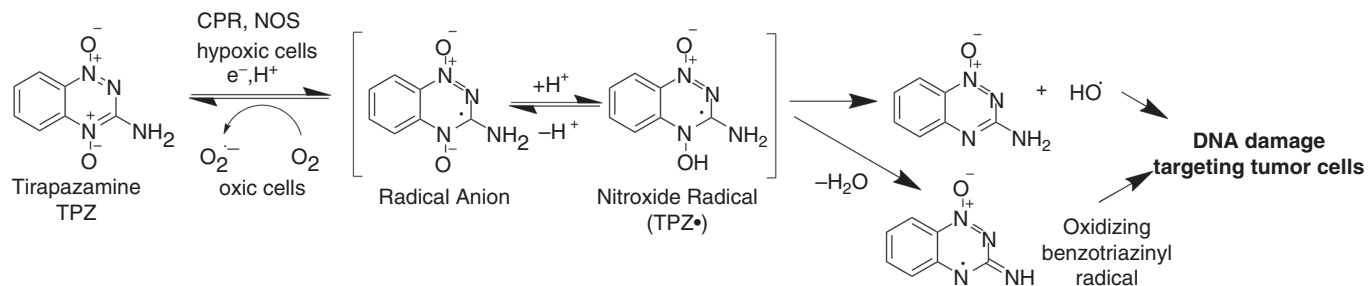
drugs must be a substrate for one-electron reductases that effectively convert the compound to a free radical (Fig. 3.20). Thus, these are considered “prodrugs.” Bioreductive activation mechanisms within tumor cells offer a novel chemotherapeutic strategy to medicinal chemists who are attempting to selectively target and kill cancerous tumor cells. The apparent selectivity of this bioactivation mechanism is due in part to tumor cells possessing hypoxic regions (e.g., lacking oxygen), which are typically resistant to chemotherapy and radiotherapy, contributing to cancer progression. In a cell with normal oxygen levels, the resulting free radical formed rapidly transfers the unpaired electron to oxygen to form superoxide (Fig. 3.20), which may be scavenged by reactive oxygen scavenging enzymes such as superoxide dismutase and catalase. Conversely, in a hypoxic environment, the generated radical may accumulate to cytotoxic levels within the cell. Interestingly, hypoxia leads to an elevation in the expression of some reductive enzymes such as CPR, relative to normal cells [130], which in addition to the hypoxic environment, may yield selectivity for this reductive mechanism in those types of cells.

3.7.3.1 Tirapazamine. TPZ is one of the most promising hypoxia-selective anti-tumor drug molecules to date [131], with 50–300 times higher toxicity observed in hypoxic cells as compared to normally oxygenated cells [132]. TPZ contains two *N*-oxide functionalities that may undergo a one-electron reduction to form the radical anion which exists in equilibrium with the protonated nitroxide radical (TPZ[•]) [131,133]. As previously mentioned, in an environment where oxygen is present, this radical can donate its electron back to oxygen to form superoxide, rendering TPZ essentially nontoxic. However, under hypoxic conditions, investigations within the Gates’ lab support a mechanism by which the neutral TPZ radical product undergoes homolytic fragmentation to produce the DNA damaging hydroxyl radical (HO[•]) [134]. It has also been reported that the TPZ radical may undergo dehydration to form the oxidizing benzotriazinyl radical (Fig. 3.20), leading to DNA damage [135]. The exact chemical mechanisms for TPZ-mediated DNA damage remain under investigation.

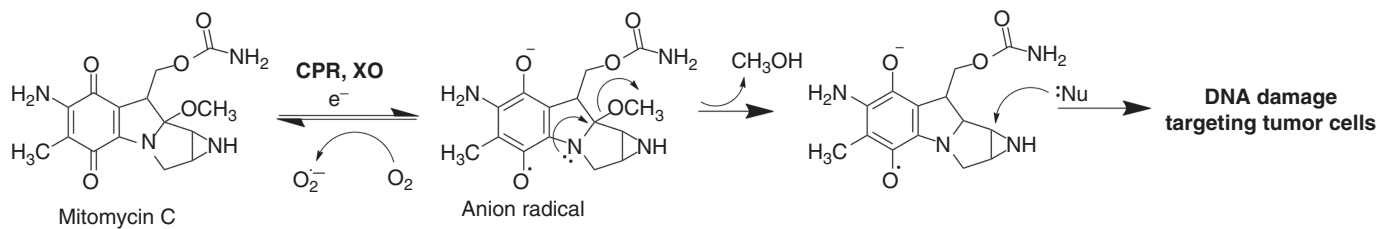
From an enzymology standpoint, a role for both CPR and NOS has been demonstrated in the reductive activation of TPZ [136,137]. It is worth noting that the (NOS) enzyme has both an oxidase and a reductase domain, with its reductase domain sharing strikingly similar homology with CPR [138], likely contributing to the observed overlapping substrate selectivity in this example. In addition, TPZ is reported to be a substrate for two- or four-electron reduction by DTD [107], which as described earlier with menadione, may lead to the nonradical inactive product, providing a potential detoxification mechanism, and complicating selective bioactivation to the necessary cytotoxic radical in tumor cells. Numerous phase II/III clinical studies have been performed evaluating the effectiveness of this bioreductive prodrug approach, with mixed results [139,140], indicating the complexity in utilizing these mechanisms in cancer therapy. Nonetheless, the promise remains that TPZ could be a viable therapeutic option in the treatment of cancer.

3.7.3.2 Mitomycin C and CB 1954. Mitomycin C and CB 1954 have also been among the bioreductive prodrug design effort, and they undergo quinone reduction and nitro reduction, respectively (Fig. 3.20). Mitomycin undergoes reduction to its radical anion by both CPR and the molybdenum-containing enzyme XO [141]. Formation of the radical anion facilitates aromatization of the quinone ring and loss of methanol,

N-Oxide Reduction



Quinone Reduction



Nitro Reduction

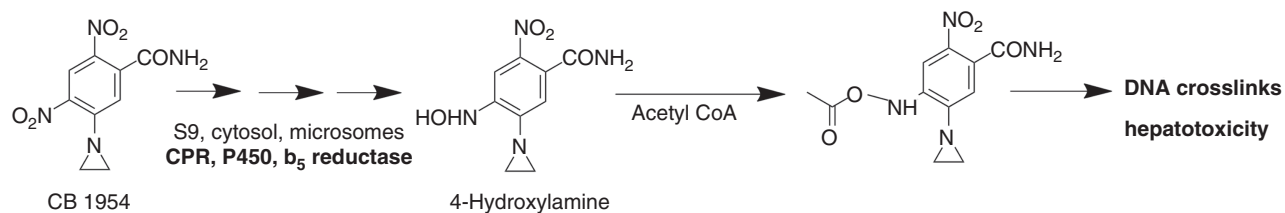


Figure 3.20 Examples of bioreductive activation of antitumor prodrugs tirapazamine, mitomycin C, and CB 1954 by numerous reductive pathways.

due in part to the resonance stabilization, which then enables nucleophilic attack at the C1 carbon and potential for DNA damage. As mentioned, a critical necessity of the bioreductive prodrug approach in anticancer therapy to minimize toxicity in non-targeted cells is selective bioactivation of the prodrug in the tumor. This challenge is highlighted by CB 1954, an antitumor prodrug that demonstrated dose-limiting hepatotoxicity in Phase I clinical studies [142]. *In vitro* studies aimed at understanding whether CB 1954 could undergo reductive bioactivation in human liver found that nitro reduction at both the 2- and 4-positions occurred in S9 and cytosolic fractions, as well as microsomes, with the potent cytotoxic 4-hydroxylamine being the predominant metabolite [143], which can react with acetyl CoA to form a potent DNA crosslinking agent (Fig. 3.20). During enzyme phenotyping studies, it was observed that carbon monoxide, an inhibitor of P450, decreased metabolite formation in human liver S9, and CB 1954 metabolites were observed in microsomes supplemented with the cofactor NADH. This supports *b*₅ reductase activity, indicating that multiple reductive hepatic enzymes are likely involved to some degree in the nitro reduction of CB 1954 [143]. Owing to the complexities of bioreductive activation, namely, competing deactivation pathways and noted species differences in the reduction of CB 1954 [144], the search for more selective bioreductive prodrugs appears extremely challenging, but is ongoing.

3.8 CONCLUSIONS AND PERSPECTIVES

While bioactivation of drug molecules to reactive metabolites is most commonly associated with the cytochrome P450 enzyme system, this chapter highlights that examples of bioactivation of xenobiotics by non-P450 drug-metabolizing enzymes are plentiful and should not be overlooked. Contributing to the potential for oversight is that drug-discovery programs typically conduct the majority of their screening for metabolism and reactive intermediates using microsomes from liver (the location where majority of drug metabolism occurs). This approach only addresses the potential risks of reactive metabolite formation in the liver by a limited number of metabolic enzymes (e.g., cytochrome P450 and FMO). Highlighted in this chapter is that extrahepatic toxicities may also occur via non-P450-mediated bioactivation event(s), particularly in the tissue where these enzymes are expressed, such as PGHS and its purported role in bioactivating APAP to its NAPQI intermediate in the kidney. Table 3.2 summarizes the additional examples described in this chapter, highlighting the xenobiotic, functional group bioactivated, reactive intermediate formed, and the target tissue for toxicity, if in fact reported. Equally relevant may be the absence of a detoxification pathway in a particular tissue, such as the lack of AO activity in brain where MPTP illicitly its untoward effects. This suggests that for more thorough profiling of the metabolism and risk of generation of reactive metabolites, different tissue fractions, or even purified enzymes should be used, in addition to those which are typically available. Overall, it is the opinion of these authors that while there is no perfect “catch-all” *in vitro* system for evaluating the risk of bioactivation of drug molecules, familiarity with the non-cytochrome P450 drug-metabolizing enzymes, in combination with an understanding of chemical moieties that are susceptible to bioactivation by these enzymes, should lead to more informed chemical modification during drug candidate optimization.

TABLE 3.2 Summary of Xenobiotics Presented in this Chapter that are Bioactivated by Non-P450 Enzymes and the Toxicities Associated with Reactive Intermediate Formation

Enzyme	Xenobiotic	Bioactivated Moiety	Reactive Intermediate	Target of Toxicity
FMO	Thiobenzamide	Thioamide	Iminosulfinic acid	Lung Skin Kidney
	Ketoconazole	Aryl piperazine	Nitrone	Liver
MAO	MPTP	4-Fluoro- <i>N</i> -methyl aniline	Quinoneimine	Brain
	Dopamine	Pyridine	Pyridinium	
SSAO	Allylamine	Amine	Aldehyde	Brain
ADH	Allyl alcohol	Amine	Aldehyde	Heart
	Felbamate	Alcohol	Aldehyde	Heart
PGHS	Abacavir	Aldehyde carbamate	2-Phenylpropenal	Liver Blood
	APAP	Alcohol	Aldehyde	Skin
	Benzidine 2-naphthylamine	Phenol	Free radical/NAPQI	Kidney
	PAH	Aromatic amines	Diimine	Bladder
	Phenytoin	Aromatic ring	Epoxide	Lung
MPO	Diclofenac	Hydantoin	Radical	Teratogen
	Ticlopidine	Secondary arylamine	Quinoneimine	Blood
	Clozapine	Thiophene	<i>S</i> -chloride	Blood
	PAH	Secondary nitrogen	Nitrenium	Blood
AKR	Aristolochic acid	Diols	<i>ortho</i> -quinone	Lung
NQO1	Paraquat	Nitro	Nitrenium	Kidney
CPR	Flutamide	Pyridinium	Radical	Lung
	Tirapazamine	Nitro	Diimine	—
CPR, NOS		<i>N</i> -oxide	Benzotriazinyl radical, hydroxide radical	Tumor cell
CPR, XO, <i>b</i> ₅ reductase	Mitomycin C	Quinone	Anion radical	Tumor cell
	CB 1954	Nitro	Hydroxylamine	Tumor cell Liver

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4 Bioactivation by Phase-II-Enzyme-Catalyzed Conjugation of Xenobiotics

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4.1	Summary	1
4.2	Introduction to phase-II-mediated bioactivation	2
4.3	Glucuronidation	6
4.4	Sulfonation	19
4.5	N-acetylation	24
4.6	Glutathione conjugation	27
4.7	Acyl-S-CoA formation	38
4.8	Conclusions	42
	Acknowledgments	43
	References	44

4.1 SUMMARY

Phase-II-enzyme-catalyzed conjugation of xenobiotics most often leads to detoxification but can also mediate their bioactivation to chemically reactive and potentially toxic metabolites. Chemically reactive metabolites of xenobiotics, including drugs and environmental chemicals, are generated most often by oxidative metabolism (e.g., phase-I-mediated cytochrome P450-mediated oxidative bioactivation), which can lead to covalent binding to protein and/or nucleic acids. In some cases, this process leads to intrinsic (acute) and/or idiosyncratic (immune mediated) toxicity and carcinogenicity. However, additional biotransformation routes, such as phase-II-mediated conjugation reactions, can also play a role in the formation of chemically reactive electrophilic conjugates that are toxic. Phase II metabolism encompasses processes including glucuronidation, sulfonation, acetylation, amino acid conjugation, glutathione (GSH) conjugation, methylation, and phosphorylation. Xenobiotics and/or xenobiotic metabolites can be conjugated with endogenous cofactors leading to hydrophilic derivatives that, in most cases, function to eliminate and detoxify xenobiotics from the body. For example, the intrinsic toxin acetaminophen is detoxified by glucuronidation

and sulfonation leading to highly water-soluble, unreactive, oxygen-linked glucuronide and sulfonate metabolites, respectively, that are efficiently excreted from the body. However, despite the well-established classical role of phase-II-enzyme-catalyzed xenobiotic detoxification, the metabolic activation of drugs or drug metabolites by phase II metabolism in some cases leads to the formation of highly reactive conjugates capable of mediating the covalent binding of drug equivalents to protein and/or DNA. The major conjugation reactions known to be involved in xenobiotic bioactivation are glucuronidation, sulfonation, acetylation, GSH conjugation, and acyl-S-CoA formation. Glucuronidation of xenobiotics leads to the formation of electrophilic glucuronides, such as acyl glucuronides formed from carboxylic-acid-containing xenobiotics as well as endogenous compounds and N- or N-O-linked glucuronides formed from arylhydroxamic acids. Metabolic activation by O-sulfonation of chemicals such as polycyclic aromatic benzylic alcohols, allylic alcohols, *N*-arylhydroxylamines, and *N*-acetyl-*N*-arylhydroxylamines leads to the formation of chemically reactive nitrenium ion and carbocation species that can covalently bind to and damage DNA. The N-acetylation of xenobiotic aromatic hydrazines and aromatic amines can function as a preliminary step in the bioactivation of protoxicants to chemically reactive intermediates that are proposed to induce hepatotoxicity or carcinogenesis by covalently binding to protein or DNA. Conjugation with GSH can lead to the formation of chemically reactive toxic conjugates that mediate oxidative stress and/or covalent binding to important macromolecules, leading to hepatic, kidney, and even CNS injury. Amino acid conjugation of carboxylic-acid-containing drugs is mediated by acyl-S-CoA formation and can lead to the production of reactive acyl-linked intermediary metabolites that covalently bind to protein via transacylation, which has been proposed to be responsible, at least in part, for mediating idiosyncratic toxicity. In summary, this chapter deals with the known mechanisms of formation of chemically reactive xenobiotic metabolites, which are mediated by phase-II-drug-conjugating enzymes that can lead to tissue toxicity rather than xenobiotic detoxification.

4.2 INTRODUCTION TO PHASE-II-MEDIATED BIOACTIVATION

4.2.1 Classification of Xenobiotic Metabolism

Xenobiotic metabolism has long been classified as occurring by two distinct phases, namely, phase I (functionalization) and phase II (conjugation) [1,2]. Phase I biotransformation reactions include the oxidation, reduction, or cleavage of a xenobiotic functional group. Phase II reactions encompass the conjugation of a functional group of a xenobiotic or a xenobiotic phase I metabolite with endogenous substances such as glucuronic acid, sulfate, acetate, GSH, or an amino acid such as glycine. Both phase I and phase II metabolic transformations function most often to provide increased aqueous solubility and, therefore, provide increased excretion efficiency of xenobiotics into urine and/or bile, thus affording mechanisms by which they can be eliminated and detoxified from the body. In his classic book "Detoxification mechanisms: The metabolism and detoxication of drugs, toxic substances and other organic compounds," R. T. Williams [1] stated "... phase-I reactions cannot be considered as detoxication mechanisms although in many cases detoxication does occur as a result of these reactions. Phase II reactions, on the other hand, appear to be largely processes of detoxication but again

exceptions occur.” It is now well known that the metabolism of xenobiotics by phase I and phase II reactions, and sometimes a combination of both, can be toxicologically significant. An important hypothesis that was established during the 1960s and 1970s proposed that in order for some xenobiotics to induce carcinogenesis, they first must undergo phase-I-mediated metabolic activation by cytochrome P450s to chemically reactive species [3]. It was stated that chemically reactive hard electrophiles, such as carbocations, nitrenium ions, or iminium ions, react with hard nucleophiles located in nucleic acids, leading to DNA damage [4]. For example, cytochrome P450 is a major phase-I-drug-metabolizing family of enzymes that can function in the bioactivation of xenobiotics to form chemically reactive metabolites, such as epoxides, quinones, quinone imines, quinonemethides, imine methides, and nitroso intermediates, that lead to covalent binding to cellular macromolecules and, in some cases, can result in organ toxicity or carcinogenesis [5]. Other phase I metabolism enzymes can also function in the bioactivation of xenobiotics; for example, the flavin-dependent monooxygenases-mediated bioactivation of 4-fluoro-*N*-methylalanine [6], the alcohol-dehydrogenase-mediated metabolic activation of abacavir [7], and the monoamine-oxidase-mediated bioactivation of haloperidol [8].

4.2.2 Phase-II-Mediated Pathways of Xenobiotic Metabolic Activation

There are numerous examples where phase II enzymes have been shown to be important in the metabolic activation of xenobiotics to toxic metabolites (Table 4.1). Phase II enzymes known to be involved in the bioactivation of xenobiotics include UDP-glucuronosyltransferases (UGT), cytosolic sulfotransferases (SULT), *N*-acetyltransferase (NAT), GSH *S*-transferases, and acyl-*S*-CoA synthetases. These phase II enzymes are increasingly being shown to mediate the bioactivation of promutagens, procarcinogens, and protoxicants, leading to the formation of chemically reactive electrophiles that bind covalently to DNA, RNA, and protein [9].

Glucuronidation functions in the conjugation of glucuronic acid to a xenobiotic, which sometimes leads to the generation of chemically reactive intermediates [30]. For example, the glucuronidation of carboxylic acid drugs leads to the formation of acyl glucuronides that are able to covalently bind to cellular macromolecules including proteins and DNA via transacylation and Schiff base formation. The *N*-glucuronidation of *N*-hydroxylated metabolites of aromatic-amine-containing xenobiotics leads to unstable *N*-glucuronides that decompose to highly reactive nitrenium-ion-containing intermediates, which covalently bind to and damage DNA. The varied types of toxicities that are proposed to be mediated by chemically reactive glucuronide conjugates include idiosyncratic hypersensitivity, bladder cancer, and gastroenteropathy [30].

Sulfonation of promutagens and procarcinogens that have chemical moieties such as polycyclic aromatic benzylic alcohol, allylic alcohol, *N*-arylhydroxylamine, and *N*-acetyl-*N*-arylhydroxylamine leads to unstable sulfonate esters that decompose to chemically reactive benzylic or allylic carbocations and nitrenium ions, respectively, which are able to react with nucleic acids leading to carcinogenesis [31].

The *N*-acetylation of protoxicants containing aromatic hydrazine or aromatic amine substructures can serve as the first step in their metabolic activation to chemically reactive intermediates, such as highly reactive acylating acetyl radical species or reactive nitrenium ions, which covalently bind to protein and nucleic acids leading to hepatotoxicity or carcinogenesis [17].

TABLE 4.1 Metabolic Reactions Involved in the Phase-II-Mediated Bioactivation of Xenobiotics

Phase II Reaction	Enzyme	Cofactor	Functional Group	Reactive Intermediate	Example	References
Glucuronidation	UDP-glucuronosyl-transferases	UDPGA	Carboxylic acids	Acyl glucuronide	Diclofenac	10
			Arylhydroxamic acids	N–O-linked glucuronide (nitrenium ion)	<i>N</i> -Hydroxy-2-AAF	11
Sulfonation	Sulfotransferases	PAPS	Benzylic alcohols	Benzylic sulfonate esters (benzylic carbocation)	7-OH-DMBA	12
			Allylic alcohols	Allylic sulfonate esters (allylic carbocation)	Tamoxifen	13
			<i>N</i> -Aryl-hydroxylamines	<i>N</i> – <i>O</i> -sulfonate ester (nitrenium ion)	<i>N</i> -hydroxy-MAB	14
			<i>N</i> -Acetyl- <i>N</i> -aryl-hydroxylamines	<i>N</i> – <i>O</i> -sulfonate ester (nitrenium ion)	<i>N</i> -hydroxy-2-AAF	15
N-Acetylation	<i>N</i> -Acetyl transferase	Acetyl CoA	Aromatic hydrazines	Acetyl radical	Isoniazid	16
			<i>N</i> -Hydroxyaromatic amines	<i>N</i> -Acetoxyaromatic amine	Benzidine	17
Glutathione Conjugation	Glutathione <i>S</i> -transferases	GSH	Haloalkanes	Episulfonium ion	Ethylene dibromide	18
				Thionoacyl halide	Sevoflurane	19,20

			Haloalkenes	Thioketene	Hexachlorobutadiene	21
			Benzoquinone	Bromohydroquinone-linked	Bromobenzene	22
				cysteine- <i>S</i> -conjugates		
			<i>o</i> -Quinone	<i>o</i> -Quinone-NAC adduct	Ecstasy	23,24
			<i>S</i> -Acyl-CoA	<i>S</i> -Acyl-GSH thioester	Clofibrilic acid	25
			Isothiocyanate	Carbamoyl–GSH	α -Naphthylisothio-cyanate	26
			Isocyanate	Thiocarbamoyl–GSH	Methyl isocyanate	27
<i>S</i> -Acyl-CoA formation	Acyl CoA synthetases	CoASH, ATP	Carboxylic acids	<i>S</i> -Acyl-CoA	Ibuprofen	28
				Acyl-AMP	Cholic acid	29

Abbreviations: UDP, uridine diphosphate; UDPGA, UDP- α -D-glucuronic acid; *N*-hydroxy-2-AAF, *N*-hydroxy-2-acetylaminofluorene; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; 7-OH-DMBA, 7-hydroxymethyl-12-methylbenz[*a*]anthracene; *N*-hydroxy-MAB, *N*-hydroxy-4-methylaminobenzene; acetyl CoA, acetyl coenzyme A; NAC, *N*-acetylcysteine; GSH, glutathione; acyl-AMP, acyl-adenylate; CoASH, coenzyme A; ATP, adenosine triphosphate.

Conjugation of reactive intermediates with GSH with or without catalysis by GSH *S*-transferases (GST) is often important for their detoxification. However, in some cases, such as in the conjugation of GSH to haloalkanes, haloalkenes, and benzohydroquinones, it instead leads to the formation of reactive intermediates that covalently bind to protein and contribute to tissue toxicity [32]. Sometimes bioactivation of xenobiotics by GSH conjugation requires further metabolism via mercapturic acid pathway enzymes as well as renal cysteine β -lyase leading to chemically reactive, potentially toxic, intermediates [33].

Acyl-*S*-CoA synthetases are involved in the activation of carboxylic-acid-containing drugs to *S*-acyl-CoA thioesters that function as necessary substrates for certain phase II conjugating enzymes that lead to their detoxification, for example, in the formation of amino acid conjugates that are eliminated into urine. However, chemically reactive *S*-acyl-CoA thioesters are known to mediate the transacylation of protein nucleophiles and, therefore, similar to reactive acyl glucuronides, have been proposed to mediate idiosyncratic allergic toxicity for some acidic drugs [34].

Thus, both phase I and phase II classes of xenobiotic metabolism, as well as their combination, can function in the metabolic activation of xenobiotics leading to chemically reactive intermediates that bind covalently to DNA or tissue proteins potentially leading to subsequent organ toxicity. This chapter covers the metabolic mechanisms involved in the phase-II-mediated bioactivation of xenobiotics to reactive metabolites and their toxicological consequences.

4.3 GLUCURONIDATION

Metabolism by phase-II-mediated glucuronidation represents a major route of clearance of xenobiotics and/or xenobiotic metabolites from the body [35,36]. The conjugation reaction leading to the formation of glucuronide metabolites is catalyzed by two families (based on amino acid sequence similarity) of UGT enzymes (EC 2.4.1.17) UGT1 and UGT2, which are further divided into three subfamilies, UGT1A, UGT2A, and UGT2B. UGT enzymes are located in high concentration in liver tissue, but they are also found in other tissues such as kidney, heart, stomach, intestine, colon, esophagus, brain, adrenal glands, and thymus [37,38]. The ability of a certain tissue to metabolize a substrate by glucuronidation depends on the presence and concentration of the appropriate UGT isoforms in the tissue [39].

UGTs are membrane-bound enzymes, in which the subcellular location is in the smooth endoplasmic reticulum (microsomal fraction) [35]. The cofactor necessary for UGT function is UDP- α -D-glucuronic acid (UDPGA), which is derived initially from the catabolism of glycogen in the cytosol leading to the formation of glucose-1-phosphate. Further reaction with uridine triphosphate (via pyrophosphorylase) leads to the formation of UDP-glucose that is finally biotransformed to UDPGA by UDP-dehydrogenase.

Xenobiotics that are metabolized by glucuronidation have nucleophilic functional groups such as hydroxyls, carboxylic acids, amines, and reduced sulfhydryls, leading to the formation of ether-, acyl-, amino-, and thioether-linked glucuronides, respectively. The mechanism of glucuronide formation catalyzed by UGTs occurs whereby the "electron-rich" nucleophile of the substrate reacts in a nucleophilic displacement

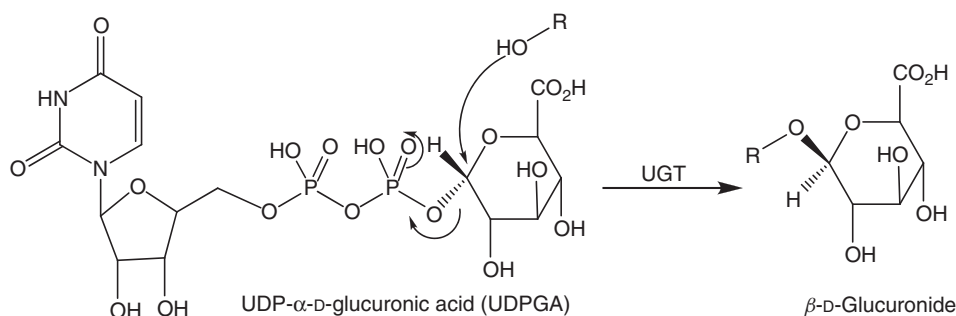


Figure 4.1 Mechanism of β -D-glucuronide conjugate formation catalyzed by UGTs.

fashion with the C-1 position of the glucuronic acid ring of UDPGA to provide the β -D-glucuronide product (Fig. 4.1).

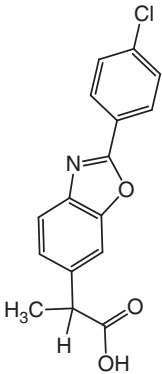
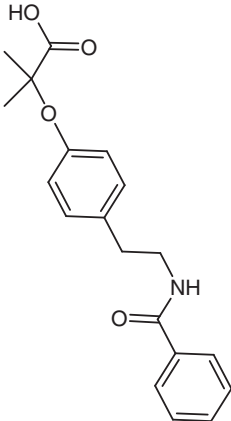
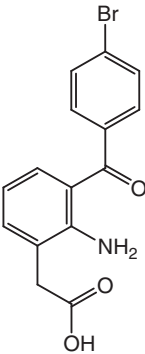
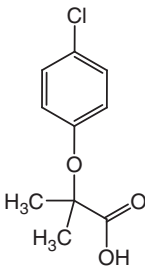
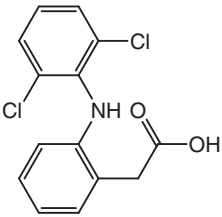
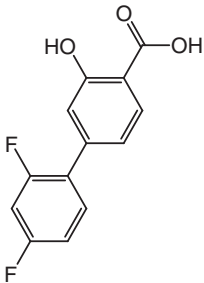
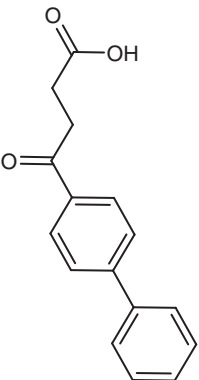
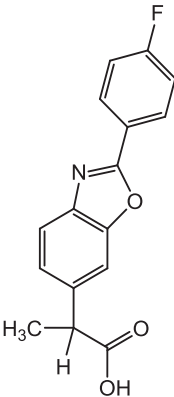
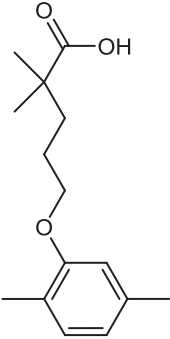
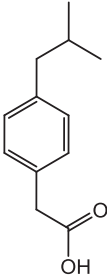
Glucuronidation of lipophilic xenobiotics and/or xenobiotic metabolite substrates, in most cases, leads to detoxification products that are chemically stable and have increased water solubility because of the high hydrophilicity of the glucuronic acid moiety, leading to their efficient excretion from the body in bile and/or urine [40]. Glucuronide conjugates of drugs can also be transported into bile and urine via efflux transporters (e.g., multidrug-resistance-associated protein 2) located in the hepatic bile canalicular and renal tubular brush border membranes [41–43]. Glucuronidation of endogenous compounds and xenobiotics sometimes results in the formation of glucuronide-linked metabolites that are pharmacologically more potent and/or toxic than their respective aglycones [30,44]. Thus, glucuronidation of drugs in most cases results in the loss of pharmacological activity, except in a few known cases where the glucuronide conjugate is sometimes more potent (bioactive) than the parent aglycone (e.g., morphine-6-*O*-glucuronide) [45].

In terms of mediating toxicity, the glucuronidation of some drugs or xenobiotics has been shown to lead to hepatic cholestasis; for example, the estrogen steroid glucuronide estradiol-17 β -D-glucuronide and the cholestatic agent lithocholate-3-*O*-glucuronide have been shown to produce cholestasis that is related to the activity and expression of Mrp2 [46–49]. In addition to the important role of UGTs in the phase-II-mediated detoxification of xenobiotics and endogenous compounds (e.g., bile acids), they can also metabolize varied types of functional groups to chemically reactive electrophiles that are proposed to mediate the toxicity of a number of chemicals via covalent binding to protein [50]. The different types of toxicities that are proposed to be mediated by chemically reactive glucuronide conjugates include hypersensitivity (e.g., hepatic, renal) [51], carcinogenesis (e.g., bladder) [52], and gastroenteropathy [53]. The types of electrophilic glucuronides that have been well studied include acyl glucuronides of carboxylic-acid-containing xenobiotics (Table 4.2) and endogenous compounds [34,54,55] and N- or N-*O*-linked glucuronides formed by glucuronidation of hydroxamic acids [17,56].

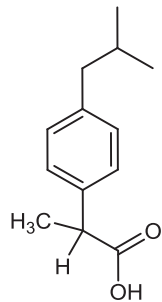
4.3.1 Acyl Glucuronidation

The conjugation of carboxylic-acid-containing compounds with glucuronic acid leading to the formation of 1- β -*O*-acyl glucuronides is an important route of metabolism

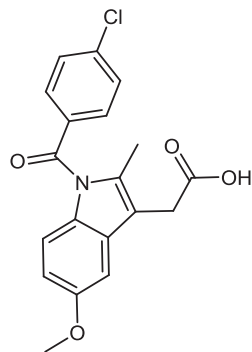
∞ TABLE 4.2 Carboxylic-Acid-Containing Compounds Cited in the Text

Benoxaprofen ^a	Bezafibrate	Bromfenac ^a	Clofibric acid	Diclofenac
				
Diflunisal	Fenbufen	Flunoxaprofen ^a	Gemfibrozil	Ibufenac ^a
				

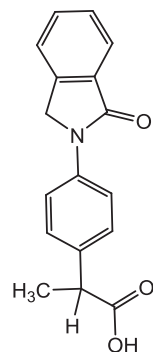
Ibuprofen



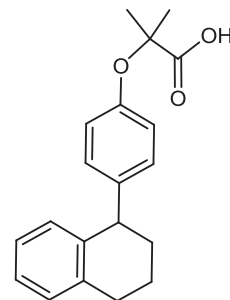
Indomethacin



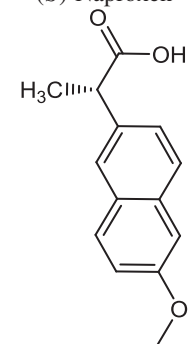
Indoprofen^a



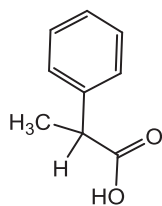
Nafenopin



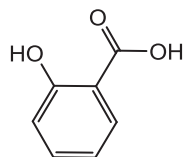
(S)-Naproxen



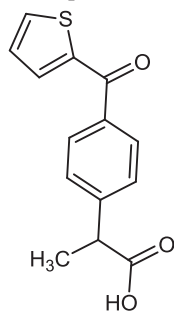
2-Phenyl-propionic acid



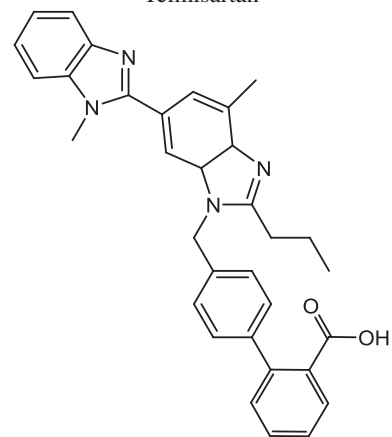
Salicylic acid



Suprofen^a



Telmisartan



Temafloxacin^a

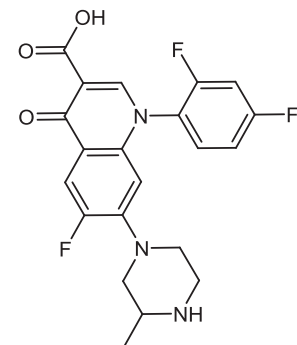
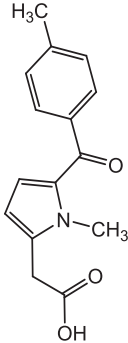
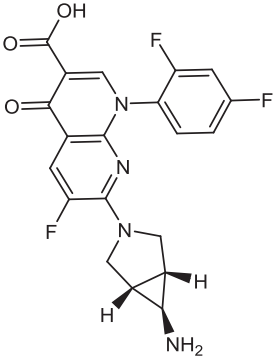
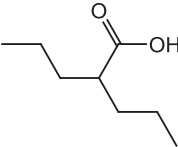
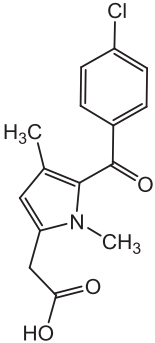


TABLE 4.2 (Continued)

Tolmetin	Trovafloracin ^a	Valproic acid	Zomepirac ^a
			

^aWithdrawn.

for endogenous compounds, drugs, and xenobiotics [35,36]. 1- β -*O*-Acyl glucuronides are chemically unstable and undergo both hydrolysis-mediated and acyl-migration-mediated degradation *in vitro* and *in vivo* [57]. During *in vitro* studies performed in buffer (pH 7.4–7.5, 37°C), authentic 1- β -*O*-acyl glucuronide derivatives biosynthesized from a range of carboxylic acids having varying substitution at the carbon atom adjacent to the carboxylic acid's carbonyl group showed that steric hindrance caused by bulky groups such as methyl (e.g., ibuprofen, clofibric acid) and propyl (valproic acid) led to increased stability (Table 4.2) [50,54,58,59]. The range of 1- β -*O*-acyl glucuronide degradation half-lives (apparent first order) is wide and can vary from 0.3 h as observed for the arylacetic-acid-type drug tolmetin to 3.3 h for ibuprofen, and up to 79 h for valproic acid. In addition, the instability of 1- β -*O*-acyl glucuronides can increase with electronic differences that lead to the carbonyl carbon of the acyl linkage being more electrophilic. For instance, the 20-fold difference between the degradation rates of the 1- β -*O*-acyl glucuronide of the benzoic-acid-containing drugs salicylic acid (1.3 h) and telmisartan (26 h) might be explained in part by the presence of the *o*-hydroxy group of salicylic acid that, unlike telmisartan, is able to hydrogen-bond to the carbonyl oxygen resulting in increased dipole moment of the carbonyl group of the acyl linkage. The presence of a heteroatom at the β -position, such as the oxygen atom present in phenoxyacetic acids, instead of a less electronegative carbon atom also results in increased degradation rates (shorter half-life) of the 1- β -*O*-acyl glucuronide. For example, the sixfold faster degradation rate of clofibric-acid-1- β -*O*-acyl glucuronide (7.3 h) than that of gemfibrozil-1- β -*O*-acyl glucuronide (44 h) is influenced by the placement of the oxygen atom present at the β -position versus the 6-position of these 2,2-dimethylalkanoic acid derivatives. One last notable structure–activity relationship with regard to 1- β -*O*-acyl glucuronide instability in buffer is for (*R*)- and (*S*)-2-phenylpropionic acid (2-PPA) 1- β -*O*-acyl glucuronides, where, owing to the α -methyl group in the (*S*)-isomer encumbering intramolecular acyl migration [60], the (*R*)-isomer degrades twofold more rapidly than the (*S*)-isomer [61].

Carboxylic-acid-containing compounds are proposed to form covalent adducts with proteins in fashion that is mediated by metabolism to chemically reactive acyl glucuronide derivatives [54,58,62]. In general, the chemical reactivity of acyl glucuronides with biological nucleophiles, such as albumin and other proteins, *in vitro* and *in vivo* decreases with increasing substitution at the carbon adjacent to the carbonyl carbon of the carboxylic acid moiety [54,63]. In experiments performed by Benet *et al.* [54], it was shown that the *in vitro* degradation rates of authentic 1- β -*O*-acyl glucuronides (due to both acyl migration and hydrolysis) of nine structurally varied carboxylic acid drugs correlated with their corresponding covalent binding to albumin *in vitro* and human plasma protein *in vivo*. Bolze *et al.* [63] demonstrated a similar correlation for eight structurally different carboxylic acid drug acyl glucuronides, where in one *in vitro* experiment, both the formation of acyl glucuronide metabolites by human liver microsomes and the assessment of corresponding reactivity with protein were achieved. A strong correlation was established between the maximal amount of covalently bound drug to protein (normalized to the amount of protein and expressed as percentage of total acyl glucuronide synthesized in the microsomal incubation) and the aglycone appearance rate constant weighted by the percentage of isomerization [63]. In general, structure–reactivity observations from these studies showed the rank

order of 1- β -*O*-acyl glucuronide instability and covalent binding to protein to be arylacetyl linked > 2-phenylpropionyl linked > benzoyl linked [54,63]. More recently, a novel quantitative LC/MS/MS technique was developed to study the reactivity of acyl glucuronide metabolites with protein by measuring the rate of reaction of structurally varied drug acyl migration isomers with the dipeptide lysine–phenylalanine (Lys–Phe) in buffer, leading to the formation of the corresponding acyl glucuronide–Lys–Phe Schiff-base-linked adducts [64]. It was proposed that the degree of reactivity of acyl glucuronides with protein would be proportional to the amount of the corresponding Lys–Phe adducts formed *in vitro*. In support of this proposal, results from their experiments showed a structure–reactivity relationship where the reaction rate rank order of forming the dipeptide adducts was acetic acid > propionic acid > benzoic acid derivatives, which was consistent with previous observations based on the steric and electronic properties of each carboxylic acid tested [54,64].

Carboxylic-acid-drug-protein adducts are hypothesized to function as immunogenic complexes that are recognized by the immune system as foreign leading to immunotoxic reactions [50,51]. The irreversible binding to albumin *in vivo* was shown in bilirubin-1- β -*O*-acyl glucuronide, leading to the formation of biliprotein as one of the first reports on acyl glucuronide-mediated covalent binding to protein [65]. Since that report, *in vitro* studies have shown that 1- β -*O*-acyl glucuronide conjugates of carboxylic acid drugs, for example, zomepirac [66], tolmetin [67], and benoxaprofen (BNX), also bind covalently to protein. Observations from mechanistic studies such as these indicated that 1- β -*O*-acyl glucuronides covalently bind to protein by two alternative reaction mechanisms (Fig. 4.2). One mechanism takes place where the electrophilic carbonyl carbon of the 1- β -*O*-acyl linkage of the acyl glucuronide reacts with protein nucleophiles (e.g., lysinyl-NH₂, tyrosine-OH and serine-OH, and cysteinyl-SH) via nucleophilic displacement of glucuronic acid leading to drug-amide-, ester-, and thioester-linked protein adducts, respectively (Fig. 4.2a). The second mechanism follows a more complex reaction route, where the 1- β -*O*-acyl glucuronide first undergoes acyl migration from 1- β -*O*-acyl position to the adjacent hydroxyl group around the glucuronic acid ring forming 2-, 3-, and 4-*O*-acyl migration isomers (Fig. 4.2b) [68]. Unlike 1- β -*O*-acyl-linked glucuronides, the acyl migration isomers are not hydrolyzed by the enzyme β -glucuronidase [69]. These acyl migration isomers in the ring tautomer form are in equilibrium with their corresponding open-chain tautomers, which are aldehydes that can react with the ϵ -amino groups of protein–lysiny residues forming Schiff-base-linked protein adducts. The 3- and 4-*O*-acyl glucuronide Schiff-base-linked adducts can then undergo a stabilization reaction via an Amadori rearrangement yielding stable 1-amino-2-keto-linked acyl glucuronide protein adducts [66,70]. The covalent products formed from these two pathways are significantly different in that, unlike the transacylated nucleophilic displacement product, the Schiff base mechanism of covalent binding leads to protein adducts that retain the glucuronic acid moiety. Such a common moiety linking proteins to drugs might function as a common neoantigen and serve as an explanation for cross-reactive allergic reactions between structurally varied nonsteroidal anti-inflammatory drugs (NSAIDs) [50,71]. From these observations, it was proposed that there may be a relationship between the *in vivo* covalent binding of acyl glucuronide metabolites of acidic drugs to protein and toxicological properties of the drugs [54,58,61,66]; however, convincing data to support this proposal is still lacking.

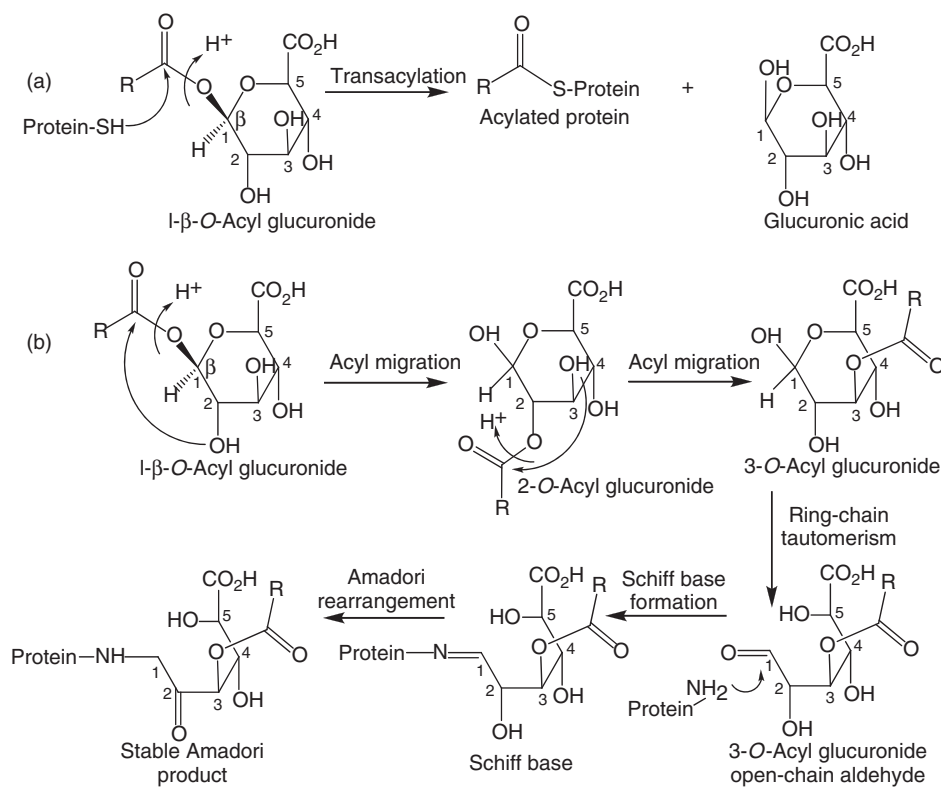


Figure 4.2 Mechanisms for (a) the transacylation of protein nucleophiles by 1-β-O-acyl-linked glucuronides and (b) stable adduct formation with lysine residues on proteins by acyl glucuronide migration isomers.

A total of 17 carboxylic-acid-containing drugs were withdrawn from the worldwide pharmaceutical market during 1960–1999 for safety reasons, including hepatotoxicity, renal failure, and/or dermatologic toxicity [72]. Some of these drugs included the 2-arylpropionic acid (profen) NSAIDs BNX, indoprofen, and suprofen; the arylacetic acid NSAIDs bromfenac, fenclofenac, ibufenac, and zomepirac; and the benzoic-acid-containing anti-infective quinolone drugs temafloxacin and trovafloxacin (Table 4.2). These drugs are biotransformed to varying extents to 1-β-O-acyl glucuronide metabolites [73–80]. In addition to these withdrawn drugs, currently used drugs that are also metabolized by acyl glucuronidation and have been characterized for covalent binding properties include diclofenac [71], diflunisal [81], valproic acid [82], tolmetin [83], clofibric acid [84], ibuprofen [85], and salicylic acid [86] (structures shown in Table 4.2).

4.3.1.1 Benoxaprofen. The NSAID BNX (2-[2-(4-chlorophenyl)-1,3-benzoxazol-5-yl]propanoic acid) was withdrawn from clinical use in 1982 when it was determined to mediate a rare but fatal cholestatic jaundice [87,88]. The mechanism of hepatotoxicity remains unexplained, but it has been postulated that the hepatotoxicity could be related to the formation of BNX–protein covalent adducts formed via a reactive acyl

glucuronide metabolite in the liver. However, no indication of immune-mediated hepatotoxicity was ever observed in BNX-treated patients. BNX-1- β -*O*-acyl glucuronide is the major metabolite (90% of the dose) of BNX formed in humans, and two-thirds of the acyl glucuronide was reported to be renally excreted, with the remaining amount eliminated in feces [89].

Tandem mass spectrometry was used to identify the mechanism of covalent binding to protein formed during incubations of with human serum albumin (HSA) [90]. Results demonstrated that BNX-1- β -*O*-acyl glucuronide mediated the formation of covalent adducts with protein nucleophiles on HSA by both nucleophilic displacement and Schiff base reactions with Lys-159 and Lys-199 ϵ -amino-lysine residues, where Lys-159 was identified as the major amino acid involved in BNX-protein adduct formation. Similar results were obtained from studies with tolmetin-1- β -*O*-acyl glucuronide, where covalent binding to HSA by both transacylation and Schiff base mechanisms was also detected; however, Lys-199 was determined to be the major site of tolmetin-protein adduct formation [67]. Other major binding sites of transacylation by BNX-1- β -*O*-acyl glucuronide included Ser-312, Ser-480, and Arg-222.

In vivo covalent binding studies in rat showed BNX to become irreversibly bound to plasma and liver proteins, and immunochemical techniques detected the presence of multiple hepatic protein targets [91]. Results showed the formation of protein adducts with masses of 70 and 110 kDa as the primary hepatic targets. *In vitro* covalent binding of BNX-1- β -*O*-acyl glucuronide (170 μ M) to HSA (30 mg/mL, pH 7.4, 37°C) was time dependent and reached 45 pmol/mg protein after 24 h of incubation. The apparent first-order degradation half-life of the acyl glucuronide during incubation was 1.55 h, compared to 1.43 h in buffer alone. No covalent binding was detected in corresponding incubations with BNX-free acid. The formation of BNX-protein adducts in rat hepatocyte cultures, which were depleted of UDPGA (by treatment with [–]-borneol), led to a 46% inhibition in protein adduct formation as detected by SDS-PAGE Western immunoblotting analysis. The partial inhibition of covalent binding to protein suggested that other bioactivation mechanisms must also be involved. Other routes of BNX bioactivation leading to covalent binding to protein in the liver might include P450-mediated oxidation, acyl glucuronidation, and/or *S*-acyl-CoA formation (discussed below). BNX contains a benzoxazole substructure potentially able to be metabolized via phase I metabolism to reactive intermediates that could account for, at least in part, some of the covalent binding to hepatic protein occurring *in vivo*. However, in a recent report, the covalent binding of [¹⁴C]BNX to protein in NADPH-fortified human liver microsomes also showed no detectable covalent binding to protein [92]. These results suggest that BNX is not biotransformed by cytochrome P450 to reactive intermediates (e.g., epoxides), and no GSH conjugates of BNX have been reported to date. In another study, BNX-1- β -*O*-acyl glucuronide formation was shown not to be a critical protein-reactive intermediate of BNX because *in vitro* experiments conducted in UDPGA-fortified human liver S-9 fraction appeared to completely block covalent binding of BNX to protein [93]. To date, evidence for the causative reactive metabolite(s) of BNX leading to hepatic cholestasis is not available. One proposal put forward is that an insoluble metabolite of BNX leads to drug-related precipitation in the bile canaliculi as the mechanism of cholestasis [88]; however, an insoluble metabolite of BNX has never been observed. Other studies in sandwich-cultured rat and human hepatocytes have demonstrated both BNX glucuronidation and BNX covalent binding to protein [94]. From those studies, it was proposed that sandwich-cultured human hepatocytes

be used as an *in vitro* tool to investigate the role of acyl glucuronidation in the covalent binding of other acidic drugs to protein as a screening method, but perhaps, the method could also be employed to investigate further the mechanism of BNX-mediated cholestatic hepatotoxicity [95].

4.3.1.2 Diclofenac. Diclofenac (2-[2-(2,6-dichlorophenyl)-aminophenyl]ethanoic acid) is an NSAID used clinically for the treatment of patients with osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and acute muscle pain [96]. Diclofenac has been implicated in several cases of severe idiosyncratic hepatotoxicity as well as enteropathy, hemolytic anemia, and immune-mediated anaphylaxis [97]. One proposed molecular mechanism mediating diclofenac-induced hepatitis is that reactive metabolites of diclofenac covalently bind to protein and are then recognized by the immune system as foreign, thus leading to an immunotoxic response [10,98]. Diclofenac is metabolized by UGT2B7 in humans and UGT2B1 in rats where 10–20% of the drug is eliminated as the acyl glucuronide [99]. The apparent first-order degradation half-life of diclofenac-1- β -*O*-acyl glucuronide (D-1- β -*O*-G) in buffer at pH 7.4 and 37°C is rapid (0.51 h), and D-1- β -*O*-G degrades primarily via acyl migration [58]. Other *in vitro* studies conducted in buffer showed that D-1- β -*O*-G is chemically reactive and transacylates GSH forming diclofenac-*S*-acyl-GSH [100]. In studies where diclofenac was incubated with rat and human hepatocytes, diclofenac-*S*-acyl-GSH thioester was also formed. However, the GSH thioester formation was not mediated by D-1- β -*O*-G because inhibition of D-1- β -*O*-G production had no effect on diclofenac-*S*-acyl-GSH formation. These results suggested that diclofenac must also be biotransformed to chemically reactive metabolites other than D-1- β -*O*-G, leading to the transacylation of GSH. Covalent binding studies performed in short-term-cultured rat hepatocytes showed diclofenac acyl glucuronide formation to be associated with covalent binding of the drug to hepatocellular proteins; however, this was not related to the acute cytotoxicity [101]. From these studies, it was proposed that protein adduct formation, and its modulation by UDPGT, can be toxicologically important for the formation of diclofenac-induced hepatitis. Further studies conducted *in vivo* in rat and *in vitro* in short-term-cultured rat hepatocytes showed that covalent binding of diclofenac to hepatocyte proteins is selective, where a 60-kDa microsomal membrane protein appeared to be the major target for covalent binding in rat liver and in the cultured hepatocytes exposed to diclofenac [102]. The covalent binding of diclofenac to protein was investigated in incubations with rat liver microsomes and was shown to be time dependent and UDPGA cofactor dependent. In addition, diclofenac covalent binding to protein was significantly increased in the presence of the imine-trapping reagent, sodium cyanide, and was significantly decreased by 7,7,7-triphenylheptyl-UDP, a specific inhibitor of UGT. Covalent binding to protein resulted in an adduct weighing 60 kDa. Overall, the results indicated that diclofenac acyl glucuronide covalently binds to hepatic microsomal proteins by both nucleophilic displacement and Schiff base mechanisms [71]. *In vivo* covalent binding studies with diclofenac in rat, using immunoblotting techniques, led to the detection of a diclofenac-labeled 50-kDa rat liver microsomal protein (P450 mediated) and 110-, 140-, and 200-kDa diclofenac-adducted plasma membrane proteins (UGT mediated) [103].

The 110-kDa diclofenac-labeled protein adduct in rat liver was later determined to be dipeptidyl peptidase IV (CD26), and it was found that the activity of CD26 in liver plasma membrane fractions was decreased after diclofenac treatment of rats. It was

proposed that the hepatotoxicity associated with diclofenac might be due, at least in part, to the covalent modification of CD26 [104]. More recently, studies on the covalent binding of diclofenac to protein in normal Wistar rats compared to that occurring in mutant transport-deficient (TR⁻) rats demonstrated that diclofenac glucuronide is selectively transported into rat bile mediated by Mrp2 and that hepatobiliary transport is necessary for diclofenac covalent binding to protein (118-kDa canalicular protein detected as the major protein adduct) in the biliary tree [105].

Results from one study showed the formation of IgM autoantibodies against the acyl glucuronide conjugate of 4'-hydroxydiclofenac that was detected in the urine of a diclofenac-dosed patient suffering from acute Coombs-positive hemolytic anemia [106]. The metabolite was shown to promote agglutination of normal red blood cells and therefore was proposed to potentially mediate hemolytic anemia. The 4'-hydroxydiclofenac acyl glucuronide was determined to be catalyzed by CYP2C8-mediated metabolism of D-1- β -O-G [107]. Along a similar line, gemfibrozil-1-O-acyl glucuronide has been shown to be bioactivated by CYP2C8-mediated hydroxylation leading to reactive metabolite formation and subsequent time-dependent inactivation of the enzyme [108,109].

The small intestinal injury caused by diclofenac has been proposed to occur by enterocyte cytotoxicity mediated by the reactive acyl glucuronide of diclofenac or the acyl glucuronide of one of its oxidative metabolites [110]. In one study, post administration of diclofenac to rats by gastric gavage, subsequent immunoblotting analysis of small intestine homogenates and isolated enterocyte subcellular fractions with drug-specific antiserum revealed 142-, 130-, 110-, and 55-kDa protein adducts [111]. The 142- and 130-kDa protein adducts of diclofenac were identified as aminopeptidase N (CD13) and sucrase-isomaltase, respectively. These authors proposed that oral tolerance to intestinal drug-protein adducts can be developed in gut-associated lymphoid tissue, leading to the downregulation of drug-induced allergic reactions in many individuals who do not have gut toxicity to diclofenac.

Diclofenac also undergoes cytochrome P450C9- and 3A4-mediated oxidation of the aromatic ring to 4'- and 5-hydroxylated products, respectively. These hydroxylated metabolites then can undergo further P450-mediated oxidation to electrophilic quinone imine metabolites that react, in a Michael acceptor fashion, with GSH and proteins [112,113]. Other mechanisms propose diclofenac-mediated cytotoxicity to be caused by mitochondrial damage, P450-mediated reactive metabolites, or oxidative stress induced by free-radical-containing metabolites [114–116]. In cytotoxicity studies with diclofenac conducted in rat hepatocytes, it was demonstrated that when diclofenac glucuronidation was inhibited with (–)-borneol, the LD₅₀ decreased from 1000 μ M in the absence of the inhibitor to 740 μ M in its presence [117], which was consistent with the results obtained by Kretz-Rommel and Boelsterli [101], where diclofenac was shown to cause P450-mediated acute cell injury in cultured rat hepatocytes. To date, clear evidence for the identity of causative hepatotoxic metabolite(s) (i.e., UGT- or P450-mediated) of diclofenac has not been obtained [98].

4.3.2 Glucuronidation of Arylhydroxamic Acids

Arylhydroxamic acids are metabolic products formed from the P450-mediated oxidative metabolism of N-acetylated N-arylamine-containing compounds, leading to a hydroxyl

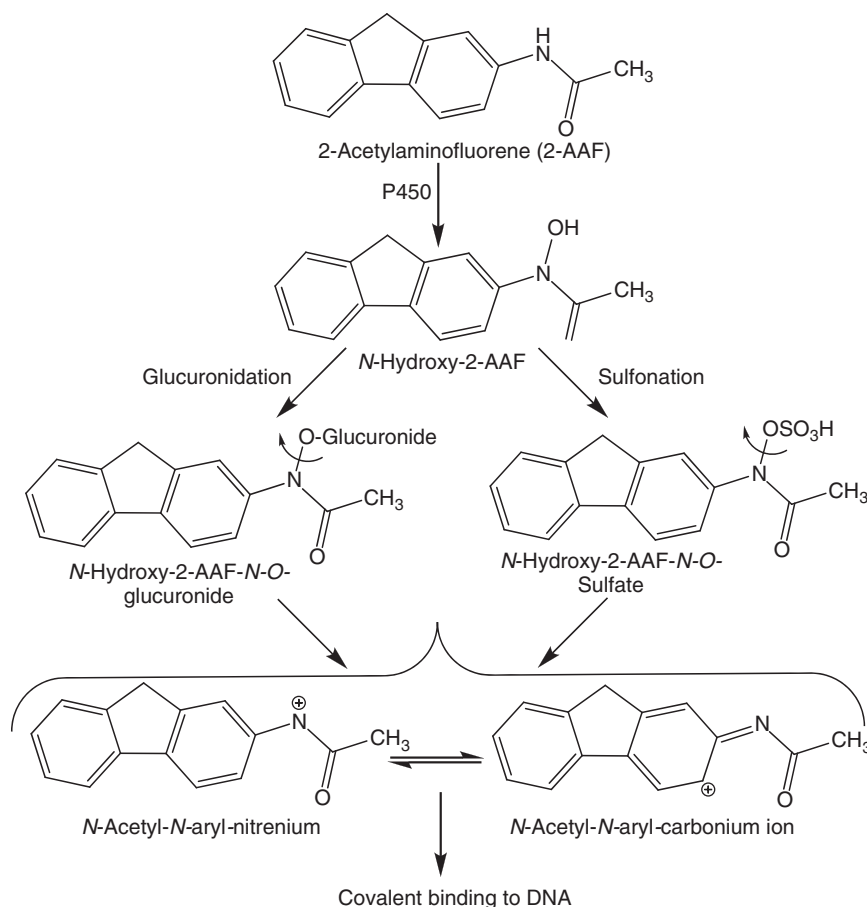


Figure 4.3 Mechanisms by which *N*-hydroxy-2-AAF can lead to DNA adduct formation via *N*-O-glucuronidation or *N*-O-sulfonation.

group attached to the nitrogen of the acetyl-amino linkage. Glucuronidation of arylhydroxamic acids forming *N*-O-linked glucuronides was discovered as the first example of chemically reactive glucuronide metabolites [11,30,118].

4.3.2.1 2-Acetylaminofluorene. From early studies performed in the 1960s on the *in vivo* metabolism of the carcinogen 2-acetylaminofluorene (2-AAF), it was shown that a major metabolite eliminated in the urine of test animals was the O-linked glucuronide of *N*-hydroxy-2-AAF (Fig. 4.3) [119]. The specific rat UGT isoforms known to catalyze O-linked glucuronide formation of *N*-hydroxy-2-AAF include UGT2B1, UGT2B2, and UGT1A6 [120]. Recombinant human UGT isoforms expressed in liver that catalyze the glucuronidation were shown to be UGT1A6 and UGT2B7 [121]. Therefore, in rats and rabbits, 2-AAF had undergone P450-mediated *N*-hydroxylation followed by O-glucuronidation before urinary elimination. The glucuronide was determined to be degradable by treatment with β -glucuronidase.

Later it was shown that the O-linked glucuronide of *N*-hydroxy-2-AAF was chemically reactive *in vitro* at pH 7 with the amino acids methionine and tryptophan as well as with the *N*²-position of the purine nucleoside guanosine forming products, where the acetyl group was retained [11]. The mechanism by which the *N*-O-glucuronide of *N*-hydroxy-2-AAF leads to adduct formation with nucleophiles is believed to proceed by the *N*-O-glucuronide undergoing heterolytic decomposition to DNA-reactive *N*-acetyl-*N*-aryl-nitrenium or carbonium ions (Fig. 4.3) [17]. Comparisons of the chemical reactivity of the *N*-O-glucuronide with the corresponding phosphate and sulfate *O*-esters (discussed below) of *N*-hydroxy-2-AAF showed lower reactivity for the glucuronide [52,122]. However, the chemical reactivity of the *N*-O-glucuronide of *N*-hydroxy-2-AAF was enhanced in incubations performed at higher pH, leading to increased adduct detection [123], which may be toxicologically significant, for instance, in bladder cancer when urinary pH is elevated [17,44]. The *N*-O-glucuronide of *N*-hydroxy-2-AAF in buffer under mildly basic pH conditions undergoes deacetylation to form the substantially more reactive electrophile *N*-hydroxy-2-aminofluorene *O*-glucuronide [17,124,125]. Structure–reactivity relationship experiments for *O*-glucuronides of *N*-acetyl-*N*-arylhydroxylamines in reactions with nucleic acids showed that the rank order of reactivity with respect to the aryl group being 2-AAF > *trans*-4-acetamidostilbene > 4-acetamidophenyl > 2-acetamidophenanthrene [126].

There is no evidence for the direct UGT-catalyzed glucuronidation of *N*-O-glucuronides of arylhydroxylamines (potentially due to their high instability), where their biological formation depends instead on the degradation of N–O-linked glucuronides of *N*-acetyl-*N*-arylhydroxylamines [30]. However, the UGT-mediated biosynthesis of N-linked glucuronides of both arylamines and arylhydroxylamines does occur, but in these cases, the product *N*-glucuronides are not perceived to be toxicologically important since they otherwise do not readily generate reactive electrophiles [17]. However, under acidic conditions (pH 5), N-linked glucuronides of *N*-arylhydroxylamines (e.g., *N*-hydroxy-2-naphthylamine) are labile and might serve as proximate carcinogens before acid-catalyzed degradation, eventually leading to highly reactive arylnitrenium ions [17,127].

In summary, *O*-glucuronide conjugates of *N*-acetyl-*N*-arylhydroxylamines may play an important role in the extrahepatic tissues' carcinogenicity induced by N-acetylated *N*-arylamines such as 2-AAF by serving as relatively stable transport species of P450-mediated N-hydroxylated precursors. In nonhepatic target organs such as the bladder, N–O-linked glucuronides can then undergo pH-dependent hydrolytic reactions to liberate chemically reactive metabolites [17,128].

In studies conducted by King and Phillips [122], they demonstrated a 105,000g supernatant protein fraction of rat liver to catalyze covalent reactions of 2-AAF with nucleic acid, and the cofactor requirements suggested, in part, that the reactive intermediates involved were the *O*-sulfonate esters (discussed below) of *N*-hydroxy-2-AAF. Sulfonate esters have been established as ultimate carcinogens in 2-AAF-induced hepatocarcinogenesis, where interspecies comparisons for tumor incidence were consistent with *O*-sulfonate ester formation [129]. The *O*-glucuronide-linked metabolites of *N*-acetyl-*N*-arylhydroxylamines have been proposed to be stable transport forms of carcinogens and as such determine the target organ of toxicity, for example, in 2-AAF-induced bladder carcinogenesis [130].

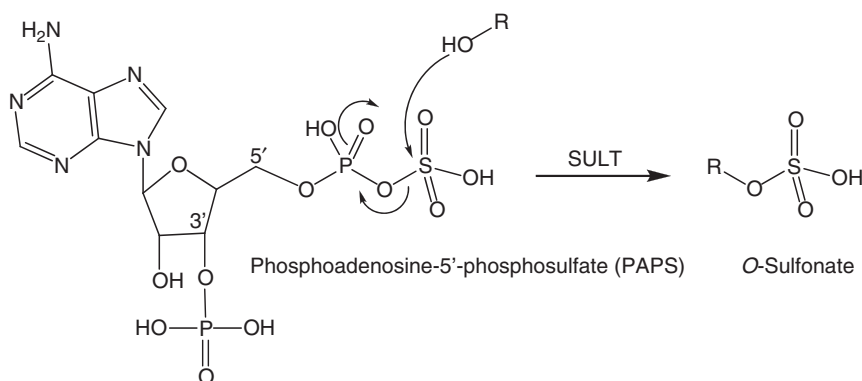


Figure 4.4 Mechanism of *O*-sulfonate conjugate formation catalyzed by SULTs.

4.4 SULFONATION

Sulfonation of a wide range of structurally varied endogenous compounds, drugs, and other xenobiotics by cytosolic SULT (EC 2.8.2) enzymes most often represents an important route of their clearance and detoxification from the body [131–133]. SULT enzymes catalyze the conjugation of a sulfonate group (SO_3^-) from the endogenous universal donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to hydroxyl, amino, or sulfhydryl groups of substrates forming sulfonates, sulfamates, and thiosulfates, respectively (Fig. 4.4) [31,131,134]. Sulfonation of a substrate hydroxyl group to produce sulfonate is the most common sulfonation reaction observed in the body [135]. The SULT cofactor PAPS is biosynthesized from SO_4^{2-} obtained from the amino acids cysteine and methionine, and two molecules of ATP are necessary for the synthesis of each PAPS molecule produced. PAPS is present in low concentrations in the cytosol, and therefore, the sulfonation pathway may be saturated at high substrate concentrations. The sulfonate group ($\text{p}K_a < 1$) is ionized at physiological pH such that the sulfate-containing conjugates are often water soluble and in most cases are incapable of binding and activating their corresponding receptors compared to their unconjugated parent molecules [31,135]. However, there are examples of drugs that undergo sulfonation leading to sulfate ester metabolites that are more pharmacologically potent than their parent drugs. Such examples include the vasodilator minoxidil [136,137] and the antihypertensive agent cicletanine [138].

Generally, conjugation of xenobiotics with a sulfonate group results in increased water solubility leading to their efficient excretion from the body into urine and/or bile. For example, *O*-sulfonation is required for detoxification and elimination of drugs such as acetaminophen (APAP), which is a leading cause of liver failure in humans [139,140]. However, *O*-sulfonation is also increasingly being shown to function as a bioactivation mechanism for promutagens and procarcinogens by leading to the formation of chemically reactive electrophiles that bind covalently to DNA, RNA, and protein nucleophiles [9,31,122,141].

SULTs have a wide tissue distribution and function as a major detoxification mechanism in adults and in the developing human fetus [142]. Two broad classes of SULTs are known. One class is membrane bound and is located in the Golgi apparatus, which

functions in the sulfonation of endogenous peptides, proteins, carbohydrates, and lipids. The second class of SULTs is soluble and is located in the cytosol where it is known to mediate the metabolism of steroids, neurotransmitters, bile acids, and xenobiotics [135,142]. Therefore, the cytosolic SULTs are the important class of SULT enzymes relevant to xenobiotics metabolism, disposition, and potential bioactivation. A nomenclature system for the cytosolic SULT superfamily has been developed [143], and at least 13 human SULTs that differ strongly in their tissue distribution and their substrate specificity have been characterized [31,142]. There are two main SULT families that are known to mediate mutagenicity and carcinogenicity by metabolism of phenolic and arylhydroxylamine-containing xenobiotics. These families include the aryl (phenol) SULTs (aryl-SULT, SULT1; EC 2.8.2.1) and alcohol (hydroxysteroid) SULTs (alcohol-SULT, SULT2; EC 2.8.2.2) [31,141].

Discussed below are some known examples of xenobiotic chemical classes that undergo metabolic activation by SULTs, including polycyclic aromatic benzylic alcohols, allylic alcohols, *N*-arylhydroxylamines, and *N*-acetyl-*N*-arylhydroxylamines.

4.4.1 Polycyclic Aromatic Benzylic Alcohols

Benzylic-*O*-hydroxy metabolites of methyl-substituted polycyclic hydrocarbons can undergo sulfonation to form extremely reactive sulfonate esters that play a role as ultimate mutagens and/or carcinogens.

4.4.1.1 7-Hydroxymethyl-12-methylbenz[*a*]anthracene. Studies have shown that benzylic-*O*-hydroxy polycyclic hydrocarbons such as 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-OH-DMBA) are bioactivated preferentially by hepatic hydroxysteroid SULT to unstable benzylic sulfonate esters that degrade to highly reactive benzylic carbocations that covalently bind to DNA, RNA, and proteins (Fig. 4.5) [12,144,145]. 7,12-Dimethylbenz[*a*]anthracene (DMBA) is a polycyclic aromatic compound determined to be a potent carcinogen. DMBA is found in the environment and produced from the incomplete combustion of organic matter.

7-OH-DMBA is a carcinogenic major metabolite of DMBA formed in the liver [144]. *In vitro* studies with 7-OH-HMBA showed that covalent binding to DNA was observed during incubations with human liver cytosol and only when fortified with PAPS [145]. Similar results were obtained with the hydroxylated DMBA metabolites 7-methyl-12-hydroxymethylbenz[*a*]anthracene (12-OH-DMBA) and 7,12-dihydroxymethylbenz[*a*]anthracene (7,12-diOH-DMBA). Results from studies also showed that the SULT-mediated activation of the methyl-hydroxylated DMBA derivatives was predominantly catalyzed by dehydroepiandrosterone DHEA-steroid SULT. Further studies showed that GST-mediated detoxification, with GSH forming the nonmutagenic *S*-(12-hydroxymethylbenz[*a*]anthracen-7-yl)methyl-GSH product, occurred during incubations of 7,12-diOH-DMBA with PAPS and GSH-supplemented hepatic cytosol, where the GSH adduct was formed directly from DHBA-7-sulfate as the obligatory intermediate [12]. Results from studies such as these strongly support the hypothesis that polycyclic-aromatic-type benzylic alcohols undergo SULT-mediated bioactivation related to the formation of DNA adducts. In addition to the bioactivation of primary benzylic alcohols as discussed above, secondary benzylic alcohols (e.g., 1-hydroxy-3-methylcholanthrene) [146] can also undergo bioactivation by SULTs leading to the formation of chemically unstable mutagenic/carcinogenic sulfonate metabolites.

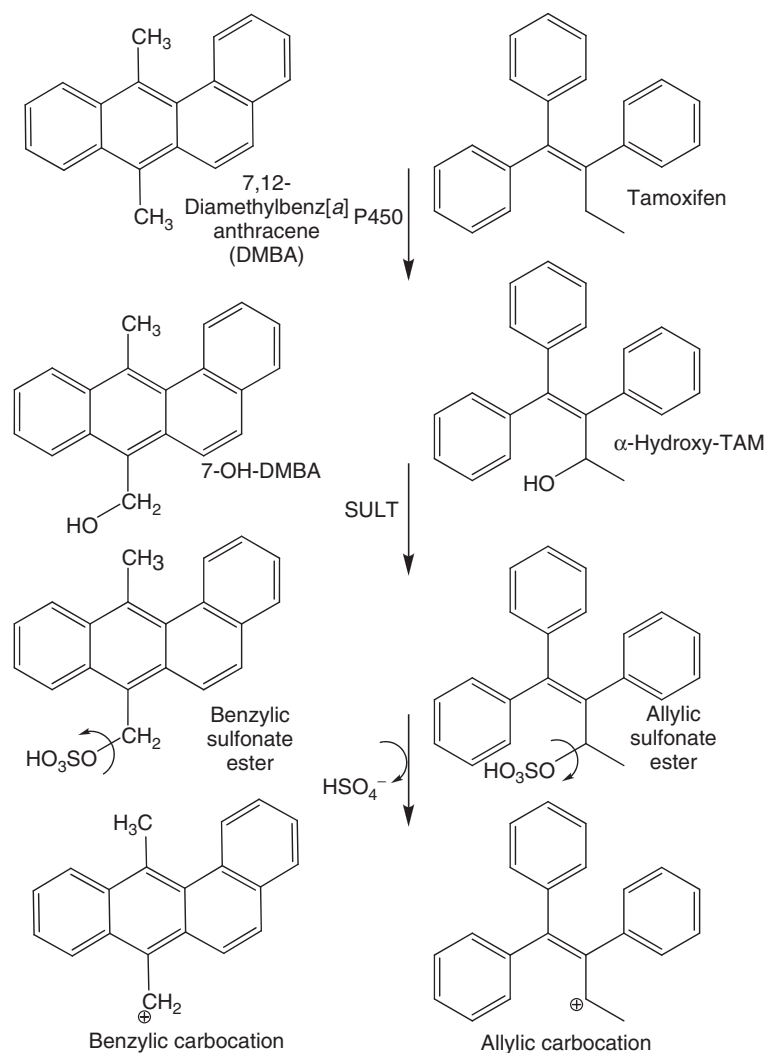


Figure 4.5 Metabolic activation of benzylic and allylic alcohols leading to the formation of unstable sulfonate esters that decompose forming benzylic and allylic carbocations capable of covalently binding to DNA.

4.4.2 Allylic Alcohols (Tamoxifen)

The nonsteroidal antiestrogen drug tamoxifen (TAM) is used as a long-term chemopreventative for breast cancer in healthy women [147]. The drug has been shown to be a potent genotoxic hepatocarcinogen in rats [148], and the increased risk, however rare, of endometrial cancer in long-term TAM-treated breast cancer patients has heightened concerns about the use of the drug [149].

DNA adducts of TAM have been observed in both rat and human tissues, and the hepatocarcinogenic effects in rat are known to follow a mechanism where DNA adducts accumulate in the liver. The pathway of bioactivation of TMA leading to DNA adduct

formation is sequential and involves cytochrome P450-mediated α -hydroxylation followed by O-sulfonation leading to an unstable sulfonate ester that decomposes to a highly reactive allylic carbocation that binds covalently to DNA (Fig. 4.5) [13].

Multiple human P450s, including P450 3A4 and, to a lesser extent, P450 2D6, P450 2B6, P450 3A5, P450 2C9, and P450 2C19, have been shown to mediate the formation of α -hydroxy-TAM [150]. Many of these P450s are found in endometrial tissue and therefore may mediate the production of reactive metabolites that play a role in the formation of endometrial cancer. The formation of α -hydroxy-TAM-sulfonate from α -hydroxy-TAM has been shown to be catalyzed by both rat liver hydroxysteroid (alcohol) SULT-a and human alcohol (hydroxysteroid) SULT enzymes (SULT2A1) [151,152]. The production of TMA-DNA adducts is known to occur during incubations of TAM with both rat and human hepatocytes [153,154]. However, TAM-DNA adduct formation occurs to a less extent in incubations with human hepatocytes than that measured in rat hepatocytes. This observation has been explained by a lower extent of TAM bioactivation (by α -hydroxylation or sulfonation) and a greater rate of glucuronidation-mediated detoxification of α -hydroxy-TAM occurring in human compared to rat.

Other mutagenic/carcinogenic allylic alcohols that are well known to be metabolically activated by SULTs include 1'-hydroxysafrole, 1'-hydroxyestragole, 5-hydroxymethylfural, and α -hydroxycyproterone acetate [141].

4.4.3 Arylhydroxylamines and Arylhydroxamic Acids

4.4.3.1 4-Dimethylaminobenzene. 4-Dimethylaminobenzene (DAB) is a hepatocarcinogenic dye that was used as an industrial chemical and shown to induce the formation of liver tumors in rat [155]. DAB undergoes sequential metabolism via cytochrome P450 by N-demethylation to 4-methylaminobenzene (MAB), followed by flavoprotein mixed-function amine-oxidase-mediated N-oxidation to the arylhydroxylamine, *N*-hydroxy-MAB [156]. Incubations of *N*-hydroxy-MAB with rat liver cytosol fortified with PAPS led to the production of adducts of methionine, guanosine, ribosomal RNA, and GSH [14,157]. The formation of these adducts was dependent on the O-sulfonation of *N*-hydroxy-MAB to the product sulfonate ester, which decomposes to a chemically reactive electrophilic nitrenium ion and sulfate (Fig. 4.6). Some other carcinogenic *N*-arylhydroxylamines that are known to be metabolically activated by human liver thermostable phenol SULTs include *N*-hydroxy derivatives of 2-aminofluorene, 4,4'-methylene-bis(2-chloroaniline), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, and 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole [158]. The thermostable phenol SULT (SULT1A1) appears to be polymorphically expressed in humans. Therefore, an important role for the enzyme has been suggested to be related to interindividual susceptibility to *N*-arylhydroxylamine-containing carcinogens [158,159].

4.4.3.2 2-Acetylaminofluorene. As discussed above, the carcinogenic N-acetylated *N*-arylamine 2-AAF is bioactivated by P450-mediated N-hydroxylation followed by O-glucuronidation leading to the unstable N-O-linked glucuronide that decomposes to the chemically reactive nitrenium ion. In addition to this metabolic activation route, *N*-hydroxy-2-AAF undergoes O-sulfonation-mediated bioactivation to an unstable O-sulfonate ester (Fig. 4.3). Just as described above for the O-sulfonate ester of

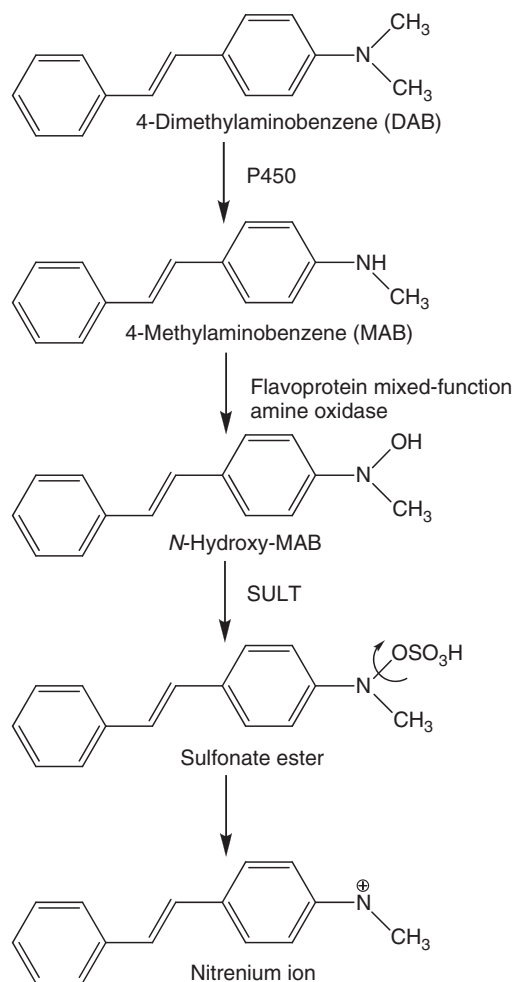


Figure 4.6 Metabolic activation of 4-dimethylaminobenzene (DAB) leading to a chemically reactive electrophilic nitrenium ion that covalently binds to DNA.

N-hydroxy-MAB, the *O*-sulfonate ester of *N*-hydroxy-2-AAF undergoes heterolytic cleavage to the electrophilic *N*-acetyl-*N*-aryl-nitrenium ion [15]. The nitrenium ion is in resonance with the aryl carbocations that also contribute to the formation of covalent adducts with DNA nucleophiles [17]. Covalent binding to DNA by this mechanism was shown to lead to the formation of 3-deoxyguanosin- N^2 -yl-2-AAF and *N*-deoxyguanosine-8-yl-2-AAF adducts [141,160]. Similar to the hepatocarcinogenic affects of arylhydroxylamines, the metabolism of *N*-hydroxy-AAF by deacetylation to *N*-hydroxy-2-aminofluorene (*N*-hydroxy-AF) has been proposed as a critical bioactivation process leading to the formation of hepatic DNA adducts and the initiation of liver tumors in 12-day-old male B6C3F1 mice [161]. In those studies, it was shown that deacetylation of *N*-hydroxy-AAF to *N*-hydroxy-AF followed by *O*-sulfonation was essential for the leading to *N*-deoxyguanosine-8-yl-2-AF DNA adduct formation

and liver tumor initiation in infant male B6C3F1 mice treated with *N*-hydroxy-AAF [160].

In summary, the observations from the mechanistic studies with 2-AAF and related arylamines that were initially conducted in the 1950s by Miller and Miller and others led to a working hypothesis stating that for some compounds to become carcinogenic, they must first be metabolically activated by cytochrome P450 followed by O-conjugation forming unstable *O*-glucuronides and/or *O*-sulfonates, which lead to the formation of chemically reactive nitrenium ion or carbocation species that covalently bind to and damage DNA [9,162]. The irreversible interactions of these electrophilic metabolites with DNA, and the carcinogenic and mutagenic effects that occur with the resulting DNA damage, have provided the foundation on which much of the current mechanistic drug metabolism has been built and for genetic toxicity testing [163–166].

4.5 N-ACETYLATION

Conjugation of amines by N-acetylation, which is catalyzed by human NAT enzymes (EC 2.3.1.5) located primarily in the cytosol of the liver and many other tissues, plays an important role in the metabolism of drugs, xenobiotics and environmental chemicals and in both the detoxification and bioactivation of procarcinogens in humans [2,167–169]. The NAT enzyme reaction mechanism requires acetyl coenzyme A (acetyl CoA) as the cofactor to provide the acetyl group for the conjugation of aromatic amines or aromatic hydrazines forming aromatic amide or aromatic hydrazide metabolites, respectively (Fig. 4.7) [170]. The xenobiotic-metabolizing enzymes that are important in humans leading to the N-acetylation of drugs are NAT1 and NAT2, and the genetic polymorphisms for both enzymes are known, for example, the “slow” and “fast” acetylators of isoniazid [171,172]. The expression of NAT1 and NAT2 in humans differs, where NAT1 is widely expressed in varied tissues and NAT2 shows expression primarily in the liver and intestine.

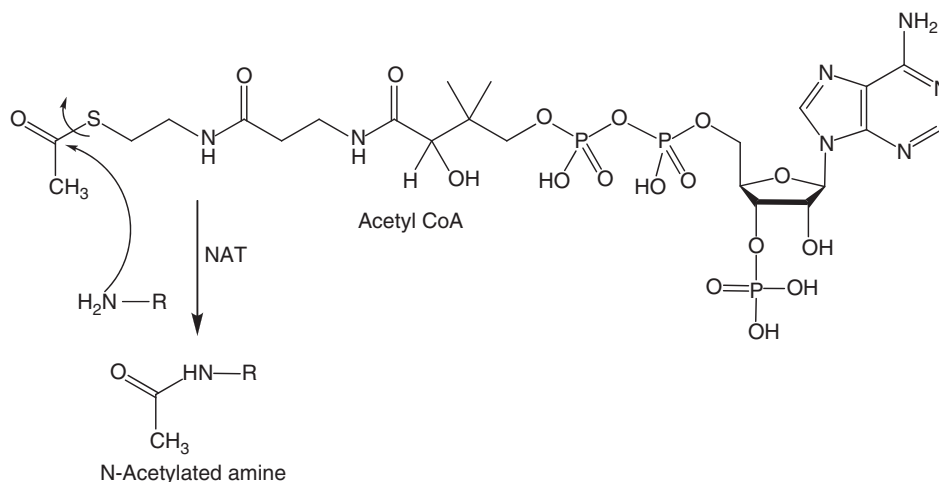


Figure 4.7 Mechanism of N-acetylated amine formation catalyzed by NAT.

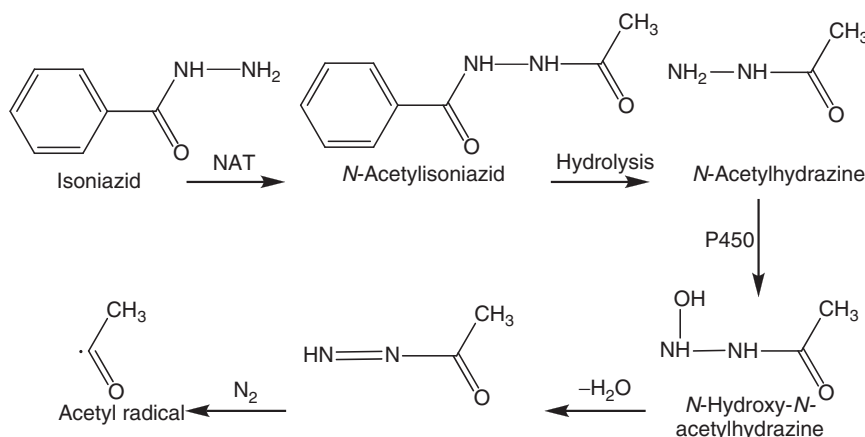


Figure 4.8 Metabolic activation of isoniazid initiated by N-acetylation, leading to the formation of reactive acetyl radical that covalently binds to protein.

The N-acetylation of xenobiotic-containing aromatic hydrazine (e.g., isoniazid) and aromatic amine (e.g., benzidine) functional groups can serve as a preliminary step in their bioactivation to chemically reactive intermediates that are proposed to induce hepatotoxicity or carcinogenesis by covalently binding to protein or DNA, respectively [17,173].

4.5.1 Aromatic Hydrazines—Isoniazid N-Acetylation

Isoniazid (isonicotinohydrazine, INH; Fig. 4.8), an aromatic-hydrazine-containing compound, is the primary drug used for the prevention and treatment of tuberculosis. INH causes hepatitis as one of its two major adverse toxicities and peripheral neuropathy is the other major toxicity. There is no clear evidence that INH-induced hepatotoxicity is immune mediated [174]. INH is metabolized primarily in the liver by NAT2-mediated N-acetylation to *N*-acetylisoniazid, where the rate of acetylation is genetically determined. Genetic polymorphisms of NAT2 correlate with fast-, slow-, and intermediate-acetylation phenotypes [175,176].

The proposed mechanism of bioactivation follows on such that *N*-acetylisoniazid is hydrolyzed to *N*-acetylhydrazine and isonicotinic acid. *N*-Acetylhydrazine then undergoes further oxidative metabolism by P450 enzymes to a highly reactive acylating acetyl radical species (Fig. 4.8) [16]. It was shown that covalent binding of this reactive species to hepatic macromolecules paralleled hepatocellular necrosis [177]. The trapped *S*-acetyl-GSH adduct has been detected from *in vitro* incubations [16]. Cytochrome P450 2E1 (CYP2E1) was shown to be important in the metabolic activation of *N*-acetylhydrazine, and CYP2E1 genetic polymorphism has been proposed to be associated with susceptibility to INH-induced hepatitis [178]. Recently, it was shown that CYP2E1 c1/c1 homozygotes are prone to developing more severe hepatotoxicity than other c1/c2 and c2/c2 genotypes [179]. Fast and slow acetylators excrete 90% and 60% of INH, respectively, as *N*-acetylisoniazid in urine; however, the effect of acetylation rate on isoniazid hepatotoxicity remains controversial [180].

4.5.2 Aromatic Amines—Benzidine

Aromatic-amine-containing carcinogens are metabolized by N-acetylation catalyzed by both human NAT1 and NAT2 enzymes [168]. N-Acetylation functions as a detoxification route of aromatic amines and as an alternative metabolic pathway to them undergoing CYP-mediated N-hydroxylation [181]. N-Hydroxylation of some aromatic amines is the first step toward their bioactivation leading to DNA-reactive electrophiles. Therefore, aromatic amines that may be inefficiently acetylated, because they are poor substrates for NAT and/or because of the slow acetylator phenotype, might “build up” and serve as substrates for oxidative enzymes such as CYP1A2 [182] leading to *N*-hydroxyaromatic amine (hydroxylamine) products, which become substrates for NAT enzymes leading to unstable aromatic *N*-acetoxyaromatic amine intermediates. *N*-Acetoxyaromatic amines are extremely reactive electrophiles that react with endogenous nucleophiles such as amino acids, proteins, RNA, and DNA [17]. Aromatic amines such as the bicyclic aromatic amine benzidine (4,4'-diaminobiphenyl), a formerly used chemical for the production of azo dyes [183], have been recognized as potential human carcinogens for many years [184]. One mechanism for the metabolic activation of benzidine that requires the participation of NAT follows on such that an initial CYP-mediated N-hydroxylation of benzidine followed by N-acetylation of the same N-hydroxylated nitrogen leads to an *N*-arylhydroxamic acid intermediate (Fig. 4.9). However, another pathway affording the *N*-arylhydroxamic acid intermediate can occur

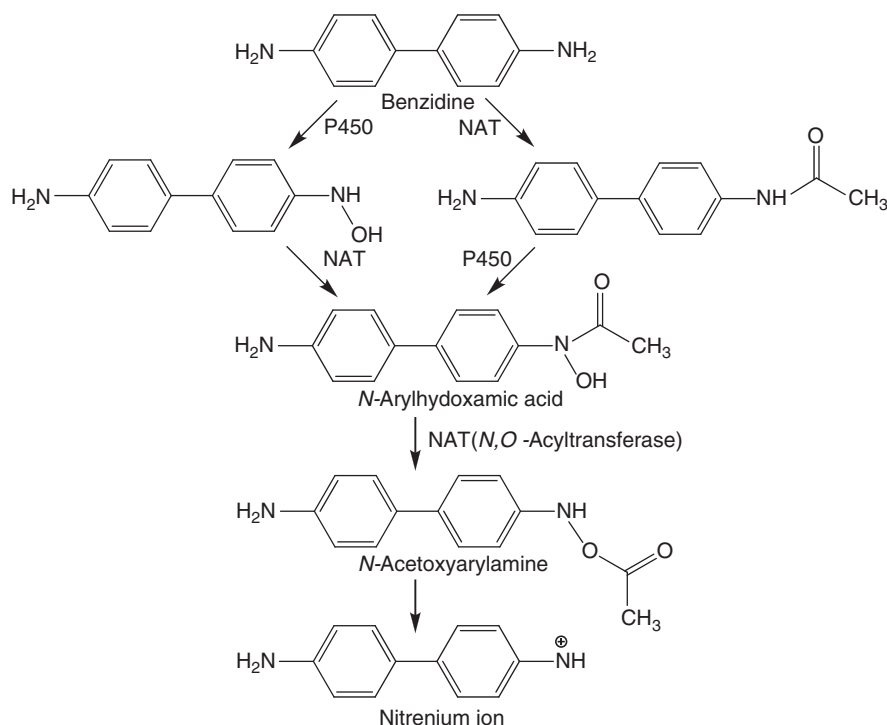


Figure 4.9 Metabolic activation of benzidine in part via N-acetylation, leading to a reactive nitrenium ion that covalently binds to DNA nucleophiles.

by initial N-acetylation of benzidine followed by CYP-mediated N-hydroxylation of the amide nitrogen, which is similar to a mechanism for the N-hydroxylation of the heterocyclic aromatic amine 2-AAF [17]. The final bioactivation step occurs when NAT functions as an *N*, *O*-acyltransferase and catalyzes the intramolecular transfer of the acetyl group from the nitrogen to the oxygen to give an unstable *N*-acetoxyarylamine intermediate that decomposes to acetic acid and the chemically reactive nitrenium ion that covalently binds to DNA nucleophiles [17]. Alternative to the *N*-arylhydroxamic acid intermediate, in another pathway, an *N*-hydroxylated arylamine undergoes direct O-acetylation by NAT to give the unstable *N*-acetoxyarylamine derivative, as shown for the NAT-mediated bioactivation of *N*-hydroxy-2-AF that leads to an unstable *N*-acetoxy-2-AF intermediate [185]. Slow acetylators are prone to increase CYP1A2-mediated N-hydroxylation of bicyclic aromatic amine carcinogens, which then undergo bioactivation by O-acetylation catalyzed by NAT1 [2]. Heterocyclic aromatic amines are poor substrates for N-acetylation by NAT1 and NAT2 and therefore undergo hepatic CYP1A2-mediated N-hydroxylation followed by NAT-catalyzed O-acetylation to the unstable *N*-acetoxyarylamine intermediate. In general, fast acetylators can detoxify bicyclic aromatic amines to amide products in the liver, whereas fast acetylators may bioactivate heterocyclic *N*-hydroxylaromatic amines to unstable *N*-acetoxyarylamine derivatives that lead to the formation of DNA adducts and the initiation of carcinogenesis [2].

4.6 GLUTATHIONE CONJUGATION

Conjugation of reactive electrophiles with the endogenous tripeptide nucleophile GSH (γ -glutamyl-cysteinyl-glycine) is an important phase II biotransformation/detoxification pathway in drug metabolism. The distribution of GSH in tissues is variable, but generally is high (1–10 mM). In hepatocytes, there are two major pools of GSH: the cytosolic pool represents ~85% and the mitochondrial pool ~15%. The structural uniqueness of GSH is due to the arrangement of one of its two peptide linkages where the γ -carboxyl moiety of the glutamyl amino acid, rather than the α -carboxylic acid moiety, is bound to the cysteinyl-amine via a peptidase-stable amide bond. The cysteinyl-sulfhydryl group of GSH is a “soft” nucleophile that is able to react with soft electrophiles such as carbon in polarized double bonds (e.g., α,β -unsaturated ketones, quinones, and quinone imines) and alkyl halides. GSH also reacts with intermediate soft electrophiles such as the carbon of epoxides and nitrenium ions.

The conjugation of GSH with chemically reactive xenobiotics can also be catalyzed by GST enzymes (EC 2.5.1.18) [186]. GSTs are located in the cytosol and microsomal and mitochondrial fractions of the cell. GSTs function by catalyzing the formation of a highly reactive thiolate anion, via an active-site tyrosine or serine located in the GSH binding site that activates the sulfur, with subsequent nucleophilic attack with a hydrophobic electrophile present in the hydrophobic substrate binding site of the GST dimer [186]. The major forms of the GST enzymes are found in the cytosol and are homodimers that are grouped into four families, namely, alpha (A), mu (M), pi (P), and theta (T).

Conjugation of chemically reactive electrophilic xenobiotics or xenobiotic metabolites with GSH most often results in the formation of GSH adducts with increased chemical stability. For instance, well-known examples of chemically stable GSH adducts,

which account for, in most cases, the clearance of reactive, potentially toxic, substances from the body, include thioether-linked GSH adducts of acetaminophen, diclofenac, and naphthalene. However, as discussed below, some GSH adducts have also been shown to function as chemically reactive electrophiles in their own right and to be capable of leading to the covalent binding of xenobiotics to biological nucleophiles resulting in varied tissue toxicities [32,33].

Enzyme-mediated degradation of GSH adducts can also lead to reactive metabolite formation. This pathway occurs initially via an extracellular process specifically by γ -glutamyl transpeptidase (γ -GT) in the liver and the kidney as major tissue sites. For example, GSH adducts can be transported out of the hepatocyte into the plasma where they undergo interorgan transport into the kidney. Once in the kidney, GSH adducts are broken down first by γ -GT (EC 2.3.2.2), the only known enzyme to cleave GSH and GSH adducts appreciably, which functions by hydrolyzing the γ -glutamyl bond [187], leading to the formation of the corresponding cysteinyl-glycine-*S*-linked adducts (cys-gly adducts). Following the γ -GT step, cys-gly adducts undergo further hydrolysis by a cys-glydipeptidase (EC 3.4.13.6), or aminopeptidase N (also known as *aminopeptidase M*; EC 3.4.11.2), leading to the formation of the corresponding L-cysteine-*S* adducts [188]. L-Cysteine-*S* adducts then serve as substrates for cysteine-*S*-conjugate NAT (EC 2.3.1.80) [189], which functions to acetylate the cysteinyl-amine residue of these conjugates (via the cofactor acetyl CoA) affording *N*-acetylcysteine (NAC) adducts before excretion in urine. Cysteine-*S*-conjugates in addition to undergoing *N*-acetylation to mercapturates can also be metabolized by the renal cysteine conjugate β -lyase (EC 4.4.1.13), a pyridoxal-phosphate-dependent enzyme located in renal mitochondria and cytosol, leading to the cleavage of the cysteinyl C–S bond and the subsequent formation of sometimes unstable thiols that are proposed to function as nephrotoxic metabolites [190].

The examples discussed below include the bioactivation of xenobiotics resulting in the formation of chemically reactive GSH adducts (e.g., ethylene dibromide (EDB), α -naphthylisothiocyanate (ANIT), and carboxylic acid drugs) and chemically reactive mercapturic acid pathway degradation products of the corresponding GSH adducts (e.g., 3,4-methylene-dioxymethamphetamine, bromobenzene, sevoflurane, and hexachlorobutadiene).

4.6.1 Ethylene Dibromide Episulfonium Ion Formation

A well-known case of the formation of a GSH adduct mediating mutagenic and genotoxic effects is that of the industrial chemical EDB (Fig. 4.10) [18]. EDB is a haloalkane bifunctional electrophile that undergoes bioactivation by GST-catalyzed conjugation forming a reactive intermediate that leads ultimately to the formation of DNA adducts *in vivo* in rat and *in vitro* in rat and human hepatocytes [191–193]. The conjugation of EDB with GSH is known to be selectively catalyzed by both GST-A and GST-T classes of enzymes [193,194]. In this reaction mechanism, the thiol of GSH reacts with EDB in an S_N2 -type reaction leading to the elimination of bromide ion and the formation of a thioether-linked half sulfur mustard, which undergoes another round of bromide ion elimination via an intramolecular S_N2 reaction (β -elimination) with the sulfur atom to provide the chemically reactive episulfonium-ion-GSH adduct. The episulfonium-ion-containing GSH adduct is reactive with DNA, where the *N*⁷-guanine residue is a major target for DNA alkylation forming *S*-[2-(*N*⁷-guanylyl)ethyl]-GSH

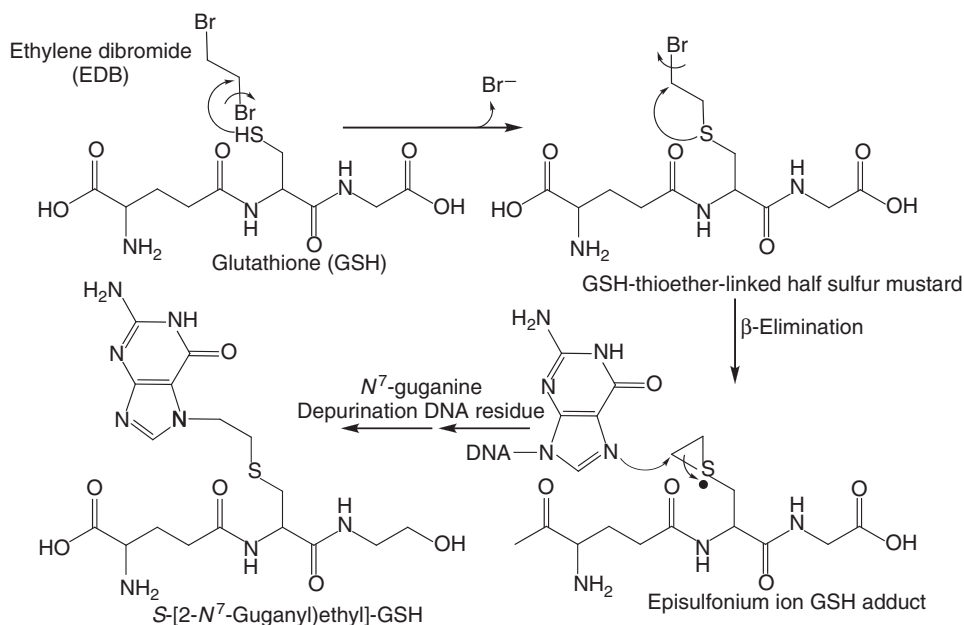


Figure 4.10 Scheme for the bioactivation of ethylene dibromide via conjugation with GSH.

leading to damaged DNA and subsequent carcinogenicity (Fig. 4.10) [18]. The corresponding NAC adduct, namely, S -[2-(N^7 -guanyl)ethyl]-NAC, has been detected in the urine of EDB-dosed rats [195]. In summary, the GST-mediated conjugation of the haloalkane EDB with GSH leads to covalent binding to intracellular nucleophiles such as nucleic acids leading to mutagenic and carcinogenic effects.

4.6.2 3,4-Methylenedioxymethamphetamine-Induced Neurotoxicity: Mercapturic-Acid-Pathway-Dependent Bioactivation

3,4-Methylenedioxymethamphetamine (MDMA), also known as *Ecstasy*, is an illicit psychoactive drug that possesses stimulant and hallucinogenic properties [196]. However, MDMA also functions as a serotonergic neurotoxicant in many species, including nonhuman primates, which is thought to be mediated in part by neurotoxic metabolites of MDMA [23,197]. A proposed scheme for the metabolism of MDMA leading to neurotoxic metabolites is shown in Fig. 4.11 [23,198]. Thus, MDMA first undergoes metabolism in the liver by cytochrome P450s to nonneurotoxic metabolites either by CYP2B6-mediated N-demethylation forming methylenedioxyamphetamine (MDA) or by CYP2D6-mediated O-demethylation forming N -methyl- α -methyldopamine (N -methyl- α -MeDA). CYP-mediated O-demethylation of MDA leads to the formation of α -MeDA. Then, both of these o -catechol metabolites undergo further oxidation yielding chemically reactive o -quinone intermediates that are reactive with GSH forming 2- or 5-(glutathion- S -yl)- N -methyl- α -MeDA and 2- or 5-(glutathion- S -yl)- α -MeDA, respectively. Subsequent oxidation of these o -catechol-GSH adducts to reactive o -quinone intermediates, followed again by reaction with GSH, leads to the formation of the corresponding 2,5-bis-glutathionyl conjugates [23]. The hepatic formation of these

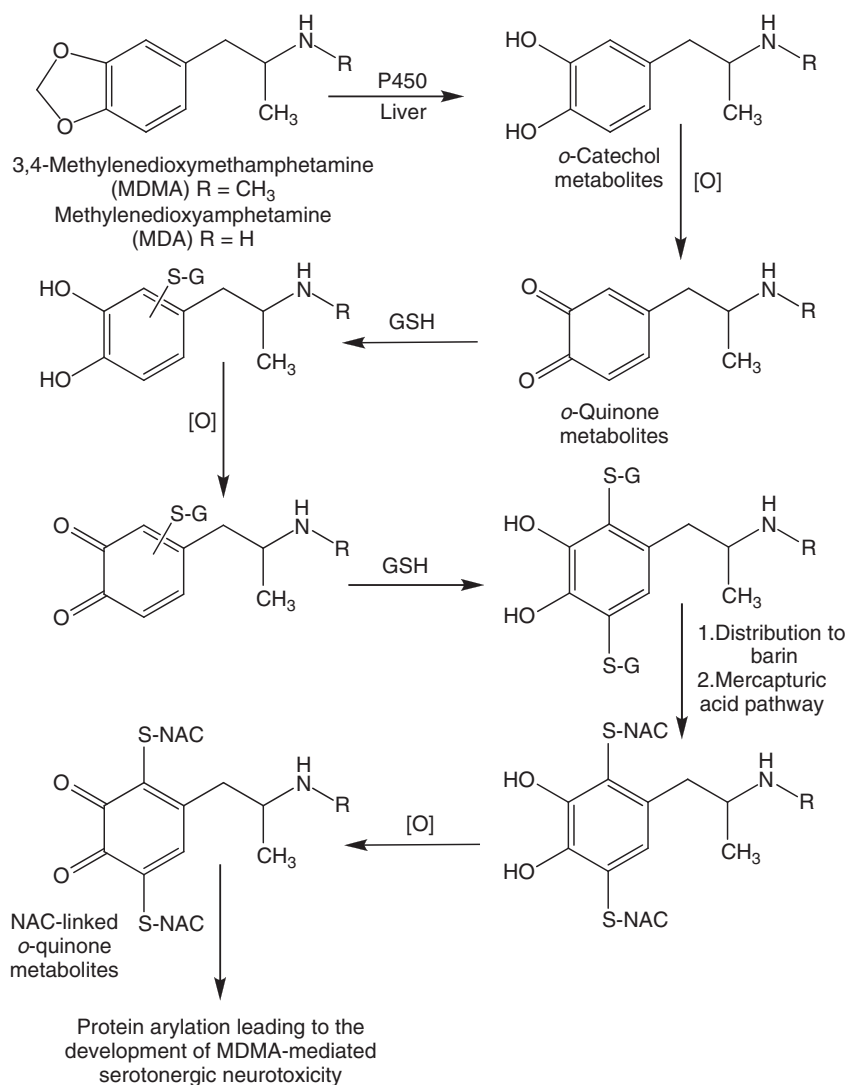


Figure 4.11 Proposed scheme for the metabolic activation of MDMA via P450, GSH conjugation, and mercapturic acid formation, leading to neurotoxic metabolites.

mono- and bis-GSH-linked adducts is followed by their interorgan transport and distribution into the brain by crossing the blood–brain barrier [199,200]. The GSH adducts can then undergo further metabolism by the mercapturic acid pathway in brain tissue or capillary lumen to the corresponding NAC derivatives, which are slowly eliminated from the brain leading to accumulation. The catechol-containing NAC adducts are proposed to undergo further oxidation in brain tissue leading to reactive oxygen species and chemically reactive *o*-quinone intermediates that arylate proteins leading to the development of MDMA-mediated serotonergic neurotoxicity. In summary, results from studies on the neurotoxic effects of MDMA showed that neurotoxic thioether-linked metabolites of MDMA derived from reactions of chemically reactive

o-quinone metabolites with GSH mediate the toxicity rather than the detoxification of the illicit drug.

4.6.3 Bromobenzene

Bromobenzene was formerly used as an industrial solvent; however, it is hepatotoxic and induces centrilobular necrosis by a mechanism consistent with P450-mediated bioactivation to chemically reactive benzoquinone-type metabolites [22,201]. In terms of bioactivation via GSH conjugation, bromobenzene also causes nephrotoxicity, where a crucial role for benzoquinone-derived GSH adducts has been elegantly established [24,202,203]. Thus, treatment of rats with radiolabeled [^{14}C]bromobenzene was shown to result in the development of renal necrosis and covalent binding of the radioactive molecule primarily to necrotic tubular cells, which provided evidence that the covalent binding to protein and necrosis are correlated [204]. The mechanism of bromobenzene-induced nephrotoxicity was proposed to occur whereby a reactive cytochrome P450-generated bromoquinone metabolite generated in the liver reacts covalently with hepatic GSH forming bromohydroquinone-linked thioether GSH adducts that undergo interorgan transport to the kidney where the GSH adducts are processed by the mercapturic acid pathway γ -GT and alanylaminopeptidase enzymes leading to bromohydroquinone-linked cysteine-*S*-conjugates (Fig. 4.12). These bromohydroquinone-linked cysteine-*S*-conjugates are known to readily undergo redox cycling leading to oxidative stress and the formation of bromoquinone-linked cysteine-*S*-conjugates that react covalently with protein. Renal cysteine conjugate β -lyase metabolism of the cysteine-*S*-conjugate was shown not to contribute to the nephrotoxicity of 2-bromo-(cystein-*S*-yl)hydroquinone in rats, where inhibition of the β -lyase did not diminish nephrotoxicity [24]. However, by contrast, in another study, inhibition of γ -GT led to diminished covalent binding to renal tissues and nephrotoxicity post administration of 2-[^{14}C]bromohydroquinone to rats [205]. In summary, the kidney necrosis observed in rats post administration of bromobenzene is strongly viewed to be due in part to 2-bromohydroquinone *S*-linked GSH adducts formed in the liver and then transported to the kidney, followed by conversion to ultimate nephrotoxic metabolite(s) by further mercapturic-acid-pathway-mediated degradation to the corresponding redox cycling sensitive cysteine-*S*-conjugates. One of the GSH adducts of bromobenzene, namely, 2-Br-3-(glutathion-*S*-yl)hydroquinone, was studied in detail *in vitro* in incubations with γ -GT. It was determined that the γ -GT-mediated cleavage of the γ -glutamate moiety leads to the freeing up of the nucleophilic cysteinyl-amine functional group in the 2-Br-3-(cystein-*S*-yl-glycine)hydroquinone product. Subsequent oxidation of the hydroquinone to the quinone derivative, which occurs readily, allows for the condensation of the α -amino-cysteine group with the carbonyl carbon of the quinone, leading to the elimination of water and the formation of the 1,4-benzothiazine product, 2*H*-(3-glyciny)-7-hydroxy-8-Br-1,4-benzothiazine. Elimination of glycine followed by coupling of two product benzothiazine derivatives leads to the formation of 2,3-bis-(1,4-benzothiazine) dimers, which can tautomerize and undergo subsequent coupling to form insoluble polymers [206]. It has been proposed that the deposition of insoluble polymers within the renal proximal tubules might contribute to the nephrotoxicity of the 2-Br-3-(glutathion-*S*-yl)hydroquinone conjugate [206].

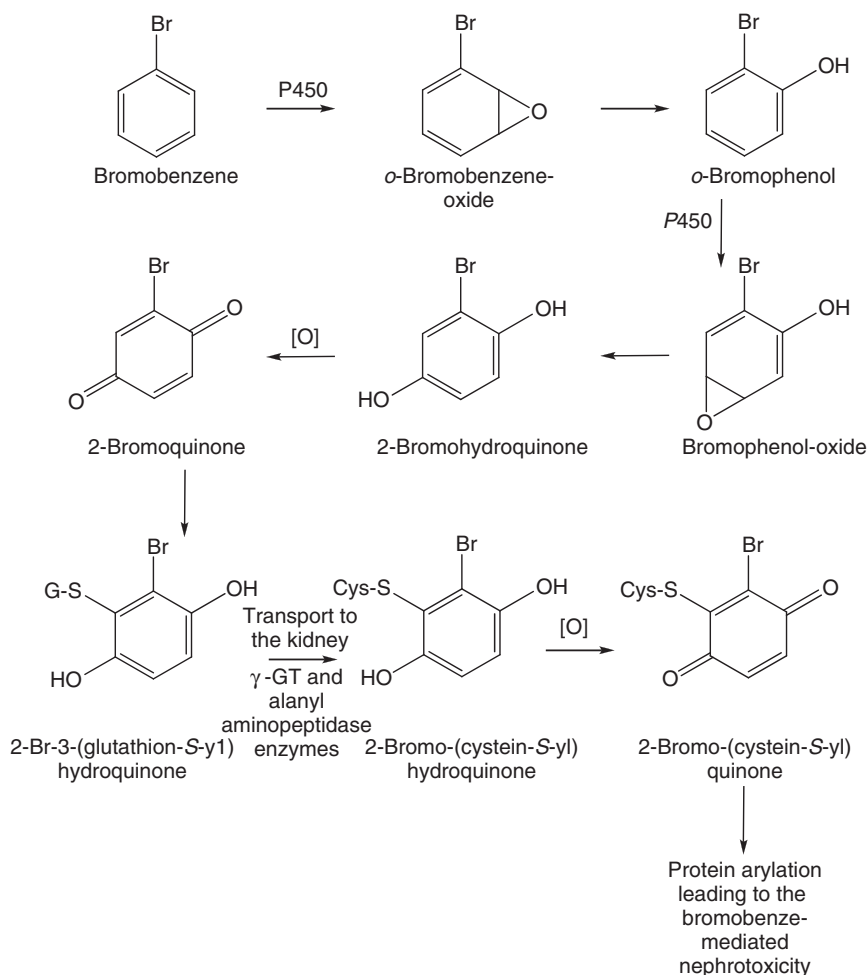


Figure 4.12 Bioactivation via GSH conjugation, leading to benzoquinone-derived GSH adducts that mediate bromobenzene-induced nephrotoxicity.

4.6.4 Sevoflurane-Induced Nephrotoxicity: Renal Cysteine Conjugate β -Lyase-Dependent Bioactivation

The renal cysteine conjugate β -lyase pathway is an important mechanism for the metabolic activation of structurally varied nephrotoxic haloalkenes [19,190,207]. The initial step in this multistep bioactivation mechanism is the GST-catalyzed formation of GSH-*S*-conjugates in liver. After interorgan transport to the kidney, the GSH adducts undergo γ -GT- and dipeptidase-catalyzed hydrolysis in the proximal tubules to the corresponding cysteine-*S*-conjugates. Cysteine-*S*-conjugates that do not undergo detoxification by cysteine *N*-acetylation and conversion to mercapturates can be bioactivated by renal cysteine conjugate β -lyase to thioacylating intermediates that react with cellular nucleophiles leading to renal tissue necrosis. An example of renal cysteine conjugate

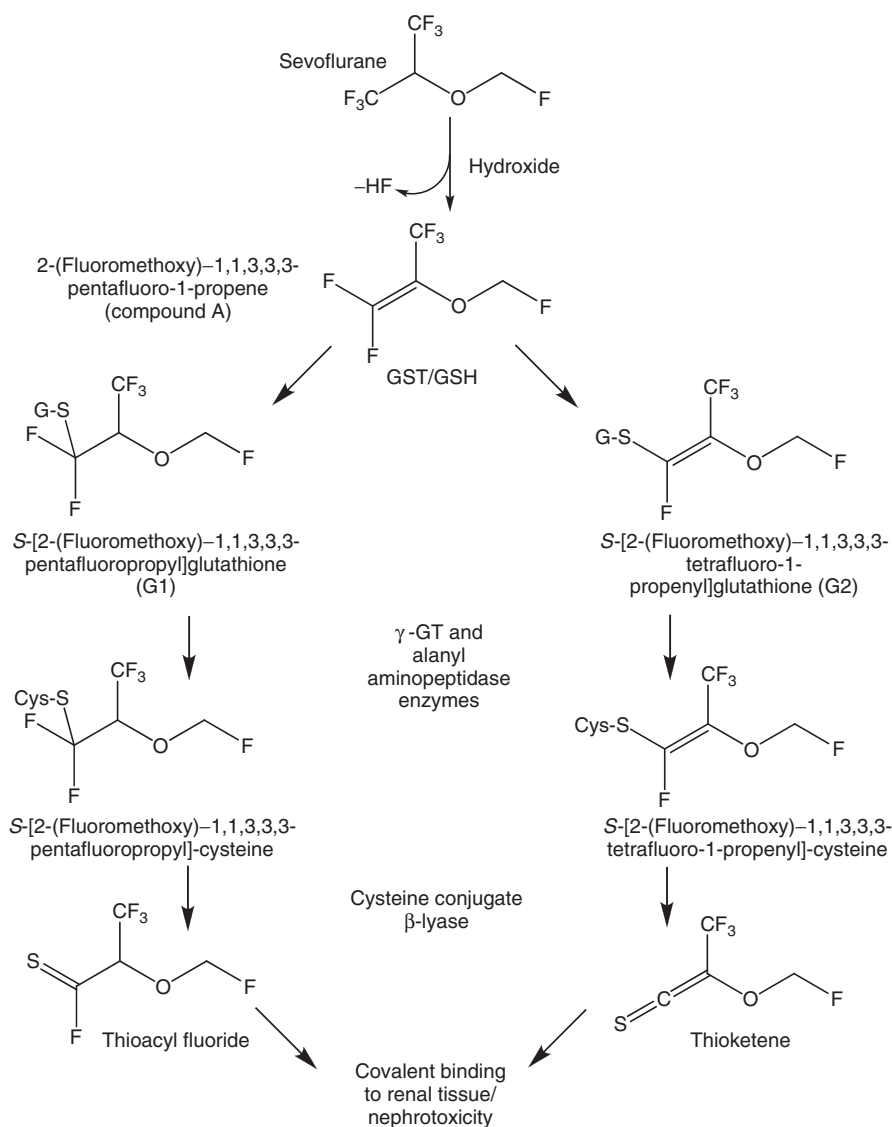


Figure 4.13 Glutathione conjugation- and cysteine β -lyase-dependent bioactivation of sevoflurane, leading to reactive metabolite formation, covalent binding to renal proteins, and nephrotoxicity.

β -lyase-dependent bioactivation is for the drug sevoflurane fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl)ethyl ether (Fig. 4.13). Sevoflurane is a volatile general anesthetic that was first used clinically in Japan in 1990 by Maruishi Pharmaceuticals and subsequently marketed in the United States and worldwide (1995) by Abbott Laboratories as Ultane[®] and Sevorane[®], respectively [208,209].

Sevoflurane has been shown to undergo chemical degradation when in contact with strong bases such as soda lime found in anesthesia circuits linked to carbon dioxide

scrubbers. This leads to the formation of 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (compound A), a halogenated alkene that is nephrotoxic in rats and mediates the formation of proximal tubular lesions leading to tubular necrosis of the outer stripe of the outer medullary layer of the kidney [20,210]. Compound A is bioactivated by GSH-dependent metabolism, and two compound-A-derived GSH adducts, namely, *S*-[2-(fluoromethoxy)-1,1,3,3,3-pentafluoropropyl]-GSH (G1) and *S*-[2-(fluoromethoxy)-1,3,3,3-tetrafluoro-1-propenyl]-GSH (G2) and the two corresponding mercapturates, *S*-[2-(fluoromethoxy)-1,1,3,3,3-pentafluoropropyl]-NAC and *S*-[2-(fluoromethoxy)-1,3,3,3-tetrafluoro-1-propenyl]-NAC, are eliminated in the bile and urine, respectively, of rats administered compound A (Fig. 4.13) [211]. The GSH adducts G1 and G2, and the corresponding cysteine-*S* adducts, have been shown to be nephrotoxic in rats [212]. Results demonstrating that the inhibition of renal cysteine conjugate β -lyase partially protected against compound A renal toxicity provided evidence that this enzyme pathway may be important in the bioactivation of compound-A-derived GSH adducts leading to nephrotoxicity in rats. Compound A GSH adducts G1 and G2 undergo mercapturic acid pathway hydrolytic degradation by γ -GT followed by dipeptidase-mediated cleavage of glycine affording the corresponding compound A cysteine-*S*-linked adducts. The cysteine-*S*-conjugates are the penultimate metabolites in this bioactivation pathway and function as substrates for bioactivation by renal cysteine conjugate β -lyase, which forms the ultimate toxic reactive thionoacyl halide and thioketene species that react with renal cellular nucleophiles. However, no immunochemical evidence for renal protein adducts was found in male Wistar rats 24 h after the administration of the mercapturates or GSH adducts of compound A, which lead to renal corticomedullary necrosis. Such results were proposed to provide evidence against the involvement of cysteine conjugate β -lyase in compound-A-induced nephrotoxicity in rats [213].

In summary, the phase II mechanism of metabolism of compound A by GSH conjugation leading to cysteine-*S*-conjugate formation is proposed to mediate nephrotoxicity in rats via subsequent renal uptake and β -lyase-dependent bioactivation with the subsequent acylation of tissue proteins. Human (and rat) kidneys are injured by compound A produced by degradation of the clinically dosed (inhalation) sevoflurane. Renal injury in volunteers dosed with sevoflurane was shown to increase with increasing duration of exposure to a given concentration of compound A [214]; however, other investigations have shown no significant changes in either standard or experimental renal markers [213]. In comparison to rats, other than the report by Eger *et al.* [214], there has been no evidence for compound-A-induced nephrotoxicity in humans [215]. The differences in toxic potential have been proposed to be due to differences between species with regard to compound A exposure, renal cysteine conjugate β -lyase activity, and the relative reactive metabolite formation versus detoxification (i.e., mercapturate formation).

4.6.5 Hexachlorobutadiene-Induced Nephrotoxicity

Like sevoflurane, haloalkenes are also bioactivated after multiple enzyme steps involving GSH, the mercapturic acid pathway, and renal cysteine conjugate β -lyase leading to reactive electrophilic thioketene intermediates that covalently bind to renal tissue protein and lead to nephrotoxicity.

Hexachlorobutadiene, a polychlorinated alkene, is not a potent hepatotoxin, but a well-known nephrotoxicant in rodents that induces necrosis in the S-3 portion

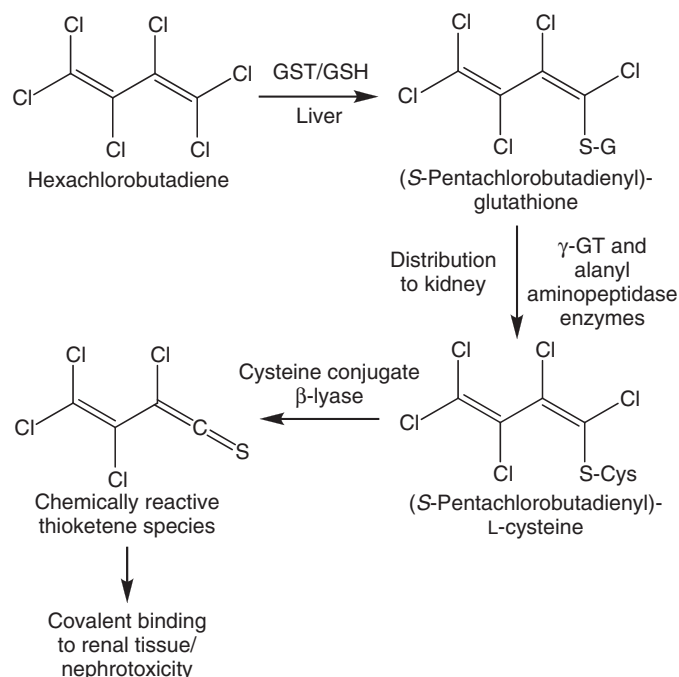


Figure 4.14 Glutathione conjugation- and cysteine β -lyase-dependent bioactivation of hexachlorobutadiene, leading to reactive metabolite formation, covalent binding to renal proteins, and nephrotoxicity.

of the renal proximal tubule [216]. The metabolism of hexachlorobutadiene to a GSH adduct occurs in the liver where GST catalyzes the conjugation with GSH eliminating one chlorine atom and forming (*S*-pentachlorobutadienyl)-GSH. Then, (*S*-Pentachlorobutadienyl)-GSH, which is not chemically reactive, either is eliminated in bile or undergoes interorgan transport to the kidney where it is metabolized further by the mercapturic acid pathway enzymes by sequential cleavage of glutamic acid and glycine residues leading to the cysteine-*S*-conjugate, namely, (*S*-pentachlorobutadienyl)-L-cysteine [21]. (*S*-Pentachlorobutadienyl)-L-cysteine can undergo detoxification by N-acetylation via NAT leading to mercapturic acid or undergo bioactivation by renal cysteine conjugate β -lyase leading to the formation of a chemically reactive thioketene species, which covalently binds to cellular macromolecules leading to nephrotoxicity (Fig. 4.14).

4.6.6 α -Naphthylisothiocyanate-Induced Intrahepatic Cholestasis

An example of hepatic cholestasis that is proposed to be mediated by phase II GSH adduct formation of a corresponding reactive chemical comes from studies with the model compound ANIT. ANIT is a chemical which induces intrahepatic cholestasis, hepatocellular and biliary epithelial cell necrosis, bile duct obstruction, and biliary epithelial cell hyperplasia in rats [217] and is often used as a model reagent for human intrahepatic cholestasis. The isothiocyanate moiety of ANIT is chemically reactive and reacts readily with GSH in the liver forming an unstable thiocarbamoyl-GSH adduct

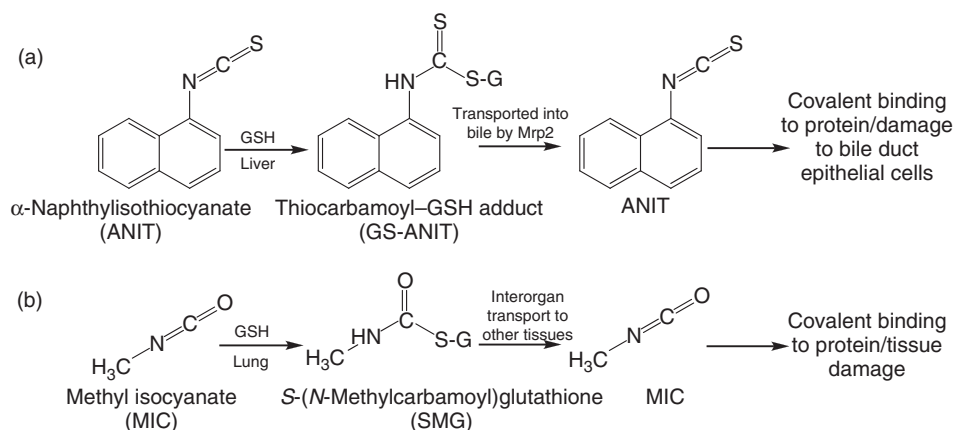


Figure 4.15 Proposed schemes for the mechanism of cholestasis and tissue damage mediated by (a) ANIT and (b) MIC, respectively, via conjugation with GSH.

(GS-ANIT) within the hepatocytes (Fig. 4.15a) [26]. ANIT is cytotoxic and induces morphological and functional changes in hepatocytes and leads to GSH depletion and the release of cytoplasmic enzymes [218]. The proposal for the mechanism of toxicity to biliary epithelial cells occurs whereby GS-ANIT is transported into bile by Mrp2, where the conjugate then dissociates back to ANIT and GSH [219]. The free ANIT becomes concentrated in the bile where it damages bile duct epithelial cells and induces cholangitis, followed by intrahepatic cholestasis [219]. Evidence toward this hypothesis of ANIT-induced cholestasis in rats comes from studies showing that Mrp2-deficient TR(-) rats were protected from ANIT-induced cholestasis. In addition, results from studies with hepatic GSH-depleted rats (via treatment with buthionine sulfoximine) showed protection against hepatotoxic doses of ANIT [220]. In summary, phase II conjugation of ANIT with GSH leads to the formation of reversible GS-ANIT conjugate that allows for the exposure of high concentrations of ANIT to the biliary epithelium and subsequent intrahepatic cholestasis.

Methyl isocyanate (MIC) is another chemically reactive, highly toxic chemical that causes multiorgan system toxicity (respiratory, cardiovascular, gastrointestinal, musculoskeletal, and immunological) in part via reaction with GSH, leading to the formation of an unstable GSH adduct [27,221]. MIC is a volatile liquid that is reactive with the cysteinyl thiol of GSH, and on inhalation of the vapor reacts with GSH in the lung forming the carbamate thioester product, namely, S-(N-methylcarbamoyl)-glutathione (SMG; Fig. 4.15b) [222]. Intraperitoneal administration of MIC to rats results in the excretion of SMG in bile and the corresponding mercapturate in urine [27,221]. The SMG conjugate is less reactive than MIC; however, it is known to be able to function as a carbamoylating agent under physiological conditions and to be cytotoxic to hepatocytes during incubation [223]. SMG formed in the lung is proposed to cross the alveolar-blood barrier and enter the systemic circulation where it undergoes interorgan transport to other tissues and mediates extrapulmonary toxicities after dissociating into MIC and GSH [221]. Thus, similar to ANIT and its thiocarbamoyl-GSH adduct, the GSH adduct of MIC formed *in vivo* may serve as an interorgan transport vehicle for MIC to target organs of toxicity separate from the site of GSH adduct origin in the lung [27].

4.6.7 S-Acyl-Glutathione Thioester Adducts of Carboxylic-Acid-Containing Drugs

As discussed in the section on acyl-CoA-thioester-mediated bioactivation, carboxylic-acid-containing drugs can be metabolically activated to chemically reactive *S*-acyl-CoA thioesters (as well as intermediate acyl-adenylates) that are able to transacylate the cysteinyl thiol of GSH, forming drug-*S*-acylated-GSH (*S*-acyl-GSH) adducts [34]. *S*-Acyl-GSH adducts are increasingly being shown as metabolites of carboxylic-acid-containing compounds *in vivo* and *in vitro*. They have been detected in the bile of dosed rats and from studies with rat and human hepatocytes incubated with bile acids [223], arylacetic acids [224,225], 2-arylpropionic acids [28], and fibric acids [226].

S-Acyl-GSH thioester derivatives are chemically reactive and have been shown to readily transacylate NAC *in vitro* [25]. Thus, it was demonstrated that when a range of 11 GSH thioesters of structurally varied carboxylic acid drugs were incubated with NAC in potassium phosphate buffer (pH 7.5, room temperature), the formation of the corresponding *S*-acyl-NAC conjugates occurred at a relative rate that depended on the extent of substitution at the α -carbon position next to the carbonyl carbon of the thioester linkage. The rank order of transacylation reactivity with NAC forming the corresponding *S*-acyl-NAC-thioesters was shown to be phenoxyacetyl- > arylacetyl- > 2-phenylpropionyl- $\approx$ α,α -dimethyl-phenoxyacetyl- > α,α -dimethyl-substituted-*S*-acyl-GSH. In these studies, the phenoxyacetyl-*S*-acyl-GSH derivatives tested were $\sim$ 3- and 26-fold more reactive with NAC than the arylacetic-acid- and profen-*S*-acyl-GSH derivatives examined, respectively, and no significant *S*-acyl-NAC thioester formation was observed with the α,α -dimethyl-substituted *S*-acyl-GSH derivative (gemfibrozil-*S*-acyl-GSH). Such results indicate that drug-*S*-acyl-GSH derivatives might be able to transacylate protein nucleophiles *in vivo*, such as on exposure to hepatobiliary proteins after excretion and concentration in bile, and thereby contribute to covalent binding of acidic drugs to tissue proteins; however, no experiments have been performed to date to test this proposal [100].

S-Acyl-GSH thioester derivatives can be degraded by γ -GT via cleavage of the γ -glutamyl group leading to *S*-acyl-cysteinyl-glycine (*S*-acyl-cys-gly) thioester-linked products that spontaneously rearrange to the corresponding *N*-acyl-cys-gly amide-linked conjugates (Fig. 4.16) [227–229]. Further dipeptidase-mediated cleavage of glycine leads to *N*-acyl-cys amide products. These cys-gly and cys amide products have been detected in the bile and urine of dosed animals. From the examples provided above, drug-*S*-acyl-linked-NAC adducts would not be expected to be the adducts of interest examined for in urine as markers of drug-*S*-acyl-GSH adduct formation occurring *in vivo*. Drug-*N*-acyl-cys or *N*-acyl-cys-gly derivatives should be employed as markers of *S*-acyl-GSH adduct formation occurring *in vivo* when studying bile and/or urine extracts obtained from preclinical species or patients dosed with carboxylic-acid-containing drugs that may become metabolically activated by *S*-acyl-CoA formation since the corresponding mercapturate derivatives, except for one literature report [230], most often are not detected *in vivo*.

In addition to the potential transacylation of protein nucleophiles by *S*-acyl-GSH thioesters, the formation of drug-*N*-acyl-cys or *N*-acyl-cys-gly free-sulphydryls and subsequent exposure to the extracellular canalicular membrane of the bile duct epithelium and/or the external surface of the proximal renal tubules of the kidney, as well as the increased concentration of γ -GT and dipeptidase enzyme activity in those tissue

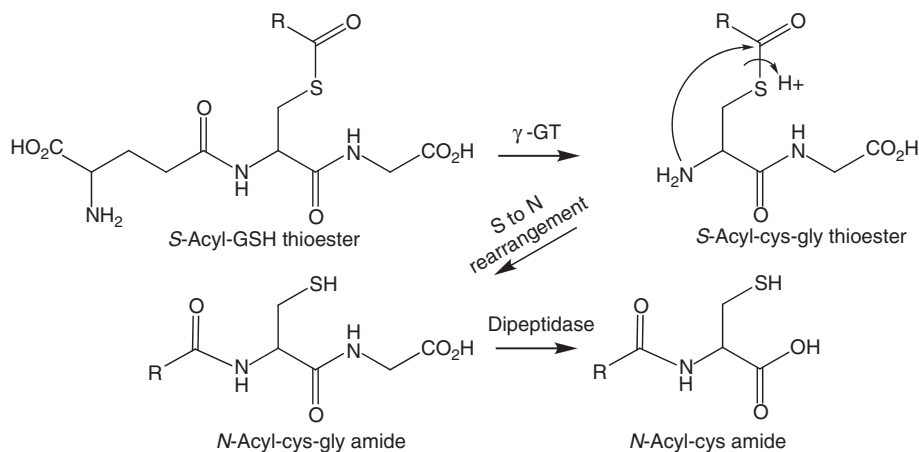


Figure 4.16 Proposed scheme for the γ -GT- and dipeptidase-mediated degradation of *S*-acyl-GSH thioesters, leading to the formation of *N*-acyl-cys-gly and *N*-acyl-cys amides, respectively.

sites [231], could potentially lead to these sulfhydryl derivatives covalently binding via disulfide bond formation to protein-cysteinyl-sulfhydryls. Examples of drugs containing a free-sulfhydryl moiety that are believed to be responsible for allergic rash due to drug–protein disulfide-linked adducts mediating immune reactions are the angiotensin-converting enzyme inhibitor captopril and the immunosuppressive agent D-penicillamine. Potentially, *N*-acyl-cys and *N*-acyl-cys-gly metabolites of acidic drugs formed from precursor *S*-acyl-GSH adducts could be contributors to covalent binding to protein *in vivo* and subsequent idiosyncratic drug reactions of some carboxylic-acid-containing drugs linked to liver and kidney toxicities, for instance, BNX [88] and diclofenac [10].

In summary, as demonstrated by the examples discussed above, the phase-II-mediated transfer of GSH to a xenobiotic does not always function to detoxification but, by contrast, sometimes leads to the formation of toxic GSH adducts that mediate oxidative stress and/or covalent binding to macromolecules, including DNA and protein.

4.7 ACYL-S-CoA FORMATION

A requirement for phase-II-mediated conjugation reactions of carboxylic-acid-containing compounds, for example, amino acid conjugation with glycine or glutamine, taurine amide formation, and carnitine ester formation, is that they must first be activated to *S*-acyl-coenzyme A (*S*-acyl-CoA) thioester-linked intermediates [232–235]. Therefore, acidic drugs are often substrates for biotransformation by *S*-acyl-CoA formation leading to the generation of chemically reactive electrophiles that serve as necessary substrates for conjugating enzymes such as acyl CoA:amino acid *N*-acyltransferase (Fig. 4.17) [34,58,62,236–238]. However, chemically reactive *S*-acyl-CoA thioesters have also been shown to mediate the transacylation of cellular protein nucleophiles and are therefore similar to reactive acyl glucuronides,

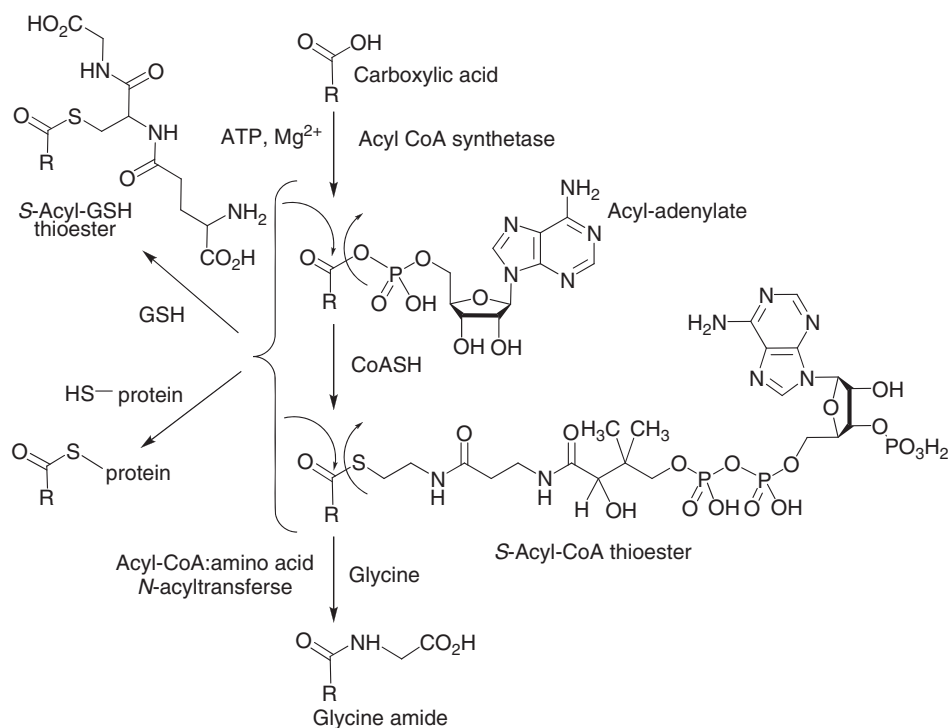


Figure 4.17 Acyl-CoA-synthetase-mediated formation of *S*-acyl-CoA thioesters via the reactive acyl-adenylate intermediate, leading to the transacylation of GSH and protein nucleophiles.

proposed to mediate idiosyncratic allergic toxicity for a range of acidic drugs [34,50,51,237,238].

S-Acyl-CoA thioester formation is catalyzed by acyl CoA synthetases (EC 6.2.1.1–EC 6.2.1.3) located in the endoplasmic reticulum, the outer membrane of mitochondria (long-chain fatty acid acyl CoA synthetases), and the mitochondrial matrix (short-, medium-, and branched-chain acyl CoA synthetases). The acyl-CoA-synthetase-mediated catalysis of *S*-acyl-CoA thioester formation requires the cofactors ATP and coenzyme A (CoASH) and proceeds via a two-step reaction mechanism involving the formation of a chemically reactive acyl-adenylate intermediate (acyl-AMP; Fig. 4.17) [239]. Unlike short-, medium-, and long-chain endogenous fatty acid *S*-acyl-CoA thioesters, which are efficiently metabolized by fatty acid β -oxidation, xenobiotic *S*-acyl-CoA thioester derivatives, such as those formed from the metabolism of profen- and arylacetic-acid-containing drugs, are not degraded by fatty acid β -oxidation. Therefore, if xenobiotic *S*-acyl-CoA thioesters are not eliminated by conjugation with glycine or taurine, these reactive derivatives might accumulate in the cell and lead to the transacylation of protein nucleophiles resulting in the formation of drug–protein adducts [240]. The potential of chemically reactive xenobiotic *S*-acyl-CoA thioesters to mediate the toxicity of certain carboxylic-acid-containing drugs is not known; however, increasing investigations on the chemical and biochemical properties of these intermediates performed over the last two decades have provided necessary insight into their potential importance

with regard to mediating covalent binding to protein, and hence potential toxicity [34,58,233,239,241].

4.7.1 *S*-Acyl-CoA Thioesters

The inherent chemical reactivity of *S*-acyl-CoA intermediary metabolites of carboxylic acid-containing drugs is due to the presence of an electrophilic carbonyl carbon at the *S*-acyl linkage [242]. Because of their large size and amphipathic properties, xenobiotic acyl CoA thioesters do not leave the intracellular space but serve as substrates for phase II metabolism by conjugation with amino acids, carnitine, and sometimes glycerol esters, yielding hybrid triglycerides [243]. Again, these transacylation-type conjugation reactions are enzyme mediated and require a chemically reactive thioester carbonyl carbon that leads to amide or ester product formation. A growing number of studies have shown that xenobiotic *S*-acyl-CoA thioesters are chemically reactive intermediates and are able to transacylate endogenous nucleophiles such as glycine; protein amino, hydroxyl, and sulfhydryl groups; and the cysteinyl sulfhydryl of GSH. For example, salicylic acid *S*-acyl-CoA thioester was shown to react nonenzymatically with glycine, leading to the formation salicyluric glycine amide [244]. Results from additional studies have demonstrated that long-chain acyl CoA derivatives including palmitoyl CoA, arachidonyl CoA, and retinoyl CoA react nonenzymatically with cysteinyl sulfhydryls of proteins and peptides [245–247]. Many recent examples of chemically reactive *S*-acyl-CoA thioesters from both endogenous and xenobiotic origins include those formed from the bile acid cholic acid [248]; the hypolipidemic drugs clofibric acid [25], bezafibrate [240], and nafenopin [240,249]; and the NSAIDs ibuprofen [28], flunoxaprofen [250], naproxen [251], tolmetin [234], and zomepirac [235]. The prediction of *S*-acyl-CoA thioesters for their ability to covalently bind to protein based on their chemical structures has been examined [252]. It was determined that similar to the chemical reactivity of acyl glucuronides (discussed above), the *in vitro* chemical reactivity of *S*-acyl-CoA thioester derivatives with biological nucleophiles, such as GSH, decreases with increasing substitution at the carbon adjacent to the carbonyl carbon of the carboxylic acid moiety [252]. Thus, the chemical reactivity of eight structurally varied synthetic *S*-acyl-CoA derivatives with GSH was tested *in vitro* [252]. The *S*-acyl-CoA thioesters studied were prepared from the carboxylic acids (*R*, *S*)-ibuprofen, clofibric acid, indomethacin, fenbufen, tolmetin, salicylic acid, (*R*, *S*)-2-phenoxypropionic acid, and 4-chloro-2-methylphenoxyacetic acid (MCPA). The chemical structures (Table 4.2) of these acids differ from one another by the substitution of methyl groups at the α -carbon, by having an oxygen atom in place of a methylene group at the β -position, and/or by the location of an *o*-hydroxy group on a benzoic acid moiety. Results from reactivity experiments showed that the relative reactivity of the *S*-acyl-CoA derivatives with GSH (0.1M potassium phosphate buffer, pH 7.4, 37°C) was dependent on (i) the increased extent of methyl group substitution at the α -carbon leading to decreased rates of reaction, (ii) the placement of an oxygen atom in place of the β -position methylene group leading to increased reactivity, and (iii) *o*-hydroxy group substitution of benzoic-acid-type acids, which leads to increased reaction rate forming the corresponding *S*-acyl-GSH derivatives. Therefore, the rank order of reactivity for the *S*-acyl-CoA thioester derivatives tested with GSH was phenoxyacetic acid > *o*-hydroxybenzoic acid

$\sim(R, S)$ -2-phenoxypropionic acid > arylacetic acid derivatives > 2-methyl- (R, S) -2-phenoxypropionic acid $\sim(R, S)$ -2-PPA. As a specific example, it was shown that the S -acyl-CoA thioester derivative of the phenoxyacetic acid MCPA was approximately fourfold more reactive than salicylic acid S -acyl-CoA and was 120-fold more reactive than ibuprofen- S -acyl-CoA in reactions with GSH, leading to S -acyl-GSH thioester adduct formation.

A strong relationship between S -acyl-CoA formation and covalent binding to protein *in vitro* and *in vivo* has been shown [238,249,253,254]. For example, mechanistic covalent binding studies in rat hepatocytes were performed in order to examine the correlation between metabolism of (R, S) -2-PPA to a reactive acyl glucuronide or acyl CoA thioester that leads to covalent binding to hepatocyte protein [238]. Results showed that the time course profile of covalent binding of radioactive molecule to protein correlated with 2-PPA- S -acyl-CoA formation and not with 2-PPA-1- β - O -G formation. In addition, *in vitro* studies in rat hepatocytes incubated with radioactive (R)- or (S)-2-PPA isomers showed enantioselective covalent binding to protein ($R/S = 4.5$) that correlated with enantioselective 2-PPA- S -acyl-CoA formation ($R/S = 7.0$), but not with 2-PPA-1- β - O -G formation ($R/S = 0.7$). Such results were consistent with data from inhibition studies where the covalent binding of 2-PPA to hepatocyte protein was found to decrease by 53% in cells treated with trimethylacetic acid that resulted in a 66% decrease in 2-PPA- S -acyl-CoA formation occurred. The complete inhibition of 2-PPA-1- β - O -G formation only led to a 19% decrease in covalent binding to protein. Corresponding studies performed *in vivo* in rats showed that covalent binding of 2-PPA to liver proteins was decreased by $\sim 50\%$ in rats pretreated with trimethylacetic acid (64% decrease in hepatic 2-PPA- S -CoA exposure), whereas pretreatment with (–)-borneol, which inhibited the exposure of 2-PPA acyl glucuronide by 95%, only decreased covalent binding to hepatic protein by 23% [238]. Such results clearly demonstrate that metabolism of 2-PPA by S -acyl-CoA formation contributed to the covalent binding to protein more than did bioactivation of 2-PPA by acyl glucuronidation.

In terms of the ability of S -acyl-CoA thioesters to transacylate GSH, the enantioselective formation of S -acyl-GSH thioesters has been investigated with the chiral profen drugs ibuprofen and flunoxaprofen [28,250]. Experiments with ibuprofen led to the detection of the ibuprofen- S -acyl-GSH thioester (ibuprofen- S -acyl-GSH) during incubation with rat hepatocytes. Results obtained from the tandem mass spectrometric analysis of extracts from incubations with enantiomerically pure (R)- and (S)-ibuprofen isomers showed that ibuprofen- S -acyl-GSH formation, in addition to having a time course of formation that was consistent with the time course profile of ibuprofen- S -acyl-CoA but not with ibuprofen-1- β - O -acyl glucuronide (ibuprofen-1- β - O -G) formation, was highly enantioselective for the (R)-antipode. In addition, data from enzyme inhibition experiments demonstrated that inhibition of ibuprofen- S -acyl-CoA formation (by coincubation with lauric acid), and not ibuprofen-1- β - O -G production (by coincubation with [–]-borneol), led to a corresponding decrease in ibuprofen- S -acyl-GSH formation. Therefore, chemically reactive ibuprofen- S -acyl-CoA thioester, and not the ibuprofen-1- β - O -G metabolite, led to the transacylation of hepatocyte GSH. Results from similar studies with (R)- and (S)-flunoxaprofen showed the highly stereoselective transacylation of GSH by (R)-flunoxaprofen forming flunoxaprofen- S -acyl-GSH during incubation with rat hepatocytes, which provided further evidence for the primary involvement of S -acyl-CoA thioester intermediates, rather than 1- β - O -acyl glucuronide

metabolites, in the bioactivation of carboxylic-acid-containing drugs leading to the transacylation of GSH [250].

4.7.2 Acyl-Adenylates (Acyl-AMP Intermediates)

The detection of intermediate mixed anhydride adenosine 5-monophosphate (AMP) adenylate derivatives of xenobiotic and endogenous carboxylic acids during the biosynthesis of their corresponding *S*-acyl-CoA thioesters has been demonstrated [255–257]. Acyl-AMP intermediates, such as those detected from the metabolism of the bile acid cholic acid, are chemically reactive because they have an electrophilic carbonyl carbon in the mixed anhydride linkage that can react nonenzymatically with nucleophiles. Recently, bile acid acyl-AMP intermediates were shown to be reactive in transacylation-type reactions with peptides and proteins and with the thiol group of GSH, leading to the formation of bile acid *S*-acyl-GSH conjugates [29,257,258]. From incubations of cholic acid with rat liver microsomes fortified with cofactors for acyl CoA formation (ATP, CoASH, and Mg^{2+}), cholyl-acyl-AMP was detected in concentrations similar to the cholyl-*S*-acyl-CoA thioester [256]. Further studies showed that the reactivity of cholyl-acyl-AMP and cholyl-*S*-acyl-CoA with GSH in buffer under physiological conditions (pH 7.4, 37°C) resulted in almost identical rates of cholyl-*S*-acyl-GSH formation. Therefore, both the *S*-acyl-CoA thioester and the acyl-AMP intermediate would be predicted to be important with respect to the transacylation of protein nucleophiles, leading to covalent binding to protein. In addition to endogenous bile acids, xenobiotic carboxylic acids, for example, of the anticonvulsant drug valproic acid and the NSAID ibuprofen are also metabolized to detectable acyl-AMP intermediates. The acyl-adenylate intermediate of valproic acid was shown to be formed in incubations with rat liver mitochondria (fortified with ATP and CoASH) and rat hepatocytes where it exists in part in the nonbound form to the acyl CoA synthetase enzyme [259]. Similar observations were made with (*R*)-ibuprofen, during incubations with rat liver mitochondria (also fortified with ATP CoASH) [255], where the corresponding acyl-adenylate intermediate was detected. For both valproyl-AMP and ibuprofenyl-AMP, their chemical reactivity with GSH or protein nucleophiles was not investigated.

In summary, carboxylic-acid-containing compounds can be bioactivated by acyl CoA synthetases leading to the generation of chemically reactive acyl-AMP and *S*-acyl-CoA intermediates that are able to mediate the transacylation of protein nucleophiles and therefore may be responsible, in addition to reactive acyl glucuronides, for contributing to covalent binding to protein that potentially leads to the initiation of untoward hypersensitivity reactions [34,58].

4.8 CONCLUSIONS

It is clear from the examples provided above that the metabolism of xenobiotics to chemically reactive intermediates is a major concern due to the potential of reactive metabolites to covalently bind to DNA or protein in the body leading to carcinogenesis, acute tissue damage, and/or immune-based idiosyncratic toxicities. In most cases, phase-II-mediated biotransformation of xenobiotics and/or xenobiotic metabolites leads to their deactivation, clearance, and detoxification by converting them to increasingly

hydrophilic substances that are efficiently excreted from the body. By contrast, this chapter has dealt with many cases of known mechanisms involved in the bioactivation of xenobiotics by phase II enzymes to chemically reactive metabolites and the toxicological significance that results from their covalent binding to cellular macromolecules including nucleic acids and proteins. Glucuronidation of carboxylic-acid-containing drugs and arylhydroxamic-acid-containing compounds can generate electrophilic acyl glucuronides and N–O-linked glucuronides, leading to allergic toxicity and carcinogenicity, respectively. There have been increasing examples of chemically reactive acyl glucuronide metabolites that have led to a greater appreciation and understanding of the potential role of glucuronidation in the carboxylic acid drug immune-mediated toxicity. O-Sulfonation of allylic and benzylic alcohol-, and arylhydroxylamine-containing compounds catalyzed by SULTs leads to highly electrophilic carbocations and nitrenium ions, respectively, that react with cellular DNA and protein and are mutagenic and carcinogenic in preclinical species and humans. N-Acetylation of hydrazines and aromatic amines, together with cytochrome P450-mediated bioactivation, leads to the formation of reactive acylating radicals and acetoxylaromatic amines, respectively, which react covalently with cellular macromolecules that mediate liver damage and carcinogenicity. Conjugation of reactive metabolites with GSH in the liver sometimes leads to GSH adducts that undergo further bioactivation after transport to the kidney by enzymes of the mercapturic acid pathway to chemically reactive, nephrotoxic metabolites. Therefore, some phase II metabolites, for instance, acyl glucuronides and GSH adducts, may distribute to organs different from the site of formation in the liver, leading to covalent binding to protein and subsequent extrahepatic toxicity. To date, there is strong evidence that the metabolism of carboxylic-acid-containing compounds by acyl CoA formation leads to the generation of reactive *S*-acyl-CoA and/or acyl-adenylate intermediates that can transacylate tissue proteins and are therefore potentially linked to immune-mediated toxicity. However, definitive evidence that these reactive intermediates mediate toxicity is not yet available, even though data on arylacetic-acid-containing NSAIDs demonstrates a causative association with reactive *S*-acyl-CoA, and/or acyl glucuronide, formation and hypersensitivity reactions in patients. The role of phase I oxidative enzymes such as cytochrome P450s often has an important part in the formation of protoxicants that has a determining effect on glucuronidation-, O-sulfonation-, N-acetylation-, and GSH-dependent bioactivation. Therefore, knowledge of the balanced coordination between phase I and phase II metabolism is important for understanding the role of reactive metabolites and subsequent toxicity in varied tissues. Just as in the case of phase I enzymes, polymorphic differences in phase II enzyme gene expression in humans are proposed to lead to differential susceptibility to potentially toxic xenobiotics. In summary, an increased understanding of the factors that influence the disposition of phase II metabolites of xenobiotics will be fundamental in understanding their potential biological, pharmacological, and toxicological effects.

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5 Covalent Drug–Protein Adducts: An Exploration of Their Role in Drug-Induced Liver and General Organ Toxicity

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5.1	Introduction	1
5.2	Intervention strategies to prevent drug-induced liver injury	7
5.3	Biomarkers of drug-induced liver injury	28
5.4	Concluding remarks	34
	References	34

5.1 INTRODUCTION

Drug-induced liver injury (DILI) represents by far the most common cause for added label warnings or drug withdrawal and accounts for ~13% of cases of acute liver failure in the United States [1–4]. Implicit in this statistic is that preclinical safety studies currently performed as part of compiling a drug registration dossier offer limited predictive value for hepatotoxicity potential in humans. Other human toxicities with poor correlations with animal studies include hypersensitivity and cutaneous reactions [5]. The form of DILI of most concern is idiosyncratic hepatocellular injury that occurs in a small percentage of patients (1/10,000–100,000) and hence is detected only in large patient populations typically after the drug has received marketing approval. Significantly, the majority of such cases (60–80%) will, without liver transplantation, result in death [6–8]. Of the drugs that have FDA black box hepatotoxicity warnings, many are known to undergo bioactivation and produce reactive metabolites capable of covalently binding to endogenous proteins (Table 5.1) leading to a hypothesis that a link exists between drug bioactivation, the formation of drug–protein adducts, and downstream toxicity [9,10]. Adding merit to this hypothesis is the finding that drugs that are poorly metabolized rarely cause idiosyncratic liver toxicity or hypersensitivity reactions. Moreover, since tissue damage (e.g., liver, kidney, blood, and skin) is typically regiospecific to the site of metabolic activation, the link between these two

TABLE 5.1 Drugs Withdrawn for Hepatotoxicity

Time Period	Drugs	Total Number
pre-1960	Cinchophen, <u>iproniazid</u>	2
1960–1969	benziodarone, ibufenac, <u>phenoxypropazine</u> , pipamazine, xenazoic acid	5
1970–1979	<u>fenclozic acid</u> , <u>mebanazine</u> , <u>nialamide</u> , oxyphenisatin	4
1980–1989	<u>benoxaprofen</u> , clomacron, clometacin, cyclofenil, <u>exifone</u> , <u>glafenine</u> , <u>isaxonine</u> , nitrofazole, <u>nomifensine</u> , perhexiline, suloctidyl, <u>tienilic acid</u> , zimelidine	13
1990–1999	<u>alpidem</u> , <u>amineptine</u> , <u>bendazac</u> , <u>benzarone</u> , bromfenac, chlormezanone, dilevalol, ebrotidine, <u>fipecide</u> , <u>moxisylyte</u> , <u>niperotidine</u> , <u>pirprofen</u> , tolrestat	13
2000–2009	exanta, pemoline, <u>nefazodone</u> , <u>troglitazone</u> , <u>lumiracoxib</u>	5

Drugs under underlined form reactive products.

Source: Adapted from Guengerich & MacDonald, 2007 [9].

events is compelling [11]. The finding, however, that drug–protein adduct formation does not always equate with toxicity provides a level of complexity which continues to provide a challenge to the pharmaceutical industry. The question essentially becomes one of how to translate such information derived in early stage drug discovery and development into a risk assessment of relevance to patients?

Idiosyncratic drug reactions generally occur within the therapeutic range, are not associated with the pharmacology of the compound, are dose dependent in susceptible patients, but often occur at disparate dose levels in different patients receiving the same drug. There is also an induction period for onset of symptoms after first exposure to the drug (greater than two weeks) but with an acute response upon drug rechallenge (<24 h) with typical immune system manifestations such as fever and eosinophilia. The unpredictable nature of these adverse events essentially results in the removal of a medication that would otherwise benefit the majority of patients. Hence, there is a desire to understand the etiology of these idiosyncratic reactions with a view to developing screening tools, which could either halt the further development of such drugs in preference for others that have an improved safety profile or tools that could prospectively identify patients who are safe to receive such medications. In the absence of these tools, the fall-back position is to design drugs with minimal potential to form reactive metabolites at least as one strategy aimed at minimizing the potential for downstream toxicities, and hence avoid the associated patient safety issues and economic consequences of drug withdrawal.

Adverse drug reactions are often referred to as either *predictable* or *unpredictable* [12,13]. Predictable adverse reactions are dependent on the on- or off-target pharmacological properties of the drug and increase in intensity with increased dose and are ameliorated when the dose level is reduced. Examples of pharmacological on-target adverse reactions include the development of hypotension (antihypertensives) or hemorrhaging (anticoagulants), essentially indicative of a suprapharmacological response. An example of a pharmacological off-target reaction is ventricular arrhythmia associated with the HERG binders, astemizole, cisapride, and grepafloxacin [14,15].

Importantly, these predictable pharmacologically based reactions are mediated by parent compound or metabolites, which are *stable* and circulate in the systemic circulation; hence metabolite quantification has recently become a more highly regulated part of the drug development process [16] and has been formalized in an International Conference on Harmonization (ICH) M3(R2) guidance (<http://www.ich.org/cache/html/501-272-1.html>). Unpredictable adverse drug reactions are considered to result from metabolic activation (“bioactivation”) of a drug (=prohaptén), which results in the formation of typically *unstable* reactive metabolites capable of binding to endogenous macromolecules; a process often referred to as *hapténization*. From an immunological viewpoint, adducted proteins are “nonself” or “modified self” products that may provoke immune responses against themselves [17]. In the case of an idiosyncratic hypersensitivity response (e.g., halothane [17,18] and tienilic acid [19]), T-cell sensitization requires that drug-modified biomolecules are taken up by antigen presenting cells and transported into the local draining lymphoid tissue, where they are processed and presented on major histocompatibility complexes (MHC). The derived antigen-experienced T-cell progeny have specific tissue-homing properties. Therefore, in the case of a hypersensitivity reaction, it is the cytotoxic mechanism elicited by T-cells and not the drug alone, which damage tissue cells [20]. This type of toxicity should be distinguished from direct, acute, reactive-metabolite-mediated toxicity, albeit they both initially involve drug bioactivation and formation of a drug–protein adduct. A good example of the latter is acetaminophen, which is very safe when taken as directed but becomes predictably toxic in overdose situations and hence does not satisfy the criteria of an idiosyncratic (highly individualized, unpredictable) immune-mediated toxicant. The mechanism of this toxicity is well understood, whereby following a high dose that depletes endogenous GSH, which would normally trap the substituted quinoneimine reactive intermediate, the quinoneimine reactive intermediate instead alkylates liver proteins some of which result in disrupting mitochondrial function leading to cell death and liver necrosis [21–23]. While not a primary focus of this review, mechanism-based inactivation (MBI) of CYP450 can represent a direct functional readout of bioactivation and can result in drug–drug interactions.

Controversy, therefore, still remains on how to view the formation of a covalent bond between drug-derived reactive intermediates and endogenous proteins since no single diagnostic test currently exists to ascertain the downstream toxicological consequences of such an event with any degree of certainty. Moreover, the mode of action of some therapeutics are actually predicated on a prior metabolic activation step [24,25] (Table 5.2), albeit for this class of compound there is awareness on behalf of medicinal chemists to focus preferentially on the drug design option of making such interactions covalent, but reversible, when possible [26]. The challenge for the pharmaceutical industry, therefore, is to develop strategies that continue to support basic science research in the area of DILI. This is especially important at a time when the currently available toolbox is largely limited to medicinal chemistry expertise to minimize the potential for drug bioactivation during lead optimization, and to the risk management strategies of the individual companies and approval bodies when assessing adverse event data arising during clinical development, pre- and post approval.

5.1.1 Idiosyncrasy of Drug Hypersensitive Reactions

The formation of a drug–endogenous protein adduct forms the basis of the haptén hypothesis of drug hypersensitivity [27]. These adducts both stimulate a T-cell

TABLE 5.2 Pharmaceuticals Covalently Interacting with Their Pharmacological Targets

Drug ^a	Reacting Functionality	Target
Amoxicillin (acylation)	B-lactam (I)	Serine-type D-Ala-D-alacarboxypeptidase
Orlistat (acylation)	Lactone (R)	Triacylglycerol lipase
Rivastigmine (acylation)	Carbamate (R)	Acetylcholinesterase
Clavulanate (acylation)	B-lactam (I)	B-lactamase
Aspirin (acylation)	Ester (R)	Prostaglandin endoperoxidase synthase
Warfarin (acylation)	Coumarin	Vitamin K epoxide reductase (warfarin sensitive)
<i>Isoniazid</i> (acylation)	Hydrazide (I)	Enol-acyl carrier protein reductase
<i>Disulfiram</i> (acylation)	Disulfide (I)	Aldehyde dehydrogenase
Fosfomycin (alkylation)	Epoxide	UDP- <i>N</i> -acetylgluc-osamine-1- carboxy-vinyl transferase
D-Cycloserine (alkylation)	Amine	Alanine racemase
Vigabatrin (alkylation)	Amine (I)	GABA-AT
<i>Exemstane</i> (metal/metabolite binding)	Methyl (I)	Aromatase
Bortezomib (metal/metabolite binding)	Boronic acid (R)	Proteasome
<i>Omeprazole</i> (disulfide bond formation)	Sulfonamide (I)	H ⁺ /K ⁺ ATPase
<i>Clopidogrel</i> (disulfide bond formation)	Thiol (I)	P2Y ₁₂ purinoreceptor antagonist
Propylthiouracil	Thiourea	Seleno-enzyme
VX-950 (hemiketal formation)	Ketoamide (R)	Serine protease hepatitis C virus NS3
<i>Gemcitabine</i> (Michael addition)	Vinyl ketone	Ribonucleosidediphosphatereductase
Floxuridine (Michael addition)	Unsaturated amide (R)	Thymidylate synthase
Finasteride (Michael addition)	Unsaturated amide (R)	5- α -reductase
<i>Selegiline</i> (Michael addition)	Aceylenic imine (I)	Monoamine oxidase-B
Vildagliptin (Pinner reaction)	Nitrile (R)	Dipeptidylpeptidase (DPP)-IV
Odanacetib (Pinner reaction) (MK-0822)	Nitrile (R)	Cathepsin K

^aProdrugs are italicized and the chemical mechanism of drug–protein interaction is placed in parenthesis: I, irreversible interaction and R, reversible interaction.

Source: Adapted from Potashman and Duggan, 2009 [25].

proliferation and provide an antigenic signal to direct the effector arm of the immune system to the tissue cells, ultimately leading to tissue damage and potential toxicity. Indeed, Castrejon *et al.* [28] recently reported on the inhibitory effect of GSH on sulphamethazole nitroso metabolite-mediated T-cell proliferation, providing evidence for the role of drug–protein adduct formation in this process. Matzinger [29] laid down the fundamental premise of the importance of a “danger signal” in the context of an immune response and essentially stated that the primary goal of the immune system is to protect against danger; in the absence of danger tolerance prevails and in the presence of danger (e.g., underlying disease state, which might be asymptomatic, or a more obvious medical condition such as a viral infection) a full immune response ensues. Uetrecht [30–32] matured this concept by proposing the “danger hypothesis” that aimed to account for the very low incidence of idiosyncratic drug reactions encountered in patients. That is, contrary to what might be a natural outcome from the hapten hypothesis, not all drugs that form protein adducts mediate an immune response that results in toxicity. Uetrecht [32] proposed that this trigger could be provided by a “danger signal” and that the susceptibility of patients is increased through a culmination of genetic, environmental, and existing health factors that make some individuals more predisposed to aberrant outcomes resulting in a subtle though significant shift of the dose–toxicity response curve to the left. In support of this conclusion, antibodies to diclofenac adducts were detected in all diclofenac-induced liver injury serum samples, but they also occurred in 60% of individuals who had received the drug without DILI [33,34]. The candidate signal(s) that could sense danger could be exogenous (e.g., bacterial antigen) [35] or endogenous, to include heat shock protein (Hsp72) and high mobility group box 1 (HMGB1), since such molecules are produced when the body is under stress (e.g., viral infections or trauma coupled to drug-induced tissue necrosis) and are able to alert the immune system to mount an appropriate immune response [36,37] (see below). In summary, drug–protein adduct formation leads to cell damage, an insult that in turn produces a “danger signal” that can ultimately result in a B-cell-mediated antibody or cytotoxic T-cell-mediated response. Interestingly, Pirmohamed *et al.* [27] speculated that in certain instances, the nature of the insult provided by the reactive intermediate to the cell may be sufficiently large so as to overcome its ability to launch an immune response [27]; as might be the case for acetaminophen. This group speculated further that it may be possible to prime patients to have greater resilience against developing immune-mediated toxicities by starting at lower doses and increasing the dose slowly, which has been a successful strategy for both lamotrigine [38] and cotrimoxazole [39]. This proposal was based on the notion that at low doses, or low levels of reactive intermediate formation, only the initial part of the immune cascade is launched, that of an interaction between a MHC-restricted antigen and a T-cell receptor, resulting in tolerance, not a full blown immune response.

In addition to the danger hypothesis proposed by Uetrecht, which is drug metabolism dependent, Pichler, Tilch, and Schnyder [20,40] have reported that drugs can interact directly with T-cell receptors *without* previous metabolism or need to bind to proteins, and have coined this as “pharmacological interaction” (p-i) hypothesis [41,42]. This was based on the observation that clones of lymphocytes from patients with a history of an idiosyncratic drug reaction to sulfamethoxazole (in the lymphocyte transformation test (LTT) *in vitro*), proliferated in response to sulfamethoxazole in the absence of metabolism. According to this hypothesis, T-cells must express a T-cell receptor that can bind drug and induce a stimulatory signal with a low threshold for activation.

In addition, the T-cell receptor needs to interact with the MHC on antigen presenting cells in order to enhance the response to the drug. Lymphocytes from CBZ- (carbamazepine) treated patients also were observed to proliferate with therapeutic concentrations of CBZ (5–10 μM [43]) *in addition to* the CBZ-10,11-epoxide and 10-hydroxy-CBZ metabolites (but not other CBZ metabolites), which indicates that patients have T-cells that are specific for CBZ stable metabolites, a finding that is compatible with the p-i hypothesis. This hypothesis is also applicable to ximelagatran, a peptide like molecule [32], which is believed to form a low affinity association directly with MHC molecules as defined in a competition peptide–MHC binding assay [44]. These overall observations have led Wu *et al.* to speculate that a T-cell response, once activated, can become more generalized across a drug structure class. They cite administration of oxcarbazepine, a prodrug of 10-hydroxy-CBZ, to CBZ-hypersensitive patients as potentially leading to reactivation of preexisting memory T-cells and the development of a hypersensitivity reaction [45], and also the potential contraindication of structurally related antimicrobial sulfonamides in sulfonamide allergic patients [28].

5.1.2 Skin Reactions

The development of a skin reaction is probably the most obvious phenotype of a hypersensitive drug reaction, but how do drugs taken orally mediate such reactions? One proposal is that following distribution to the skin, skin cells are capable of metabolically activating drugs leading to allergic skin reactions [46], which is consistent with the hypothesis that tissue damage is regiospecific to the site of metabolic activation (see above). This has been postulated for the anti-HIV pharmaceutical, nevirapine (nevirapine $\rightarrow$ 12-hydroxylation $\rightarrow$ quinonemethide) [47,48], and the anticonvulsants lamotrigine (lamotrigine $\rightarrow$ 4'-5'- or 5'-6'-epoxidation $\rightarrow$ thioether conjugates) [49] and CBZ (CBZ $\rightarrow$ CBZ-10,11-epoxide and CBZ-2,3-epoxide [50,51]) (Fig. 5.1). In a second proposal, selective migration of T-cell populations, potentially arising from drug bioactivation in the liver, kidney, or blood, to the skin, is believed to be mediated via the skin-homing receptor and cutaneous lymphocyte antigen, which has been shown to be present on T-cell clones from patients hypersensitive to, for example, lamotrigine [52]. Clearly it is difficult to weigh the relative importance of these last two pathways for those compounds that are metabolized to reactive intermediates in both the skin and liver. Lastly, chemokine receptors (CCR) are also believed to play a role in cell-selective cutaneous migration. Toxic epidermal necrolysis (TEN) is a blistering reaction caused by the cutaneous infiltration of cytotoxic CD8^+ T cells [53], whereas a different population, CD4^+ T cells, appear to be associated with generalized

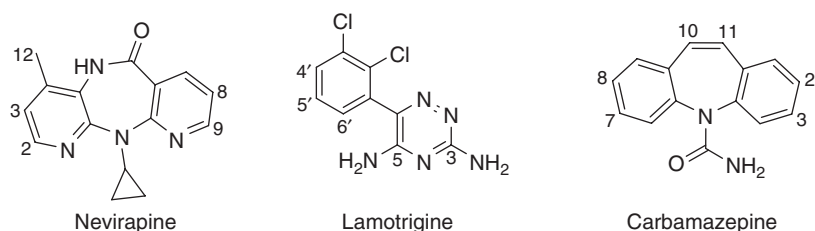


Figure 5.1 Structures of nevirapine, lamotrigine, and carbamazepine.

exanthematous pustulosis [42,54]. Wu *et al.* [55] have investigated the CCR-mediated trafficking of CD8⁺ and CD4⁺ T cells into epidermal and dermis cells where such cells were shown to persist in blood of CBZ-hypersensitive patients. While CCR8 was determined to be responsible for the steady-state trafficking of memory T cells into healthy skin [56,57], CCRs such as CCR4 and CCR10 were determined to mediate selective entry of T cells into inflamed skin [55]. It appears, therefore, that both direct (bioactivation in the skin) and indirect (bioactivation by internal organs) mechanisms are potentially at play in the mediation of skin reactions.

5.2 INTERVENTION STRATEGIES TO PREVENT DRUG-INDUCED LIVER INJURY

The foregoing text should lead the reader to conclude that the science describing the link between drug–protein adduct formation and downstream toxicity is rapidly developing. Researchers agree that the formation of drug protein adducts has the potential to represent a pivotal first step in a spiral toward endpoint toxicity and that forming reactive intermediates is, therefore, an undesirable occurrence and should be avoided when selecting drug candidates. Pragmatically, this could be rephrased to state that in the absence of a general understanding of the link between bioactivation and toxicity, the formation of reactive intermediates should be minimized as much as possible within reasonable discovery timeframes during the selection of drug candidates. In 2004, Merck & Co. translated these statements in to a strategy that could be adopted by the pharmaceutical industry [10]. Of note, Merck & Co. no longer routinely conducts covalent binding studies, but the relative merit and limitations of such studies are discussed since they are used by other companies (see below). Regardless, the core of this strategy, that of minimizing the potential for metabolic activation during the lead optimization stage of drug design, remains intact and has been broadly adopted in the pharmaceutical industry. The functional execution of the strategy, however, has been tailored by individual companies to meet their specific organizational needs, scientific expertise, and resource availability, some of which is now described.

5.2.1 Strategy to Minimize Potential for Reactive Intermediate Formation

The intent of the strategy initially proposed by Merck & Co. was to attenuate the potential toxicity risks associated with compounds capable of eliciting both the direct *and* idiosyncratic forms of reactive-intermediate-mediated liver toxicities. This was achieved by measuring the extent of the bioactivation process *in vitro* (covalent binding studies) in a variety of preparations (liver microsomes and hepatocytes from preclinical species and human to account for the full range of metabolic reactions and potential species differences in metabolism) and to elucidate the chemical mechanism by which bioactivation occurred. This latter process was facilitated by the use of chemical trapping agents, which enabled stabilization of otherwise highly reactive electrophilic species allowing their structures to be characterized and, thereafter, mechanisms postulated so as to infer how such species were initially formed. In so doing, lead chemical structures, otherwise already optimized for pharmacological potency *in vitro* and efficacy in preclinical studies were, in addition, optimized with respect to having minimal potential to form reactive intermediates. Covalent binding studies were also performed

in vivo in the rat in order to gain an understanding of how covalent binding observed *in vitro* in liver preparations translated into covalent binding observed in the liver homogenate and plasma of a whole animal following oral dosing. Other factors such as the anticipated dose level, the target population, and the severity of the disease were all additional considerations of this strategy—one which aimed to reduce metabolic activation in a systematic structure–reactivity mode of operation, but not one aimed necessarily at predicting toxicity since there are many other mediators of toxicity that are not dependent on drug bioactivation (e.g., inhibition of mitochondrial function, disruption of intracellular calcium homeostasis, activation of apoptosis, and inhibition of specific enzymes or transporters [58]), and it is known that covalent binding does not always track with toxicity. For these reasons, which are admittedly complex, the intent was for research groups to minimize covalent binding to <50 pmol drug equivalents per milligram protein, and to view this as a target threshold and not a “go” or “no-go” criteria. This value was chosen based on an ~20-fold margin over a covalent binding level of ~1 nmol drug equivalents per milligram liver protein associated with some model hepatotoxins in rodents (e.g., APAP, bromobenzene, furosemide, or 4-ipomeanol). This value also corresponded to a level of radioactivity that was ~10 times that of the experimental background under normal conditions, and thus provided a suitable dynamic range for measurement of covalently bound drug–protein adducts. The approach of BMS in using a fluorescently tagged trapping agent (dGSH) highlights the trend to move away from routinely synthesizing numerous radiolabeled compounds to conduct covalent binding studies [59–63].

At the outset of formulating this strategy, it was known that some compounds bind covalently to proteins yet fail to cause tissue damage. In what has now become a hallmark study, Tirmenstein and Nelson [64] evaluated the hepatotoxicity of APAP (4'-hydroxyacetanilide) and its regioisomer 3'-hydroxyacetanilide (AMAP) in the mouse and determined that APAP was hepatotoxic whereas AMAP was not; this despite the electrophilic metabolite of AMAP (a substituted benzoquinone) providing similar levels of covalent binding to that of the reactive product of APAP oxidation (a quinoneimine derivative). This lack of concordance between covalent binding and toxicity led skeptics of the strategy proposed by Merck & Co. [10] to point out that, in adopting this strategy, a company could potentially “throw out” perfectly good compounds. Proponents of this strategy, however, highlight that they acknowledge this discordance but armed with the experimental tools and means to rapidly overcome such a potential liability; it is better to bring a compound forward for expensive, regulated, drug development studies that is optimized in all regard as a pharmaceutical but with limited potential to form reactive intermediates, even in the absence of truly understanding the actual toxicological risk associated with forming a drug–protein adduct. Indeed, the medicinal chemistry “tool set” used to overcome metabolic activation is essentially the same as that used to optimize structure–pharmacokinetic/pharmacodynamic relationships [11,14] and can be performed very rapidly. The intent of the strategy proposed by Merck & Co. could therefore be summarized by the following statements.

1. The focus of the strategy was on “minimizing” bioactivation and not on “predicting” toxicity. The supposition was that by reducing covalent binding to <50 pmol drug equivalents per milligram protein (or as low as reasonably possible) in a variety of metabolism vectors *in vitro* and *in vivo*, one would markedly attenuate the safety risk potentially associated with metabolic activation (see taranabant

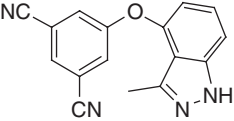
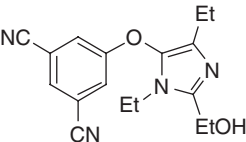
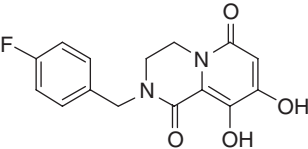
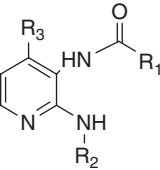
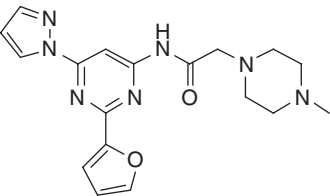
example; see below). As a consequence, the origin of this target threshold of binding was clearly somewhat arbitrary, not least because it related *in vitro* binding to *in vivo* binding without any regard to kinetically modeling the correlation.

2. To account for those instances where the bioactivation of a particular chemotype was inextricably linked to its pharmacology, or where there was overlap between pharmacological structure–activity relationship and bioactivation-mediated structure–reactivity relationship, additional qualitative factors were used to assess safety risk. This qualitative approach was premised on the notion that predictive toxicology based on covalent binding studies was not considered at the time to be feasible, not least because the APAP/AMAP example (see above) had shown this to be the case. When needing to undertake qualitative estimates of safety risk, however, this group did leverage *in vivo* parameters (e.g., estimated dose level, metabolic routes) when assessing covalent binding derived from *in vivo* studies, and metabolic turnover (e.g., extent and routes) when assessing *in vitro* covalent binding [65]; indeed such efforts generally date back to the 1990s [66]. Recently, more stringent attempts have been made to leverage covalent binding data to *predict* hepatotoxicity and the relative merits of these approaches are discussed (see above).
3. The strategy acknowledged that some compounds bind covalently to proteins, yet fail to cause tissue damage. This is probably attributable to multiple factors, which include:
 - a. Subcellular targets for adduct formation are different [67]. The binding of “critical” proteins will disrupt cell function, but the binding of “noncritical” proteins will not. A web site now exists (http://tpdb.medchem.ku.edu:8080/protein_database/) that catalogs the known protein targets of reactive intermediates for 18 well-studied chemicals and drugs of known toxicity [68], but the challenge still remains to discern which proteins, when disrupted, represent robust prognostics of cell function and potential downstream toxicity. Of note, to create an effective immunogen, an adducted protein of >50 kDa is considered to be required.
 - b. Protein adducts may differ in their “signaling” of an electrophilic insult, and recruitment of appropriate cellular defenses.
 - c. Some covalent binding may represent a detoxification or cytoprotective process. For instance, in mice dosed with APAP, GSH levels decreased 60% before serum-derived acetaminophen-cysteine (APAP-Cys; derived from enzyme-hydrolyzed serum protein) adducts were detected. In APAP overdose patients, serum-derived APAP-Cys adducts were detectable before large increases in prototypic liver function tests were observed [69].
 - d. Some drug–protein adducts may differ in their stability. The stability or persistence of a drug–protein adduct may be a critical determinant of toxicity. Studies with model electrophile probes have indicated that instability of an adduct corresponded with lack of toxicity *in vitro* [70].
4. Covalent binding is a quantitative measure of the bioactivation process and only provides an assessment of reactivity of the putative reactive intermediate toward a protein nucleophile.
 - a. When the parent drug is radiolabeled, one can assess the potential to form drug–protein adducts *in vivo* and *in vitro*.

- b. When covalent binding studies are conducted with chemical trapping agents, such studies serve the dual purpose of providing a quantitative estimate of the amount of adduct formed (e.g., by using a fluorometric assay to monitor adducts that react with dansyl glutathione (dGSH) [71] or monitoring radioactive levels of adduct formed with ^{35}S -GSH [72] or ^{14}C -cyanide [73]), and access to products that lend themselves to characterization (notably by mass spectrometry) as the precursor step for defining the mechanism by which bioactivation has occurred. This underpins a considered chemistry strategy to overcome reactive intermediate formation.
5. In isolation, covalent binding is a measure of bioactivation, not a predictor of toxicity. Of note, liver microsomes are “CYP450 rich” and represent an ideal model system to exacerbate metabolic phase I bioactivation reactions and in which to chemically trap reactive intermediates for subsequent characterization. Attenuation of metabolic activation in liver microsomes, therefore, represents a high stringency hurdle for medicinal chemists.

The following projects represent examples where metabolic activation has been overcome as part of pharmaceutical lead structure optimization. The important distinction is that for both the taranabant and the selective estrogen receptor modulator (SERM) examples, covalent binding was reduced as a part of drug optimization *without* any toxicology studies having yet initiated. This approach by Merck & Co. was premised on the fact that it is not possible to predict *a priori* whether a drug candidate that undergoes metabolic activation will cause toxicity in animals or humans. Moreover, even if a drug candidate undergoes metabolic activation, but fails to cause tissue damage during preclinical safety assessment, there remains the concern that it may elicit immune-mediated adverse events in humans (idiosyncratic toxicity, see above). This is a concern since no animal model exists for human immune-mediated toxicities, the clinical incidence of immune-mediated adverse events is vanishingly small, and hence safety issues are identified only after the patient population has become relatively large (phase 3 clinical trials and beyond), a time by which significant financial investment has already been made in the product. Indeed, this proactive approach to mitigating metabolic activation has been widely adopted by both the pharmaceutical industry and academia and representative, although by no means exhaustive, examples of this are presented in Table 5.3 [74–92] to demonstrate the broad-based adoption of this strategy across a wide range of therapeutic areas. This approach is undoubtedly related to the recognized deficiencies using preclinical species to predict clinical hepatotoxicity [5]. This is exemplified by a cross-pharmaceutical company retrospective review of candidate drugs in clinical development where 31/238 (14%) cases of hepatotoxicity were observed. Of these 31 compounds, 13 (42%) were negative in preclinical safety studies and 18 (58%) were positive, and this led to 14 compounds (45%) being terminated and 17 compounds (55%) continuing in clinical development [93]. Since compounds continued in clinical development, despite toxicity in preclinical studies, highlights the medical benefit–risk management process evident in all pharmaceutical companies. The fact that drug withdrawals have occurred as a consequence of drug-induced idiosyncratic hepatotoxicity, or clinical drug development has been stopped as a consequence of liver toxicity being observed in man, but not in preclinical species, highlights the limitations of animals as models of human toxicity mechanisms. The remaining two examples discussed, those of optimizing either a leukotriene- or an $\text{A}_{2\text{A}}/\text{A}_1$ -receptor

TABLE 5.3 Reducing Potential for Metabolic Activation as a Consideration During Drug Design

Structures	Therapeutic Area, Observation, and Outcome
	HIV (NNRTI, Pfizer): Speculated that 3-methyl-indazole could undergo proton abstraction from the methyl followed by single electron oxidation to form the imine methide. The 3-ethyl-analog was stable to reactive intermediate formation [74]
	HIV (NNRTI, Pfizer): Capravirine analog. Reactive metabolites trapped (GSH) but no further reports yet published [75]
	HIV (Integrase inhibitor, Merck & Co.): Dihydropyridopyrazine-1,6-dione series optimized for reduced covalent binding: (<50 pmol drug equivalents/mg protein/60 min incubation in rat and human liver microsomes; <5 pmol drug equivalents/mg protein in rat liver homogenate <i>ex vivo</i> [76]):
	Pain (Bradykinin B1 receptor antagonist, Merck & Co.): 2,3-Diaminopyridine (2,3-DAP) analogs were determined to be CYP3A metabolism dependent inhibitors and were also shown to extensively bind to rat and human liver microsomes (2–4 nmol drug equivalents/mg protein/60 min). Oxidation of the 2-amino group produced the highly electrophilic species, pyridine-2,3-diimine, which led to the discontinuation of this series [77,78]
	Neurodegenerative disorders, including Parkinson's and Huntington's diseases (A _{2A} receptor antagonist, Neurocrine Bioscience & Amirall Research): Concern for unsubstituted furan bioactivation led to methylation α to the furan oxygen (cf KW-6002) and bis-methyl substitution of the pyrazole moiety. This later change, in addition to replacement of the furan with a thiophene, was optimal for pharmacokinetics and pharmacodynamics [79]

(continued overleaf)

TABLE 5.3 (Continued)

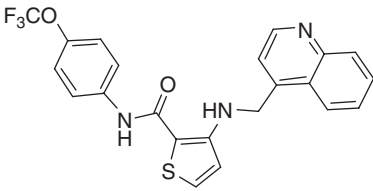
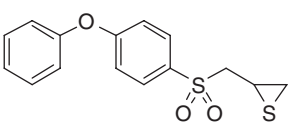
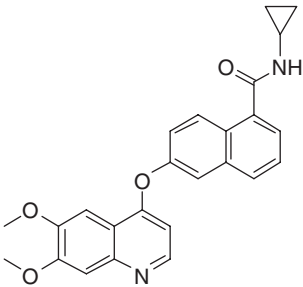
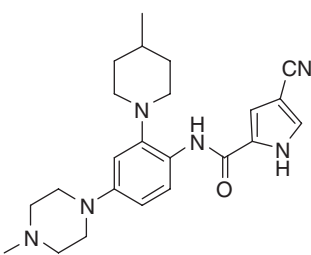
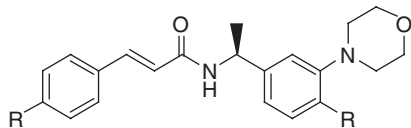
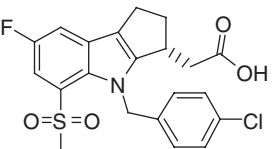
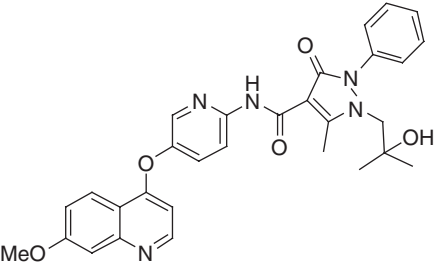
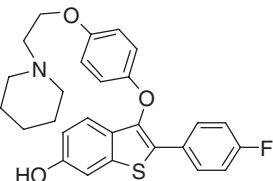
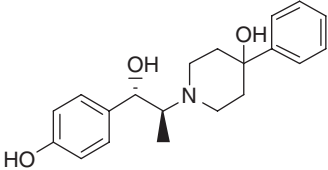
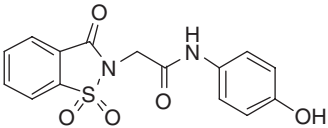
Structures	Therapeutic Area, Observation, and Outcome
	Cancer (OSI Pharmaceuticals): OSI-930 is well tolerated and efficacious in animal models and humans. GS-conjugates was consistent with thiophene bioactivation (S-oxidation to the sulfoxide). Given the severity of the therapeutic indication and the finding that a radiolabeled close was cleared from rat tissue with a half-life of 1–1.5 days, this bioactivation was not considered, in isolation, to be a major concern [80]
	Cancer & Stroke (Selective gelatinase inhibitor, University of Notre Dame, IN, USA): GS-conjugates were characterized for SB-3CT indicating that the thiirane ring had undergone bioactivation. Methyl-substituted analogs were investigated to attenuate bioactivation [81]
	Angiogenesis (Vascular endothelial growth factor-2 tyrosine kinase (VEGF) inhibitor, Amgen): This cyclopropylamide analog provided levels of covalent binding <i>in vitro</i> (human liver microsomes) of ~350 pmol drug equivalents/mg protein/60 min. The unsubstituted naphthamide had markedly attenuated levels of covalent binding (<40 pmol drug equivalents/mg protein/60 min) with retained pharmacokinetic and pharmacodynamic properties [82]
	Inflammation, including arthritis (FMS inhibitor, Janssen Research & Development): Potential to form quinonediimine type reactive intermediates led this group to (i) replace the methylpiperazine moiety with a C-linked piperidine and (ii) replace the N-linked piperidine with a cycloalkene substituent. Pharmacokinetic and pharmacodynamic properties were commensurately optimized [83]

TABLE 5.3 (Continued)

Structures	Therapeutic Area, Observation, and Outcome
	Migraine (KCNQ2 potassium channel opener, Bristol-Myers Squibb): When R=H, reactive metabolites were formed that were also implicated in the metabolism dependent inhibition of CYP3A. When R=F, CYP3A inhibition was overcome. Only F-substitution adjacent to the morpholinyl moiety significantly reduced CYP3A inhibition, indicating that CYP450 inhibition was attributable to formation of quinoneimine- and/or quinonemethide-type intermediates [84]
	Seasonal allergic rhinitis (Prostaglandin D ₂ receptor (PGD ₂) antagonist, Merck & Co.): MK-0524 was the product of optimizing earlier lead structures that formed an iminium cation. The methylsulfone in MK-0524 was postulated to destabilize the putative iminium cation due to its powerful electron withdrawing properties [85,86]
	Cancer (cMet, a receptor tyrosine kinase inhibitor, Amgen): For AMG-458, the O-aryl moiety was observed to be displaced, forming a methoxyquinoline GS-conjugate. Replacement of O with N as the linker virtually eliminated any detectable adducts. Substituents that interfered with oxygen lone pair delocalization to the aromatic system, thereby reducing potential of the alkyl substituent to serve as a leaving group, also reduced potential for GS-adduct formation [87]
	Breast cancer (SERM, University of Illinois, USA): 4'-Fluoro-desmethylarzoxifene (4'-DMA) exemplifies how 4'-fluorination resulted in a marked reduction of oxidative bioactivation to quinone-type products [88–90]

(continued overleaf)

TABLE 5.3 (Continued)

Structures	Therapeutic Area, Observation, and Outcome
	Pain (NR2B-selective antagonist, Pfizer): Phenol bioactivation was observed for CP-101,606 with commensurate formation of quinone-type products. Various phenol bioisosteres were evaluated and a pyrazole substituent was determined to be a successful replacement [91]
	Pain (Neuroscience Center of Excellence, Louisiana State University Health Center, LA, USA): Efforts were made by this group to attenuate metabolic activation and optimize the general safety profile of APAP. The 3-oxo-1,2-benzisothiazol-2-yl-1,1,-dioxide acetamide analog was evaluated in limited acute toxicity studies and demonstrated an improved liver function test profile in mice [92]

antagonist, represent a response to an *observed* toxicity, but nevertheless used the same tool box (understand the chemical mechanism of bioactivation by chemically trapping reactive intermediates and thereafter mitigate against this biotransformation) to overcome their respective structural deficits and provide a successful outcome for the project.

5.2.1.1 Optimization of Taranabant. During the discovery of taranabant (MK-0364, Table 5.4), a novel acyclic amide cannabinoid-1 inverse receptor agonist (CB1R) that was formerly in clinical development as a low dose (<10 mg once daily) compound for the treatment of obesity [94–97], reactive intermediate formation was attenuated using a structure–reactivity approach deployed across a series of closely related chemical analogs. While in this study radiolabeled analogs were prepared using a common tritiated precursor (which expedited the time lines of these studies), it might be expected that the use of dGSH [71] will also have produced similar rank order data for the extent of metabolic activation observed. During the optimization of this aryloxy series of compounds, sites of metabolic activation were identified rapidly using online LC-MSⁿ on an ion trap mass spectrometer. Following incubation of the initial phenyl analog with rat or human liver microsomes, reactive intermediates were trapped as their corresponding glutathione conjugates. Mass spectral characterization of these conjugates allowed potential sites and mechanisms of bioactivation for this compound to be proposed (phenyl oxidation → *ortho*-quinone). One strategy to reduce metabolic activation was to explore aromatic fluorine substitution as a means of blocking sites of oxidative metabolism, the aim being to take advantage of the electron-withdrawing effects of fluorine to reduce the π -electron density of the aromatic ring and hence render this moiety less susceptible to CYP450 catalyzed metabolism [98,99]. In addition, the carbon–fluorine bond is stronger than the corresponding carbon–hydrogen bond, and thus is less prone to oxidative cleavage. Unfortunately, fluorinated analogs, such as the 3,5-diF-phenyl derivative, underwent extensive metabolic activation that

TABLE 5.4 Optimization of Taranabant (MK-0364): Irreversible Binding of Radioactivity to Human and Rat Liver Microsomal Protein upon Incubation with a Series of Tritiated CB1R Inverse Agonists at a 10 μ M Concentration for 1 h in the Presence of an NADPH-Regenerating System

X	Ar	Covalent Binding to Liver Microsomal Protein ^a (pmol drug equivalents/mg protein)			
		Human		Rat	
		No GSH	5-mM GSH	No GSH	5-mM GSH
H	Phenyl (Ph)	3900 $\pm$ 300	650 $\pm$ 64	1500 $\pm$ 130	330 $\pm$ 16
H	3,5-diF-Ph	1700 $\pm$ 320	470 $\pm$ 76	840 $\pm$ 94	120 $\pm$ 47
H	2-pyridinyl	910 $\pm$ 110	290 $\pm$ 18	540 $\pm$ 21	140 $\pm$ 50
H	5-Cl-2-pyridinyl	300 $\pm$ 81	100 $\pm$ 19	190 $\pm$ 43	54 $\pm$ 17
H	5-CF ₃ -2-pyridinyl	88 $\pm$ 4	27 $\pm$ 11	110 $\pm$ 21	24 $\pm$ 5
CN	5-CF ₃ -2-pyridinyl (MK-0364)	27 $\pm$ 2	Not reported	Not reported	Not reported

The irreversible binding was assessed both in the absence and presence of 5 mM GSH.

^aTritium was incorporated in to the bi-phenyl moiety of the molecule.

was accompanied by oxidative dehalogenation and formation of glutathione adducts. Therefore, an alternative strategy was pursued whereby a pyridine was incorporated as a phenyl isostere replacement; this modification resulted in reduced levels of metabolic activation. Further improvements were made via chlorination (5-Cl-2-pyridine-analog) or trifluoromethylation (5-CF₃-2-pyridine-analog) of the pyridine. Additional structural refinements in other parts of the molecule (nitrile analog; taranabant) provided a compound with minimal bioactivation properties, while retaining desirable pharmacokinetic and pharmacodynamic characteristics. The clinical use of centrally acting CB1R inverse agonist has, however, stopped because of unacceptable central side effects for this class of compound [100].

5.2.1.2 Optimization of a SERM. A similar approach was taken in optimizing a SERM, a class of compound that treats breast cancer and postmenopausal symptoms, where the initial tritiated lead was determined to covalently bind to proteins in a time- and NADPH-dependent manner following incubation with human liver microsomes (1100 pmol drug equivalents per milligram protein) [101] (Fig. 5.2). At least three bioactivation pathways were determined and included oxidative cleavage of the dihydrobenzoxathiin moiety to give a hydroquinone/*para*-benzoquinone redox couple, hydroxylation at position 5 or 7 of the benzoxathiin moiety leading to an *ortho*-quinone intermediate, and metabolism of the piperidine ring to give an iminium ion. The latter reactive intermediate was identified as its bis-cyano adduct when sodium cyanide was included in the incubation mixture as a reactive intermediate trapping agent. Structural

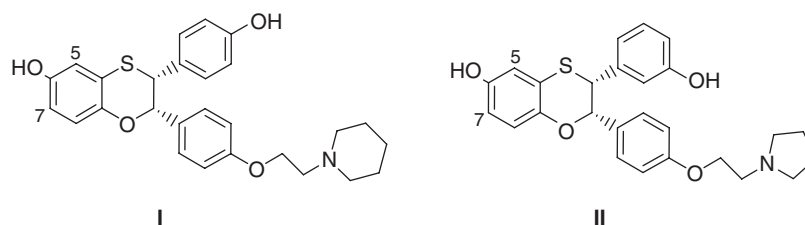


Figure 5.2 Optimization of a SERM.

modification of **I**, including a replacement of the piperidine with a pyrrolidine group, led to **II**, which did not form a reactive iminium ion. Following the incubation of **II** with human liver microsomes, covalent binding was reduced to 450 pmol drug equivalents per milligram protein. When covalent binding studies were conducted using human hepatocyte preparations, the level of binding for **I** and **II** was 170 and 50 pmol drug equivalents/mg protein, respectively, again highlighting how structure-covalent binding optimization of a series of compound was achieved with a good understanding of the bioactivation mechanism.

5.2.1.3 Optimization of a Leukotriene Receptor Antagonist. The development of CP-85,958 was discontinued because of liver toxicity in monkeys [102]. The underlying mechanism of toxicity was considered to be related to the formation of the lactol that could ring open to produce a reactive hydroxy-aldehyde intermediate capable of alkylating endogenous proteins (Fig. 5.3). Subsequent chemical modifications to CP-85,958 involved methylation at the 2-position to block lactol formation, chiral resolution to give the dextrorotatory enantiomer, and replacement of the carboxylic acid with a trifluoromethylsulfonamide to afford CP-195,494. This compound was pharmacologically active in animal models and had the same order of potency as zafirlukast and pranlukast and was an order of magnitude more potent than its predecessor, CP-85,958. An alternative approach to optimizing CP-85,958 was also investigated and involved introducing chemical groups that increased both pharmacological potency and metabolic lability at a site other than that leading to lactol formation [103]. The pharmacokinetics of these alternative compounds, CP-199,330 and CP-199,331, were investigated in rats and monkeys, with CP-199,331 having the better profile (low clearance, moderate half-life, 8–10 h, and good oral bioavailability, 46–72%). For both compounds, O-demethylation to the corresponding phenol was the major metabolite observed, thereby providing some evidence of metabolic switching away from formation of the lactol. When dosed to monkeys for five days, no liver toxicity was observed at plasma concentrations in excess of an order of magnitude higher than those predicted for clinical efficacy.

5.2.1.4 Optimization of a Dual A_{2A}/A_1 Receptor Antagonist. JNJ-27631734 (Fig. 5.4) is an A_{2A}/A_1 receptor antagonist and represented a lead compound being considered for development as a potential treatment for Parkinson's disease [104]. When assessed in the standard battery of genotoxicity assays, it was shown to be Ames positive in the *Salmonella typhimurium* strain TA1537, but only after the activation by induced (aroclor 1254) rat liver S9, indicating a role of metabolism in

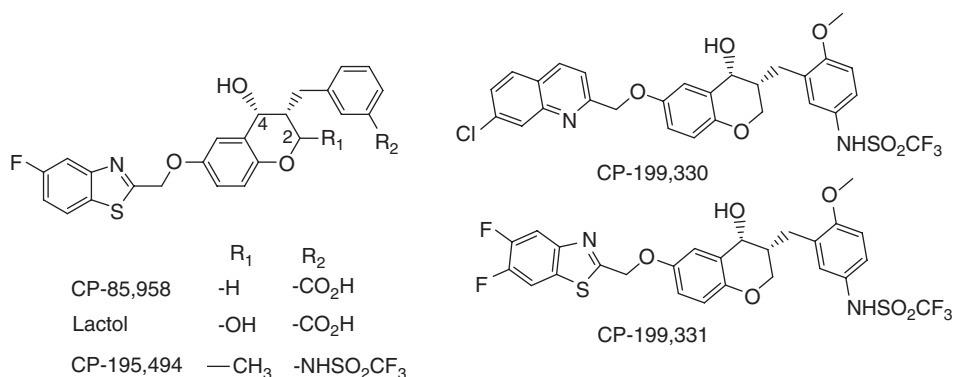


Figure 5.3 Optimization of a leukotriene receptor antagonist.

the positive genotoxicity outcome. The compound was similarly positive in the mouse lymphoma assay, again only in the presence of induced rat liver S9. Experiments in which sodium cyanide was used to trap putative reactive intermediates in the presence of either induced rat liver S9 or human liver microsome preparations were conducted and yielded comparable qualitative and quantitative results indicating that in both species *in vitro*, the pyrrolidine group had undergone sequential two-electron oxidations to form iminium ion intermediates as precursors of a bis-cyano final product. A test compound whereby the two carbon atoms α to the pyrrolidine nitrogen were substituted with methyl groups did not form cyano adducts and were shown to be Ames negative providing evidence that pyrrolidine activation had been a key bioactivation step for genotoxicity. Further optimization of the compound (pyrrolidine $\rightarrow$ pyridine) yielded a product that was optimized for potency, efficacy, pharmacokinetic properties, and lack of genotoxicity.

Notwithstanding the fact that there is much information in the scientific literature to increase the awareness of medicinal chemists to chemical structure alerts prone

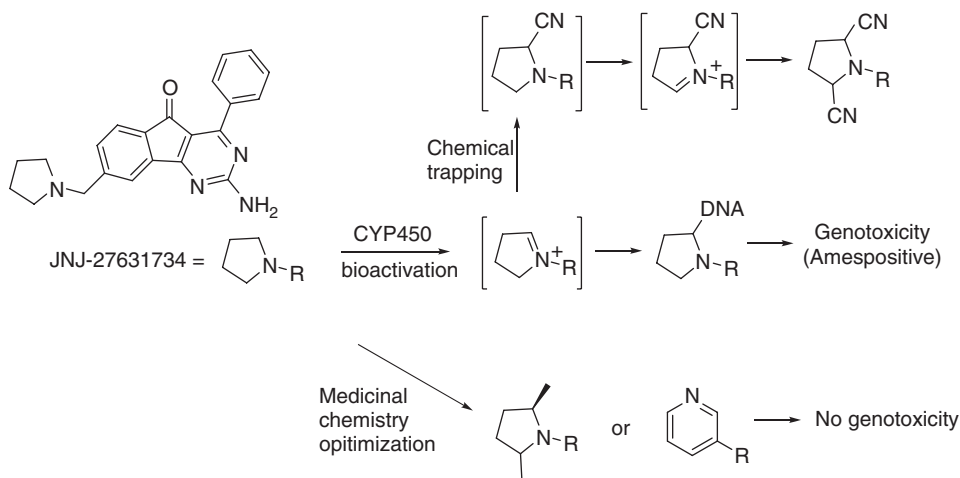


Figure 5.4 Optimization of an A_{2A}/A₁ receptor antagonist.

to metabolic activation [105–107], the foregoing examples indicate that the simple structure modifications can attenuate metabolic activation while maintaining or improving the pharmacokinetic and pharmacodynamic properties of the compound [11] and include

- isostere replacements—for example, pyridine for phenol
- metabolic blocking/steric hindrance—for example, introduction of a methyl group
- metabolic switching—for example, introduction of a methoxy moiety
- electronic effects—for example, phenyl substitution with fluorine.

5.2.2 Evaluation of Covalent Binding as a Component of Predicting Hepatotoxicity

The role of covalent binding studies, whether using liver protein to trap radiolabeled drug-related material or nucleophilic “trapping agents” (labeled or not) to trap drug-related reactive intermediates, have been used effectively to reduce the extent of metabolic activation during lead structure optimization. Recently, however, efforts have been made to leverage these data as a part of an integrated approach to *predict* the risk of developing hepatotoxicity. A number of groups have done this, notably Roche, AstraZeneca (data not shown), BMS, Pfizer, and Daiichi Sankyo (see below) where the learning is that prediction of hepatotoxic risk is improved when one integrates the amount of bioactivation derived from covalent binding studies with an understanding of how much exposure there is to the reactive intermediate, which is a function of inputs such as metabolic turnover *in vitro* and the daily dose level encountered in the clinic. In recent years, these more integrated approaches have been more widely investigated by the industry and are now presented in greater detail, notwithstanding the recognized limitations of this approach.

Researchers at Pfizer performed a retrospective investigation of whether integrating covalent binding data with pharmacokinetic data *in vitro* (intrinsic clearance, CL_{int}) and *in vivo* (pharmacologically effective human dose) could distinguish between known clinical hepatotoxins and nonhepatotoxins. This group evaluated a quantitative approach to derive a hypothetical estimate of “total daily dose of covalent binding” equivalents (Dcb; mg/day) for 18 radiolabeled compounds (9 known hepatotoxins and 9 nonhepatotoxins) in human liver microsomes [108] or cryopreserved human hepatocytes [109], where Dcb in this latter metabolic system was defined as

$$Dcb = fCL_{cb} \cdot \text{daily dose}$$

in which fCL_{cb} is the fraction of metabolism comprised by covalent binding,

$$fCL_{cb} = \frac{CL_{int, cb}}{CL_{int} + CL_{int, cb}}$$

and CL_{int} is the intrinsic clearance obtained using cryopreserved human hepatocytes:

$$CL_{int} = \left(\frac{[S]}{AUC} \right) \cdot \left(\frac{V}{\text{Number of cells}} \right)$$

where $[S]$ was the initial substrate concentration, V is the incubation volume, and AUC is the area under the $[S]$ versus time curve.

The level of covalent binding for these 18 compounds to *human liver microsomes* was determined not to be discriminatory with respect to their classification as either a hepatotoxin or nonhepatotoxin [108]. Indeed, seven of the nine nonhepatotoxic drugs demonstrated binding to liver microsomes. However, when a Dcb cutoff value of 1 mg/day was used, as determined using *human hepatocyte* preparations, 6 of 9 hepatotoxins (67%) were correctly categorized as having Dcb > 1 mg/day (exceptions were hepatotoxins sudoxicam, felbamate, and CBZ, which had Dcb < 1 mg/day), and 12 of 17 (71%) compounds were correctly categorized as either nonhepatotoxins or hepatotoxins (the additional exceptions were the nonhepatotoxins buspirone and propranolol, which had Dcb values > 1 mg/day) [109].

Similarly, researchers at BMS evaluated dGSH as a trapping agent to quantify adduct formation following the incubation of 50 drugs (10 associated with toxicity, 40 that were not) in *human liver microsomes* [110]. Unlike covalent binding studies with radiolabeled drug that will provide information on the capability of all types of reactive intermediate to bind to liver macromolecules, the use of dGSH is more restrictive and typically will not provide stable conjugates with, for example, “hard” electrophiles such as iminium ions. Nevertheless, the use of a dansylated chemical trapping agent does allow for both downstream chemical characterization of the precursor-reactive intermediate (allowing a plausible bioactivation mechanism to be postulated) and for relatively high throughput fluorescence and UV quantification of adducts formed relative to the overall turnover of the compound (f_m). Similar to the Pfizer group, BMS also used known human pharmacokinetic values, notably dose level (D), fraction of dose absorbed (f_a), and fractional clearance via oxidative metabolism (f_m) to provide an integrated assessment of approximate daily burden of thiol-reactive metabolites (D_{rm}):

$$D_{rm} = D \cdot f_a \cdot f_m \cdot f_{rm}$$

When focusing only on the percentage of thiol adducts formed (as would be the case during lead optimization), a number of “safe” drugs formed adducts (including paroxetine, losartan, montelukast, raloxifene, omeprazole, and lansoprazole). Conversely, a number of “toxic” drugs did not form adducts (including CBZ, flutamide, phenytoin, terbinafine, and zileuton) reinforcing the limitation of using such data as stand-alone criterion for decision making. However, when using the more integrated approach described for D_{rm} , nonhepatotoxic drugs (risperidone, simvastatin, rosiglitazone, paroxetine, losartan, and raloxifene) and hepatotoxic drugs (acetaminophen, clozapine, and troglitazone) were separated with D_{rm} values of less or greater than 10 mg, respectively, highlighting the predictive merit of this approach. Intermediate values of D_{rm} (1–10 mg) were obtained for one nonhepatotoxic drug (duloxetine) and two hepatotoxic drugs (diclofenac and nimesulide). At BMS, no absolute threshold is set for compound rejection, although for compounds projected to be administered at relatively high doses, <1% (~1000 pmol thiol adduct/h/mg protein) of adduct formation is deemed acceptable as long as there is reasonable metabolic turnover of the compound. Follow-up studies to assess reactive intermediate formation *in vivo*, representing a fully integrated biological system, are also performed regularly. As for other companies, BMS will rarely use covalent binding data as stand-alone criteria for stopping the progression of a candidate compound; they instead preferring to view these data as only one part of overall risk assessment.

Researchers at Daiichi Sankyo have also expended considerable effort in understanding the value of covalent binding studies as predictors of toxicity. Takakusa *et al.* [111] initially investigated 16 drugs that were associated with a variety of toxicities (hepatotoxicity, agranulocytosis, or hypersensitivity reactions) that either carry a Black Box warning, or have been removed from the market, and assessed their covalent binding in protocols initially proposed by researchers at Merck & Co. [10,112]. Daiichi Sankyo also assessed four “safe” drugs such as amlodipine, caffeine, valsartan, and warfarin (Table 5.5). This group chose compounds based on the diversity of the reactive intermediates implicated in their toxicities, and included quinoneimine derived from amodiaquine [113], *ortho*-quinone from benzbromarone [114], quinone-methide from tacrine [115], nitrenium ion from clozapine [116], iminium ion from nevirapine [105] and zafirlukast [117], nitroso species from erythromycin [118], phenacetin [119], procainamide [120] and sulfamethoxazole [121], arene oxide for imipramine [122], acyl glucuronide for valproic acid [123] and zomepirac [124], and radical species from aminopyrine [125], flutamide [126], and phenytoin [127]. While values of covalent binding in rat and human liver microsomes for the “safe” drugs were all <50 pmol drug equivalent/mg protein, values of covalent binding in human liver microsomes were determined to be a poor predictor of toxicity for “withdrawn” drugs (aminopyrine and zomepirac) and Black Box drugs (clozapine, nevirapine, and valproic acid), since they also provided levels of covalent binding of <50 pmol drug equivalents/mg protein. However, most (seven of eight) of the drugs in the withdrawn or Black Box warning category exhibited levels of covalent binding higher than 50 pmol drug equivalents/mg protein when assessed collectively in the *in vitro* assay performed using human liver microsomes and in the rat *in vivo* assay providing some basis for a reasonable risk assessment. Of note, long term retention of radioactivity in the bone marrow of rats was observed for some drugs associated with severe agranulocytosis (e.g., amodiaquine and clozapine), possibly as a consequence of drug-related material binding to stromal cells in the bone marrow [111]. The same research group progressed to assessing the covalent binding potential of a broader range of drugs in three test systems; namely, human liver microsomes, human hepatocytes, and *in vivo* in rat liver [128]. While covalent binding in either of the test systems were in of themselves not predictive of human toxicity, Nakayama *et al.* [128] did propose a “zone classification system” where 30 of the 37 drugs tested were correctly assigned to their respective safety categories where covalent binding in human hepatocyte preparations adjusted for daily dose was the best predictor among the three test systems evaluated.

Overall, there appears a good opportunity during early stage drug structure optimization to use trapping agents to direct medicinal chemistry efforts toward compounds with reduced potential for metabolic activation without the need to necessarily synthesize numerous radiolabeled compounds—this has been the approach at Janssen Research & Development. Once a lead compound emerges, however, certainly if it still carries some bioactivation risk, synthesis of a radiolabeled analog and evaluation of its bioactivation potential in a variety of protocols (*in vivo* and *in vitro*) do have merit as part of an integrated risk assessment toward defining organ toxicity risk. Covalent binding studies performed in hepatocyte preparations, with adjustment of derived data for metabolic turnover and anticipated daily dose level, appear to increase the ability to predict risk of a hepatotoxicity outcome.

TABLE 5.5 Covalent Binding Data for Black-Box and Withdrawn Drugs or Drugs Associated with Hepatotoxicity or Hypersensitivity Reactions

	Daily Dose (mg)	CB <i>In vitro</i> (+NADPH) ^a (pmol equivalent/mg protein)		CB <i>In Vivo</i> (pmol equivalent/mg protein)	
		RLM	HLM	Liver	Plasma
<i>Withdrawn or Black-Box</i>					
Aminopyrine (W)	130–3000	26	54	560	790
Amodiaquine (W) ^b	1750–2450	258	94	130	4
Benzbromarone (BB)	50–150	390	306	14	3
Clozapine (BB)	100–900	25	58	160	13
Flutamide (BB)	750	166	77	60	9
Nevirapine (BB)	200–400	0	33	80	2
Valproic acid (BB)	400–4200	4	0	140	180
Zomepirac (W)	200–600	3	4	28	7
<i>Known Hepatotoxicity or Hypersensitivity</i>					
Erythromycin	1000	53	32	55	100
Imipramine	75–300	43	104	330	530
Phenacetin	900	9	7	17	2
Phenytoin	300–600	2	16	34	3
Procainamide	1000–4000	0	23	27	1
Sulfamethoxazole	800–1600	1	6	13	1
Tacrine	40–160	134	17	46	3
Zafirlukast	40	35	43	14	1
<i>Safe</i>					
Amlodipine	<10	7	2	0	0
Caffeine	200–900	8	0	21	7
Valsartan	80–320	1	1	6	6
Warfarin	<10	9	36	17	11

Abbreviations: W, withdrawn; BB, Black-Box.

^aNADPH-dependent covalent binding (CB).

^bNADPH-independent CB.

Source: Adapted from Takakusa *et al.* 2008 [111].

5.2.3 Covalent Binding Data as Part of an Integrated Risk Assessment

In the foregoing examples of covalent binding studies conducted by Pfizer and BMS, there were outlier results in the context that some drugs were purposefully included in the study as examples of being “safe” or “nonhepatotoxic” yet were not classified correctly. The examples included propranolol, buspirone, paroxetine, and raloxifene. Conversely, there were also drugs evaluated that were examples of being “not safe” or “hepatotoxic” yet were incorrectly categorized as “safe” and included CBZ, phenytoin, felbamate, and sudoxicam. These examples are discussed from the viewpoint of points to consider when integrating data from several sources as part of the aforementioned risk assessment, as would be the case during lead selection, but helps also in highlighting the deficiencies that still exist with respect to predictive hepatotoxicology.

5.2.3.1 Propranolol. Innopran; daily dose 160–480 mg [128]; Dcb > 1 mg/day in cryopreserved human hepatocytes [109], Fig. 5.5.

Propranolol is widely used for the treatment of hypertension or arrhythmias as an adrenoceptor blocking agent. While classified by Nakayama *et al.* [128] as a drug without a Black Box warning but with a warning for hypersensitivity reactions and agranulocytosis, propranolol is generally regarded as having an excellent clinical safety record and was defined as a prototypic nonhepatotoxin in the Pfizer study [109]. In rat, it was reported that 4-hydroxy metabolite can generate 1,4-naphthoquinone (trapped with GSH), which can potentially inactivate rat CYP2D and covalently bind to proteins present in rat liver microsomal preparations [129]. Propranolol 4-hydroxylation is controlled by polymorphic CYP2D6 in humans and the same downstream quinone reactive intermediate pathway is considered to be relevant in rats and humans as being implicated in CYP2D inactivation [130]. Nakayama *et al.* [128] derived levels of covalent binding (pmol drug equivalents/mg protein) for propranolol, which were 70 (human liver microsomes), 9 (human hepatocytes), and 87 (rat liver homogenate *ex vivo*) highlighting potential for competing phase 2 metabolic conjugation reactions to attenuate the level of binding in human hepatocytes relative to human liver microsomes. This

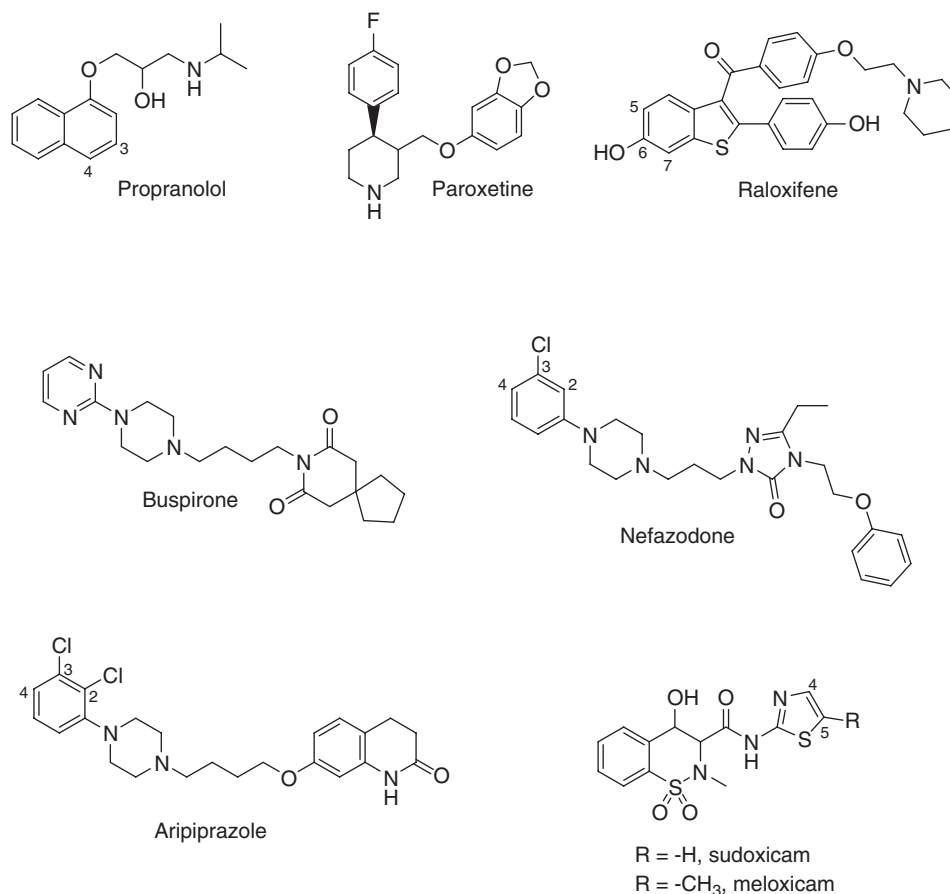


Figure 5.5 Structures of compounds that were not classified correctly as either hepatotoxins or nonhepatotoxins in covalent binding studies.

was also supported by the attenuation of covalent binding in human liver microsomes to which UGT cofactors had been added [108], and borne out, albeit post hoc in the context of a discussion occurring during lead drug optimization or a final decision as a development candidate, by the observation that 17% of the drug undergoes direct glucuronidation in humans [131]. The negative side of the risk assessment is the potentially high daily dose and a well-defined mechanism of bioactivation that translates to measurable covalent binding *in vitro* and *in vivo*. The importance of alternative (non-bioactivating) metabolic pathways, notably glucuronidation, typically does weigh high when attempting to assess hepatotoxicity risk and is presumably why, at least in part, propranolol turned out to have such a good clinical safety record. Overall, however, the integrated classification approach proposed by both Pfizer and Daiichi Sankyo consistently categorized propranolol as a potential hepatotoxicant (a false positive), highlighting the fallible nature of this predictive exercise.

5.2.3.2 Buspirone. BuSpar; daily dose 60 mg; Dcb > 1 mg/day in cryopreserved human hepatocytes [109], Fig. 5.5.

Buspirone is used to treat anxiety disorders and is considered to have a good clinical liver safety record. The Pfizer group also investigated related analogs, nefazodone (Serzone; daily dose 200–400 mg typical, 600 mg encountered, [132]) and aripiprazole (Abilify; daily dose 10–30 mg [133]), with a view to rationalizing their potential to mediate toxicity based on their bioactivation potential, covalent binding in liver microsome preparations and cryopreserved hepatocytes, and general pharmacokinetic properties. Nefazodone was withdrawn from the market by the manufacturer in 2003 [134] when the marketing company acknowledged a worldwide total of 28 reports of liver failure resulting in 18 deaths [135–137]. Since this time, research efforts have focused on the effects of nefazodone on drug transporter and mitochondrial function, as well as investigating its potential to undergo metabolic activation to gain a mechanistic understanding of its toxicity. Kostrubsky *et al.* [138] reported that nefazodone caused strong inhibition of the human bile salt export pump (BSEP) in human hepatocyte preparations and that transient increases in bile acids were detected in rat serum following oral dosing. Buspirone did not exhibit such effect on the bile acid transport system in this study. Similarly, nefazodone was determined to strongly inhibit mitochondrial function *in vitro*, whereas buspirone showed minimal toxicity [139]. Regarding potential for metabolic activation, the CYP3A4-mediated 4-hydroxylation of the 3-chlorophenyl-piperazinyl moiety in nefazodone, and the subsequent two-electron oxidation of this product to a quinoneimine reactive intermediate, was speculated to be involved in the etiology of liver toxicity in humans. Buspirone contains a pyrimidine ring in place of the 3-chlorophenyl moiety and while it also undergoes 4-hydroxylation, no glutathione conjugate of a putative downstream analogous quinoneimine reactive metabolite was detected, although the experimental finding of Dcb > 1 mg/day supports a conclusion that a bioactivation event has occurred but not yet mechanistically characterized. These researchers rationalized that a two-electron oxidation of *para*-hydroxybuspirone was less energetically favorable than that of *para*-hydroxynefazodone [132]. Similarly for aripiprazole (Abilify), a compound with a good safety profile, the 2,3-dichlorophenyl-piperazinyl moiety can undergo *para*-hydroxylation, but no further aberrant metabolism of this product was observed [133]. The conclusion from this line of investigation was that pyrimidinyl- and 2,3-dichlorophenyl-substituted piperazinyl analogs were preferable (“safer”) structural motifs compared to a 3-chlorophenylpiperazinyl moiety present

in nefazodone. On the negative side of the risk assessment, therefore, buspirone had $D_{cb} > 1$ mg/day. Conversely, buspirone had minimal potential to undergo “nefazodone-like” bioactivation, and demonstrated minimal potential to inhibit either BSEP or mitochondrial function.

5.2.3.3 *Paroxetine.* Paxil; daily dose 20 mg, Fig. 5.5.

Paroxetine, an antidepressant that contains a structure alert in the form of a 1,3-benzodioxole (methylenedioxyphenyl), provides high levels of covalent binding in liver microsomes [108] yet has been prescribed for over a decade, where life-threatening adverse drug reactions have been extremely rare [140,141]. Its bioactivation has been well characterized [142] (paroxetine $\rightarrow$ paroxetine catechol $\rightarrow$ two-electron oxidation to the *ortho*-quinone), which is consistent with its well-characterized and clinically relevant MBI of polymorphic CYP2D6. On the negative side of the risk assessment, therefore, there is evidence of MBI *in vitro*, a plausible mechanism of bioactivation, and significant levels of covalent binding in liver microsomal preparations. Conversely, the paroxetine catechol is efficiently conjugated during phase 2 metabolism (*O*-methyl, sulfate, and glucuronide conjugates have been identified) and the parent drug has a low daily dose (20 mg). Indeed, when integrating data on dose level and the fraction of metabolism in hepatocytes resulting in covalent binding, where phase 2 metabolism can be accommodated, the risk associated with metabolic activation for paroxetine was deemed minimal ($D_{cb} < 0.1$ mg/day [109]).

5.2.3.4 *Raloxifene.* Evista; daily dose 60 mg, Fig. 5.5.

Raloxifene is an SERM, which is effective in the treatment of osteoporosis in postmenopausal women. Overall, raloxifene has a good safety record in the clinic albeit for a case of hepatitis in a 49-year-old woman who had jaundice accompanied by elevated liver enzymes following oral administration of the drug for 30 days [143]. The toxicity was suggested to result from an immune mechanism based on observations of skin rash and eosinophilia [144], but the role of chemically reactive metabolites of raloxifene in the process remains unknown. When incubated with human liver microsome preparations, raloxifene elicits high levels of covalent binding [108,110]. Glutathione adducts of raloxifene have been identified in such preparations; substitution with GSH occurring at the 5- or 7-position of the benzothiophene moiety or at the 3'-position of the phenol ring, with the 7-glutathionyl derivative being most abundant based on LC/MS and NMR (nuclear magnetic resonance) analyses. These adducts are postulated to derive from addition of GSH to raloxifene arene oxides followed by dehydration and aromatization. Alternatively, raloxifene may be oxidized to an extended quinone intermediate, which is then trapped by GSH conjugation. The bioactivation of raloxifene is catalyzed by CYP 3A4 [145]. To balance these negative properties, an inspection of the chemical structure of raloxifene highlights two phenol moieties (potential to form 6- β - and 4'- β -glucuronides), which are likely to be available for metabolic phase 2 conjugation. Indeed, in cryopreserved human hepatocytes, the level of covalent binding was markedly attenuated ($D_{cb} < 1$ mg/day) [109]. What would not be known at the preclinical stage of development is the actual dose level or the expected degree of first pass glucuronidation. As determined post hoc, the dose turned out to be relatively low (60 mg) and the extent of intestinal first pass glucuronidation turned out to be extremely high, accounting for 95% of the dose [146]. What was known preclinically, however, was that biliary clearance of phenolic glucuronide conjugates of raloxifene

in the rat was high (~50%) [147], and the level of covalent binding in cryopreserved human hepatocytes was low [109] which, coupled to an understanding of metabolic routes *in vitro* (preclinical species vs human), would have helped rationally ameliorate the concern over the observed high levels of covalent binding observed in liver microsome preparations (Dcb > 1 mg/day) [108].

5.2.3.5 *Sudoxicam*. Daily dose 50 mg, Fig. 5.5.

Sudoxicam is a nonsteroidal anti-inflammatory that exerts its pharmacological action via cyclooxygenase inhibition but has been associated with cases of severe hepatotoxicity resulting in its withdrawal from clinical use [148]. Despite this, sudoxicam was shown to be indistinguishable from meloxicam, a nonhepatotoxin with a good safety record (daily dose, 15 mg), in regard to derived covalent binding observed following incubation in human hepatocyte preparations (Dcb values of “zero” [109]). This result is quite striking given that meloxicam differs from the structure of sudoxicam in only a 5'-methyl substituent on the thiazole ring. The Pfizer group speculated that this methyl substituent represents a metabolic “soft spot,” undergoing oxidation to 5'-hydroxymethyl and 5'-carboxy products as major products of metabolism in human and preclinical species [149], rather than is the case for sudoxicam where thiazole C4-C5 epoxidation is a major route of metabolism, relative to total metabolism, ultimately leading to ring scission and generation of the acylthiourea product [150]. This product can undergo S-oxidation to reactive sulfenic or sulfinic acid intermediates that can adduct to endogenous proteins leading to toxicity [151]. Despite this mechanistic rationale, it remains that sudoxicam was not effectively highlighted as a hepatotoxin by covalent binding methodologies alone. While the dose level of sudoxicam is approximately fourfold higher than meloxicam, and the identity of the proteins adducted by sudoxicam may be very relevant to its toxicity, these represent two parameters that are not easily accessible during decision making in early phase lead optimization. As such, sudoxicam represents a false negative outcome in these investigations.

5.2.3.6 *Antiepileptics—Felbamate, Carbamazepine, and Phenytoin*. Felbamate is typically used at very high doses (600 mg, titrated to 3600 mg daily) and its use was restricted via a Black Box warning due to 34 cases of aplastic anemia that resulted in 13 deaths, and 23 cases of hepatic failure that resulted in 5 deaths. The metabolism of felbamate is well understood and is characterized by the esterase-mediated formation of the monocarbamate, which can then be metabolized further by alcohol dehydrogenase to the aldehyde carbamate (Fig. 5.6) [152–154]. In a competing pathway to that leading to either the acid carbamate or the cyclic carbamate, the aldehyde carbamate can undergo β -elimination to afford the α,β -unsaturated aldehyde (2-phenylpropenal), a highly reactive electrophilic species. The lack of toxicity of felbamate in rats relative to humans was suggested to be related to the finding that only a third as much felbamate is converted to the monocarbamate in rats compared to humans, which translates to mercapturate levels of felbamate metabolites in humans being approximately sixfold higher than in rats [152]. However, the observation that dosing felbamate monocarbamate to glutathione depleted rats failed to produce hepatotoxicity still indicates the existence of a potential confounding explanation for the species difference in toxicity. Regardless, mercapturate conjugates of felbamate-related material have been observed in human urine and their structures were consistent with 2-phenylpropenal formation representing a significant metabolic pathway [153,154]. Indeed, the cyclic carbamate was suggested

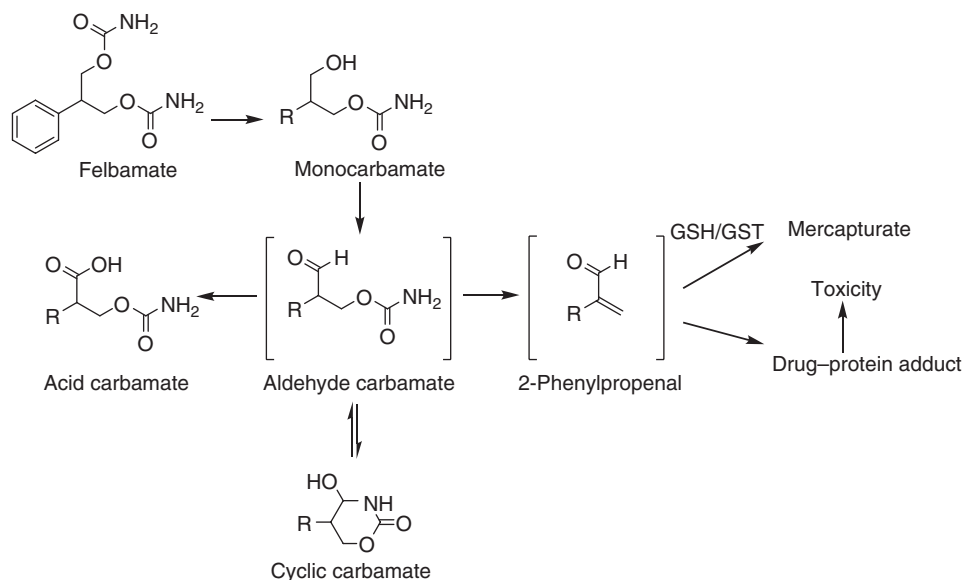


Figure 5.6 Felbamate bioactivation.

to represent a latent precursor of 2-phenylpropenal capable of travelling to sites distal from the liver to cause, for example, blood dyscrasias [153,155]. Since the proposed bioactivation of felbamate is non-CYP450 mediated, and its metabolic turnover in human hepatocytes is minimal, Dcb values in the Pfizer protocols were <0.1 mg/day in both liver microsome and hepatocyte preparations. The conclusion from this work that felbamate is a nonhepatotoxin, therefore, represents a false negative outcome.

CBZ (Carbatrol, daily dose up to 1200 mg, Fig. 5.1) is probably better known for mediating severe adverse reactions of the hemopoietic system (aplastic anemia and agranulocytosis) and skin (Stevens-Johnson syndrome (SJS) and TEN [131]). CBZ bioactivation is believed to be CYP450 mediated with potential for this to occur in both the liver and skin [50]. Similarly, DHP (Phenytek, diphenylhydantoin (DPH), daily dose up to 600 mg) provides severe adverse events of the hemopoietic system, some of which have been fatal, and are believed to be related to its bioactivation by CYP450, prostaglandin synthetase, and myeloperoxidase enzymes leading to products that covalently bind to proteins capable of eliciting a hypersensitivity response [156]. Unlike felbamate, which elicits minimal toxicity in preclinical species, CBZ and DHP are known hepatotoxins in rat at high doses. CBZ and DHP provided robust levels of covalent binding in rat and liver microsomes (225–730 pmol drug equivalents/mg protein) in protocols used by Janssen, albeit at high concentrations *in vitro* (~300 μM) [157], but to lesser extents by other investigators where significantly lower concentrations were used [108,110,111]. CBZ and DHP are both capable of undergoing epoxidation (CBZ $\rightarrow$ CBZ-10,11-epoxide [51] and CBZ-2,3-epoxide; DPH $\rightarrow$ 4'-OH-DPH [156]) to form products that can either bind covalently to cellular macromolecules or drive oxidative stress via, for example, catechol-semiquinone cycling. Furthermore, bioactivation of 3-OH-CBZ and 4'-OH-DPH to free radicals by peroxidases (myeloperoxidase representing the major oxidative enzyme in neutrophils) will form reactive

oxygen species as a by-product and hence may represent an additional mechanism by which these drugs cause idiosyncratic drug reactions [158]. Covalent binding for CBZ and DHP in human hepatocytes is consistently lower than observed in liver microsomes and in the absence of an important role for metabolic conjugation reactions, one is led to speculate that these compounds, for example, may simply not enter the hepatocytes efficiently *in vitro* or that neutrophil activation should be weighted highly. Regardless, a prospective assessment of both CBZ and DPH in these protocols could quite reasonably result in a false negative categorization of these compounds.

These prior examples highlight both the fallible nature of predictive toxicology and the need to collect data from multiple sources with the aim of conducting the best integrated safety assessment possible. Such an approach is not founded only on a formulaic assessment of covalent binding and exposure to reactive intermediates (false negatives included sudoxicam and felbamate, a false positive was propranolol) but also on data emerging from a variety of technologies that provide ancillary information that collectively provide a qualitative evidence-based approach to risk management. Clearly, if one is able to eradicate metabolic activation during lead drug design, then the need to predict hepatotoxicity as a consequence of bioactivation is not relevant and the focus then shifts to assessments of other mechanisms of toxicity.

5.2.4 Deficiencies of Covalent Binding Studies

On the basis of the foregoing sections, a number of deficiencies can be highlighted with the conduct and interpretation of covalent binding studies and data, respectively, and include the following.

1. If a drug is extensively but slowly metabolized, standard incubation conditions may underestimate the potential generation of a reactive intermediate *in vitro*. Attention to incubation time and extent of metabolic turnover are therefore important [65,85], and are accounted for by approaches that integrate hepatic intrinsic clearance [108] and/or metabolic turnover [66].
2. Compounds that undergo non-CYP450-mediated bioactivation (e.g., by esterases) will not be “flagged” by assays that utilize liver microsomes. Similarly, drugs bioactivated in tissues other than the liver will not be detected in standard covalent binding assays that are based solely on the use of liver preparations.
3. Covalent binding studies performed with radiolabeled compound are often performed with and without the addition of chemical trapping agents in order to determine if binding can be attenuated. For MBIs of CYP450, the efficiency of this process can be very poor, presumably due to such highly reactive intermediates binding to constitutive amino acid residues in the active site of the CYP450 enzyme. At worst, the reactive intermediates formed can potentially escape the trapping process, thus interfering with the elucidation of the chemical mechanism of bioactivation or, at best, the efficiency of chemical trapping is significantly reduced, which markedly hampers the structure assignment of the trapped reactive intermediate [65]. Clearly, however, the finding that a candidate drug is an MBI highlights bioactivation has occurred. An example of this phenomenon is the MBI of CYP3A4 by mifepristone (RU486) where the stoichiometry of irreversible modification of the apoprotein at the active site was determined to be 1 mol of mifepristone bound per 1 mol CYP3A4 inactivated [159].

4. Some GSH adducts are not stable and are subject to chemical degradation/rearrangement or enzymatic degradation, thereby escaping conventional detection [160]. In certain cases, *N*-acetyl cysteine (NAC) can be used in place of GSH as a nucleophilic trap, although this agent may be less efficient than GSH if the conjugation reaction in question is catalyzed (even in part) by glutathione-*S*-transferase enzymes.
5. Glutathione-*S*-transferase activity may not be optimal in, for example, liver microsomal preparations and may need supplementing to support those conjugation reactions that are enzyme catalyzed.
6. Hepatocytes contain the full complement of metabolic enzymes (e.g., phase 1 and phase 2 enzymes), which addresses a common concern of using only a “CYP450-rich” liver microsomes experimental system for those research groups wishing to conduct a quantitative hepatotoxicity risk assessment. Since a significant perturbation of genes encoding for antioxidant enzymes, drug biotransformation enzymes, and drug uptake/efflux transporters occurs following hepatocyte isolation, it is important to control for this when selecting reagents to deploy toward covalent binding assays [161] by, for instance, appropriately characterizing such reagents and using a common pool of reagents deployed across multiple experiments (also applicable to liver microsomes).
7. During the conduct of covalent binding studies, a question of whether the nonrecoverable radioactivity is covalently bound or simply not extracted using the washing solvent system employed is sometimes encountered. This can be addressed using standard gel electrophoresis protocols and determining whether radioactivity comigrates with protein [159]. This approach may shed light on a discussion of relative toxicity following adduction of a few relative to multiple proteins. Integrating data on factors such as the magnitude of binding, the relative rate of binding, and the spectrum of proteins bound by reactive electrophiles is relatively under-researched. The La assay published by researchers at Abbott Laboratories allows for the detection of reactive molecules by NMR spectroscopy, where they concluded that slowly reacting compounds may be more likely to cause adverse events [162]. This is consistent with investigations into the role of arylation in quinone toxicity where cytotoxicity was governed by the rate of adduct formation *in vitro*; that is, paradoxically, higher chemical reactivity equated with lower cytotoxicity [163]. It is tempting to conclude that reactive intermediates that are slowly reacting are more capable of leaving the active site and perturbing a broader array of cellular proteins. This equates with a “more shots on goal” hypothesis of covalent-binding-mediated toxicity, which clearly needs to be balanced with factors such as whether the proteins adducted are “critical” for cell function in addition to the extent/amount to which exposure has occurred to the reactive intermediate and the downstream adducted proteins.

5.3 BIOMARKERS OF DRUG-INDUCED LIVER INJURY

In addition to having chemistry-based strategies to minimize potential for bioactivation, there is a clear need to understand the biochemical cascade of host cell defense and

repair in order to define robust biomarkers of DILI that are amenable for routine analysis—this forms the basis of the integrated “evidence-based approach” to risk management. Such assays bridge the intent behind rational structure-based drug design, which is “safety” for the purpose of this discussion, with a functional correlate that can be monitored preclinically and have relevance clinically; essentially addressing the deficit that currently exists for hepatotoxicity [5].

Measuring serum alanine aminotransferase (ALT) represents a widely used biomarker approach to detecting hepatocellular injury, yet it is neither specific to the liver nor do fairly high elevations always indicate or predict serious DILI and hence the degree of risk to the patient [164]. Watkins *et al.* have highlighted the limitations of ALT measurements using the example of tacrine where 25% of patients developed ALT greater than three times to that of upper limit of normal (ULN) with no associated cases of symptomatic liver injury or jaundice yet for troglitazone, a withdrawn drug, <2% patients developed ALT greater than three times to that of ULN. Satisfying Zimmerman’s (Hy’s) [165,166] rule (serum ALT >3X ULN and serum total bilirubin levels >2X ULN) as a criterion for discontinuing drug therapy is not tenable since the etiology of total bilirubin level increases resides with a major and often irreversible loss of functioning hepatocytes.

Toxicogenomics has some merit in highlighting hepatotoxicity risk but has struggled to realize its full potential since it represents a qualitative tool, data from which is difficult to translate to a risk assessment. McMillian *et al.* were able to select a 69-gene set to maximally separate compounds in control, macrophage activator, peroxisome proliferator, and oxidative stress/reactive metabolite classes of compound in rodents *in vivo* [157,167,168]. A key goal of this research was to detect as many classes of toxicant as possible before their pathologies were manifest and before gene changes becoming secondary to the pathology; essentially identifying gene expression changes that predict damage and are not as a result of cell damage. The Nrf-2 (nuclear factor-erythroid 2-related factor 2) regulated genes that were identified in rat liver, which were upregulated by oxidative stressors or reactive intermediates included NAD(P)H: menadiene oxidoreductase, microsomal epoxide hydrolase, glutathione-S-transferase, heat-shock protein 90, and γ -glutamylcysteine synthetase [168]. Roth *et al.* have also deployed microarray analysis on rat livers following prior modest induction of inflammation using lipopolysaccharide (LPS). This research has suggested a role for pro-inflammatory cytokines and neutrophils in trovafloxacin-induced liver injury [169]. This work complements findings from research conducted with cytokine knockout animals using APAP as the model hepatotoxin where, for example, anti-inflammatory IL-10 protects against toxicity (IL-10 null mice have increased susceptibility to APAP toxicity), whereas the pro-inflammatory IFN γ (interferon), FasL (Fas ligand), and TNF (tumor necrosis factor) promote an injurious outcome [170,171]. However, the role of animal models to study drug-induced hypersensitivity reactions overall has been limited [172]. Making these research efforts more relevant to humans is clearly the goal and the opportunity now exists to investigate this *in vitro* in human hepatoma cell lines [173]. In this regard, Takakusa *et al.* have investigated the induction profiles of Nrf2-related genes in human hepatocytes using the prototypic idiosyncratic hepatotoxicants, ticlopidine [174], diclofenac [175,176], clozapine [177,178], and tienilic acid [179,180] and concluded that induction of heme oxygenase-1 mRNA (HO-1; catalyzes the degradation of heme resulting in the production of biliverdin that can suppress oxidative stress by scavenging reactive oxygen species (ROS) [181]) is correlated

with the occurrence of reactive metabolite-mediated drug toxicity. This conclusion was supported by the finding that HO-1 induction could be significantly attenuated by the pan-CYP450 inhibitor, aminobenzotriazole [182].

High content screening (HCS) enables kinetic monitoring *in vitro* of live cells in real time for multiple cellular biomarkers using epifluorescence microscopy and image analysis [58,183]. Xu *et al.* [184] have conducted HCS in human hepatocyte cultures and measured mitochondrial damage, oxidative stress, and intracellular GSH as markers of mitochondrial energy and cellular redox states, since these have all been implicated as important predictors of DILI [185,186]. In isolation, this testing strategy had a positive rate of 50–60% when applied to >300 drugs including many that cause DILI, and a very low false positive rate of $\leq 5\%$. Of note, while there was not a big overlap between the compounds investigated by the two Pfizer groups ([108] vs [184]), the cellular imaging approach did correctly classify buspirone, paroxetine, propranolol, and raloxifene as non-DILI positive (this was not always the case when using only covalent binding protocols, see above), suggesting HCS to represent an important member of the preclinical repertoire of safety assays.

To reiterate, mitochondrial energy and cellular redox states have all been implicated as important predictors of DILI [185,186]. Mitochondria are a major source of ROS where superoxide anion radicals, produced as a by-product during respiratory reactions, are scavenged by superoxide dismutase (SOD) producing hydrogen peroxide and molecular oxygen. Boelsterli and Hsiao and Lee *et al.* have shown that hepatotoxicity consistent with necrosis was observed following troglitazone administration to *Sod2*^{+/-} mice, but not in control animals [187–189]. These heterozygous mice contain ~50% of the SOD activity compared to wild type animals, but activities are normal for catalase and glutathione peroxidase, and it was proposed that troglitazone-mediated toxicity was a consequence of increased levels of ROS in the livers of *Sod2*^{+/-} mice [190]. Similar investigations have been conducted with flutamide [191] and nimesulide [192] indicating that the *Sod2*^{+/-} mouse is a sensitive model to assess mitochondrial toxins and complements the HCS model already discussed.

Continuing on the general theme of impaired mitochondrial function, hepatic microsteatosis is considered to be caused by inhibition of fatty acid β -oxidation, which results in lipid accumulation. Metabonomic approaches have been used to indicate that APAP can disrupt fatty acid β -oxidation in wild-type versus *Cyp2e1*-null mice using a mass spectrometric approach to investigate the serum metabolome from these animals [193]. Similarly, ¹H-NMR of urine and liver samples has also been leveraged as a metabonomic approach to identify impaired fatty acid metabolism as the mechanism of DILI in rats [194], highlighting the usefulness of metabonomics in general to investigate toxicity mechanisms.

In the context of reactive metabolite-mediated toxicity, the discovery of Keap1 (kelch-like ECH-associated protein 1) as an endogenous “sensor” of chemical/oxidative stress is probably one of the most important discoveries of recent times; it also represents an example of a drug–protein interaction, which mediates detoxification. Human Keap1 contains 27 cysteine residues in its 624 amino acid structure and plays a pivotal role as a cytosolic repressor protein that binds the transcription factor Nrf2 and keeps it in the cytoplasm and targets it for proteosomal degradation. Upon challenge, which includes an electrophilic insult and/or GSH depletion, Nrf2 is released for nuclear translocation and binding to the antioxidant response element (ARE) [195]. In a competing transcriptional regulatory mechanism, Bach1 (BTB and CNC homology 1, basic

leucine zipper transcription factor 1) heterodimer binds to the ARE and represses defensive gene expression in what has been described as a competitive interplay between Bach1-containing repressor dimers and Nrf2-containing activator dimers [196]. The binding of Nrf-2 containing activator dimer to the ARE activates transcription of a myriad of gene products, which catalyse direct inactivation of oxidants (e.g., catalase and superoxide dismutase) and detoxification of electrophiles (e.g., glutathione S-transferase, epoxide hydrolase, and quinone reductase 1(NQO1)) as part of the “host defense” response [197]. In elegant studies performed in murine models by researchers at the University of Liverpool, UK, the ability of the Keap1-Nrf2 pathway to “sense” and respond to electrophilic metabolites/oxidative stressors appears to be via mechanisms other than direct modification of Keap1. This group demonstrated that Nrf2 can be activated by molecules that do not deplete GSH but do modify Keap1, by molecules that deplete GSH to <15% of basal levels and modify Keap1 and, thirdly, by depletion of GSH only [198]. Of note, these researchers were able to demonstrate that *N*-acetyl-*p*-benzoquinoneimine (NAPQI, the reactive metabolite of acetaminophen) selectively modifies Keap1 residues *in vitro* and that activation of the Nrf2-ARE pathway at non-toxic doses of acetaminophen occurs indicating that this pathway can “sense” and respond to chemical stress before the onset of cytotoxicity [198,199]. These are the first studies to elaborate how the processes of drug bioactivation, reactive metabolite formation, GSH depletion (Nrf2 activation), and reactive metabolite binding to cellular machinery (Keap1), can all integrally be involved in evoking a cell defense response. Similar findings have been reported for the *ortho*-quinone products of estrogen catechol metabolites which have been shown to activate Nrf2 via covalent binding to Keap1 [200].

In summary, while there are a battery of assays currently deployed in the current “reactive metabolite mitigation arsenal”, to include covalent binding assays (in hepatocytes corrected for daily dose and metabolic turnover), functional assays such as MBI and genotoxicity, and additional assays that evaluate BSEP inhibition and mitochondrial toxicity, there continues to be a diligent focus on supplementing this further. The foregoing discussions on the roles of liver function tests, toxicogenomics (+/– LPS), HO-induction, HCS, animal models (IL-10 null mice, *Sod2*^{+/-} mice, CYP450-specific knock-out animals), metabolomics and Nrf2 activation, ushers in the next logical question of whether there are additional screening platforms or early indicator biomarkers of liver toxicity that are available or emerging that highlight when cell defense mechanisms are becoming overwhelmed, but precede the onset of massive hepatocellular injury or a hypersensitive response, and include the following.

1. A Nrf2 luciferase-reporter assay has recently been developed in human HepG2 cells, which is amenable to high throughput screening [201].
2. Hepatic depletion of glutathione can be monitored by measuring increases in ophthalmic acid (L-γ-glutamyl-L-α-aminobutyryl-glycine; an analog of glutathione in which the cysteine moiety is replaced by L-α-aminobutyrate [202]) levels in plasma and hence has the potential to represent a relatively noninvasive probe to assess the redox status of the liver GSH system in humans [203].
3. HMGB1 (high mobility group box protein 1) is a chromatin component and has been shown to be released in a *hypo*-acetylated form from *necrotic or damaged* cells [204]. HMGB1 is ubiquitous at 10⁶ molecules per typical mammalian

cell, which is not, however, released from *apoptotic* cells. This finding is consistent with apoptosis not representing a present and immediate danger and, as such, there is no commensurate need to trigger an inflammatory response [205]. HMGB1 is also released in a *hyper*-acetylated form from activated immune cells and, therefore, may have use in identifying patients at risk of developing hepatotoxicity before the onset of overt injury [12,204]. Consistent with this finding, *Hmgb1*^{-/-} necrotic cells have a greatly reduced ability to promote inflammation [204]. Of note, in the context of experiments conducted by Roth *et al.* (see above), macrophages and monocytes secrete HMGB1 as a delayed response to activation by LPS [206]. HMGB1 also has been proposed as a “danger signal” (see above) to trigger an idiosyncratic toxicity [36,207].

4. Heat shock protein-72 (Hsp72) has also been proposed to be an endogenous “danger signal” and, like HMGB1, is released from necrotic cells but, unlike HMGB1, is also released by cellular stress [36]. Hsp72 stimulates production of proinflammatory cytokines by monocytes and macrophages [208,209] and surface-bound Hsp72 can be chemotactic for NK cells to induce cytolytic activity [210]. HMGB1 and Hsp72 detection is rudimentary currently and is typically based on Western blot analysis [211].
5. CK18 (cytokeratin-18) is widely expressed in epithelial and endothelial cells and has been proposed as a serum biomarker of necrotic (full length CK18) or apoptotic (caspase cleaved CK18) cell death [12]. Analysis of CK18 is performed by ELISA [212].
6. The disadvantages of ALT, as a specific serum biomarker of liver function, were discussed previously. MicroRNAs (mir) are small, noncoding RNAs that are relatively stable in plasma and have merit as diagnostically sensitive and specific markers of tissue injury [213]. Specifically, mir-122 and mir-192 are enriched in liver tissue and exhibit dose- and exposure duration-dependent changes in the plasma that parallel serum aminotransferase levels and the histopathology of liver degeneration following APAP administration to mice, but their changes can be detected significantly earlier. These findings suggest the potential of using specific circulating mir’s as sensitive and informative biomarkers for DILI [213,214]. Moreover, the sequence of miR122 is conserved from mammalian species back to, for example, zebra fish, rat, dog, and monkey, making findings in early pre-clinical studies potentially more translatable to humans [215].
7. The concept that patients are genetically predisposed to various toxicities is clearly enticing; the supposition being that patients “at risk” of developing a drug-related toxicity could be proactively screened in advance and potentially redirected to a safer drug therapy option. There exists highly significant association between single nucleotide polymorphisms (SNPs) in the MHC with skin and liver toxicity [216] as exemplified for skin toxicity induced by abacavir [217] and CBZ [218,219], and hepatotoxicity induced by amoxicillin-clavulanate [220], ximelagatran (Exanta) [44], ticlopidine [221], and flucloxacillin [222] (Table 5.6). If DILI genes all turn out to be in the HLA (human leukocyte antigen) region, it would be one of the strongest rationale for why animal testing offers no predictive value for most DILI in humans [223]. For many of these examples, the statistical significance of this genetic association is high when performed retrospectively. That is, when blood samples are taken from affected patients and

TABLE 5.6 HLA Genetic Markers of Liver and Skin Toxicity

Drugs	Allele or Haplotype	Comments
Abacavir	HLA-B*5701	Patients of this genotype can avoid abacavir and be prevented from developing toxic epidermal necrolysis (TEN) or Stevens-Johnson Syndrome (SJS)
Flucloxacillin	HLA-B*5701	For suspected cases of flucloxacillin induced liver injury, an estimated 85% of these patients would carry this allele
Carbamazepine	HLA-B*1502	This allele is strongly associated with carbamazepine-induced SJS and TEN in Han Chinese, but not in Caucasian populations
Amoxicillin-clavulanate	DRB1*1501- DRB5*0101- DQB1*0602	Study performed in white native patients originating from Belgium. This haplotype was present in ~12% of the normal control which is considerably higher than the 1/1000–1/10,000 incidence of hepatitis observed for this drug. HLA association cannot therefore be the only factor responsible to explain the pathogenesis of this disease
Ximelagatran	DRB1*07- DQA1*02	Ximelagatran does not undergo metabolic bioactivation, and it is therefore conjectured that this drug forms a low affinity association with MHC. DRB1 alleles have a carrier frequency of 11% in Scandinavia and 0.3% in Japan
Ticlopidine	HLA-A*3303	Ticlopidine-induced hepatotoxicity has an increased rate in Japanese compared with Caucasian patients and is strongly associated with this allele, which has a frequency of 9.7% in Japanese and 0.53% in Caucasians

analyzed *post-hoc* to determine if there is evidence of a genetic predisposition, the correlation is high. However, when the observation that many patients who do not develop an adverse reaction also carry this genetic association is coupled to the fact that the frequency of idiosyncratic drug reactions are low, the outcome is that using HLA association cannot typically be used prospectively to identify patients at risk of developing such reactions due to the high false positive rate of such an analysis.

8. The LTT does not fall into the category of a biomarker assay that can preempt the onset of drug hypersensitivity reaction, but is highlighted here as an assay

that has utility in understanding particular immune mechanisms in different drug allergy diseases, and has been reviewed elsewhere [40,224].

5.4 CONCLUDING REMARKS

Attenuating metabolic activation during drug design and defining biomarkers that are symptomatic of host cell defense machinery perturbation can collectively contribute to bringing safer and much needed drugs to the market for the long term benefit of patients. The body is clearly adept at handling electrophilic species resulting from drug bioactivation, and thiol chemistry (GSH, Keap1) is central to this cascade defense mechanism. Instances where this defense is overcome highlight the need to target compounds with as low a dose as possible, with minimal potential to form reactive metabolites, as one strategy to mitigate against potential toxicities. Utrecht [30] has reported that drugs given at a daily dose of 10 mg or less are rarely, if ever, associated with a significant degree of idiosyncratic drug reactions. In those instances, where compounds are progressed into clinical development despite their known ability to covalently modify endogenous macromolecules, a range of methodologies have been described in this chapter, which help to provide an evidence-based approach to an integrated safety assessment. A proposed specific goal for such programs would be to bring forward back-up compounds where further attenuation/eradication of metabolic activation has successfully been achieved while maintaining all other favorable properties of the compound. This investment is necessary to avoid those very late stage clinical development or postmarketing discussions with clinical investigators and regulators where liver toxicity and/or hypersensitivity adverse event data arise and sponsor company project teams are left with the benefit of hindsight wishing that a more proactive stance had been taken to overcome metabolic activation at the outset in the discovery phase, at least as one potential causative event in the toxicity outcome. This is especially relevant at a time when drug differentiation based on safety is so crucial to product success.

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6 Safety Testing of Drug Metabolites: A Practical Approach for the Implementation of the MIST Guidance in PKDM

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6.1	Introduction	1
6.2	Scope of the chapter	2
6.3	Impact of MIST guidance on industrial ADME research	2
6.4	Guidance harmonization between the FDA and ICH	3
6.5	A MIST strategy	4
6.6	New approaches to nonradioactive metabolite quantitation: focus on plasma sample pooling, high resolution MS, and mass defect filter mass spectrometry	6
6.7	The human ^{14}C ADME study	11
6.8	Accelerator mass spectrometry (AMS)-based human ADME studies	12
6.9	Implementation	12
6.10	Conclusion	14
	Acknowledgments	14
	Abbreviations	14
	References	15

It should be emphasized that there is no straightforward or one standardized approach to the evaluation of the safety of drug metabolites. A case-by-case approach should be exercised when the information in the guidance does not apply.

—Aisar Atrakchi, *Chem. Res. Toxicol.*, 2009, 22, 1217–1220

6.1 INTRODUCTION

The Safety Testing of Drug Metabolites Guidance, also known as the *Metabolites in Safety Testing* or *MIST Guidance*, was issued by the FDA Center for Drug Evaluation and Research (CDER) in February 2008. This new guidance defined thresholds for

metabolite exposure in humans and preclinical species that would assure regulators that the preclinical species employed to define the safety of a drug candidate were in fact exposed adequately to all quantitatively significant human metabolites [1]. The US guidance was followed in June 2009 with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) M3(2) guidance, which differed in important ways with respect to metabolite exposure thresholds, but sought to achieve the same result. Both guidances acknowledged the complexity of the issue and advocated for “case-by-case” strategies that are developed with Agency consultation [2].

Since July 2009, the pharmaceutical industry focus has shifted from the thresholds and logic of MIST, to practical implementation of bioanalytical aspects of plasma metabolite analysis. For example, a “holistic” strategy for practical implementation of MIST compliance was described [3], bioanalysts have started to address best practices [4], and reviews on the evolution of scientific thinking about MIST are now appearing [5–7]. Collectively, these efforts recently culminated in a special issue of *Bioanalysis* in July 2010 [8]. The number of MIST-related articles has grown from 17 in July of 2009 [7], to approximately 30 in Sept of 2010. Therefore, it is timely to look at what drug metabolism practitioners are likely to do to address MIST, against a plethora of possible approaches that mix some old and standard activities, such as *in vitro* cross species comparisons, with new approaches focused on plasma metabolite analysis.

This chapter outlines the elements of the roll out of a strategy to address the MIST guidances at Amgen. Our objectives were twofold: first, to highlight a strategy for implementing a MIST plan in Translational Sciences [an organization within research and development (R&D) divisions of some pharma and biotechnology companies that oversees activities approximately from the time a candidate is selected for advancement to first-in-human (FIH) through end of phase 2] and second, to illustrate the relative advantages of plasma pooling strategies and cross species semi-quantitative metabolite comparison by liquid chromatography–high resolution mass spectrometry (LC-HR-MS).

6.2 SCOPE OF THE CHAPTER

The history and pharmacokinetics and drug metabolism (PKDM) issues in the pharmaceutical industry’s implementation of the MIST guidance were reviewed up to July 2009 by Slatter and several others [4,6,7]. This chapter covers practical implementation and growing experience with the FDA and ICH guidances that occurred across the industry from the summer of 2009 to the end of 2010. Our experience with the development and implementation of a Translational-Sciences-wide MIST strategy will be illustrated with examples of a practical analytical strategy.

6.3 IMPACT OF MIST GUIDANCE ON INDUSTRIAL ADME RESEARCH

The 2008 CDER guidance flowchart is reproduced in Fig. 6.1 with an overlay that shows the numerous organizational codependencies that must be addressed in a MIST plan rollout [7]. The key effect of the MIST guidance was to shift most of the burden of

Contains nonbinding recommendations

Appendix A:
Decision tree flow diagram

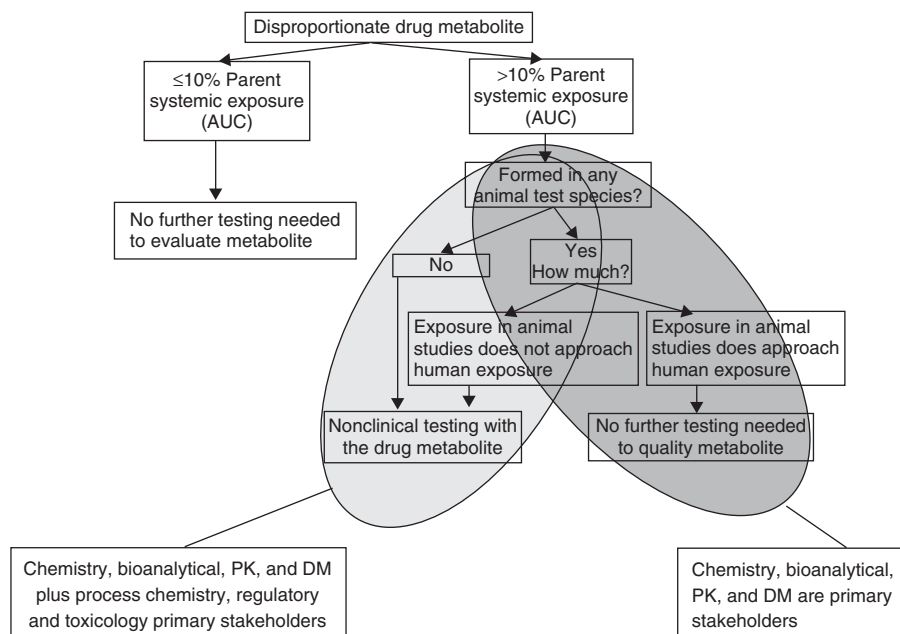


Figure 6.1 Adapted from the MIST guidance decision tree [1], modified with workload codependencies related to metabolite and internal standard synthesis, bioanalysis, regulatory interactions, and safety studies on a disproportionate metabolite. *Source:* Reprinted with permission from Ref. 7.

compliance to absorption, distribution, metabolism, and excretion (ADME) practitioners. It was now PKDM's job to prove that any metabolite characterized would be "not disproportionate." In the less-than-likely event that a metabolite(s) was disproportionate, this had to be known early enough to alert drug safety and get metabolite synthesis, qualification, activity, and a safety testing strategy completed by the end of phase 2.

6.4 GUIDANCE HARMONIZATION BETWEEN THE FDA AND ICH

One key aspect of MIST compliance is the clarification in the past year of the quantitative threshold for metabolite scrutiny. The CDER threshold for a disproportionate metabolite was set in the guidance at 10 % of parent drug area under the plasma concentration–time curve (AUC) in human plasma at steady state. This threshold had the potential to increase the number of metabolites warranting scrutiny. ICH M3R2 defined the disproportionate metabolite threshold at 10 % of plasma drug-related material in drugs that were dosed at 10 mg or higher [2]. The latter threshold was more aligned with conventional ADME practice, as it was anchored to absolute dose mass, while the former threshold created a sliding scale relative to parent drug in circulation. As the relative AUC of circulating parent drug decreased relative to metabolites, more metabolites were above the 10 % of parent threshold [7,9,10]. Recently, FDA authors

Robison and Jacobs [11] clearly indicated, with the usual disclaimers, that when the CDER guidance threshold resulted in more metabolites warranting scrutiny than the ICH M3R2 guidance, threshold set by the latter could be used. This was scientifically rational and further enabled the industry to focus on metabolites that were quantitatively significant, relative to the mass of drug material administered.

The ICH S9 draft guidance “Draft Consensus Guideline Nonclinical Evaluation For Anticancer Pharmaceuticals S9” [12], like the CDER guidance, had an exemption for the evaluation of drug metabolites of cancer drugs. The ICH S9 guidance states “for disproportionate metabolites of anticancer agents, a separate general preclinical safety evaluation might not be warranted for patients with late stage or advanced cancer, as human safety of the metabolite would have been assessed in phase 1 clinical trials. If the parent compound is positive for embryofetal toxicity or genotoxicity then separate studies for the disproportionate metabolite might not be warranted. Unless there is a specific cause for concern, nonclinical testing of the metabolite is not warranted.”

6.5 A MIST STRATEGY

In the time following the availability of the CDER guidance in February 2008, content experts across the industry monitored the implementation of the guidance. At the PhRMA Drug Metabolism Technical Group’s (DMTG) Annual Roundtable with FDA in 2008, both the positive aspects and some of the shortcomings of the guidance were discussed. PKDM program representatives conducted individual candidate risk analysis and immediate course corrections for their current clinical programs. Over the ensuing year, as they monitored the rapid evolution of MIST guidance thinking in the public domain and tested key technologies internally, companies developed MIST plans by assessing and selecting best practices for metabolite analysis. The last step was soliciting input on the draft plan inside and outside the PKDM unit before a roll out to partner organizations (such as toxicology, clinical development, and regulatory).

6.5.1 Modifications of the ADME Development Plan

Several key departures from conventional ADME development were added to small molecule drug candidate development plans in order to enable the shift from an *in vitro* and excreta focused approach to a plasma focused plan. As shown in Fig. 6.2, pre-FIH activities, such as cross species metabolite profiling and metabolite identification in microsomes and hepatocytes, were performed to discern the second species that would have metabolism most similar to humans. Significant human metabolites in microsomes and differences in profiles between human microsomes and hepatocytes were used to predict the most likely human metabolites [13,14]. Overall, the predictive value of *in vitro* screening using human hepatic microsomes and hepatocytes is best for single oxidations and decreases for sequential metabolism and for phase II metabolites. *In vitro* metabolism data can be used to reassure stakeholders that PKDM’s efforts will generate a prognosis of “not disproportionate” in the majority of cases and provide lead time in those rare cases where more data are needed. The most common complication at this stage comes as a result of lead enhancement to reduce metabolism, and some leads with good pharmacokinetics (PK) are slowly or negligibly metabolized *in vitro*. Nonetheless, with a pending focus on plasma analysis, consultation with chemists and

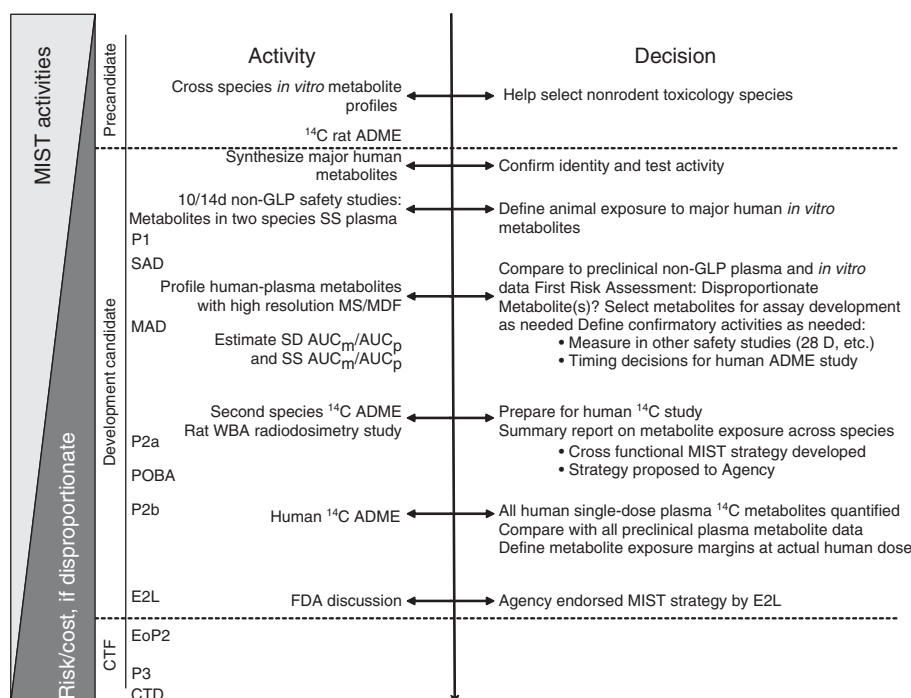


Figure 6.2 Strategy: build a data-driven proactive MIST development plan for each small molecule candidate. Timescale and order of operations are approximate; to be adjusted based on program needs. P1, phase 1; SAD, single ascending dose; MAD, multiple ascending dose; P2a, phase 2a; POBA, proof of biological activity; P2b, phase 2b; E2L, early to late stage development; EoP2, end of phase 2; P3, phase 3; CTD, common technical document; CTF, commit to file.

pharmacologists on the feasibility of synthesis and activity testing of the most probable human metabolites begins after candidate selection for advancement to FIH.

The most significant departure from excreta-based development is the need to obtain plasma for metabolite analysis and compare metabolite profiles semi-quantitatively across species. The two preclinical options are a separate dose escalation PK study in the rodent and nonrodent species, or alternatively, to obtain unused plasma from toxicokinetic (TK) analysis in non-GLP or good laboratory practice (GLP) studies. The latter option has only recently become feasible due to the recent availability of sensitive and selective high resolution mass spectrometry (HR-MS) techniques. As such, retasking of unused TK and clinical study plasma was discussed with Drug Safety and Clinical opinion leaders. Factors in implementation were study timelines, maintaining GLP compliance, protocol revisions, sample archiving, metabolite stability, reporting results, and verification of the new HR-MS techniques (Section 6.9). New protocol/informed consent language and plasma sample handling processes for unused FIH single ascending dose (SAD) and multiple ascending dose (MAD) plasma were developed.

Agreement on the need for the new plasma metabolite analyses developed quickly, since the consequence of doing metabolism “the old way” could include a clinical hold at the end of phase 2. It was recognized that a proactive approach gained time for metabolite synthesis, qualification, and safety testing, in the unlikely event that a

disproportionate metabolite was observed. Initial plasma metabolite profiling focused on identifying human steady-state metabolites in MAD samples that exceed 10 % of drug-related materials (DRMs). If no metabolites were above this quantitative threshold, subsequent comparisons to preclinical species would become unnecessary. Proximity of metabolites to the 10 % threshold would determine how prudent it would be to rely on similar observations in the definitive human ^{14}C ADME study. According to the guidance, the human ^{14}C ADME study is still the definitive proof for human metabolite exposure, albeit from a design that is typically single dose. We anticipate that HR-MS will supplant the human ^{14}C study as the definitive determination of circulating human metabolites because most researchers in the industry are moving toward HR-MS techniques to profile nonradioactive metabolites earlier in clinical development. These techniques were not as well established when the guidance was developed. As such, case-by-case arguments to regulatory authorities for the absence of disproportionate metabolites will probably be advanced before the completion of the human ^{14}C ADME study. Suitable protocol language to enable these analyses was developed proactively for clinical and preclinical safety protocol templates.

The observation in MAD samples of human plasma metabolites that exceed the 10 % of DRM threshold would trigger the analysis of unused TK samples from preclinical safety studies, or samples from a separate PK dose-ranging study, conducted to address this issue.

PKDM development team representatives are now expected to provide an ongoing MIST guidance risk assessment to their management, educate their development team, and refine their development plans to allow time to react after pivotal MIST data from the human MAD study become available. When there are no unique human metabolites, or the human metabolites greater than 10 % of DRM are unambiguously “not disproportionate,” these data can be included in a dedicated metabolism report that integrates all the available metabolism data and makes a clear case that further metabolite monitoring or metabolite assessments are not needed. This final report can be submitted in the routine annual update to the common technical application (CTA). In the alternate scenario, a disproportionate metabolite triggers dialogue with drug safety, clinical, regulatory, and pharmaceutical sciences to address the issue in a case-by-case way. The actual *in vivo* testing of disproportionate metabolites is complicated, as the ADME of separately administered metabolites may not be equivalent to the ADME of the metabolite generated *in vivo* [15,16]. There are also concerns over the potential toxicological irrelevance of any study on a separately administered metabolite [17].

The main values of the phase 1 centric plasma analysis approach are the lead time garnered for dealing with either an unique or disproportionate metabolite(s) and the ability to defer the human ADME study to after clinical proof of concept, with minimal risk of a surprise.

6.6 NEW APPROACHES TO NONRADIOACTIVE METABOLITE QUANTITATION: FOCUS ON PLASMA SAMPLE POOLING, HIGH RESOLUTION MS, AND MASS DEFECT FILTER MASS SPECTROMETRY

6.6.1 Plasma Availability and Plasma Pooling Strategy

Preclinical TK and SAD/MAD PK plasma bioanalysis do not consume all the available sample. It is therefore possible to use unused plasma for exploratory metabolite

profiling and avoid dedicated preclinical PK studies or additional human subject cohorts. The plasma volumes that are typically unused from different PK and TK studies after routine parent drug are shown in Table 6.1. Plasma from no observed adverse effect level (NOAEL) doses in animals, or maximum feasible dose and the best estimate of the effective dose in human SAD/MAD studies (if this can be predicted by target coverage) are appropriate to show metabolite coverage across species.

A semi-quantitative estimate of all the drug-related components that contribute to the total DRM AUC is obtained from time-proportional pooling of left over plasma volumes from different time-points across the sample collection period. This method is referred to as the *Hamilton-pool* or the *AUC-pool* [18] and is well documented [19]. An example of the necessary pooled sample volumes for a hypothetical time course of sample collection is shown in Table 6.2. A plasma aliquot volume from each time-point is selected based on the interval between time-points and the pooled sample is created volumetrically or gravimetrically.

The AUC-pool approach has several advantages. The metabolite profile from an AUC-pool provides a valid semi-quantitative estimate of each metabolite's contribution to total drug-related exposure (AUC) or to a separately measured parent drug exposure, as required by the guidances. The approach requires only a single liquid chromatography–mass spectrometry (LC-MS) injection from a single pooled sample, rather than the separate analysis of individual points in the concentration versus time

TABLE 6.1 An Example of Unused Plasma Sample Volumes from PK and TK Analysis

Species and Study Type	n/Dose-group	# Time-points	Plasma Volume (μL)	Volume for Metabolite Profile (μL)/Animal
Rat PK	3	9–12	100–120	50
Dog/monkey PK	3	10–12	225	175
Rat 4-d TK	3	5	80	50
Rat 10-d to 6-mo TK	4–5	5–6	120–160	70
Dog/monkey 10-d to 6-mo TK	3–4	6	200–400	70
Human PK	>4	9–12	700–1000	500–900

The remaining volumes can be used for a relevant metabolite profiling, avoiding the expense, time, and additional animal usage involved in separate studies. Unused plasma can be pooled across time and/or across animals with no changes to current PK and TK sampling approaches. If dried blood spot analysis is required or becomes common, separate plasma samples from animals or subjects may be needed to enable metabolite analyses.

TABLE 6.2 The Time-Proportional Plasma Pool

Time (h)	0	1	2	4	8	24	Sum
Δt - pool (μL)	5	10	15	30	100	80	230

The “AUC-pool” measures metabolite contribution to total AUC. These principles are well established in the literature and enable the use of unused plasma from TK analysis to afford a reasonable estimate of exposure in one LC-HR-MS run. Pooling across animals/subjects further enables measurement of an “average” exposure to the full slate of plasma metabolites. The plasma volumes would be available from either a PK or TK study.

curve. The plasma volume requirements are well within the range of unused volumes from completed quantitative GLP analyses of parent drug (Table 6.1). The AUC-pool may be built for each individual animal or subject in the study or the plasma from different animals/subjects may be pooled to comprise a single average exposure estimate for all the individuals in the study. If plasma volumes are too limited or time-points are sparse, individual time-points could be analyzed or a separate study or sample cohort would be necessary.

6.6.2 High Resolution Mass Spectrometry (HR-MS) Methods

New HR-MS methods now provide a unique ability to discern all metabolites in biofluids with high resolution and sensitivity without dosing a radiolabeled drug. High resolution mass spectrometers with time-of-flight and Orbitrap [20] architectures have the analytical and data analysis capabilities to conduct HR-MS screening of plasma samples. These mass spectrometers allow an accurate determination of mass/charge ratio (m/z) of both the base mass spectrometry (MS) peak and its collision-induced dissociation (MS^n) fragments down to millimass or higher accuracy. After acquisition of LC-HR-MS data, techniques such as mass defect filtering, background subtraction, and isotope ratio filtering can discern both parent drug and drug-related metabolite peaks in the chromatogram.

The mass defect of a pseudomolecular ion is the difference between its high resolution m/z and its nominal m/z (e.g., if an analyte's high resolution m/z was 500.1234, its mass defect would be 0.1234 Da). Mass defects of a drug and its metabolites are similar, within predictable ranges, and importantly, are different from most endogenous interferences [21,22]. A suitable range above and below the mass defect is selected (e.g., ± 100 mmu) based on the parent drug accurate mass and logical biotransformations and ions are "mass defect filtered" to distinguish the drug and its metabolites from endogenous interference ions [23,24]. When control sample such as predose plasma is available, comparing its high resolution liquid chromatography–mass spectrometry (HR-LC-MS) profile with a dosed sample allows for every metabolite to be found based on the mass defect filter (MDF) alone without use of any biotransformation templates. Figure 6.3 illustrates the sensitivity and selectivity of the approach with plasma collected after a single dose of a [^{14}C]-labeled compound. The top and middle panels are an ultra performance liquid chromatography (UPLC) unfiltered total-ion chromatogram and corresponding MDF chromatogram, respectively. The bottom panel is the radiochromatogram obtained on a conventional high performance liquid chromatography (HPLC) with an in-line radiometric detector. The HR-MS-MDF profile recapitulates the radio-profile.

Background subtraction is another way to process the LC-HR-MS data. A difference chromatogram is obtained by subtracting the dosed plasma profile from a predose profile. Historically, this method has been sensitive to retention time shifts that can occur between the two LC runs. A new algorithm tolerant of retention time shift and that has additional noise filtering has been described recently [25]. Isotope ratio filtering can also be applied if the parent drug and metabolites have atoms with high abundance +2 isotope ions (e.g., Cl and S) [26].

Metabolite related information evolves throughout development and there is a sequential accumulation of both qualitative and quantitative data that eventually are integrated to discern any disproportionate metabolite. The plasma MDF data can be

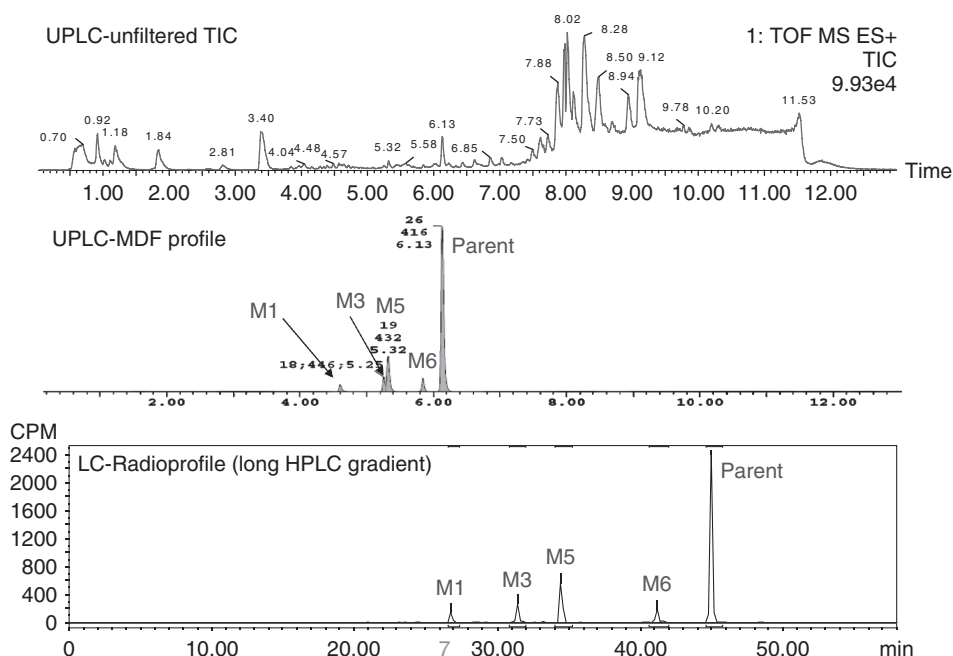


Figure 6.3 Metabolite observation by LC-HR-MS-MDF profiling. TIC, total ion chromatogram. (See color insert.)

compared to human and preclinical *in vitro* metabolite profiles and to the available preclinical *in vivo* ^{14}C ADME data.

Unfortunately, MDF metabolite profiles are not quantitative, and due to a number of factors, the MS response of the parent drug and metabolites can be different. These include electrospray ionization differences, ionization quenching by co-eluting endogenous compounds, or differences in solvent gradient composition at different elution times. When synthetic standards are available, the MDF profile peaks can be semi-quantitatively compared to metabolite mass by calculating response factors. MS response can be compared with response factors calculated from the diode-array detector (DAD) response across a wavelength range [19], with a radiolabeled metabolite peak area [27,28], or based on NMR quantitation of an isolated metabolite [29]. Each type of MS response factor calculation has its own limitations [30,31]. MS ionization methods employing lower flow rates appear to mitigate the ionization differences and new methods such as captive-spray ionization are being developed [31,32].

The HR-MS-MDF approach described here uses parent drug concentrations that were separately determined by validated LC-MS methods to benchmark the MDF response of each metabolite. Benchmarking to parent drug concentration enables semi-quantitative comparison of every human metabolite to comparably benchmarked pre-clinical plasma data. This approach to the MDF metabolite profile is illustrated in Fig. 6.4. The top panel shows a hypothetical HR-MS-MDF profile from an AUC-pool of plasma obtained from preclinical safety species at steady state. The bottom panel shows a human circulating metabolite profile obtained from an AUC-pool of plasma collected following either a single or a multiple oral dose. A qualitative examination

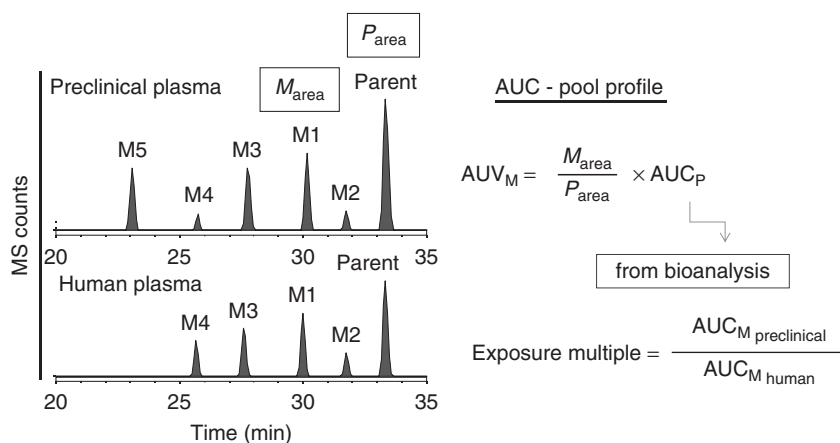


Figure 6.4 Estimation of circulating human metabolite coverage by obtaining HR-MS-MDF profiles of AUC-pooled plasma from human and safety species; and compute and compare the relative “AUC_M” from each profile.

reveals whether the metabolites observed in human were also found in preclinical safety species for the program. For a given metabolite, relative coverage can be estimated from the same data set without determining its actual concentration. Because the profile was obtained from an AUC-pool, the relative AUC of metabolite (“AUC_M”) can be computed:

$$AUC_M = \frac{M_{\text{area}}}{P_{\text{area}}} \times AUC_P$$

$$\text{Exposure multiple} = \frac{AUC_{M_{\text{preclinical}}}}{AUC_{M_{\text{human}}}}$$

where M_{area} and P_{area} are the peak areas of the metabolite and parent from HR-MS-MDF profile and AUC_P is the parent AUC, a parameter estimated already available from prior analysis using a qualified or validated bioanalytical method. Exposure multiple is then computed for each metabolite using the equation shown above. Metabolite coverage is satisfied if exposure multiple is equal to or greater than unity. The principal advantage of this method is that there is no requirement for any metabolite reference standard (synthetic, radiolabeled, or isolated). Furthermore, any interspecies metabolite abundance comparisons are focused only on metabolites that are quantitatively above the 10 % of DRM threshold in humans.

An analogous approach of estimating exposure multiple by simply computing the ratio of metabolite area in preclinical versus human from their HR-MS profiles was published recently [33]. In contrast to the approach advocated here, these authors do not benchmark metabolite abundance to the parent AUC in their exposure multiple calculations. In another variation, Gao *et al.* [34] have proposed measurement of relative plasma exposures. After qualitative metabolite profiling of AUC-pooled plasma discerns useful metabolite multiple reaction monitoring (MRM) transitions, a ratio of each metabolite and parent drug to an internal standard is measured by MRM on a triple-quadrupole mass spectrometer. In both methods [34,33], the dosed plasma is

mixed with the undosed comparator plasma, for example, dosed human plasma would be mixed with equal volume of undosed preclinical safety species plasma (or vice versa) to account for any potential endogenous interference in peak intensity that may arise from the mass spectrometric analysis. The drawback of this approach is unknown sample stability because addition of the undosed plasma could potentially alter metabolite levels due to species differences in plasma borne enzyme activities (e.g., esterases and amidases).

For any human metabolite that is at or near the 10% of DRM threshold, the higher the AUC_M in the preclinical safety species relative to human, the greater the confidence that the preclinical safety species covered the human metabolite. If the animal to human AUC_M ratio is close to 1 or lower, a synthetic standard and qualified bioanalytical method for the metabolite in question will probably be applied to enable a decision of whether the metabolite is disproportionate. A decision tree has been published that determines when a more rigorous analytical determination of a potentially disproportionate metabolite is needed [34].

Best practices incorporating a tiered analytical validation approach to metabolite quantification has been proposed by the European Bioanalysis Forum [4] and others [30,31]. Semi-quantitative estimation of metabolites by the HR-MS-MDF methods described above represents a screening method that will discern obvious and borderline disproportionate metabolites, but these methods do not constitute either qualified or validated assays that would be needed for further investigation of borderline cases.

6.7 THE HUMAN ^{14}C ADME STUDY

According to the CDER guidance, the human ^{14}C ADME is still the definitive study for human metabolite exposure and the impact of the guidance on the timing of the human ADME study has been discussed by Anderson *et al.* [30]. However, the Guidance did not foresee the technological advance of HR-MS-based mass defect filtering, which enables the visualization and semi-quantitative measurement of a slate of metabolites with minimal interference from endogenous peaks. The case-by-case provisions in the guidance enable a data-based argument to maintain the status quo for timing the human ADME study after clinical proof of concept and to use HR-MS-MDF analysis of plasma to drive earlier decisions on potentially disproportionate metabolites. There are specific cost advantages here, given the unfortunately high probability of compound termination at or before clinical proof of concept. There is still value in the human ADME study for discerning the rate and routes of excretion of radioactive metabolites and the complete recovery of the DRM. The non-ambiguous, quantitative measurement of radioactive metabolites and parent drug in plasma after a single radioactive dose in half a dozen healthy volunteers or patients can be used to corroborate the risk-based decisions on human metabolites that were built on the human MAD plasma profiles and by cross species comparison. The advantage of using the human ADME study for MIST strategy validation rather than MIST strategy development is completely avoiding the inferred need for a steady-state answer from a type of study that under almost all circumstances should be run as a single dose.

6.8 ACCELERATOR MASS SPECTROMETRY (AMS)-BASED HUMAN ADME STUDIES

Most companies conduct the human ^{14}C ADME study using a conventional radiochemical (ca. $100\text{ }\mu\text{Ci}$) dose and apply a liquid scintillation counting (LSC)-based detection whenever sensitivity allows. In cases where circulating radiochemical concentrations are low, accelerator mass spectrometry (AMS) will be necessary to obtain the requisite quantitative plasma metabolite profile. Accelerator mass spectrometers are essentially ^{14}C atom counters that enable plasma drug or metabolite quantitation many orders of magnitude below the limit of detection of LSC-based measurement [35]. Plasma obtained from a conventional $100\text{ }\mu\text{Ci}$ dose of a potent drug that affords very few radiocounts in plasma can be analyzed by AMS to obtain quantitative metabolite profiles. Alternatively, clinical human ADME studies can be conducted using radiochemical doses in the hundred nanocurie range, without a prerequisite rodent whole-body autoradiography-based radiodosimetry or the need for good manufacturing practice (GMP) radioformulation [36].

One drawback of AMS for quantitative metabolite profiling is that identification of isolated radioactive HPLC peaks is done by retention time only, as graphitization destroys the sample and affords only the quantitative data on ^{14}C atom concentration. Second, the analysis must be outsourced by most companies and AMS can be expensive when metabolism is extensive. The AUC-pool strategy can be used to decrease the cost of metabolite profiling by AMS. AMS vendors are all versed in the implications of the MIST guidance and have a unique ability to generate quantitative plasma metabolite profiles at plasma concentrations of radioactivity that are not measurable by any other method.

AMS vendors have proposed that AMS-based clinical designs could accelerate the human ADME study into phase 1 to address MIST. With the availability of the HR-MS techniques discussed above, this may be unnecessary except under unusual circumstances that might include exploratory investigational new drug (eIND) approaches for lead selection [37]. In the eIND approach, human metabolite profiles could be obtained by AMS from select superior leads being advanced to a full CTA. As such, AMS approaches within the eIND approach may enable earlier human plasma metabolite quantitation in select programs.

6.9 IMPLEMENTATION

After the final MIST guidance issued in February 2008, at Amgen, we monitored and contributed to the discussions occurring throughout the industry [7,38]. Some aspects of the guidance elicited extensive discussion in the 17 MIST-related papers published in 2009. Many of these publications focused on different analytical approaches to metabolite assessment and also made data-driven comparisons of our ability to predict the structure and relative importance of human metabolites in plasma (reviewed in Ref. 7). Some literature and company database meta-analyses on the probability that a metabolite will be disproportionate were developed [9,39]. An opinion paper from a CDER representative reiterated the case-by-case aspects of guidance compliance [40]. By the end of 2009, PKDM groups were testing and implementing strategies to comply with the FDA guidance when the December 2009 ICH M3(R2) guidance

became effective. It set a different and less conservative threshold of 10 % of DRM and dose greater than 10 mg. In papers and seminars from 2010, the focus shifted to the role of bioanalysts in MIST strategy and the industry converged on MS-based approaches [31].

Our initial strategy within PKDM was to triage discovery and development of small molecule programs and work with PKDM scientists to identify programs at risk for disproportionate metabolites. Development plans of affected programs were then modified to address the MIST guidances and team PKDM scientists embarked on case-by-case development team education and data generation. Meanwhile extramural and intramural experience grew and consistent internal procedures were developed. Opinion leaders in drug safety, early clinical development, regulatory affairs, and pharmaceutical development previewed the PKDM MIST strategy and helped develop best practices for plasma procurement and metabolite profiling. Discussions with chemists and pharmaceutical development revolved mainly around responsibility for an earlier synthesis of the major *in vitro* human metabolites to enable activity testing and use as analytical standards. In some cases, depending on synthetic complexity, biogenesis of the metabolite(s) [41] may be required and purification and determination of analytical purity can be done by NMR [29] or a variety of MS techniques [31].

Good laboratory practice/good clinical practice (GLP/GCP) compliance and metabolite stability aspects of procuring unused plasma from preclinical safety and human SAD/MAD studies were worked out. The use of unused plasma and sample pooling is efficient and avoids the time involved in conducting separate studies. When clinical plasma becomes available, unused plasma could be obtained from GLP studies by amendment or from a separate PK study. Owing to uncertainties regarding metabolite stability, protracted cold storage of plasma for metabolite analysis is less desirable than the use of more recently procured samples. Non-GLP analysis of the plasma samples was preferred, with a bioanalytical report as the deliverable. After conducting the initial human plasma screening of the SAD and MAD samples, steady-state human metabolites at or near the 10 % of DRM threshold would be the reason for, and focus of, subsequent analysis of preclinical plasma samples.

Similar discussions with clinical development centered on mechanisms for the transfer of samples after PK analysis, suitable protocol, and informed consent language.

As in the analysis of preclinical safety samples, non-GLP analysis would be pursued and a bioanalytical report would be generated. Ultimately, a MIST summary report encompassing all the *in vitro* and *in vivo* metabolism data would be assembled along with a recommendation for any necessary subsequent activities. With adequate preclinical coverage, the recommendation would be no further metabolite profiling is necessary until the human ADME study. The report could be submitted in late phase 1, coincident with the next annual report to the CTA. Regulatory endorsement that all MIST issues are resolved would be requested. Clearly disproportionate metabolites or metabolites at or near the threshold would entail more interactive discussions on the path forward with teammates and regulators.

The responsibilities of the PKDM project leader were as follows: Develop a MIST action plan for their candidate, include a MIST risk analysis statement and related activities in monthly highlights, and engage development team partners. PKDM project leaders were to leverage available pre-IND samples for preliminary method development and analysis, arrange synthesis of key metabolite(s), and arrange determination of their pharmacological on target activity, with a team dialogue on how much activity data

were appropriate. Protocols were to be checked for appropriate sample retention, storage, and transfer. The PKDM project leader would discern human metabolites greater than 10 % of DRM first, compare other species as necessary, and then write MIST bioanalytical reports to compile into the consensus metabolism report and action plan. Recommendations might include subsequent metabolite(s) monitoring in TK studies and/or in larger clinical studies. When this is necessary, bioanalysts could be engaged in GLP method development, and regulatory and early development partners would then help define the more complicated, case-by-case path forward by discussion with regulators.

The rollout was conducted at unit meetings in preclinical safety and regulatory and clinical safety units. All the planning and rollout of these activities took about one year from initial discussions to endorsement and routine application. The plan evolved significantly over time as many new publications became available that discerned how the industry as a whole would deal with MIST guidance compliance.

6.10 CONCLUSION

This chapter overviewed the development and rollout of a plan to comply with new regulatory guidances on metabolites. A proactive, data-driven, best-practice approach was developed by consensus with key development partners. Changes to traditional metabolism approaches included nonradiometric profiling of plasma metabolites across species and the development of a data-driven plan to preclude the existence of disproportionate metabolites. When potentially disproportionate metabolites are observed, under this plan, there is still time to conduct subsequent metabolite assessments, well in advance of the end of phase 2.

Suddenly a mist fell from my eyes and I knew the way I had to take.

—Edvard Grieg, Composer (b1843–d1907) [42]

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ABBREVIATIONS

AMS	accelerator mass spectrometry
ADME	absorption, distribution, metabolism, excretion
AUC	area under the plasma concentration versus time curve
CDER	Center for Drug Evaluation and Research
CTD	common technical document

DDI	drug–drug interaction
DRM	drug-related material
IND	investigational new drug
eIND	exploratory investigational new drug
FIH	first-in-human, the development phase when the drug candidate is first dosed in human subjects
HR-MS	high resolution mass spectrometry
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
MDF	mass defect filter
MIST	metabolites in safety testing (safety testing of metabolites)
MAD	multiple ascending dose
m/z	mass/charge ratio
NOAEL	no observed adverse effect level
SAD	single ascending dose
PhRMA	DMTG Drug Metabolism Technical Discussion group of the Pharmaceutical Research and Manufacturers Association
R&D	research and development
PKDM	pharmacokinetics and drug metabolism organization (DMPK in some organizations)
POBA	proof of biological activity
PK	pharmacokinetic
TK	toxicokinetic

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7 *In Vitro* Toxicity Screening in Early Drug Discovery: Importance of Metabolism and Reactive Metabolites

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7.1	Summary	1
7.2	Introduction	2
7.3	Phase I and phase II metabolism in the gastrointestinal tract	3
7.4	<i>In vitro</i> approaches for determining the role of intestinal metabolism	10
7.5	Subcellular fractions (microsomes)	11
7.6	Precision cut tissue slices from the intestine	11
7.7	Hepatic metabolism and toxicity	13
7.8	<i>In vitro</i> determination of CYP inhibition and potential drug–drug interactions	16
7.9	<i>In vitro</i> detection of reactive metabolite formation	18
7.10	<i>In vitro</i> determination of covalent protein binding	21
7.11	<i>In vitro</i> determination of oxidative stress	22
7.12	<i>In vitro</i> determination of mitochondrial membrane permeability	23
7.13	Primary hepatocytes in sandwich culture	24
7.14	Future <i>in vitro</i> models for assessing hepatic drug metabolism and toxicity	24
7.15	Conclusion	25
	Acknowledgments	25
	References	25

7.1 SUMMARY

This chapter focuses on the role of metabolites in determining drug efficacy and toxicity. Most drugs are administered orally, and therefore, the organs with the most immediate impact on their biological fate are the intestine, where absorption occurs, and the liver, where metabolism, elimination into bile, and movement into general circulation occurs. The primary enzymes responsible for phase I metabolism of drugs and

chemicals belong to the cytochrome P450 (CYP) family of proteins. Although these important enzymes are found throughout the body, they are in highest concentration in the liver. In recent years, the presence of CYP enzymes in the intestinal enterocytes and their effect on drug bioavailability, efficacy, toxicity and differences between species have been recognized.

This chapter presents the important role of intestinal and hepatic metabolism in determining the amount of parent drug and metabolites that can enter general circulation. Phase I metabolism can yield nontoxic (more) water-soluble products that can be subsequently conjugated to glucuronic acid, sulfate, or glutathione (GSH) by phase II enzymes. Phase I metabolism can also generate highly reactive (electrophilic) intermediates that are capable of covalently binding to and altering the function of essential cellular proteins, which can lead to cytotoxicity. The relative abundance and distribution of CYP enzymes and transporters in the intestine and liver are important determinants of these effects. Several examples of drug metabolism and bioactivation in the intestine and liver will be presented.

An essential component of any drug discovery program is to understand the potential adverse effects associated with new drug candidates. Initially, laboratories focused on the parent compound, but it is now clear that metabolic stability and bioactivation in the intestine and liver can have a significant impact on the safety and efficacy of new drugs. Extensive presystemic metabolism can reduce bioavailability, and production of reactive metabolites can increase the risk of toxicity. Many new compounds can be enzymatically converted to reactive intermediates capable of binding to, and altering the function of, essential cellular proteins or DNA. *In vitro* methods for characterizing metabolism, bioactivation, and cytotoxicity are presented. Emphasis has been placed on the relationship between the *in vitro* concentration that causes adverse effects and the expected *in vivo* maximum therapeutic plasma concentration. This provides a logical means of using *in vitro* models to identify potential hazards while at the same time linking these effects to an *in vivo* parameter. Finally, a road map for the collection and incorporation of *in vitro* data into a risk assessment and decision-making process, will be provided.

7.2 INTRODUCTION

The development of new drugs requires 12–15 years of research and nearly \$1 billion. One reason for the high cost is the high percentage of promising new drug candidates that are dropped because of unanticipated adverse effects in either preclinical or clinical safety trials. Over the past 10 years, there has been a significant change in the standard process of drug discovery and development. The incorporation of *in silico* and *in vitro* test systems into the hit-to-lead and lead optimization phases of drug development has significantly reduced the attrition rate. The key to the successful utilization of *in vitro* data to predict adverse outcomes in animals and humans is incorporating a disciplined *in vitro* testing strategy that includes multiple endpoints, tissue-specific models, multiple exposures, and a method for including the data obtained into the decision-making process. Nearly all pharmaceutical companies now include some form of an early toxicity screening process into their discovery process; however, many of these groups are not consistent in how they use the information obtained. A failure to understand the biology of the cell model being used as the test system, the use of limited endpoints

for toxicity, and exposure concentrations that are far above those expected *in vivo* under therapeutic conditions have made it difficult to consistently extrapolate the *in vitro* data to *in vivo* effects. Relating the *in vitro* exposure concentration to the actual plasma concentrations during repeated dosing in animals and humans is an essential component of predictive *in vitro* screening. In order to accurately use *in vivo* plasma concentration as an anchor for *in vitro* studies, it is important to understand how a new chemical entity (NCE) is metabolized. The enzymes involved, the metabolites formed, and the role of presystemic metabolism provide important insights that enable more accurate predictions of *in vivo* events from *in vitro* data. Knowledge of drug metabolism is a critical component to the development and evaluation of new drug candidates. Although the information from *in vitro* models for absorption, distribution, metabolism, and elimination (ADME) is typically acquired, the information is often left out of *in vitro* toxicity screening approaches where the goal is to identify liabilities and predict the probability of an adverse event not only in animals but also in humans. The inclusion of *in vitro* kinetic data greatly improves the models' predictive power. The purpose of this chapter is to provide an understanding of why and how *in vitro* endpoints designed to define metabolic stability, presystemic metabolism, and the formation of reactive metabolites can improve the process of hazard identification and risk assessment. Although drug metabolism can occur throughout the body, this chapter focuses on intestinal and hepatic metabolism, as the intestine and liver are the primary sites of absorption, metabolism, and presystemic transformation of orally administered drugs.

7.3 PHASE I AND PHASE II METABOLISM IN THE GASTROINTESTINAL TRACT

The oral route is one of the most common means of administering medicine. Efficacy and toxicity are determined by the corresponding concentration of the drug in plasma and tissues. The fraction of the dose absorbed across the intestine into portal circulation and the fraction that passes through the liver into systemic circulation determine the bioavailability of the drug. Bioavailability is determined by three distinct phases. The first phase is the fraction of drug that leaves the intestinal lumen (F_a) and does not degrade in the lumen or end up excreted in feces. The second phase defines the amount of drug that survives metabolism within the intestinal cells (F_g). The third phase is the fraction of the parent drug that leaves the liver without metabolic transformation (F_h) [1]. Loss of drug in the lumen (F_f) and metabolism in the liver (F_h) have long been considered to have the most significant impact on bioavailability (F). It is now clear that intestinal phase I and phase II metabolism of drugs also have a significant impact on the systemic exposure of orally administered drugs.

Metabolism of drugs in the intestinal enterocytes by phase I and phase II enzymes and active transport out of cells by P-glycoprotein (Pgp) are important in determining oral bioavailability [2]. Although several CYPs have been identified in human intestine (CYP1A1, CYP2C8–10, CYP2D6, CYP2E1, CYP3A4, and CYP3A5), by far, the most abundant and most important with respect to drug absorption is CYP3A4. This enzyme represents only 30–40% of the total hepatic CYP pool but 70–80% of the intestinal CYP pool and has activity that is equal to or greater than that found in the liver [2–5] (Fig. 7.1). Determining the portion of the administered oral dose that reaches general

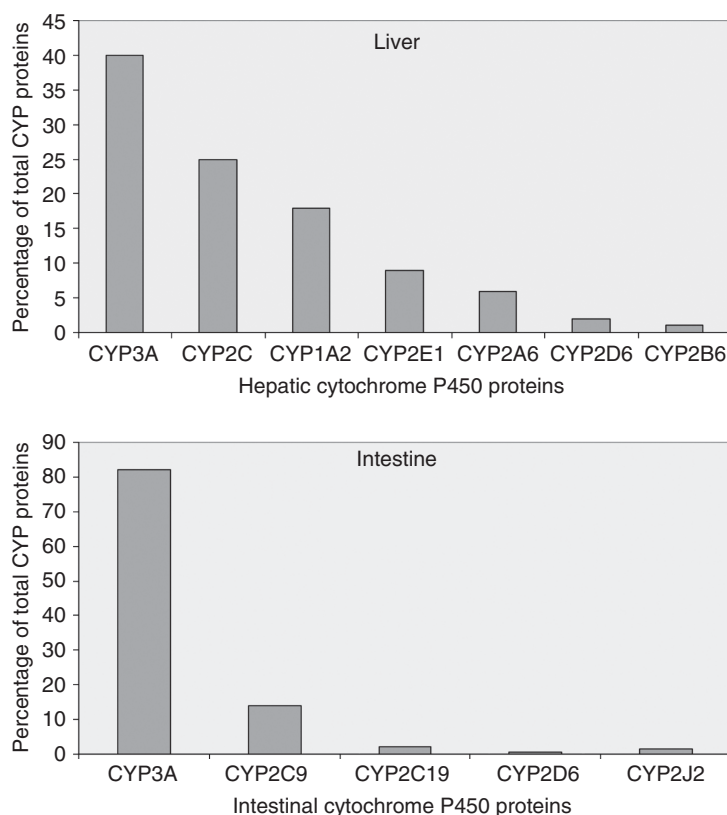


Figure 7.1 Comparison of the relative abundance of CYP proteins in human liver and intestine.

circulation (%*F*) is important for determining an efficacious or therapeutic dose, tissue interactions, and toxicity. When orally administered drugs are rapidly metabolized before entering general circulation, they are said to undergo first-pass metabolism. Intestinal metabolism by phase I and phase II enzymes participates in biotransformation of absorbed drugs before reaching the liver. In the liver, the parent drug (as well as primary metabolites) is subject to additional (secondary) metabolism. As a result, orally administered drugs that are substrates for CYP enzymes can undergo substantial metabolic transformation before ever reaching systemic circulation. Although intestinal metabolism of drugs has been known to occur, it was generally believed that the liver played the primary role in first-pass metabolism. This belief changed when it was discovered that CYP3A4 existed not only in the liver but also in the intestinal mucosa [4]. Cyclosporine and midazolam are rapidly metabolized by gut CYP3A4 before reaching the liver and therefore the gut plays an important part in first-pass metabolism. Midazolam metabolites (1-hydroxy and 4-hydroxy) can then be conjugated with glucuronic acid by UDP-glucuronosyltransferases 1A4, 2B4, and 2B7 (Fig. 7.2) [6]. Because CYP3A4 is known to metabolize and detoxify more than half of the prescribed drugs, its presence in both the intestine and liver cannot be overlooked.

The anticancer drug docetaxel (Taxotere) provides an excellent example of the significant role that intestinal CYP3A4 can have on drug efficacy and toxicity. This drug

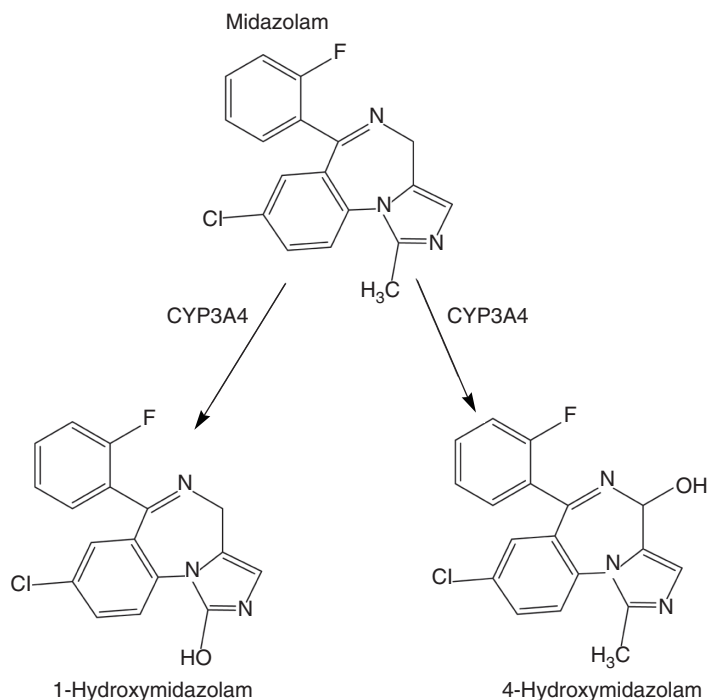


Figure 7.2 Midazolam metabolic pathways.

is used to treat advanced breast cancer. Like most cytotoxic antitumor drugs, docetaxel has a narrow therapeutic window and large interindividual variation in exposure and clearance. Therefore, any change in its absorption or elimination will have a significant impact on its efficacy and toxicity. The use of knockout mice that express CYP3A in liver, but not in their intestine, to study the uptake and metabolism of docetaxel revealed a dramatic increase in plasma levels reflected by an increase in $C_{\max}$, $\%F$, and AUC_{oral} relative to wild-type mice that have both intestinal and hepatic CYP3A4. When the situation was reversed, mice that lacked hepatic CYP3A, but expressed intestinal CYP3A, had much less total absorption into the general circulation, with significant reductions in $\%F$, Cl , and AUC_{oral} . It was concluded that the presence of CYP3A in the intestine had a greater impact on the amount of drug entering general circulation than CYP3A metabolism by liver alone. However, the hepatic expression of CYP3A was most important in determining the clearance of drug [7]. Clinically, docetaxel is given as a 1-h intravenous drip. This route of administration bypasses gut metabolism and relies on liver metabolism for clearance (Fig. 7.3).

The sleep aid triazolam was also evaluated in a mouse CYP3A knockout model. Triazolam is a classical substrate for CYP3A, but not for the export pump Pgp [8]. In knockout mice that lacked all CYP3A, the metabolism of triazolam was significantly reduced. Like docetaxel described above, intestinal metabolism by CYP3A played an important role in controlling drug exposure. When the CYP3A inhibitor ketoconazole was coadministered with triazolam, there was a substantial increase in triazolam exposure, which was due to the inhibition of CYP3A4 metabolism. If CYP3A expression

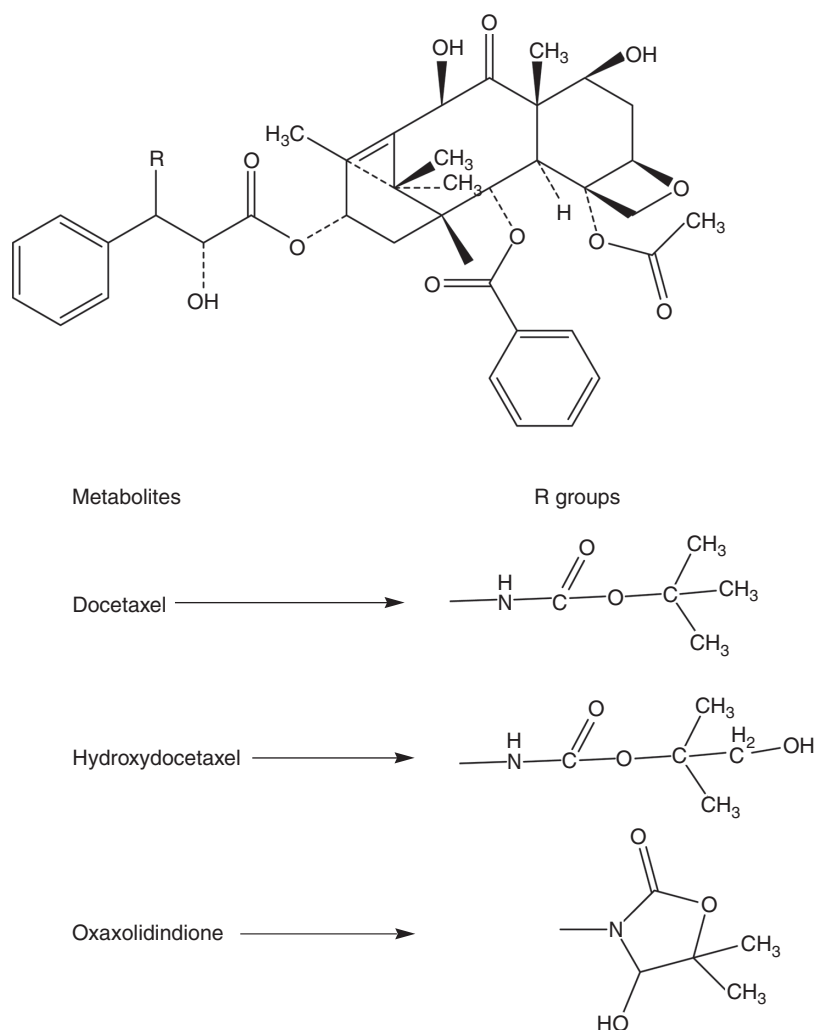


Figure 7.3 Metabolism of docetaxel.

was induced by the administration of a drug such as gefitinib, the level of systemic exposure was lower due to increased metabolism [8]. These findings are important because they demonstrate the barrier role that intestinal metabolism can play. This type of drug–drug interaction (DDI), when one drug induces the metabolism of another, is termed *heterotropic positive cooperativity*. Knowing how coadministered drugs affect CYP induction and inhibition is important for predicting adverse events [8–10].

Warfarin has been used extensively to prevent thromboembolic complications associated with atrial fibrillation. Because this drug has a narrow margin of safety, serious DDIs can occur when administered with drugs that inhibit its clearance. In humans, CYP3A4 is the primary enzyme responsible for warfarin metabolism, which produces the 3-hydroxy and *N*-oxide metabolites. Inhibition of CYP3A4 by fluconazole and

miconazole caused elevated plasma concentrations of warfarin and enhanced anticoagulation effects. If the drug was administered with compounds that induce drug-metabolizing enzymes, warfarin metabolism increases and plasma concentrations can decrease below therapeutic levels, resulting in a diminished (efficacy) anticoagulant effect [9].

Rifabutin is a broad-spectrum antibiotic used to treat infections in AIDS patients. The oral bioavailability of the drug is low and extremely variable ranging from 3% to 42% [11]. When drugs known to be potent inhibitors of CYP3A (e.g., fluconazole, ketoconazole, and clarithromycin) were coadministered with rifabutin in humans, both the peak plasma concentration and the AUC were substantially increased. The inhibition of CYP3A activity by ketoconazole or fluconazole in microsomes prepared from both intestinal enterocytes and liver showed that enzyme inhibition in the microsomes from the enterocytes was similar to inhibition observed in microsomes prepared from liver [12]. Interestingly, while CYP3A4 represents only 30% of the total human hepatic P450 content, it represents more than 70% of the CYP content in human enterocytes. This example as well as those already presented, indicate that the metabolism of orally administered drugs by intestinal CYP3A4 represents an important barrier to absorption.

A study in human patients undergoing liver transplant provides additional evidence supporting the importance of intestinal metabolism to bioavailability. In this example, cyclosporine was administered directly to the small intestine during the procedure. Subsequent analysis of portal blood showed that 25–50% of the parent drug had been metabolized [5]. A similar experiment was also done with midazolam, and the results showed that only 12% of the parent drug entered portal blood [2,13].

Clearly, the ability to separate intestinal metabolism from hepatic metabolism of a drug would be important in terms of predicting systemic availability. This separation was achieved in clinical pharmacokinetic studies by administering cyclosporine by i.v. and oral routes. The drug was given alone or in combination with a known inducer of CYP3A4 (rifampicin) or an inhibitor of CYP3A4 (ketoconazole). In this way, it was possible to separate the relative contributions of intestinal and hepatic metabolism to oral bioavailability [14,15]. Differentiation was based on the observation that the observed oral bioavailability (F) could be determined by taking the product of the fraction absorbed as parent drug by the intestine (F_a) and multiplying it by the fraction of parent drug that entered portal circulation (F_g), and by the unmetabolized portion of the drug that passes through the liver and into systemic circulation (F_{hep}) (Eq. 7.1) (Fig. 7.4) [16].

$$F = F_a \times F_g \times F_{\text{hep}} \quad (7.1)$$

The fraction that passes through the liver unmetabolized was determined by dividing i.v. drug clearance by hepatic blood flow. The results of experiments conducted in patients with cyclosporine revealed that cyclosporine bioavailability was controlled primarily by intestinal metabolism (F_g).

In clinical practice, there can be wide interindividual variation in the disposition or bioavailability of orally administered drugs that can be metabolized by CYP3A4. These changes can not only impact plasma concentrations and efficacy, but also influence compound toxicity.

As we have seen in the discussions thus far, the safety profile of a drug can be altered if its metabolism and clearance are inhibited, or induced because these effects

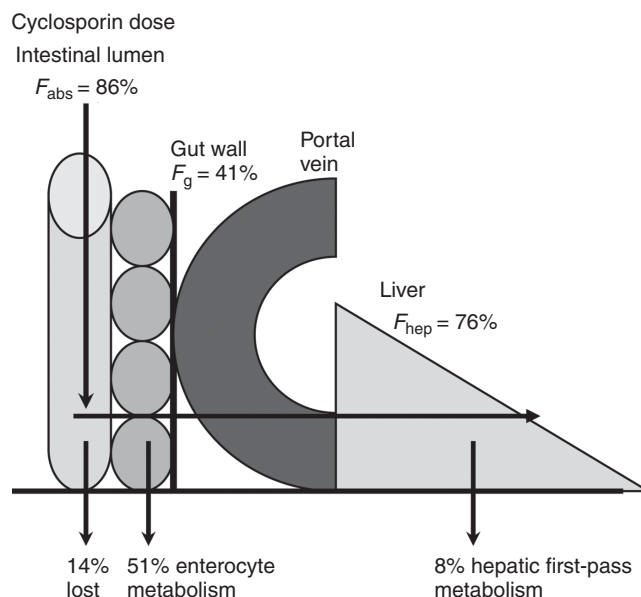


Figure 7.4 Diagrammatic representation of cyclosporine bioavailability.

can increase plasma concentrations above a safe level or decrease plasma concentrations below an efficacious level. Adverse events can also occur when a drug is metabolized to a reactive intermediate, which can bind to cellular nucleophiles (such as proteins) and alter their function. Orally administered drugs that undergo metabolic activation via CYP3A4 or other CYP enzymes can form reactive metabolites during absorption by the gut enterocytes and by CYP metabolism in the liver.

Nevirapine is a non-nucleoside HIV-1 reverse transcriptase inhibitor and was the first of its type to be approved by the US Food and Drug Administration (FDA) for combination therapy. This drug is widely used throughout the world and represents one of the most prescribed antiretroviral drugs in the developing countries. Despite its success, nevirapine has been associated with hepatotoxicity and skin rash. Although the exact mechanism(s) responsible for the observed toxicity has not been clearly defined, one possibility is the formation of reactive metabolites via CYP metabolism. It is known that this drug undergoes extensive metabolism in the liver, forming 2-, 3-, 8-, and 12-hydroxyl metabolites followed by phase II glucuronidation or sulfation. Formation of GSH adducts can be an indication of the formation of reactive metabolites. GSH adducts formed in human liver microsome experiments were found after metabolism by CYP3A4, 2D6, 2C19, and 2A6, with the greatest portion of reactive intermediate formed by CYP3A4 [17]. 12-Hydroxynevirapine has been associated with multiple DNA adducts [17–19] and with the formation of skin rash when dosed at concentrations lower than those of the parent drug. It is believed that reactive intermediates play a role in the liver toxicity of nevirapine and that metabolism to reactive intermediates can occur via two pathways (Fig. 7.5).

One pathway involves the formation of GSH adducts in the absence of phase II conjugation by a dehydrogenation mechanism to yield the quinone methide species.

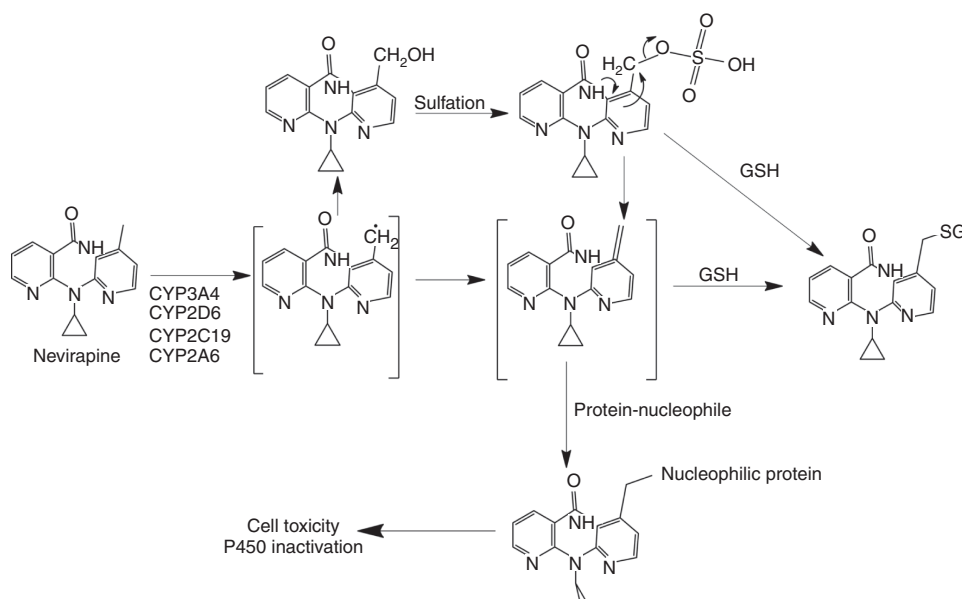


Figure 7.5 Nevirapine metabolic pathways.

In vivo, the most likely pathway is sulfation, followed by spontaneous loss of sulfate and formation of the quinone methide, which binds to cellular nucleophiles. It is now known that sulfotransferases are present in the intestine. Therefore, the primary *in vivo* reaction described for nevirapine is likely to occur before the drug reaches the liver. This implies that high CYP3A4 activity in the intestine combined with the presence of intestinal sulfotransferases could increase the amount of 12-hydroxynevirapine and its sulfation product that reaches the liver. This combined with the fact that most patients receiving the drug are HIV positive, take multiple drugs, and have induced intestinal and hepatic CYPs or compromised liver health could explain at least in part interindividual differences in hepatic toxicity observed in this patient group.

Phase II conjugation enzymes which are responsible for glucuronidation and sulfation are present in the intestinal enterocytes [20–22]. These enzymes are now known to play a significant role in the bioavailability of drugs. For example, the bioavailability of orally administered salbutamol is quantitatively affected by the activity of sulfotransferase 1A3. Studies have shown that presystemic intestinal availability of salbutamol was lower than its presystemic hepatic availability [23]. These data provide evidence for a quantitative effect of sulfotransferases on the oral bioavailability of salbutamol.

When drugs have a high degree of cell permeability, the fraction lost in the intestinal lumen is low. An example is ethinylestradiol (EE), which has a $\log P$ of 4.52 and high cell permeability. As a result, nearly the entire amount of dosed drug enters the enterocytes, but <50% of the parent molecule remains after intestinal metabolism ($F_g = 0.479$). The fraction of the remaining parent drug then enters the liver where only a small fraction undergoes metabolism ($F_h = 0.874$) before reaching systemic circulation [1]. These data clearly indicate that for this drug, intestinal metabolism is the primary determinant of bioavailability. The presence of drug transporters, such as

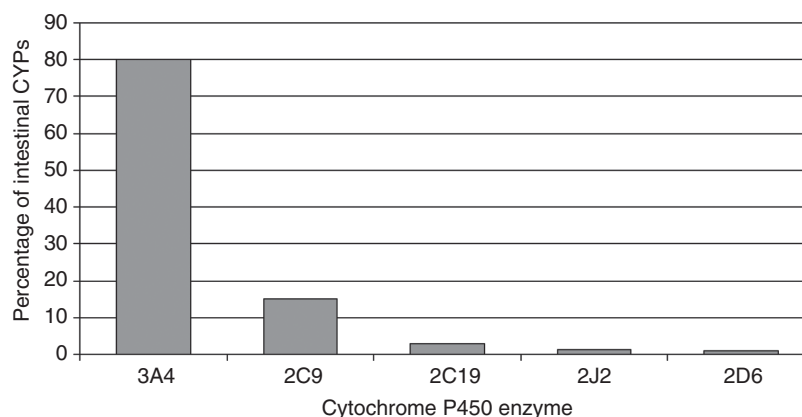


Figure 7.6 Percentage of total CYP proteins in the human intestine.

MDR1, MRP, and OAT, can also have a significant impact on drug absorption. The reader is referred to a review by van de Kerkhof *et al.* [24] and others to begin learning about transporters in the intestine and their impact on drug absorption.

The presence of drug-metabolizing enzymes in the GI tract is highest in the more proximal regions. The duodenum and jejunum have higher activity than the ileum and colon, and this is true in both rats and humans. In humans, there are considerable interindividual differences in the relative abundance of each CYP isoform. This is due to polymorphisms, age, gender, diet, and the presence of medications. In general, CYP3A4 represents the greatest portion of the intestinal CYP protein (80%), followed by CYP2C9 (15%), CYP2C19 (2.9%), CYP2J2 (1.4%), and CYP2D6 (1.0%) (Fig. 7.6). Unlike the liver, CYP1A1/2 and CYP2D6 are either lacking or in very low abundance [24,25]. In general, the level of phase II conjugating enzymes present in the intestine decreases in the distal direction, although their abundance is not the same in all parts of the intestine. Sulfatase activity is high in the more proximal regions of the small intestine, but the highest activities have been reported in the colon. Glucuronidation activity for UGT2B1, 2B3, and 2B6 decreases in the distal direction; however, UGT1A1 and 1A6 activities are homogeneous throughout the small intestine. The colon possesses the highest activity of UGTs (UDP-glucuronosyl transferases) 1A1, 1A3, 1A6, and 1A8. Glutathione *S*-transferase (GST) activity has also been demonstrated in the intestine.

7.4 IN VITRO APPROACHES FOR DETERMINING THE ROLE OF INTESTINAL METABOLISM

Understanding the quantitative effects of intestinal and hepatic metabolism early in the drug discovery process is important for lead optimization and selection of a candidate molecule for preclinical safety studies. *In vitro* test systems that utilize subcellular fractions (e.g., microsomes), primary cells, whole-organ perfusion, and immortalized intestinal cell lines can be used (reviewed by van de Kerkhof *et al.* [24]). The Ussing chamber is primarily used to study permeability. The method involves the dissection of a small section of intestine, which is then placed into the chamber. An important

limitation of this system is that tissue viability is only for 2–4 h, making it difficult for use in toxicity studies. Primary intestinal epithelial cells can also be isolated; however, the yields are typically low and successful culture is difficult to optimize. Two other important *in vitro* methods for evaluating intestinal metabolism and drug toxicity are microsomes and precision cut intestinal slices (PCIS).

7.5 SUBCELLULAR FRACTIONS (MICROSOMES)

An excellent example that demonstrates the importance of characterizing intestinal and hepatic metabolic pathways before making predictions about clinical risk is illustrated in the work reported by Dalvie *et al.* [22] for the drug raloxifene (Evista). Raloxifene is an estrogen receptor modulator and is used therapeutically to treat osteoporosis and breast cancer. Raloxifene undergoes first-pass metabolism with an oral bioavailability of only 2%. *In vitro* systems have demonstrated the formation of a reactive metabolite and the subsequent formation of GSH and protein adducts. Bioactivation occurred in both human and rat liver microsome experiments (Fig. 7.7).

Interestingly, despite the *in vitro* data, no significant drug-related adverse events have been observed in humans who received the drug by oral administration. This apparent disconnect between the *in vitro* data and *in vivo* reality can be explained by understanding presystemic metabolism in the intestine and liver.

The *in vitro* experiment designed by Dalvie *et al.* [22] involved an initial incubation of raloxifene with human intestinal microsomes (HIM) and rat intestinal microsomes (RIM). This was followed by another incubation in either human liver microsomes (HLM) or rat liver microsomes (RLM) (Fig. 7.8). The data generated from this experiment clearly showed that intestinal phase I and phase II metabolism greatly reduced the amount of raloxifene that reached the liver. As a result, the intrahepatic concentrations of reactive metabolites were below those that would cause an adverse event.

The use of both intestinal and hepatic microsomal metabolism data can greatly improve the interpretation of the *in vitro* data. It is important to remember that microsomal systems do not possess the capacity to measure phase II conjugation metabolism. Therefore, either these enzymes and cofactors must be added or a more complete subcellular fraction, such as an S9 fraction, should be included. The primary advantages of using subcellular fractions are that they can provide information on the metabolic stability, metabolic activation, and relative metabolism of the intestine and liver both rapidly and at low cost. The information obtained can be used to identify drug inhibition, DDI issues, and species differences.

7.6 PRECISION CUT TISSUE SLICES FROM THE INTESTINE

Simple *in vitro* methods that enable the study of drug metabolism and toxicity in the intestine are limited when compared to those for other organs such as the liver. The intestine is highly heterogeneous with structural and functional differences between the duodenum, jejunum, ileum, and colon. PCIS provides a power tool for studying the impact of these regions on drug metabolism, bioavailability, and toxicity. Nonsolid tissue, such as from the intestine, can be prepared for tissue slicing by cutting a section of the intestine from the region of interest, ligating one end, and then filling it

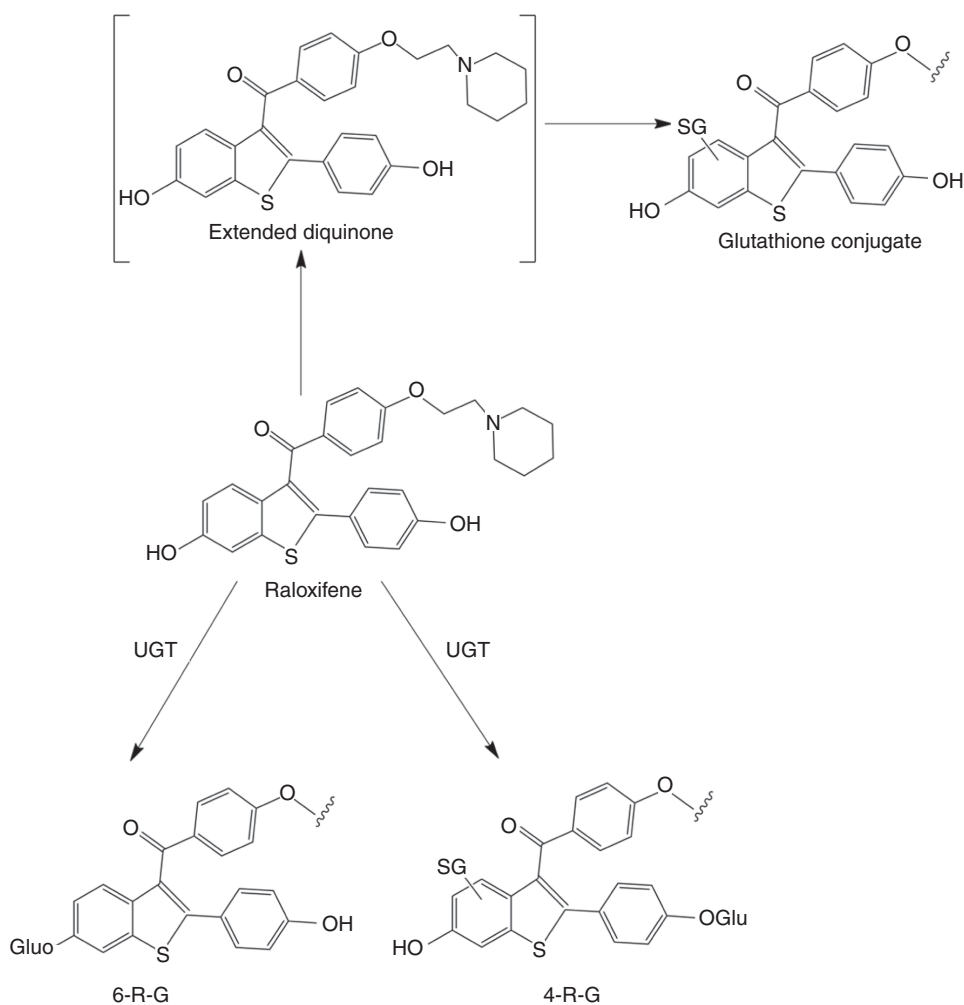


Figure 7.7 Metabolic pathway of raloxifene.

with agarose maintained at 37°C. The agarose-filled section is allowed to solidify by cooling in an ice-cold medium. The section filled with agarose is then placed into the embedding unit and covered with low melt agarose to form a solid cylindrical organ base. Tissue embedding can be done using the tissue embedding unit from Alabama R&D (Munford, AL) [26]. The embedded tissue can then be sliced using either a Krumdieck *et al.* [27] or Brendel-Vitron [28] slicer (Fig. 7.9).

Studies comparing the quality of slices prepared from each slicer showed no appreciable differences [29]. The Krumdieck slicer is fully automated and can be set to a continuous mode. The Brendel-Vitron slicer is manual but has the ability to oxygenate tissues, which the Krumdieck slicer does not have. The use of these slicers enables the preparation of reproducible tissue slices with uniform thickness. For intestinal tissue, the slices are typically between 150 and 400 µm in thickness. A standard culture medium consists of Williams medium E with glutaMAX supplemented with antibiotics.

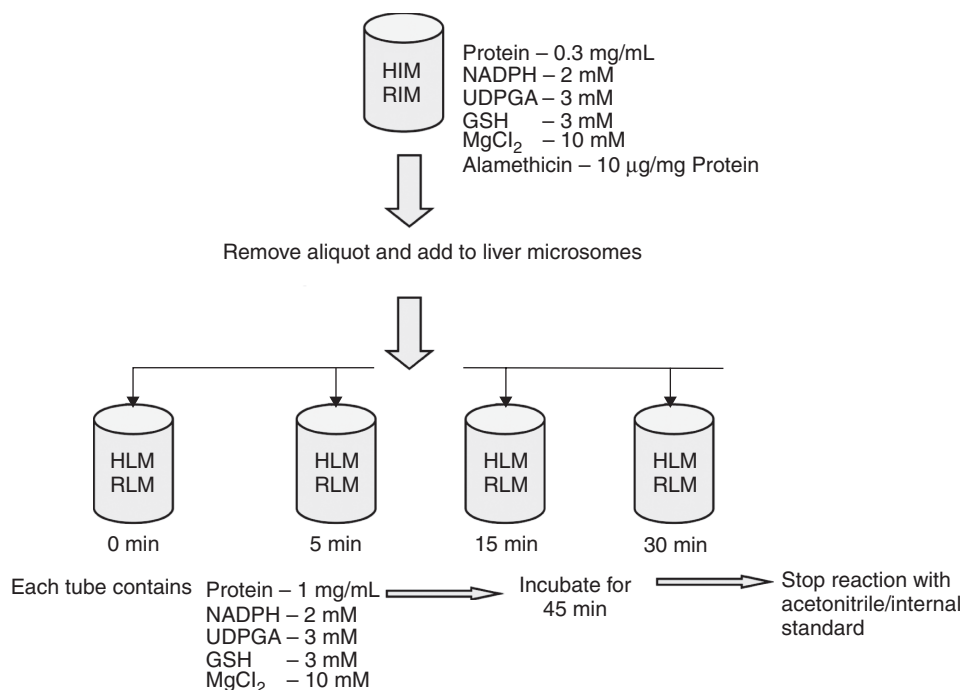


Figure 7.8 Sequential exposure of drug to rat and human intestinal and liver microsomes. UDPGA, UDP glucuronic acid.

Additional supplements have been suggested as a means of improving viability and extending culture time, including insulin, hydrocortisone, methionine, and fetal calf serum [30,31].

PCIS possess good viability for up to 24 h and have both phase I and phase II metabolizing enzymes. The viability of rat and human PCIS has been well studied and shown to have good morphology, to maintain constant ATP levels, and to provide a steady metabolic rate for both phase I and phase II enzymes for at least 3 h [26,32–33].

Precision cut tissue slices (PCTS) have several advantages over primary cell culture, cell lines, and subcellular fractions. They maintain tissue architecture and the relative abundance of different cell populations typically found *in vivo* and possess both phase I and phase II enzymes. Many slices can be prepared from a single animal, thus extending the endpoints that can be monitored in a single experiment. Finally, the method allows for slices to be prepared from other organs in the same animals (e.g., liver and intestine) and from multiple species. For a detailed review of PCTS and a well-described protocol, see the article by de Graaf *et al.* [30].

7.7 HEPATIC METABOLISM AND TOXICITY

The liver controls many key functions *in vivo*, which include maintaining plasma glucose levels, energy storage, fat metabolism, uptake and secretion of lipoproteins, cholesterol synthesis, bile formation, and the metabolism and elimination of exogenous

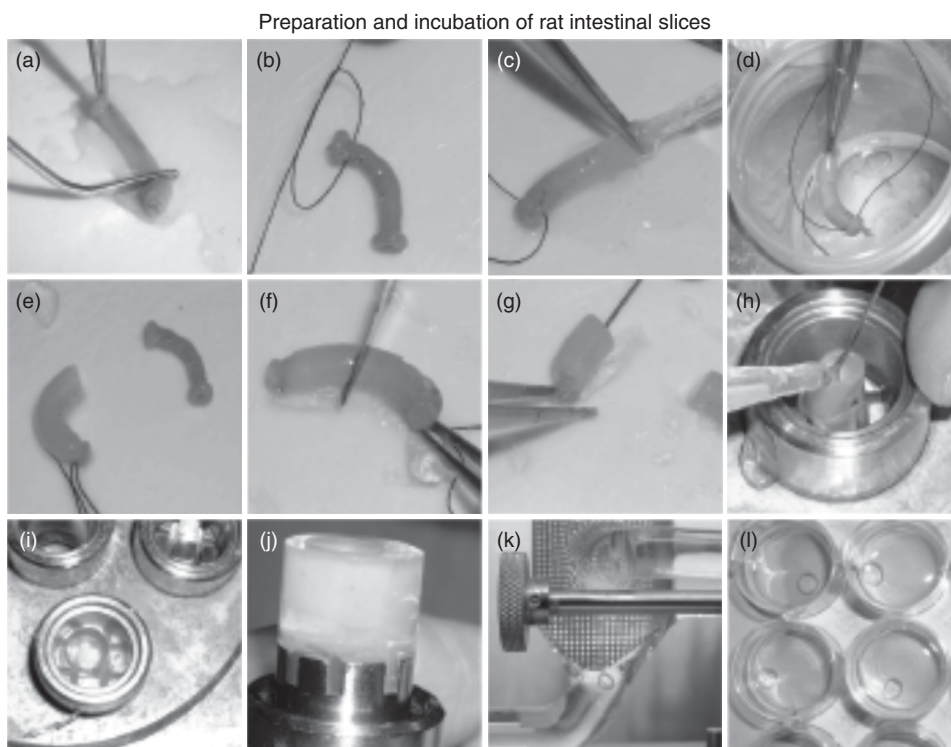


Figure 7.9 (a–l) Diagrammatic representation of precision cut intestinal slices. (See color insert.)

chemicals. The liver is situated anatomically in a unique position to control the systemic exposure of drugs and chemicals absorbed from the intestine. As such, it effectively separates presystemic from systemic circulation. The liver is the primary site for drug metabolism, a process that in most instances reduces drug toxicity, increases solubility, and speeds elimination from the body. However, as already discussed in this chapter, there are many examples where phase I metabolism can produce a highly reactive metabolite that can covalently modify essential cellular proteins, resulting in toxicity. Therefore, it is generally recognized as a good practice to determine whether NCEs can be metabolized to reactive intermediates.

P450 enzymes are ubiquitous proteins with highest concentrations in the liver. The five major classes of CYP enzymes involved with drug metabolism in humans are CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 [34]. These enzymes carry out phase I reactions that can lead to the formation of innocuous metabolites and elimination of the drug or they may result in the formation of reactive metabolites that may cause adverse effects. Reactive intermediates can alter normal cellular function by covalently binding to macromolecules, such as DNA/RNA, proteins, and lipids. Many drugs can be metabolized by more than one CYP enzyme. It is important to know not only that a reactive metabolite is formed, but also which CYP enzymes are responsible. The identification of a reactive intermediate is not the only determinant of *in vivo* toxicity. It is also important to identify the enzyme pathway involved and its prominence in the tissue where an adverse event is detected. This will determine the

levels of metabolite that can be achieved in the target tissues. It is also important to know how a patient's age, gender, current health, and drug regimen may increase the toxic potency of the metabolite. This knowledge can then be used to assess the risk of an adverse event in humans.

Acetaminophen (paracetamol or APAP) is generally considered to be a safe drug. It is used to reduce pain and fever. It has no anti-inflammatory properties and thus is not a member of the class of drugs known as *nonsteroidal anti-inflammatory drugs* (NSAIDs). Brodie and Axelrod (1948) were the first to suggest that APAP can be used for medicinal purposes, and in 1955, APAP went on sale in the United States under the brand name Tylenol. APAP can cause liver toxicity when taken at doses higher than those used clinically in normal individuals. Toxicity has also been observed in alcoholic individuals who take prescribed therapeutic doses. APAP can be directly conjugated with glucuronic acid by UGT or with sulfate by sulfotransferases, which inactivate the drug and enhance its excretion into urine. A small amount of APAP is metabolized by CYP enzymes to a reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Fig. 7.10). At normal therapeutic doses, NAPQI can be detoxified by conjugation with GSH. At high doses or when CYP enzymes are induced by other medications or alcohol, the percentage of NAPQI can increase thus leading to a reduction in GSH levels. This in turn allows NAPQI to covalently bind to proteins causing liver toxicity [35]. The reduction in GSH levels in the liver triggers the activation of the Nrf2/Keap1/ARE signaling pathway, which induces the expression of cytoprotective proteins. APAP metabolism can deplete cellular GSH levels, and activation of this pathway increases the expression of proteins responsible for GSH synthesis, such as γ -glutamylcysteine synthetase [35].

Identifying whether a new drug can undergo metabolic conversion to reactive intermediates is important for understanding the risk of human toxicity. The reactive intermediate is the hazard, but the risk of human liver toxicity is determined by having the ability to predict the dose that will result in over production of the undesired metabolite which can cause a depletion of the natural defense mechanisms. Factors such as age, gender, disease status, the use of other drugs, degree of covalent protein binding, specific proteins targeted, and high alcohol usage can predispose individuals to APAP toxicity [36,37]. The balance between metabolic activation of a drug and the liver's ability to detoxify determines whether tissue toxicity will occur. The primary defense of the liver against the reactive electrophilic compounds is the tripeptide GSH. GSH is present in high (millimolar) concentrations in the liver and is the first line of defense against electrophilic molecules. If the concentration of reactive metabolites exceeds the capacity of the liver to detoxify, then toxicity may ensue. Reactive intermediates may also damage mitochondrial function, which could result in the generation of reactive oxygen species (ROS), leading to oxidative stress and lipid peroxidation. Oxidative stress can induce the Nrf2/Keap1/ARE signaling pathway, which regulates the expression of several protective proteins [38,39] (Fig. 7.11).

An important lesson learned through understanding the mechanisms of APAP-mediated liver toxicity is that the presence of the reactive intermediate alone is not the sole determinant of tissue toxicity. The cellular concentration of the metabolite is important, but other cytotoxic mechanisms induced by the initiating event can also dramatically increase the risk for tissue damage. It is now known that hepatocytes, Kupffer cells, and endothelial cells all participate in APAP-induced liver toxicity [36]. The formation of NAPQI at high levels reduces GSH levels, which leads to covalent

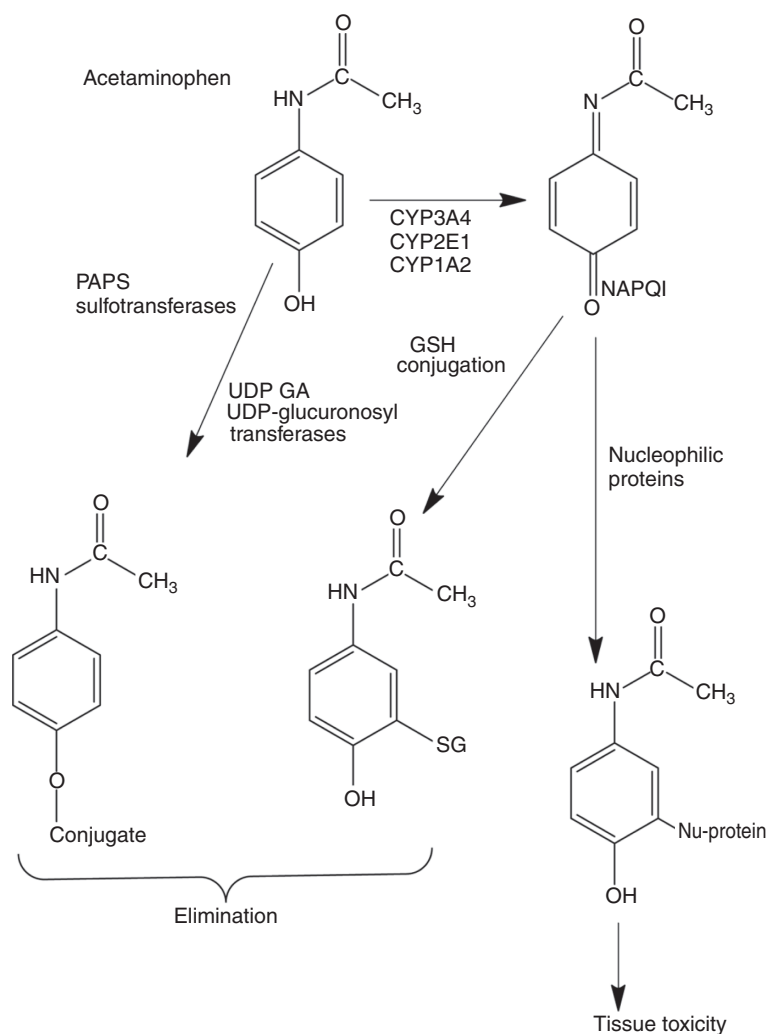


Figure 7.10 Acetaminophen metabolic pathways.

protein binding, disruption of mitochondrial function, and generation of ROS produced by each of the cell types mentioned. The result is the formation of peroxynitrite radical and superoxide anion. High levels of the peroxynitrite radical lead to protein nitrosylation, while high levels of superoxide lead to lipid peroxidation (Fig. 7.12). Both pathways result in liver toxicity.

7.8 *IN VITRO* DETERMINATION OF CYP INHIBITION AND POTENTIAL DRUG-DRUG INTERACTIONS

It is now generally recognized that the fraction of the dose metabolized by CYP enzymes is a major factor that determines the extent of drug interaction. When one drug

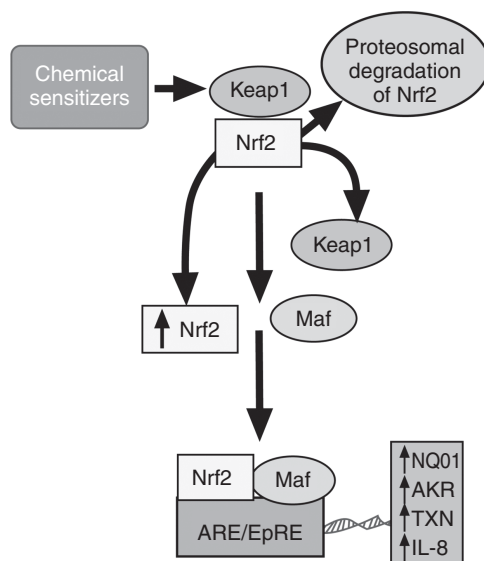


Figure 7.11 Nrf2/Keap1/ARE signaling pathway. IL-8, interleukin 8 AKR, aldoketoreductase; TXN, thioredoxin.

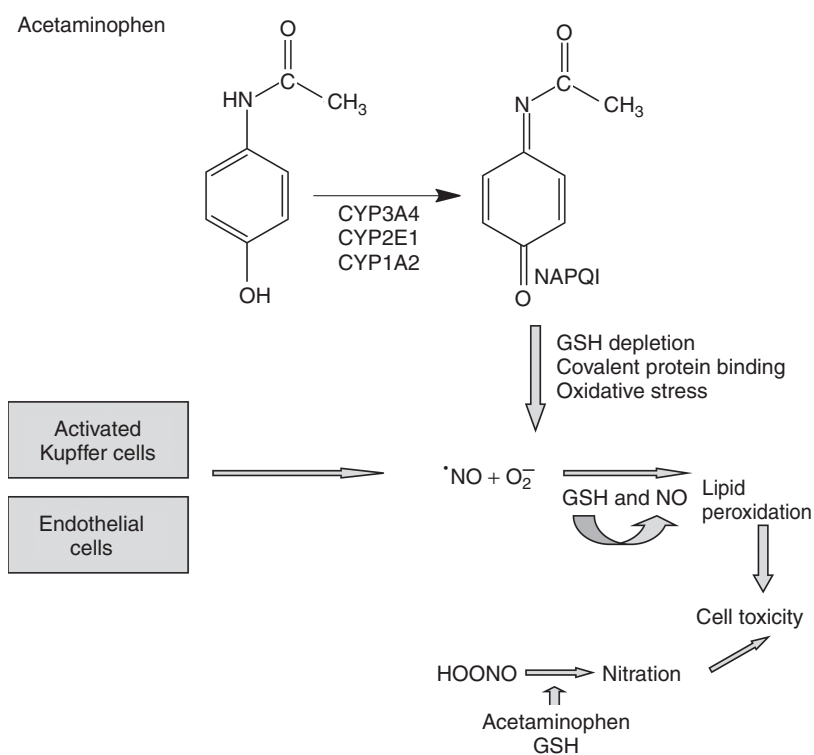


Figure 7.12 Mechanisms of acetaminophen toxicity.

inhibits the metabolism of another drug, there are changes in basic pharmacokinetic parameters that can reduce efficacy or increase toxicity and this is defined as a *DDI*. Terfenadine, bromfenac, alosetron, cerivastatin, and serzone are only a few examples of drugs that have been withdrawn from the market because of CYP-mediated DDIs [34]. A comprehensive list of clinical drugs reported to be mechanism-based CYP inhibitors can be found in the review by Zhou and Zhou [40]. It is important to understand which CYP enzymes are responsible for a drug's metabolism as well as the potential of a drug or its metabolites to induce or inhibit CYP enzymes.

Determining the CYP enzymes capable of metabolizing a drug is known as *cytochrome P450 reaction phenotyping*. For a comprehensive review, the reader is referred to the article by Zhang *et al.* [41]. Reversible or competitive inhibition of CYP enzymes is also an important component of early drug safety evaluations. The most important type of inhibition with respect to metabolites is time-dependent inhibition (TDI). TDI occurs when there is a change in drug potency during an *in vivo* exposure period. Some mechanisms underlying TDI are formation of inhibitory metabolites and mechanism-based inhibition (MBI). Three types of MBI have been reported: (i) the formation of inhibitory metabolites that inactivate CYP enzymes by covalently binding to the heme moiety, (ii) covalent binding to the apoprotein, and (iii) the formation of chelates or coordinates with the heme moiety [42]. This type of inhibition is clinically considered to be of far greater risk than competitive and reversible inhibition [40]. The greatest effects are seen under conditions of multiple dosing. Moreover, the formation of reactive metabolites may also predict TDI. Most mechanism-based irreversible inhibition is by definition due to the formation of a reactive metabolite that covalently binds to the CYP and inactivates it. Several *in vitro* methods have been described for identifying competitive inhibition and TDI [40,42].

7.9 *IN VITRO* DETECTION OF REACTIVE METABOLITE FORMATION

The incorporation of *in vitro* methods for the detection of reactive metabolites and oxidative stress early in the lead optimization phase of drug development provides important data that can be used to select the candidate with the highest probability of success in the preclinical safety studies.

Compounds undergoing metabolic activation may form soft or hard electrophiles. Soft electrophiles such as quinones, epoxides, quinone imines, and quinone methides can be trapped by GSH [43]. Hard electrophiles such as iminium species and aldehydes do not bind well to GSH but can be trapped using potassium cyanide (KCN) and the products detected by mass spectrometry or LC/MS/MS [44,45]. A rapid indirect method for determining bioactivation of new drug candidates is to monitor GSH depletion instead of GSH adducts. This is accomplished by preparing a standard reaction mixture containing potassium phosphate buffer (100 mM; pH 7.4), hepatic microsomes (0.5–1 mg/mL), NADPH (1 mM), test drug (10 μ M), and GSH (1 mM). The reaction is initiated by the addition of NADPH, and the incubation is allowed to continue for 30–60 min. The reaction is stopped with either an equal volume of ice-cold acetonitrile or 10% trichloroacetic acid added in a volume that is 15% of the total reaction volume. The samples are centrifuged at $10,000 \times g$ for 15 min at 4°C to precipitate protein, and the supernatant is processed for the detection of total GSH equivalents using the recycling method described by Tietz [46] and later modified by Griffith and

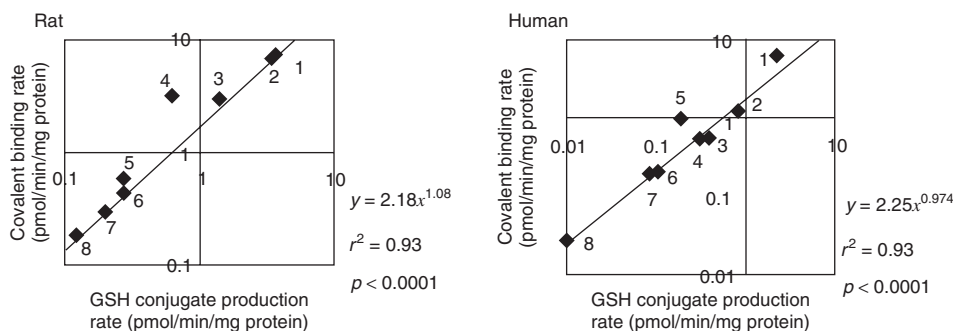


Figure 7.13 Correlation between covalent protein binding and GSH conjugate formation in rats and humans.

Meister [47]. Depletion of free GSH molecules is used as an indication of reactive metabolite formation and subsequent binding of GSH (Fig. 7.13) [48–50].

The formation of GSH conjugates has been shown to correlate with covalent protein binding *in vitro* and *in vivo* in both human and rat models (Fig. 7.13) [49]. When the data are expressed as a covalent binding rate in min/mg protein, it is possible to compare the relative binding between drugs in the same group. In Fig. 7.14, the toxicity profiles of three compounds are shown after exposure to rat hepatoma (H4IIE) cells. Buthionine sulfoximine (BSO) is a prototypical inhibitor of GSH synthesis, and the depletion of cellular GSH levels follows a concentration-dependent pattern. Azathioprine is a purine synthesis inhibitor that results in the loss of ATP. It is also known to disrupt mitochondrial function, leading to oxidative stress and depletion of GSH. Leflunomide treatment caused a significant loss in cellular GSH levels at low exposure concentrations. It is important to note that in each of these examples, cell viability remained high, as determined by membrane integrity (MemTox). Endpoints that indicate mitochondrial effects (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) or ATP) and cell proliferation (Cell #) can be affected by exposure concentrations and times that do not cause cell death. MTT can be reduced intracellularly by reduction reactions in mitochondria, cytosol, endoplasmic reticulum, and lysosomes. Therefore, it is important to include other markers of energy production as indicators of mitochondria function. These could include ATP or mitochondrial membrane potential ($\Delta\Psi$). These endpoints are commonly used as markers for assessing cytotoxicity, yet the interpretation of effect is greatly influenced when more than one endpoint is used. This example illustrates the importance of using multiple endpoints in the *in vitro* model for correct interpretation of the data obtained [50].

Phenothiazines, such as thioridazine and chlorpromazine, were introduced in the 1950s as tranquilizers and antipsychotic drugs. Because of their antipsychotropic effects, these drugs are often administered together with antidepressants to treat many disorders including psychotic depression and depression associated with schizophrenia. Despite the clear benefits of these drugs, there have been reported cases of rare, but severe, hepatic injury [51,52]. The hepatic injury associated with these drugs was labeled idiosyncratic in nature. Recent studies have demonstrated that many of the idiosyncratic drug toxicities in liver can be traced to the formation of reactive metabolites [53–55]. Chlorpromazine and thioridazine undergo extensive “first-pass”

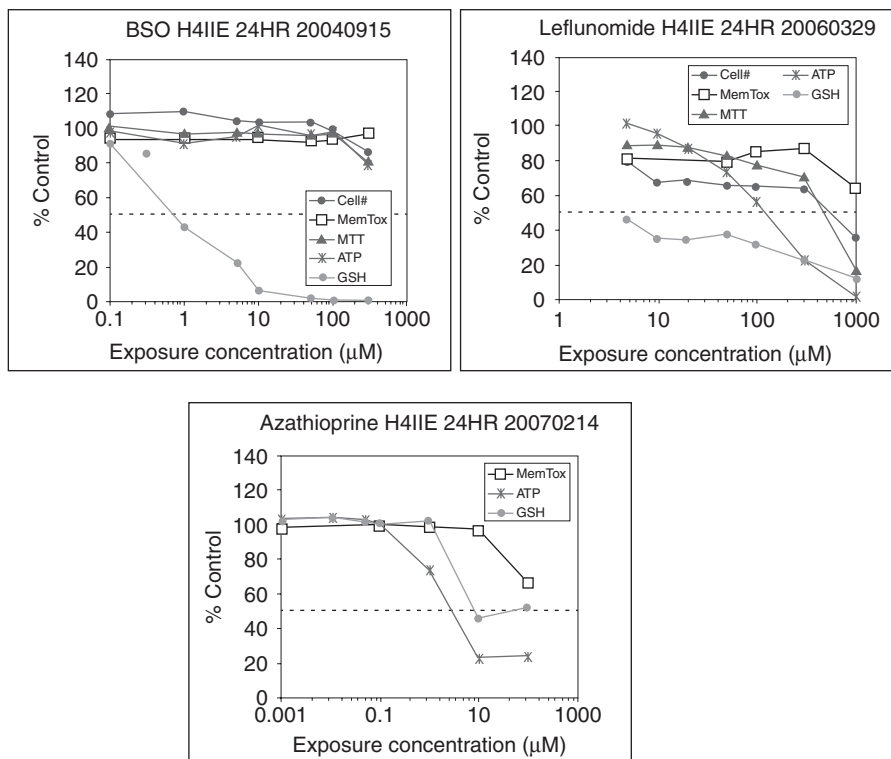


Figure 7.14 The importance of multiple endpoints for the interpretation of *in vitro* cytotoxicity data.

metabolism. The primary metabolites generated from phase I P450 enzymes are the products of hydroxylation, N-dealkylation, N-oxidation, and S-oxidation. The 7-hydroxy form of both the drugs can undergo additional oxidation to form an electrophilic quinone imine (Fig. 7.15).

The enzymes involved in the formation of the reactive intermediates are CYP2D6 and CYP1A2, with the former being more active than the latter. One possible explanation for the idiosyncratic nature of the liver toxicity associated with these drugs could be that CYP2D6 represents a small percentage of the total hepatic P450 enzymes under normal conditions. Therefore, in order for toxicity to occur, a larger amount of drug would need to enter the liver following absorption from the GI tract because CYP2D6 is in very low levels in the intestine. Metabolism in normal individuals by CYP3A4 in the intestine to nonreactive metabolites could greatly reduce the amount of parent drug that the liver would receive and hence reduce the formation of the reactive intermediate mediated by CYP2D6. In elderly patients, or patients on drugs that could inhibit CYP3A4 metabolism in both the intestine and the liver, the amount of drug reaching the liver would be greater. Thus, more drug would be shunted to secondary pathways that produce toxic metabolites, such as CYP1A2 and CYP2D6, leading to a higher tissue concentration of the toxic intermediates, which results in liver injury.

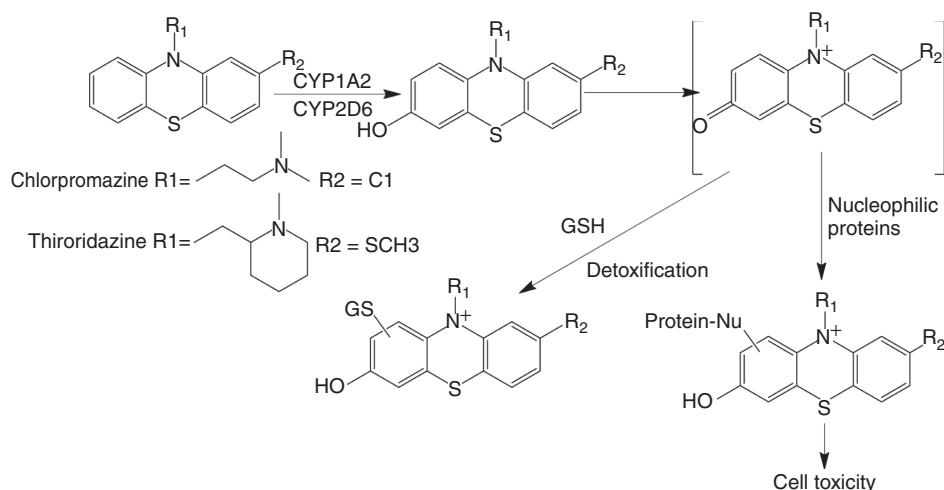


Figure 7.15 Bioactivation pathway for chlorpromazine and thioridazine.

GSH binding can serve as a surrogate for binding to more complex proteins. When the electrophilic intermediate formed is relatively stable, this method works well; however, some reactive products do not bind to GSH. This is true for amodiaquine, isoniazid, and valproic acid [56]. In addition, some metabolites lead to the generation of oxidative stress via an increase in ROS or nitrogen species, which would also not bind directly to GSH. Therefore, in addition to determining bioactivation potential, it is important to monitor the formation of ROS (superoxide anion and hydrogen peroxide), lipid peroxidation, and redox enzymes (GSH peroxidase, GSH reductase, catalase, and superoxide dismutase). Drug-induced changes in mitochondrial membrane potential ($\Delta\Psi$) can cause a release of superoxide anion and hydrogen peroxide into the cytosol. As a result, it is also important to monitor compound effects on mitochondrial membrane potential $\Delta\Psi$.

7.10 *IN VITRO* DETERMINATION OF COVALENT PROTEIN BINDING

The formation of highly reactive drug metabolites can cause cytotoxicity by the covalent binding of the metabolites to key cellular proteins and disrupting normal cell function. Adverse drug reactions are positively correlated with covalent protein binding. By quantifying the amount of protein binding that occurs following metabolic activation, it is possible to assess the risk of an adverse event *in vivo*. Clearly, the target proteins affected, as well as the site of binding, are important factors for developing a causal relationship between metabolic activation, protein binding, and cell or organ toxicity [57–59].

To determine covalent protein binding of a new drug candidate, radiolabeled drug is incubated in a standard reaction mixture containing potassium phosphate buffer (pH 7.4), EDTA, and MgCl_2 . Radiolabeled drug at a final concentration of $10\text{ }\mu\text{M}$ and specific activity of $10\text{ }\mu\text{Ci/mg}$ microsomal protein, and NADPH are added to

the mixture. The reaction mix is then incubated for 45 min at 37°C. The reaction is quenched by adding an equal volume of ice-cold acetonitrile, sonicating for 5 min, and then centrifuging at $20,800 \times g$ for 10 min. The supernatant is then removed and the protein pellet extracted with methanol–water (3:1 v/v) twice. The extracts are then combined with the supernatants and evaporated to dryness under an inert gas, such as nitrogen at room temperature. The dried residues are then solubilized in solvent for HPLC and quantified by LC/MS/MS [59]. A threshold value of 50 pmol drug equivalent per milligram protein has been proposed as a means of selecting drug candidates with the lowest activation liability.

Predicting *in vivo* adverse outcomes from *in vitro* data requires caution and a well-defined and disciplined approach for data collection and evaluation. For example, ethinyl estradiol (EE) is the active estrogenic component of birth control pills. EE has a high level of covalent protein binding *in vitro* (1200 pmol equivalents per milligram protein) but very low *in vivo* binding levels (<5 pmol). This is due to the fact that the primary routes of metabolism *in vivo* are phase II conjugation with sulfate and glucuronic acid. This, combined with low oral dose and high gut metabolism, significantly reduces the *in vivo* toxicity. This example underscores the importance of a detailed evaluation and elucidation of a new drug candidate's metabolic pathways in the presence of both phase I and phase II systems [58]. In addition, more accurate *in vitro* to *in vivo* extrapolations are possible when multiple methods are used to identify the hazard and assess risk. In the case of EE toxicity, a clear understanding of the relative contributions of phase I and phase II metabolic pathways, combined with metabolic stability experiments in microsomes from rat and human intestine and liver, would add the needed perspective for assessing the risk of *in vivo* toxicity. Moreover, this series of evaluations can be done using preparations from rat, dog, monkey, and human in order to gain an understanding of species-specific risk.

7.11 IN VITRO DETERMINATION OF OXIDATIVE STRESS

The accumulation of ROS can be measured using the fluorescent probe 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (CM-DCFDA) [60]. In this assay, nonfluorescent DCFDA readily crosses the cell membrane. Once in the cell, the acetate moiety is cleaved by endogenous esterases, which prevents leakage out of the cells and activates dichlorofluorescein (DCF) for binding to ROS (e.g., hydroperoxides and lipid peroxides which produces a fluorescence signal) [60–63]. The cleaved molecule cannot cross cell membranes and is therefore trapped inside the cell. The formation of ROS occurs rapidly, so it is important to conduct this experiment over shorter exposure periods (e.g., 1–4 h). The loading concentration of the DCFDA is typically 2–10 μM for 60 min, but this should be optimized every time the cell model is changed. An increase in fluorescence due to ROS binding is measured at an excitation wavelength of 503 nm and an emission wavelength of 529 nm. An example of data obtained from this assay in rat hepatoma (H4IIE) cells can be seen in Fig. 7.16. Two hydroperoxides, *t*-butyl hydroperoxide and cumene hydroperoxide, produced rapid increases in the DCF signal. Rotenone inhibits mitochondrial respiration, which leads to a release of superoxide anion. This process requires more time, which is detected by the assay as a delay in the increase in fluorescence. In comparison, a chemical like menadione

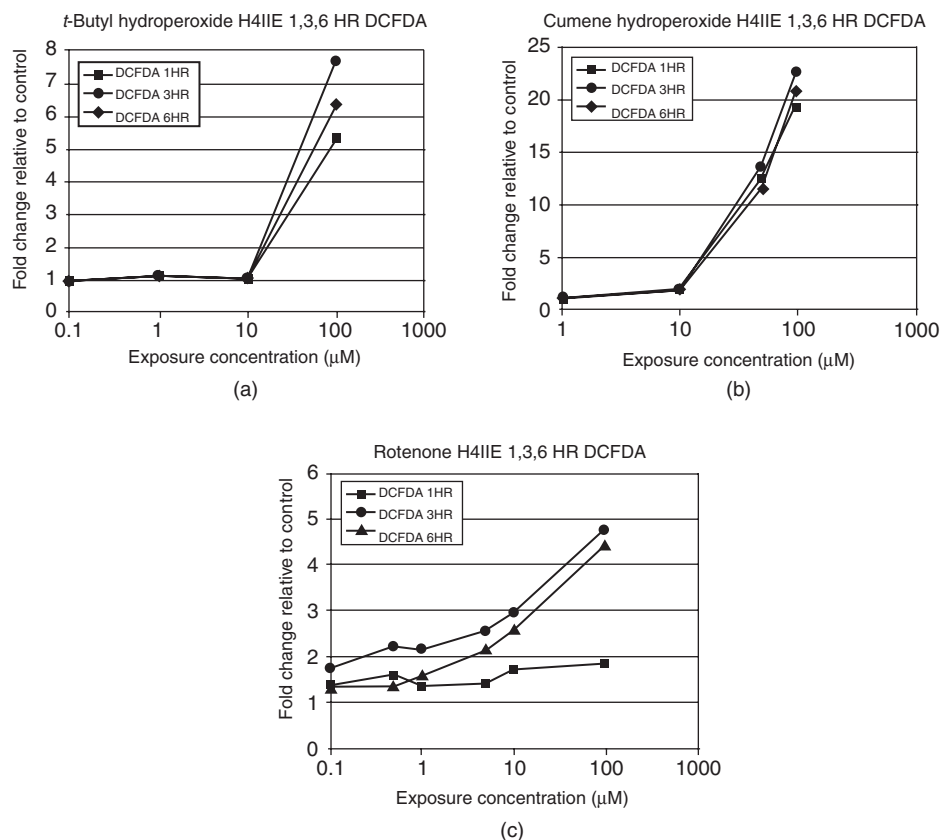


Figure 7.16 (a–c) Measuring intracellular reactive oxygen species with DCFDA.

(data not shown), which requires metabolism via CYP1A1/2 to the reactive quinone, required more time to increase the oxidative stress signal.

7.12 IN VITRO DETERMINATION OF MITOCHONDRIAL MEMBRANE PERMEABILITY

Mitochondrial damage can also cause an increase in ROS. This can occur when the membrane potential is disrupted, which allows superoxide anion to leak into cell cytosol. Therefore, it is also important to evaluate the effects of new drug candidates on mitochondrial membranes. A commonly used assay is based on the uptake of the fluorescent dye 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1). JC-1 is taken up by healthy mitochondria where it forms aggregates as the inner membrane becomes polarized. The ratio of the monomer to aggregate JC-1 is measured and used to determine changes in $\Delta\Psi$. The data are collected by using an excitation wavelength of 490 nm and then quantifying the monomer at 535 nm and the aggregate at 595 nm. The ratio of aggregate to monomer fluorescence determines the effect on mitochondrial membrane potential [60].

7.13 PRIMARY HEPATOCYTES IN SANDWICH CULTURE

All the endpoints discussed above can be measured in a single *in vitro* model. Primary hepatocytes in sandwich culture are considered to be the gold standard for determining drug metabolism and toxicity *in vitro*. The isolated hepatocytes are seeded onto culture plates that have been coated with a collagen matrix. After an attachment period, the cells are covered with another growth matrix, which can be either collagen or Matrigel. Cells cultured in this manner provide more sustained metabolic capacity and possess both phase I and phase II metabolism, which can be determined by monitoring disappearance of the parent drug and formation of metabolites. This model provides a means of measuring metabolic stability, reactive metabolite formation by measuring GSH conjugates and depletion of GSH, and oxidative stress using DCFDA.

Cell culture models should be used with multiple endpoints that measure cell health and a broad range of exposure concentrations. These include ATP levels, cell proliferation, reduction of MTT, and membrane integrity [50]. These parameters combined with the information described above will provide important information on a drug's potential for causing an adverse event *in vivo*. Hepatocytes from rat, dog, and human can provide insight into potential species differences and may guide the selection of the nonrodent species required in preclinical drug safety studies.

7.14 FUTURE *IN VITRO* MODELS FOR ASSESSING HEPATIC DRUG METABOLISM AND TOXICITY

Recent advances in cell culture technologies have allowed the introduction of 3D liver models. These models incorporate all the major hepatic cell types and can be sustained in culture for three to four weeks with stable metabolic capabilities. InSphero Inc. (Zurich) has introduced rat and human liver models in which hepatic spheroids

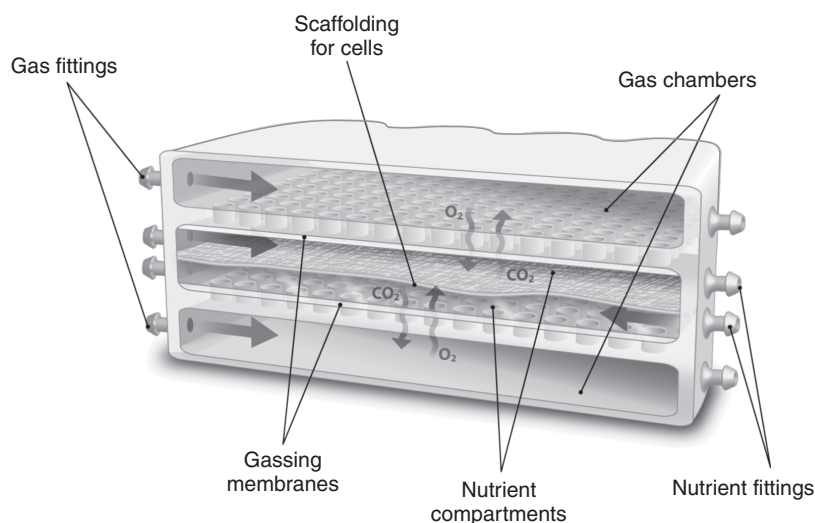


Figure 7.17 Diagram of the bioreactor designed by RealBio Inc.

containing all liver cell types, functional canalicular membranes, and good metabolic capacity can be used in 96-well format for early drug evaluations of efficacy and toxicity. Bioreactors, such as the model distributed by RealBio, Inc., Kalamazoo, MI, offer the potential to have not only extended culture times, and all major cell types present, but also the ability to control fluid/nutrient flow and oxygen content. In this system, isolated liver cells (a heterogeneous slurry) are perfused into the reactor. The cells settle onto and into the porous scaffolding and begin to aggregate and grow in a manner similar to that *in vivo*. This model is still under development for liver work, but preliminary data is promising (Fig. 7.17).

7.15 CONCLUSION

This chapter has focused on the role that metabolism and drug metabolites play in determining the safety of a new drug candidate. Emphasis has been placed on intestinal and hepatic metabolism and how drug metabolism in these organs contributes to first-pass metabolism, bioavailability, efficacy, and toxicity. *In vitro* approaches for obtaining many of the key parameters necessary for predicting *in vivo* effects were presented. It is important to realize that the use of multiple *in vitro* methods and endpoints provides more robust data sets. A careful evaluation of *in vitro* data that takes into consideration the intended therapeutic area of the drug, duration of exposure, maximum plasma concentrations for efficacy, patient population, age, gender, and health status will enable discovery scientists to make go-no-go decisions with confidence. When an intelligent and disciplined approach to the acquisition of *in vitro* data is incorporated into the drug discovery process, it is possible to select drug candidates that are not only efficacious but safe.

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8 The Application of Preclinical Toxicogenomics for Predicting and Understanding Drug-Induced Toxicity and Metabolism

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8.1 Summary	1
References	23

8.1 SUMMARY

The pharmaceutical industry is challenged by high rates of attrition during development because of preclinical and clinical toxicities that are not adequately predicted using current preclinical testing paradigms. In order to decrease late-stage attrition and improve success rates, it is necessary to consider on- and off-target-mediated toxicities at an earlier stage in product development in order to shift attrition upstream in the process. This should reduce resource-intensive developmental activities, such as preclinical toxicology and human clinical studies, on compounds that are ultimately destined to fail. Large-scale gene expression profiling technologies, such as toxicogenomics, are being applied preclinically during drug discovery and lead optimization to diagnose and predict drug-induced effects, including efficacy, toxicity, and pharmacokinetics. This includes the prediction of common hepatic effects, such as hepatic necrosis and hepatocarcinogenicity, and the mechanistic understanding of common drug-induced effects, such as hepatomegaly, cholestasis, and oxidative stress. The effect of nuclear receptor activation, particularly of pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), can be studied by evaluating the gene expression response to nuclear receptor activation, both *in vitro* and *in vivo*. When used together with classical pharmacokinetic parameters, toxicogenomics can provide an understanding of the pathways (phases I and II) of drug metabolism induced by drug treatment. A more thorough understanding of drug safety and metabolism at an earlier stage of drug discovery and development can improve our ability to translate findings to higher species, including humans. Such information can also help define appropriate screening strategies to avoid offending toxicophores, or even to avoid targets that are associated with intolerable side effects at efficacious doses. Finally, applying gene expression

profiling to *in vitro* cell-based models may allow for the prediction of hepatic effects, such as phospholipidosis, and for hazard identification by evaluating pathways diagnostic of common mechanisms of toxicity. This chapter focuses on the application of toxicogenomics in drug discovery for the prediction and mechanistic understanding of drug-induced toxicity, as well as understanding mechanisms of drug metabolism for small-molecule therapeutics.

8.1.1 Introduction to Toxicogenomics

Toxicogenomics can be broadly defined as the application of large-scale gene expression profiling technologies for the prediction and mechanistic understanding of chemical-induced toxicity [1]. The practical applications of toxicogenomics are broad and include the safety assessment of drugs [2], environmental pollutants [3], industrial chemicals [4], nanotechnology [5], and natural products [6]. In addition to the safety assessment of drugs, it has also been applied to understand drug pharmacology and off-target pharmacodynamic effects that affect drug metabolism and drug–drug interactions [7,8].

The technologies available for measuring and statistically analyzing mRNA levels at a genome-wide level using microarrays and related to global gene expression have previously been well described [8–11], and the reader is encouraged to consult these reviews for more detailed technical information. There is also a plethora of public databases of chemical–biological interactions and gene and pathway annotation to facilitate the biological interpretation of gene expression changes [12]. This chapter focuses on the practical applications and interpretation of large-scale gene expression profiling for safety assessment in the field of drug discovery and development; encompassing both prediction and mechanistic assessment of common drug-induced toxicities. The utility of gene expression profiling to understand pharmacokinetics, drug–drug interactions, and other metabolism-mediated toxicity is also discussed.

8.1.2 Gene Expression Profiling Technologies

The oligonucleotide microarray platform [13] has emerged as one of the most common and widely available methods for measuring mRNA abundance in human cell and tissue samples. The availability of complete DNA sequences for many model organisms has also made whole genome expression profiling possible with oligonucleotide-based microarray platforms. This includes most animals commonly used for preclinical drug safety testing such as the mouse, rat, canine, and nonhuman primates. One of the major drawbacks of using microarrays for some preclinical species, particularly canines and nonhuman primates, is the relatively low level of sequence annotation in these model species compared to the human or rodent genome, which is more fully characterized. However, by virtue of comparative sequence analysis and identification of gene orthologs, the function of many poorly annotated genes can be hypothesized and potentially contribute to the understanding of gene function in model organisms [14].

More contemporary gene expression profiling technologies, the so-called next generation sequencing (NGS), have recently emerged and are based on high throughput sequencing of individual RNA molecules [15,16]. The ability to sequence individual RNAs and count the number of those sequences provides a higher resolution and possibly more accurate account of the transcriptome. It also has the advantage of detecting

and quantifying small noncoding RNAs (ncRNAs) [17] such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs) that are not typically represented by oligonucleotide or complementary DNA (cDNA) microarrays, which have historically relied on gene-prediction algorithms for identifying protein-coding genes and their expressed sequences.

The advancement of NGS is rapidly changing the options available for quantitating RNA and is likely to revolutionize the way genomes are analyzed. The application of NGS to toxicological research, however, is only beginning as our understanding of the biological significance of ncRNAs is only just emerging. Numerous publications indicate that these small ncRNAs play an important role in regulating mRNA stability and function, thereby indirectly regulating protein expression and cellular functions relevant to cancer, metastasis, fibrosis, and development [18]. The miRNAs have also been reported to be a potential new source of biomarkers for diagnosing tumors [19] or for noninvasively detecting drug-induced toxicity as a result of their enhanced plasma stability relative to mRNA [20]. While exciting new efforts have emerged in this field, our understanding of the role of miRNA in regulating mechanisms of toxicity is in its infancy. Their putative role in a number of important diseases implies that they may become important players in our understanding of chemical-induced toxicity [21,22]. The field of toxicogenomics continues to evolve as do new technologies and analytical approaches; however, the majority of our understanding of predictive and mechanistic toxicology has come from cDNA and oligonucleotide microarrays combined with, or anchored to, phenotypes and pathways derived from traditional or more established molecular, biochemical, and pathological endpoints [23]. This evolution has positioned toxicogenomics as an established tool within drug discovery and development to advance our ability to predict toxicity and understand the mechanisms of drug action.

8.1.3 The Challenges of Drug Discovery and Development

A number of reports have attempted to calculate the cost of drug development and have reported a general decline in productivity based on the decreasing number of new chemical entities that have reached the market in recent years [24]. The cost of drug development has been hotly debated; however, based on one study, it has been estimated to cost approximately US\$800 million to US\$1.1 billion to develop a new drug [25]. The majority of these costs are associated with the expense on other development compounds that ultimately fail because of late-stage preclinical or clinical failure and the trial and error process of drug discovery. As a result, the effect of reducing late-stage attrition can considerably improve productivity and ultimately lower the cost of developing new drugs.

The low success rate and the failure of the pharmaceutical industry to improve productivity offered by advances in new technologies and our increased understanding of disease biology have provoked government and industry to reevaluate the process of drug discovery and development. Alternative means to improve productivity of the industry and expedite the pace of research to bring effective and safe therapies to market as efficiently as possible have ushered in new collaborative efforts across industry and government [26]. In response to these challenges, the pharmaceutical industry has concentrated on improving the critical aspects of drug discovery and

development that have historically contributed to late-stage attrition, namely, human and animal toxicity [27].

It is common for previously unidentified adverse effects in humans to be identified in later stages (phase II or III) of clinical development when tens of millions of dollars have already been invested. Detecting toxicity in earlier (phase I) clinical trials is challenging as a consequence of the limited number of healthy volunteers or patients enrolled in these early trials. The low sample size limits our ability to detect low incidence events such as liver toxicity. Statistically significant increases in the incidence of adverse events are often only identified after marketing approval when a larger number of patients have been observed. However, these postmarketing conditions are not well controlled as in a clinical trial and the adverse effects can be confounded by other factors such as disease state or other drugs.

Before human trials, the only other means of assessing human safety is to utilize animal toxicity studies, which are limited in their ability to detect toxicities that are relevant to humans. For instance, a retrospective evaluation of the concordance between animal and human toxicity indicates that rodent toxicology studies only predict 43% of human toxicities [28]. The use of nonrodents (i.e., nonhuman primates or dogs) improves the predictivity (63%) somewhat, while the combination of both rodents and nonrodents provides only 71% predictivity [28]. These results, however, are misleading in that compounds that induce an unacceptable level of toxicity in animals will never reach the clinic for evaluation in humans. As a result, many drugs may be unnecessarily halted in development due to toxicities in animals that are not relevant to humans. However, findings in animal toxicology studies are assumed to be predictive of human toxicity unless proven otherwise. Further exacerbating the challenge in bringing safe and effective therapeutics to the market is the increasingly competitive landscape in the pharmaceutical industry, and the number and types of studies necessary to demonstrate a new drug is sufficiently more efficacious and safe than standard care or otherwise provides additional value over current therapies.

In response to these challenges of late-stage attrition, the industry has taken steps to develop, evaluate, and implement new technologies and biomarkers at earlier stages in the drug discovery pipeline to shift attrition upstream in the process and before formal development begins [29]. By considering toxicity at an earlier stage, compound selection criteria can be applied to avoid further efforts on compounds destined for failure. Additionally, medicinal chemistry has an opportunity to make appropriate changes to the molecule to improve its characteristics and limit potential hazards. A combination of *in silico*, *in vitro*, and *in vivo* approaches judiciously applied at early stages of discovery has the potential to shift attrition-based decisions upstream in the process where corrective action can be implemented at lower costs (Fig. 8.1). It is here that predictive tools such as toxicogenomics have been applied to select safer clinical candidates and rapidly identify mechanisms of toxicity to aid in early human risk assessment.

8.1.4 Use of Toxicogenomics for Predicting Toxicity

The use of toxicogenomics for predicting toxicity is based on the hypothesis that changes in gene expression precede the microscopically observed tissue damage. Efforts to identify gene expression changes that precede and ultimately predict the onset of tissue damage have focused on the liver, kidney, and heart (Table 8.1). This likely reflects the greater incidence of drug-induced toxicities in these organs, in addition

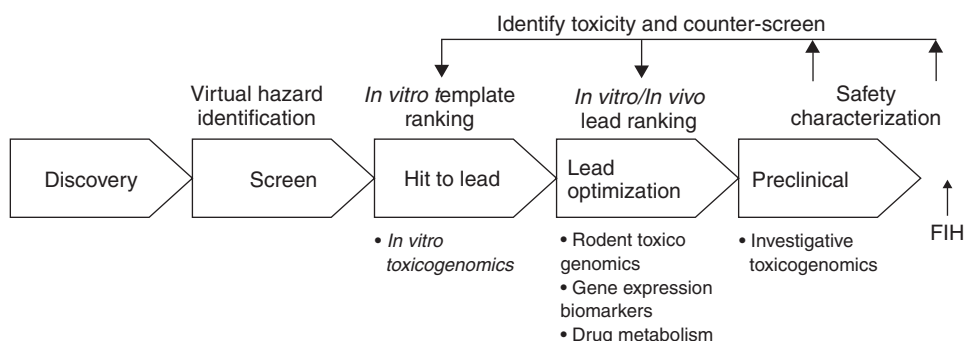


Figure 8.1 Application of toxicogenomics during drug discovery. At the screening stage, very little compound is available to support direct experimentation and toxicogenomics. At the hit to lead stage, a more thorough characterization of the compounds is undertaken to identify potential off-target toxicities. Cell-based toxicogenomics experiments could be used to differentiate and prioritize lead scaffolds to direct resources toward scaffolds that have less safety hazards before initiating more resource-intensive *in vivo* studies. At the lead optimization stage, medicinal chemistry attempts to modify the lead scaffold to improve a variety of features. At this stage, toxicogenomics approaches can be used to further characterize and predict potential hazards that may emerge under longer-term dosing conditions. Once compounds reach preclinical development, investigative toxicogenomic experiments can be used to understand the mechanism of unanticipated toxicities and develop counter-screens to aid subsequent efforts.

TABLE 8.1 Examples of Studies Designed to Identify and Validate Predictive Genomic Biomarkers of Organ Toxicity in Rats

Predicted Endpoint	Species	Study Design	References
<i>Hepatotoxicity</i>			
Various liver pathologies (necrosis, cholestasis, and steatosis)	Rat	6-h to 14-d oral repeat dose	Steiner <i>et al.</i> [30]
Oxidative stress/peroxisome proliferators/macrophage activators	Rat	24-h single oral dose	McMillian <i>et al.</i> [31]
Acute hepatotoxicity	Rat	24-h single oral dose	Zidek <i>et al.</i> [32]
<i>Nephrotoxicity</i>			
Proximal tubular toxicity	Rat	7-d oral repeat dose	Thukral <i>et al.</i> [33]
Renal tubular toxicity	Rat	28-d oral repeat dose	Fielden <i>et al.</i> [34]
Renal tubular toxicity	Rat	28-d oral repeat dose	Kondo <i>et al.</i> [35]
<i>Cardiotoxicity</i>			
Cardiomyopathy	Rat	7-d intraperitoneal dose	Brown <i>et al.</i> [36]

to a number of well-described mechanisms of toxicity in these organs. Experimental approaches typically utilize tissues from rats following short-term (i.e., 24 h to 14 days of repeated daily drug administration) treatment to identify gene expression changes in target tissues that precede the histological appearance of tissue injury. These putative biomarkers are then evaluated independently against other toxicants and negative

controls with the goal of evaluating how accurately the biomarker can diagnose or predict the pathological event. While histological identification of tissue injury will remain the gold standard for evaluating drug-induced toxicity, the ability to predict the onset of tissue injury before it is observed microscopically can aid in the early identification (approximately in days and weeks) of toxic effects that may not manifest until longer-term studies (approximately in months and years) are conducted. As a result, toxicogenomics can be applied in the early stages of lead candidate selection as a means of predicting tissue injury or identifying hazards that may not manifest until longer duration and more resource-intensive toxicology studies are conducted to support clinical development. In addition to predicting toxicity, gene expression changes have often been shown to coincide with tissue damage and can be used to understand the mechanism of action (see below), in addition to confirming the diagnosis of toxicity made with traditional endpoints. This is valuable during early screening toxicity studies when it is often difficult to ascribe a histological finding as being treatment related when the incidence or severity is low and the number of animals per group is necessarily limited. While toxicogenomics is not viewed as a replacement for traditional endpoints such as clinical and anatomic pathology, it may be able to differentiate multiple compounds on the basis of predicted toxicity where traditional methods may not. This can help support the selection of clinical candidates for subsequent testing and ultimately shift attrition upstream in drug discovery.

8.1.4.1 Predicting Hepatotoxicity. One of the most frequently used biomarkers for hepatic injury, both clinically and preclinically, is the elevation of alanine aminotransferase (ALT) activity in the serum. ALT is a cytosolic enzyme expressed selectively in hepatocytes and is released into the blood following the loss of membrane integrity as a result of drug-induced hepatic necrosis. The value of measuring serum ALT elevation for diagnosing hepatotoxicity is that the test is noninvasive, easy to measure, and a direct consequence of cell damage. However, the major limitation of this biomarker is that it is rather insensitive, as an elevation in serum ALT activity is not detected until after a sufficient level of cell damage has occurred. Premonitory signals in the liver are needed that can identify subpathological damage before histological evidence. This would permit the prediction of hepatotoxicity in longer-term studies based on short-term animal studies. Thus far it has been difficult to identify a single predictive biomarker, or group of biomarkers, that has general applicability to predict longer-term hepatotoxicity.

Zidek *et al.* [32] utilized oligonucleotide microarrays containing a focused set of liver-expressed genes to identify the expression changes that preceded the onset of histological injury. The predictive screen utilized rats that were dosed with a number of well-known hepatotoxicants, such as carbon tetrachloride and acetaminophen, and negative controls that are not hepatotoxic. Livers were evaluated for gene expression 6, 24, and 72 h following a single dose. After 24 h, hepatic gene expression was able to differentiate the hepatotoxicants from the nonhepatotoxicants, even though the livers were histologically normal at this early time point. The putative biomarker genes were associated with functions well known to be involved in the hepatic stress response, thus providing a degree of biological plausibility to support these genes as predictive biomarkers. While this study confirms that gene expression changes can precede the development of histological evidence of drug-induced toxicity, it does not address the need for being able to predict liver damage that occurs only after longer-term

dosing (i.e., weeks or months). Predicting toxicity that occurs only a few days after the biomarker signal provides only marginal practical benefit in terms of addressing late-stage attrition. It may serve to increase the sensitivity for detecting toxicity where single-dose acute rat toxicity studies are utilized for screening; however, this approach is not routinely utilized.

While the prediction of long-term toxicity may be challenging, a number of studies do support the early assessment of a compound's mode of action, which may be useful in the selection or prioritization of compounds for further study. For instance, Thomas *et al.* [37] first demonstrated that hepatic transcript profiles from mice treated with 24 prototypical toxicants from five different modes of action classes could be accurately distinguished from each other. Similar work by Hamadeh *et al.* [38] confirmed that hepatic transcript profiles can correctly distinguish peroxisome proliferators from enzyme inducers. Subsequent publications extended these initial observations with bigger data sets and additional classes, including oxidative stress [39], and various hepatic pathologies including cholestasis, necrosis, and steatosis [30,40]. While this classification provides information on the potential mode of action, it is not a formal prediction and the degree to which these classifications predict longer-term outcomes is unproven. Nonetheless, understanding a compound's mode of action can aid in the early risk assessment since some modes of action, such as oxidative stress, are inherently more hazardous and could lead to cell damage under longer-term dosing or when combined with other precipitating factors. Understanding the mode of action can also be used to guide the selection and application of *in vitro* or cell-based counter-screens for lead optimization.

8.1.4.2 Predicting Carcinogenicity. Carcinogenesis is a common adverse outcome for chemicals and drugs tested in the two-year rodent bioassay, and the most frequent target organ for chemical-induced tumors in rodents is the liver [41]. However, while many compounds increase the incidence of liver tumors in mice and rats, the findings are often not relevant to human risk because of the high doses used in the two-year rodent bioassay or the unique mechanism of action of the drug in rodents [42]. While all known agents that are carcinogenic to humans can be shown to be carcinogenic in rodents, the converse is not always the case. Therefore, the carcinogenicity findings in rodents must be assessed for their relevance to humans based on the level of exposure, duration of exposure, and the mechanism of action of the drug.

It is well understood that direct-acting genotoxic compounds present a significant risk for carcinogenicity across species. Therefore, drugs are efficiently screened early in drug discovery for their ability to mutate DNA. Compounds that cause chromosomal damage are also screened early in drug discovery to avoid potential carcinogenic risks. Numerous attempts have been made to develop *in vitro* and alternative short-term or medium term *in vivo* assays that attempt to provide a less resource-intensive means of identifying chemicals with the potential to induce tumors through nongenotoxic mechanisms [43–45]. Despite these significant efforts, there are no well-validated assays available that reliably predict the ability of nongenotoxic compounds to cause cancer. As a result, it is often not until very late in development when the two-year rodent bioassay is performed that cancer liabilities emerge as a result of nongenotoxic mechanisms of action.

The carcinogenic activity of many nongenotoxic agents can have species, strain, and tissue specificity, thus making human risk assessment a challenge. Furthermore,

a number of carcinogenic modes of action in rodents are not relevant for humans owing to species-specific effects and the high doses often used in rodent studies. However, compounds that cause hormonal perturbation (e.g., increased prolactin levels) [46] or immunosuppression (e.g., cyclosporine) [47] could be carcinogenic to humans under certain conditions of exposure. As a result, it is important to not only determine the potential for carcinogenicity but also understand the mode of action in order to extrapolate the findings and assess human risk.

Carcinogenicity in rodents has been well studied with toxicogenomics, and a number of publications have attempted to identify early gene expression changes that are predictive of tumor outcome in a two-year rodent bioassay (Table 8.2). Most studies have focused on predicting carcinogenicity induced by nongenotoxic mechanisms of action; however, other efforts have focused on differentiating genotoxic from nongenotoxic modes of action both *in vitro* and *in vivo* [48–51]. The underlying premise of using toxicogenomics for carcinogenicity prediction is that the gene expression changes in the liver or target tissue precede and presumably contribute to tumor development. If so, these expression changes can be detected following short-term *in vivo* exposure and predict longer-term carcinogenic outcome after chronic dosing. While such a prediction would likely not obviate the need for a two-year rodent bioassay without further validation, the advanced knowledge may contribute to compound selection before development or lead to the initiation of critical investigative studies that begin to understand the mode of action of the drug to aid in human risk assessment. This has the potential to avoid lengthy delays as a result of investigative studies that would only ensue following the detection of a cancer risk at very late stages of drug development.

Early experiments using microarrays and other genome-wide expression profiling technologies focused on the identification of single genes as predictive biomarkers of carcinogenicity. Although certain genes could be identified as being significantly and reproducibly induced by carcinogenic chemicals, they were found not to be specific to carcinogens [53]. Instead, unique patterns of multiple genes were found to be necessary to differentiate carcinogenic from noncarcinogenic compounds, and it was quickly appreciated that no single gene by itself could adequately predict a heterogeneous class of compounds that cause tumors by diverse mechanisms [53,55].

With the availability of large gene expression databases of well-known carcinogenic compounds and negative controls (i.e., compounds that do not induce carcinogenicity), it was feasible to use supervised classification algorithms to identify multiple-gene biomarkers, or signatures, that were able to classify compounds as being carcinogenic or not. One of the first comprehensive efforts to identify a gene expression signature to predict carcinogenicity was by Nie *et al.* [56]. Using cDNA microarray data generated from the livers of Sprague-Dawley rats treated with a single dose of one of 52 compounds for 24 h, Nie *et al.* identified a set of 6 biomarkers that correctly predicted 89% of the compounds when evaluated by crossvalidation. Subsequent testing on independent samples verified that the signature could predict other nongenotoxic carcinogens. The utility of this signature for predicting nongenotoxic compounds that induce tumors in tissues outside the liver has not been widely tested, although preliminary results indicate it may have potential. While mechanisms of cell transformation are likely to be tissue specific, the liver may respond at the gene expression level to physiological or pathological changes in other tissues by virtue of its vital role in detecting and regulating changes in systemic metabolism.

TABLE 8.2 Summary of Toxicogenomic Studies Focused on the Identification and Validation of Genomic Biomarkers of Carcinogenicity

Prediction Endpoint	Species	Genomic Technology	Study Design	References
Liver tumor promotion	Rat	Subtractive cDNA hybridization	Modified Ito medium term rat liver bioassay	Shibutani <i>et al.</i> [52]
Genotoxic and nongenotoxic carcinogens	Mouse	Custom cDNA microarray	14-d and 1-, 3-, and 6-mo dietary exposure	Iida <i>et al.</i> [53]
Genotoxic carcinogens	Rat	Affymetrix RGU34A oligonucleotide array	14-d oral repeat dose	Ellinger-Ziegelbauer <i>et al.</i> [48]
Genotoxic and nongenotoxic hepatocarcinogens	Rat	Incyte Rat GEM 1.0 cDNA microarray	5-d oral repeat dose	Kramer <i>et al.</i> [54]
Genotoxic and nongenotoxic carcinogens	Rat	Affymetrix RGU34A oligonucleotide array	14-d oral repeat dose	Ellinger-Ziegelbauer <i>et al.</i> [49]
Genotoxic versus nongenotoxic carcinogens	Mouse	Agilent Mouse Oligo array	14-d dietary exposure	Iida <i>et al.</i> [55]
Nongenotoxic carcinogens	Rat	Custom cDNA microarray	Single dose	Nie <i>et al.</i> [56]
Liver and lung carcinogens	Mouse	Affymetrix 430 v2.0 oligonucleotide array, serum NMR	90-d oral repeat dose	Thomas <i>et al.</i> [57]
Lung carcinogens	Mouse	Affymetrix 430 v2.0 oligonucleotide array	90-d oral repeat dose	Thomas <i>et al.</i> [58]
Nongenotoxic hepatocarcinogens	Rat	Codelink RU1	5-d oral repeat dose	Fielden <i>et al.</i> [59]
Genotoxic and nongenotoxic hepatocarcinogens	Rat	Affymetrix RAE230A oligonucleotide array	14-d oral repeat dose	Ellinger-Ziegelbauer <i>et al.</i> [60]
Nongenotoxic hepatocarcinogens	Rat	Affymetrix RAE230A oligonucleotide array	28-d oral repeat dose	Uehara <i>et al.</i> [61]
Lung carcinogens	Mouse	Affymetrix 430 v2.0 oligonucleotide array	90-d repeat dose exposure (oral, feed, and inhalation)	Thomas <i>et al.</i> [62]

Similar studies reported by Fielden *et al.* [59] have demonstrated the utility of a large gene expression database for generating and validating hepatic gene expression signatures for the prediction of nongenotoxic hepatocarcinogenicity in rats. To adequately test the accuracy of the model, the signature was tested against hepatic gene expression data from rats treated for up to 5 days with 47 independent compounds distinct from those used in the training set. This resulted in a sensitivity of 81% and a specificity of 84%. By comparison, the signature was superior in accuracy relative to more traditional endpoints that have previously been proposed and evaluated as predictors of liver tumors, including hepatomegaly, hepatocellular hypertrophy, necrosis, serum ALT activity, and induction of cytochrome P450 genes [63,64]. In addition, the gene expression profile of the test compound could be compared against nongenotoxic carcinogens of known mode of action in order to provide hypotheses as to its mode of action. Such compounds include P450 inducers, peroxisome proliferators, steroidal compounds with estrogenic and androgenic activity, and hepatotoxic carcinogens. These results indicate that hepatic gene expression data from short-term rat studies can be used not only to predict nongenotoxic hepatocarcinogens but also to understand a compound's mode of action to aid human risk assessment.

In addition to the many studies investigating the prediction of hepatocarcinogenicity, other work suggest that lung gene expression data from subchronic toxicology studies can also predict lung tumor outcome in the two-year rodent bioassay [58,62]. Initial efforts were focused on evaluating the relative utility of gene expression data and metabolomic data for predicting either liver or lung carcinogenicity in 90-day repeat-dose studies in mice [57]. This study utilized a number of environmental chemicals, including both genotoxic and nongenotoxic chemicals. Results from this study indicated that individual genes or metabolites were not highly predictive; however, the overall profiles did classify carcinogens and noncarcinogens. However, this study utilized a small number of chemicals, and it was not until a larger study using 13 diverse chemicals that a signature was developed and more broadly tested [58]. Subsequent work by the same group extended these initial findings to a larger set of compounds and provided a more thorough characterization of the signature and its ability to predict lung carcinogenicity, its dose dependency, and the effect of experimental variables on assay performance [62]. In contrast to previous efforts, these studies of lung carcinogenicity relied on lung gene expression from mice exposed for 90 days. While this approach would not identify carcinogens from short-term screening studies typically used in early drug discovery, it is practical in the sense that 90-day toxicology studies are often performed on chemicals in advance of longer-term chronic and lifetime rodent bioassays. Therefore, genomic results could be incorporated into these standard testing paradigms and provide prediction in advance of a lengthy two-year carcinogenicity study. As a result of using this later time point for signature generation and application, it is likely that these signatures are detecting gene expression changes that reflect neoplastic transformation rather than mechanistic events that are linked with carcinogenicity. This may provide a more robust predict of the likelihood of lung tumors in longer-term studies and their relationship to dose. However, it may not readily lead to an understanding of a compound's mode of action to enable human risk assessment and would not provide an early prediction (i.e., less than two weeks) in advance of more resource-intensive toxicology studies.

8.1.5 Use of Toxicogenomics to Understand Mechanisms of Toxicity

Although the interpretation of toxicity endpoints in preclinical studies is usually well understood, the predictivity of the findings in animal models for humans is often not clear. This could be due to underlying differences in pathophysiology between species, as well as due to differences in pharmacokinetics and drug metabolism. A number of examples have been reported demonstrating rodent-specific toxicities due to differences in drug metabolism [65], hormonal regulation [66], or mechanisms of tumor formation due to high dose tissue accumulation [67]. Chemical-induced toxicity in animals is often observed in routine screening studies during lead optimization; however, when the mechanism of toxicity and its relevance to humans is unknown, the development of that compound is typically stopped in the interest of time and resources in order to focus efforts on identifying additional compounds free of that liability. However, by understanding the mechanism of toxicity in susceptible animal models, a risk assessment can be made to understand the relevance of the finding in humans. In addition, the knowledge obtained by a thorough understanding of the mechanism of toxicity can be used to guide the application of *in vitro* assays or *in vivo* biomarkers. These can be applied to screen additional molecules and identify the offending toxicophore in order to design future chemicals that are devoid of the liability. Improvements to human risk assessment and the selection of the right drug candidates for clinical development are not made by conducting long-term toxicity studies earlier in drug discovery, but by obtaining a deeper understanding of the mechanisms of toxicity before conducting human studies. With that in mind, we now consider how toxicogenomics can contribute to an understanding of common mechanisms of liver toxicity.

8.1.5.1 Hepatomegaly and Hepatocellular Hypertrophy. Microarray analysis can be used to elucidate mechanisms of toxicity based on the fundamental concept that drug-induced toxicities are accompanied by gene expression changes mechanistically linked to the toxicity. Furthermore, compounds with a common mechanism of toxicity will produce similar gene expression changes and their profiles should cluster together and should be distinct from compounds inducing a similar toxicity but through different mechanisms. However, the identification of such distinct gene- and pathway-based information should not be taken as the final endpoint in identifying the mechanism but rather should be used in conjunction with histopathology and/or clinical pathology data for hypothesis generation, followed by additional biochemical or cell-based investigations to confirm the hypothesis. Once discriminating gene expression changes or “signatures” have been identified and validated, they can be used to classify unknown toxicants to understand their mechanism of toxicity.

An increase in liver weight, or hepatomegaly, is a frequent observation in preclinical rodent toxicologic studies. Increased liver weight can be caused by a number of mechanisms, including an increase in the number of cells (hyperplasia) or an increase in hepatocyte size (hepatocellular hypertrophy). Hyperplasia is of obvious concern for tumor formation; however, hepatocellular hypertrophy is a common adaptive response of the liver to xenobiotics or other metabolic challenges or stimuli [68,69]. In response to xenobiotics, the liver induces a characteristic spectrum of drug-metabolizing enzymes. This may be of significance for drug–drug interactions and pharmacokinetics. By contrast, the liver responds to fatty acids by increasing enzymes

involved in their metabolism. This is often of no toxicological significance for humans. Therefore, it is important to understand the mechanism underlying the increase in liver weight and hypertrophy.

Early toxicogenomic studies established that gene expression data from livers can be used to differentiate distinct mechanisms of hepatocellular hypertrophy induced by different chemicals. For example, analysis of rat liver tissues from animals treated with two classes of compounds leading to hepatomegaly and hepatocellular hypertrophy through different mechanisms resulted in distinct gene expression signatures [38]. Treatment with a peroxisome proliferator (Wyeth 14643, gemfibrozil, or clofibrate) or the enzyme inducer phenobarbital resulted in hepatocellular hypertrophy with minor histological differences between the classes after two weeks of treatment. However, only 24 h after treatment, the two classes of compounds demonstrated distinct gene expression profiles with the peroxisome-proliferator-treated samples clustering together and distinct from the phenobarbital-treated liver samples. Additionally, the observed gene expression changes were related to previously described metabolic, pharmacologic, and toxicologic effects of the compounds, thus providing relevant mechanistic information. While the peroxisome-proliferator-treated livers had increased expression of genes involved in triglyceride hydrolysis, fatty acid uptake, oxidation, and conversion to acyl CoA derivatives, phenobarbital treatment resulted in increased cytochrome P450 genes, microsomal epoxide hydrolase, glutathione transferases and a decrease in carnitine palmitoyltransferase 1 mRNA. Further investigation was performed to determine if the peroxisome-proliferator- and phenobarbital-specific gene signatures could be used to classify blinded samples based on the mechanism of toxicity of the compound [70]. Using the previous peroxisome-proliferator- and phenobarbital-treated livers as a training set, a group of 22 highly discriminatory genes were identified. These were then used to successfully classify blinded liver samples from phenytoin-, diethylhexyl phthalate-, and hexobarbital-treated rats as having a similar mechanism of action to phenobarbital, peroxisome proliferators, and neither, respectively.

These studies, along with others, helped establish that toxicogenomic analyses can be used to identify gene expression changes causally linked to a phenotypic endpoint. Although both the peroxisome proliferators and enzyme inducers result in hepatocellular hypertrophy, they produced distinct gene expression changes that could be used to differentiate between the mechanisms leading to the same phenotype. However, one must be cautious in interpreting results and in dissociating causal gene expression changes from associative changes through further investigation or consideration of other evidence. Additionally, a given toxicity may be driven by multiple mechanisms with different implications for human risk. Differentiation between peroxisome proliferators and enzyme inducers as described above may have implications for the clinic as it is often desirable to avoid enzyme inducers due to the potential for drug–drug interactions in humans. By contrast, hepatocellular hypertrophy induced by peroxisome proliferators is typically considered to be rodent specific and without relevance to humans [71,72]. Thus, gene expression signatures can be used to guide compound selection early in discovery as well as aid in the interpretation of preclinical effects, such as hepatocellular hypertrophy, and their relevance to humans.

8.1.5.2 Hepatotoxicity and Cholestasis. Drug-induced hepatotoxicity is a major hurdle in drug development and accounts for many adverse drug reactions and black box warnings in marketed drugs. Examination of terminated clinical development

projects from 1992 to 2002 found that 43%, 25%, and 35% of drugs in phases I, II, and III, respectively, were terminated as a result of toxicity, with many of these compounds classified as hepatotoxicants [73]. The susceptibility of the liver to toxicity can be partially attributed to its exposure to relatively high concentrations of drugs as a result of portal vein exposure following intestinal absorption, as well as its critical role in drug metabolism and clearance.

The liver is particularly susceptible to disruption of bile flow, which can result in cholestasis and liver injury. The liver is responsible for the synthesis of bile salts from cholesterol precursors, some of which are recycled via uptake at the basolateral membrane of hepatocytes adjacent to the portal blood supply. Hepatic uptake of bile salts primarily occurs via the sodium-dependent transporter sodium-taurocholate cotransporting polypeptide (Ntcp; *Slc10a1*) and to a lesser degree by a family of sodium-independent anion-transporting polypeptides (Oatps; *Slcos*). Canalicular excretion of biliary constituents such as bilirubin, xenobiotics, and bile salts also occurs through transporters such as the bile salt export pump (Bsep; *Abcb11*), the multidrug resistance-associated protein-2 (Mrp2; *Abcc2*), multidrug resistance-1 (Mdr1; *Abcb1*), and Mdr3 (*Abcb4*) [74]. Inhibition of Bsep, the primary bile salt efflux transporter, can lead to increased intracellular levels of bile salts, which are cytotoxic to hepatocytes at elevated concentrations. In fact, drug-related disruption of Bsep function is implicated in liver injury associated with many marketed and withdrawn drugs such as bosentan, glyburide, and erythromycin estolate [75–77]. Given that preclinical species are generally considered to be poor models of human liver injury related to Bsep inhibition, it can be difficult to preclinically predict Bsep-inhibition-mediated liver injury [75,78]. However, toxicogenomic monitoring of bile acid signaling pathways has the potential to be useful in identifying obstructions of bile flow, such as those caused by drug-induced Bsep inhibition. In particular, increased intracellular bile acid levels can lead to adaptive responses leading to altered transcription of bile acid synthesis genes and hepatocellular transporters. These gene expression changes are mediated by one or more nuclear receptors and can be monitored through gene expression analyses. For example, activation of the farnesoid X receptor (FXR) by bile acids results in decreased expression of the cytochrome P450 genes *Cyp7a1* and *Cyp8b1*, as well as the hepatocellular uptake transporter Ntcp leading to decreased bile acid synthesis and hepatocellular uptake, respectively [74,79,80]. Gene induction is also observed for Bsep and Mrp2, which serve to increase canalicular and basolateral efflux of bile acids [81–83]. Additional nuclear receptors such as the PXR and CAR also serve to regulate expression of bile acid synthesis, metabolism, and transport genes such as *Cyp3a4* and *Oatp2* (Fig. 8.2) [74]. Microarray analysis of livers from rodent bile duct ligation models of obstructive cholestasis confirms altered gene expression of bile-acid-related genes such as decreased *Ntcp* and *Cyp8b1* [84,85].

Monitoring expression of genes involved in bile acid synthesis, metabolism, and transport in preclinical models can provide important information regarding bile flow obstruction. However, as with most toxicogenomic studies, inference regarding the mechanism of cholestasis must be done cautiously, as there are many confounding factors and conditions that may result in similar responses or can mask these diagnostic expression changes. For example, a compound may directly activate PXR and/or CAR, thus resulting in altered expression of bile-acid-related genes in a manner similar to what would be expected from Bsep inhibition. Inflammation has also been documented to alter transcription of hepatocellular transporters. For example, endotoxin

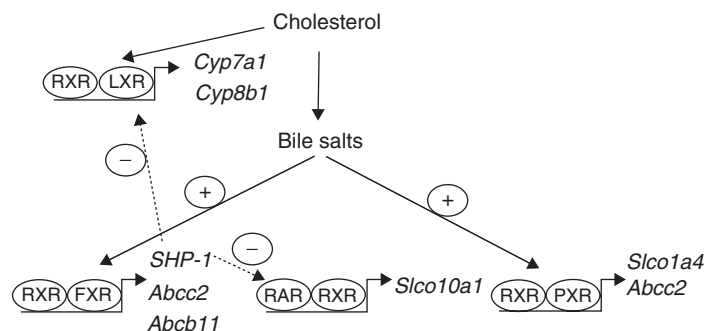


Figure 8.2 Transcriptional regulation of bile acid synthesis and hepatocellular transporters. Cholesterol activation of liver X receptor (LXR) mediates transcription of *Cyp7a1* and *Cyp8b1*. Bile acids upregulate transcription of hepatocellular transporters and inhibit their own synthesis via the activation of PXR and FXR. *Source:* Figure modified from Trauner and Boyer [74].

and interleukin-1 β have been shown to downregulate transcription factors, resulting in decreased Ntcp and Mrp2 expression [74,86]. In the case of inflammation-induced decreases in bile acid transporter genes, toxicogenomic data may provide evidence pointing to an inflammatory response such as altered expression of cytokines or acute phase proteins such as albumin or fibrinogen. In order to determine mechanisms related to transporter inhibition or nuclear receptor activation, one can supplement the *in vivo* toxicogenomic data with *in vitro* assays such as a Bsep inhibition or nuclear receptor activation assay. These assays can be used to measure potency on these off-targets, which can help rank and prioritize compounds for testing and guide medicinal chemistry efforts to avoid these off-target effects. Thus, toxicogenomics can be utilized early in drug discovery in conjunction with additional assays in order to monitor hepatocellular transporter changes indicative of transporter inhibition and cholestatic conditions. This information can help drug discovery teams evaluate the risk to humans and select appropriate drug candidates to be advanced to clinical studies.

8.1.5.3 Oxidative Stress and Reactive Metabolite Formation. Oxidative stress occurs when pro-oxidants such as reactive oxygen species (ROS) exceed the capacity of a cell's antioxidant systems to detoxify the ROS. When this occurs, it increases the risk of cellular damage and associated pathologies [87–90]. Generation of oxidative species such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical occurs through the reduction of molecular oxygen. This can occur through both endogenous processes such as oxidative phosphorylation, the inflammatory respiratory burst, cytochrome P450 metabolism, and peroxisome proliferation as well as exogenous sources including xenobiotics that undergo redox cycling and metals that participate in the Fenton reaction. Production of cellular ROS is then counterbalanced by the cellular antioxidant defense systems such as superoxide dismutase, glutathione transferase, and vitamins E and C. However, when cellular oxidants exceed the antioxidant defense system, oxidation of DNA, proteins, and lipids can occur. This would disrupt vital cellular processes and could ultimately lead to cell death. Additionally, formation of the reactive drug metabolites during metabolism or redox cycling of a chemical can lead to protein and DNA adducts. This too can lead to disruption of cellular process and cell death. Protein–drug adducts can also serve as

antigens and precipitate an adverse immune response [91–93]. Therefore, detecting the ability of a chemical to induce an oxidative stress response *in vivo* can be used to identify potential hazards that could be relevant to humans.

Given the liver's role in energy and xenobiotic metabolism and the high exposure to drug via the portal vein, the liver has an increased susceptibility to oxidative stress and reactive metabolites formed by metabolic bioactivation. The resulting cellular damage can ultimately manifest in altered clinical chemistry endpoints such as increases in serum transaminases. However, drugs can lead to increased oxidative stress through different mechanisms, each with different consequences for human risk. Unfortunately, standard endpoints such as histopathology and clinical chemistry do not provide mechanistic information. For example, oxidative stress can be a result of macrophage activation, secondary to peroxisome proliferation, or due to the generation of free radicals or reactive metabolites. Drug-induced macrophage activation or endotoxin leakage through a damaged gastrointestinal tract, as demonstrated by certain nonsteroidal anti-inflammatory drugs, leads to the production of ROS as a defensive response [94–96]. By contrast, peroxisome-proliferator-activated receptor α (PPAR α) agonism is typically followed by an oxidative stress response that is considered to be a normal physiological response to increased oxidative metabolism [97,98]. Reactive drug metabolites can lead to oxidative stress through redox cycling or free radical generation. This can overwhelm the endogenous antioxidant supply and lead to cell death. Therefore, early differentiation between these mechanisms is beneficial in decision making during drug discovery.

Analogous to the application of gene expression profiles to distinguish mechanisms of hepatocellular hypertrophy, gene expression signatures have been shown to differentiate mechanisms of oxidative stress. McMillian *et al.* [31,39,99] describe a toxicogenomic analysis of a collection of 50 in-house proprietary compounds and 100 paradigm compounds. This analysis facilitated the identification of distinct liver gene expression signatures from known peroxisome proliferators, macrophage activators, and reactive metabolite generators. The signatures were then used to categorize additional unknown compounds on the basis of their gene expression profiles. For example, the macrophage activator signature generated from lipopolysaccharide and zymosan A clustered together with compounds such as carbon tetrachloride, allyl alcohol, and galactosamine, which have previously been reported to activate macrophages in rat liver. As one may expect, the macrophage activator signature contained genes mechanistically linked to macrophage activation, such as acute phase response and immune activation genes, in addition to genes related to endoplasmic reticulum stress and chaperone genes. Interestingly, some of the genes regulated by the macrophage activators were observed to be inversely regulated by the PPAR α compounds. Similarly, the reactive metabolite signature contained many genes regulated by the nuclear factor-like 2 (Nrf2) transcription factor, which included many antioxidant and phase II metabolism genes. Selection of a subset of these genes such as NAD(P)H dehydrogenase (Nqo1), glutathione transferase μ type 2, heat shock protein 90 α , and glutamate–cysteine ligase led to the separation of a majority of the reactive metabolite compounds from compounds of the other classes [31,39,99]. Studies such as these clearly demonstrate that there are distinct transcriptional changes associated with the treatment of compounds that induce oxidative stress by different mechanisms. Furthermore, many of these genes are linked to the mechanism rather than just associative. In addition, new mechanistic insight can be gained through observation of previously unreported gene expression

changes, such as with the macrophage activator induction of endoplasmic reticulum stress-related genes and the inverse regulation of genes by macrophage activators and PPAR α agonists.

8.1.6 Use of Toxicogenomics to Understand Hepatic Drug Metabolism

Exposure to xenobiotics, whether via diet, environmental exposure, or prescription and over-the-counter drugs, can lead to enzyme induction in the liver and other organs. Xenosensors such as the nuclear receptors PXR and CAR, as well as the aryl hydrocarbon receptor (AhR), are critical mediators of defense against xenobiotics and can all mediate distinct and sometimes overlapping patterns of xenobiotic-induced enzyme induction. Upon receptor activation, there is an induction of numerous phases I, II, and III metabolism enzymes and transporters that generally serve as an adaptive and/or protective mechanism for the organism to metabolize and eliminate the xenobiotic. However, in humans undergoing single or multiple drug therapies, enzyme induction through these various sources can cause altered drug metabolism leading to uninvited consequences. It has been reported that half of the drugs withdrawn from the US market between 1999 and 2004 were associated with drug interactions [100,101]. Clearance of the primary drug or a coadministered drug can be increased or decreased, or a prodrug can be activated at altered rates leading to altered pharmacokinetic and pharmacodynamic effects. It is also well documented that enzyme inducers can exacerbate or lead to new toxicities of drugs by enhancing or initiating formation of a toxic metabolite. A classic example is ethanol-induced CYP2E1 induction, which results in the increased formation of the reactive acetaminophen metabolite, *N*-acetyl-*p*-benzoquinone imine. Enzyme induction can also alter metabolism of endogenous compounds, leading to deleterious effects. This is the case of individuals undergoing long-term treatment with enzyme inducers. For example, long-term induction of CYP3A4 can lead to increased metabolism of the active metabolite of vitamin D₃, 1 α , 25-dihydroxyvitamin D₃, thus causing vitamin D deficiency and osteomalacia [102]. Even in a more controlled environment such as preclinical drug development, enzyme induction can lead to undesirable effects such as reduced drug exposure after repeated doses (i.e., autoinduction) due to induction of the enzyme responsible for its own metabolism or clearance. It may also lead to enhanced toxicity when the metabolism enzymes bioactivate the drug or enhance clearance of endogenous molecules, such as steroid hormones. As a result of these potential interactions and effects on hepatic drug metabolism, the ideal drug candidate is one that does not affect cytochrome P450 levels or activity. The US Food and Drug Administration (USFDA) has also recommended that candidate drugs be assessed for induction or inhibition of major cytochrome P450s to determine the potential for metabolic interactions [101]. Thus, early understanding of enzyme induction in preclinical drug development can help in the design of preclinical studies and in the prediction of potential issues that may arise in humans.

8.1.6.1 PXR, CAR, and AhR. PXR and CAR are both members of the nuclear receptor family 1 and are expressed predominantly in tissues highly exposed to xenobiotics, such as those of the liver and small intestine. Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor (RXR) and binds xenobiotic response elements in the promoters of target genes, leading to increased transcription. CAR-mediated transcription occurs in a similar manner with the exception that CAR

resides in the cytoplasm and undergoes nuclear translocation upon ligand binding and activation [103]. Activation of PXR and/or CAR leads to the induction of mRNA, protein, and enzyme activity for a number of xenobiotic metabolism enzymes, including CYP3A4 and CYP2B1 in humans (Fig. 8.3). Activation of these receptors in rodents is also associated with hepatomegaly as a result of both hepatocellular hypertrophy and increased replicative DNA synthesis, resulting in hepatocyte proliferation. As a result, chronic exposure of rodents to PXR or CAR agonists, such as chlordane or phenobarbital, is also associated with liver tumor formation [68,69,104]. In humans, however, there is no evidence to indicate that PXR or CAR activation is associated with an increased risk for liver tumors [68,105,106]. Investigation of phenobarbital or chlordane administration to humanized huPXR/huCAR mice confirmed that despite the enzyme induction, liver enlargement, and hepatocellular hypertrophy, there was no increase in replicative DNA synthesis and therefore no hepatocellular

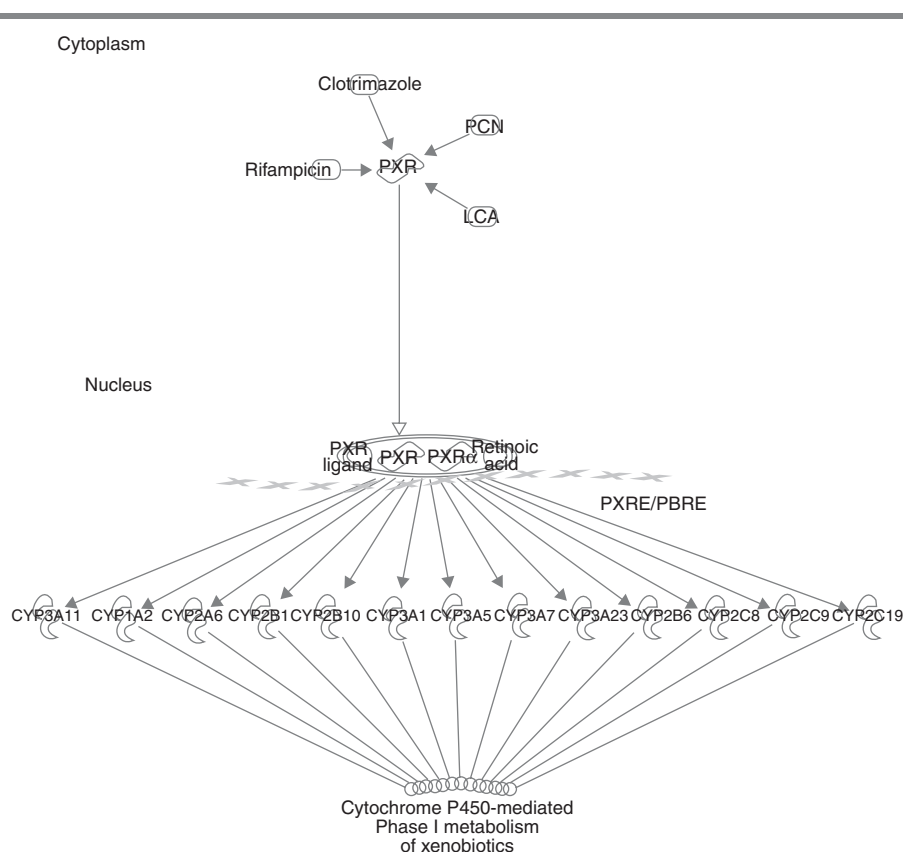


Figure 8.3 (a) PXR activation leads to the transcription of phases I, II, and III xenobiotic metabolism and transporter genes. (b) Infections and inflammatory conditions can decrease the expression of xenobiotic metabolism genes induced by PXR activation. The pathway was generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, <http://www.ingenuity.com>). PCN, pregnenolone 16 α -carbonitrile; LCA, lithocholic acid; PXRE, pregnane X receptor element; PBRE, phenobarbital response element; LPS, lipopolysaccharide.

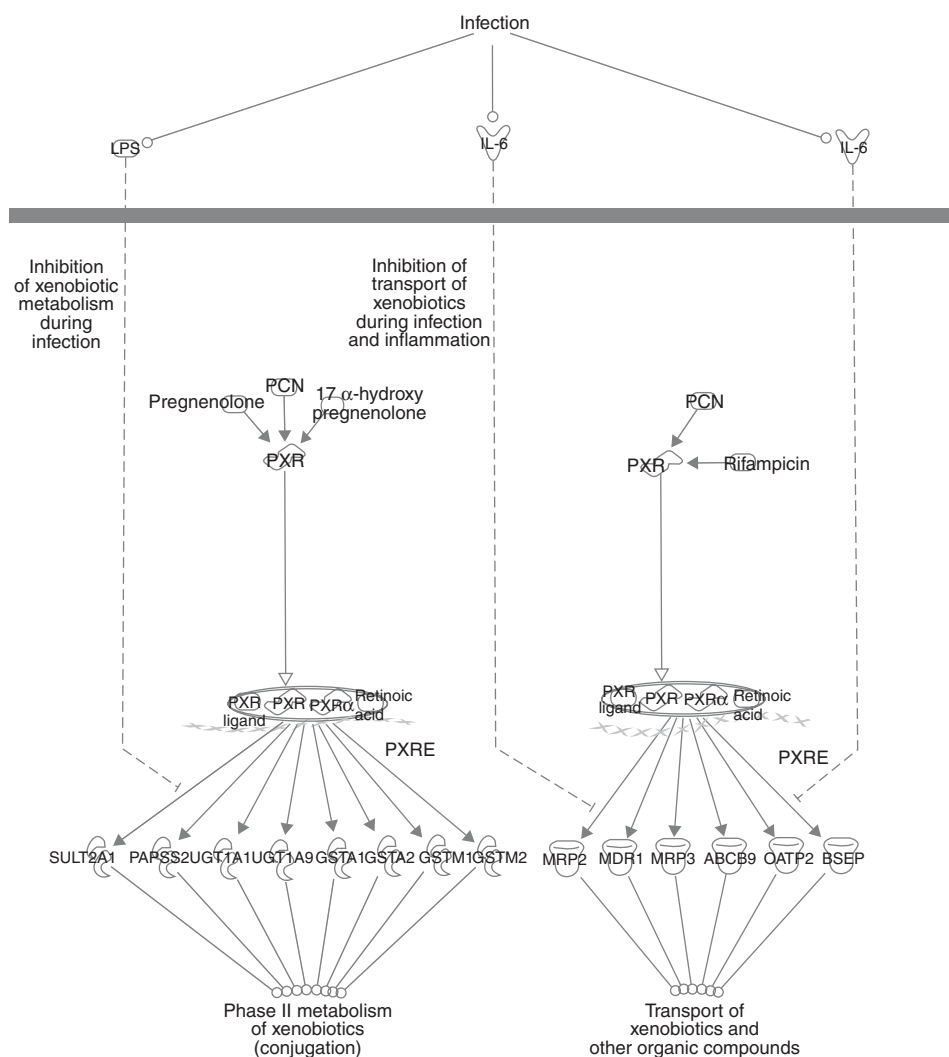


Figure 8.3 (b) (Continued)

proliferation [68]. Despite there being no increased risk for liver tumors, activation of these nuclear receptors remains an area of interest in drug development because of the enzyme induction and potential to affect metabolism of other xenobiotics or endogenous molecules. Of particular importance in hepatic metabolism is the PXR-mediated induction of *Cyp3a* genes in rodents and *CYP3A* genes in humans as demonstrated by the prototypical rodent and human PXR activators pregnenolone 16α-carbonitrile and rifampicin, respectively [107]. CYP3A4 is one of the most abundant cytochrome P450 enzymes in human liver and accounts for the metabolism of approximately 50–60% of approved drugs [108,109]. Similarly, CAR activation leads to increased transcription of the classical CAR target genes *CYP2B* and *Cyp2b* in humans and rodents, respectively. In addition to cytochrome P450s, activation of

PXR and CAR leads to altered expression of phase II/III metabolism and transport genes such as sulfotransferases, UDP-glucuronosyltransferases (e.g., *Ugt1a1*), glutathione-*S*-transferases, *BSEP*, *NTCP*, and *MRP3* [7,110]. As with PXR and CAR, the AhR regulates the expression of xenobiotic metabolism genes including the cytochrome P450 genes *Cyp1a1*, *Cyp1a2*, and *Cyp1b1*, in addition to *Ugt1a1* and *Nqo1* [111,112]. The AhR is typically retained within the cytoplasm as a complex with heat shock protein 90, hepatitis B virus X-associated protein 2, and p23 [111,113,114]. Upon ligand binding, it undergoes nuclear translocation and heterodimerization with the AhR nuclear translocator followed by transcription of target genes [115]. Effects of exposure to the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (i.e., TCDD) include hepatomegaly, hepatotoxicity, weight loss, immunosuppression, and liver tumors [116]. However, there are a number of weak AhR ligands, such as omeprazole and flutamide, that have been approved by the USFDA and do not exhibit TCDD-like toxicities in rats or humans [117]. Thus, as with PXR and CAR agonists, the major concern for drugs that are AhR agonists is the metabolic enzyme induction and potential for drug–drug interactions.

Toxicogenomics can be advantageous in the assessment of PXR, CAR, and/or AhR activation as microarrays can be used to measure the induction nuclear receptor target genes *in vitro* or *in vivo* as surrogate measures for receptor activation. For example, increased *Cyp3a*, *Cyp2b*, or *Cyp1a* expression can be considered markers of PXR, CAR, or AhR activation, respectively. The high content nature of microarray analysis allows for efficient screening of numerous genes in parallel, and when applied to *in vivo* preclinical models, it can permit the assessment of the effect of both the parent compound and any potentially active metabolites.

The mechanistic information derived from the evaluation of nuclear receptor target genes may aid in the development of more specialized assays to counter-screen additional chemicals, as well as provide data on structure–activity relationships to guide medicinal chemistry. However, understanding the specificity of nuclear receptor action is challenging owing to the complexities of gene regulation. For example, *in vivo* *Cyp1a1* induction may not be a specific marker of AhR agonism, and while it provides information regarding enzyme induction, additional information and assays may need to be considered to determine true AhR agonism [117]. Additionally, PXR and CAR can be activated by some of the same ligands, they have overlapping target genes, and they can also transcriptionally regulate each other, therefore making it difficult to determine what is controlling target gene expression [118,119]. Consequently, one may observe increased expression of both *Cyp3a* and *Cyp2b* in addition to other phase II metabolism genes, following administration of a candidate drug in a preclinical rodent study. While these observations are important for understanding the effects of the candidate drug on metabolism and possible changes in serum bilirubin or bile acid levels, more quantitative and specific methods such as real-time polymerase chain reaction (RT-PCR) analysis or cell-based nuclear receptor activation assays can be used to confirm findings based on microarray data.

There have been attempts to further identify nuclear receptor responsive gene sets that can then be used to assess individual nuclear receptor activation [120]. However, these studies do not take into account overlap or cross talk between pathways. For instance, other factors and conditions may confound interpretation of gene expression data, such as cholestasis or inflammation. Given that bile acids can be ligands for PXR, one may not be able to distinguish between PXR activation due to a cholestatic

condition or as a direct result of the drug itself. Inflammatory conditions and cytokines, on the other hand, have generally been accepted as suppressors of drug-metabolizing enzymes, possibly through the interaction of nuclear factor κ B with RXR α [121]. Therefore, a drug that leads to an increase in inflammatory cytokines, in addition to PXR or CAR activation, could lead to suppression of nuclear receptor target genes and a masking of the PXR- or CAR-mediated activity. It is also important to note that there are many species differences between nuclear receptor expression, ligands, and target genes, making it difficult to extrapolate drug–drug interaction risk from preclinical models to humans. While toxicogenomic data from *in vivo* preclinical models can be used to understand preclinical observations, it is best used in conjunction with human-specific nuclear receptor activation assays to guide clinical candidate drug selection.

8.1.7 Use of Toxicogenomics *In vitro* for Predicting Toxicity and Drug Metabolism

Applying toxicogenomics to cell-based systems represents another means of facilitating differentiation of compounds at a much earlier stage of drug discovery than *in vivo* testing would permit due to limited compound supply. Furthermore, it may offer the ability to predict *in vivo* toxicity as a means of ensuring that resources are allocated to chemical scaffolds that are least likely to be plagued by toxicity [122]. The use of toxicogenomics at this stage has not been as widely implemented because of the technical challenges of reproducibility and the questionable concordance to *in vivo* systems [123,124]. However, research in this area continues, and promising results in niche areas have demonstrated cases where toxicogenomics in cell-based systems can provide practical advantages when compared to *in vivo* models. An example of *in vitro* toxicogenomics is the prediction of phospholipidosis using human HepG2 cells [125,126].

The application of predictive toxicology models is limited not only by the degree of validation in the assays but also due to the practical need of synthesizing enough chemical material to perform *in vivo* studies, which often requires grams of material. As a means of shifting attrition even earlier in drug discovery, *in vitro* and cell-based methods are needed to take advantage of their adaptability to automation to enable higher throughput and their reliance on small amounts of test compound (approximately in milligrams). Toxicogenomics has been applied to studying drug-induced gene expression changes in a variety of cell models as a means of predicting *in vivo* toxicity or understanding mechanisms of action. Primary cells are often used because they are not transformed, as are continuous cancer cell lines, and thus maintain cellular activities more similar to cells in their native and intact tissue. However, transformed cell lines have also been evaluated, as they are easier to obtain and propagate. Comparisons of gene expression profiles of transformed, primary, and native tissues have revealed extensive differences in basal mRNA expression [123]. The differences are likely to be a result of environmental factors inherent to the culturing conditions, as well as the loss of multicellular interactions that are only obtained *in vivo*. In spite of these differences, certain drug-induced pharmacological and toxicological changes in gene expression have been shown to be reproduced *in vitro* [127]. Taking advantage of this, numerous groups have attempted to identify gene-expression-based biomarkers *in vitro* that accurately predict the ability of the compound to induce mechanisms of toxicity and pathological effects *in vivo*. Most of this work has focused on *in vitro*

cell-based models of liver and kidney toxicity owing to the importance of these tissues in drug-induced toxicity and the availability of well-characterized cell-based models.

To address the challenge of trying to recapitulate mechanisms of toxicity that may involve, or be augmented by, complex multicellular interactions *in vivo*, a number of researchers have attempted to utilize rat liver slices [128] or advanced culture models that incorporate cell–cell heterotypic interactions, perfusion flow, and three-dimensional interactions [129]. For example, liver slices have been utilized for mechanistic studies to understand the pathways relevant for the induction of liver fibrosis [130]. Although similarities in certain pathways of collagen formation were identified, other comparative studies have shown that *in vivo* models of hepatic fibrogenesis are more complex and not well represented by studying isolated hepatic stellate cells *in vitro* [131]. So while certain pathways may be reproduced in a variety of cell-based models, they fail to represent the full extent of signaling in the intact liver. As a result, the use of toxicogenomics in cell-based models is likely best reserved for studying the molecular consequences of perturbing known pathways in defined cell types, such as phospholipidosis in hepatocytes, rather than trying to fully recapitulate complex pathologies such as hepatic fibrosis.

Phospholipidosis is a lipid storage disorder that has the potential to lead to cellular necrosis. It can be confirmed only via electron microscopy, which is a time-consuming and expensive method and is not amenable to screening. To address this limitation and establish a more facile screening assay, Sawada *et al.* [125] utilized microarrays to identify gene expression changes in human HepG2 cells, a human hepatoma cell line, which would predict the induction of phospholipidosis. A number of compounds known to cause phospholipidosis *in vivo* were evaluated in cell culture for 72 h. The gene expression changes were then analyzed by microarrays to identify biomarkers that were specifically regulated by phospholipidosis-inducing compounds and not by other negative controls. A total of 17 candidate biomarkers were identified as being significantly increased or decreased in expression, and 12 of these were confirmed to be significantly changed by RT-PCR. To validate the predictivity of these biomarker genes, they were measured by RT-PCR following the treatment of HepG2 cells with an additional 14 test compounds with known phospholipidosis-inducing activity. Additionally, the changes in gene expression as measured on the microarray also suggested that alterations in lysosomal function and cholesterol metabolism may contribute to the development of this lipid storage disorder. Subsequent validation in independent laboratories confirmed the predictive nature of the signature in the same cell line [132,133]. This example demonstrates how *in vitro* expression profiling using microarrays can contribute to both a mechanistic understanding and a relatively quick and efficient means of screening compounds for a pathological effect. It is noteworthy to mention, however, that alternative and potentially higher throughput and more efficient *in vitro* methods to assess phospholipidosis in cell culture have been proposed [134]. While gene-expression-based endpoints are routine in modern molecular biology laboratories, they require multiple steps of RNA extraction, reverse transcription, and subsequent detection (microarray, PCR), and so they may not be the most economical and expeditious means of predicting toxicity.

In addition to phospholipidosis, other cell-based approaches have shown potential for predicting or mechanistically understanding a variety of pathologies. For example, gene expression profiling has been used in primary hepatocytes to differentiate compounds that do or do not induce steatosis *in vivo* [135]. Some efforts have also evaluated

cell-based assays and toxicogenomics as a means to predict hepatocarcinogenicity; however, the results suggest more work is needed to overcome limitations with system variability [136]. Nonetheless, efforts have demonstrated that genotoxic carcinogens can be differentiated from nongenotoxic carcinogens [50,51].

In order to study the mechanisms of nephrotoxicity, cell-based models such as rat primary renal cortical tubular cells are often used [137]. Consistent with other *in vitro* and *in vivo* genome-wide expression profiling experiments, nephrotoxicants clustered together and distinct from negative controls, which consisted of compounds known not to significantly affect kidney function *in vivo*. Cellular responses to stress were common. However, it is noteworthy that cisplatin, a nephrotoxic compound, and a structurally related analog carboplatin that is much less nephrotoxic *in vivo* produced a similar gene expression response regardless of dose or time. This indicates that a similar on-target effect was being recapitulated *in vitro*; however, the unique off-target effects of cisplatin that lead to toxicity were not readily detected in this cell model by gene expression. Alternatively, other extrinsic factors (cell–cell heterotypic interactions, humoral factors, etc.) are necessary to recapitulate the *in vivo* toxicity of cisplatin. Other researchers have utilized human renal tubular cell lines to study the mechanisms of nephrotoxicity of immunosuppressants such as cyclosporine and sirolimus [138,139]. New insights into mechanisms of action can be derived from well-controlled experiments; however, it has been shown that critical experimental variables can significantly impact the results when data is compared across different laboratories [138].

In addition to predicting *in vivo* toxicity and understanding mechanisms of toxicity, human and rat hepatocytes are used routinely for studying phases I and II drug metabolism and mechanisms of drug–drug interactions [140]. Evaluating induction of phases I and II metabolism enzymes can also be diagnostic of nuclear receptor activation and provide a means of assessing the relevance of animal findings to humans. While this is often done *in vivo*, hepatocyte models can provide a rapid means of assessing PXR-, CAR-, or AhR-mediated P450 expression in both rodent and human models. This can facilitate an early understanding of potential drug–drug interactions that may contribute to a prioritization of compounds or guide medicinal chemistry to avoid such off-target effects. In rodents, this may also provide an early indication of the potential of a compound to induce liver weight elevation [141]. While hepatomegaly secondary to P450 enzyme induction is often not considered adverse, it may give rise to liver tumors under chronic dosing conditions.

Cell-based models continue to improve and may ultimately provide an accurate portrait of *in vivo* gene expression and function. It is envisioned that the utility of gene expression profiling in these models should further advance our understanding and prediction of drug-induced toxicity. While extrahepatic metabolism, protein binding, and other *in vivo* pharmacokinetic considerations may modify the response in animals, the *in vitro* models should provide a means to study mechanisms of toxicity and identify potential *in vivo* hazards. However, the current use of *in vitro* toxicogenomics to predict *in vivo* responses has not been adequately demonstrated to replace or supplement the use of short-term *in vivo* models in the early evaluation of drug candidates. Hopefully, advances in cell models, such as the use of three-dimensional culture systems or coculture systems, may improve the translatability of *in vitro* gene expression measurements [129].

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9 Metabonomics in Understanding Drug Metabolism and Toxicology

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9.1	Introduction	1
9.2	Analytical platforms	2
9.3	Metabonomics in understanding drug metabolism	8
9.4	Metabonomics in understanding drug-induced toxicity	13
9.5	Conclusions and future perspectives	19
	References	20

9.1 INTRODUCTION

Metabonomics, also known as *metabolomics* or *metabolic profiling*, is defined as the measurement of low molecular weight (typically <1500 Da) metabolites and their intermediates in unicellular to multicellular biological systems that reflect the dynamic response to stimuli such as diet, lifestyle, environment, genetic effects, and exogenous factors [1]. The ability to monitor and interpret the multiparametric changes that an organism will experience is of great diagnostic and prognostic value to better understand the metabolic status of the organism and its relationship with induced stimuli. While metabonomics is often referred to studies of the endogenous metabolites, this term has been extended to the environmental chemicals and drugs to which we are exposed [2,3]. Like other “-omics” sciences, which target systems biology at a global level, metabonomics is closely related to complement genomics, transcriptomics, and proteomics [4–7]. In addition, the term *metabolome* has been coined to represent the entire metabolite collection within an organism and is analogous to the genome, transcriptome, and proteome terms already in common use [8].

At the cellular level, exogenous challenges such as drugs, dietary changes, and environmental chemicals can provoke a sequence of biochemical reactions involving receptor activation, gene transcription, and translation to protein products [6–9]. The myriad of cellular biochemical interactions generates a vast and complex array of metabolites. The fact that metabonomics measures the “downstream products” of multiple genes, proteins, and environmental interactions make it a particularly good and reliable reporter of an organism’s phenotype or physiology [10]. This is partially

due to the very different timescales required for the course changes in other “-omics” approaches. For example, gene switching can be very rapid, whereas protein synthesis is typically measured in days or weeks. Pharmacological and toxicological events at the metabolic levels can induce subsequent adaptation and/or feedback effects at the proteomic or transcriptomic levels. Therefore, metabonomics measurements can potentially maximize the probability of capturing meaningful and relevant biochemical changes, and yield important physiological information that is not accessible with gene chips, protein gels, or tissue biopsies [10,11]. Additionally, metabonomics offers the advantage of being applicable to samples collected in noninvasive (e.g., urine), or minimally invasive ways (e.g., serum, plasma, and tissue) [12,13]. This makes dynamic measurement easier without increasing patient morbidity or cost in animal lives. For example, the continuous, individual-specific time course of response can be achievable with urine sampling, providing a full profile of the toxic lesion. Meanwhile, the fact that imaging technologies, especially magnetic resonance imaging (MRI), can detect changes at the metabolic level means that tissue biomarkers generated by nuclear magnetic resonance (NMR)-based profiling can potentially be measured noninvasively *in vivo* [14–18].

The field of metabonomics has experienced a period of exponential growth in recent years primarily due to the technological advances [19,20]. With its unique focus on small molecule characterization, metabonomics is now finding widespread use in disease diagnosis, disease monitoring, nutritional science, and other clinical applications. Metabonomics also provide an effective, inexpensive route to addressing critical issues in the fields of drug metabolism, and drug safety assessment, specifically in regard to, metabolic polymorphism screening [21], toxicity screening [22,23], and biomarker identification [24–28]. These efforts, driven by drug discovery needs, are intended to enhance the quality of lead optimization decisions, decrease attrition with better candidate selection, provide safety screening for better selection of back-up compounds, and provide a tool for continuous safety assessment that can be translated from early development to the clinical studies [29]. While some of the applications have yet to become part of routine pharmaceutical or clinical practice, many of them have including the screening and identification of novel biomarkers of renal and hepatic toxicity [30–33]. The intention of this chapter is to focus on the latest advances in the applications of metabonomics to drug metabolism and toxicology. In particular, we demonstrate how metabonomics has been applied to (i) identification of novel metabolites and metabolic pathways; (ii) screening human metabolic polymorphism; (iii) preclinical toxicity screening; (iv) identification of biomarkers; and (v) investigation of mechanisms of toxicity.

9.2 ANALYTICAL PLATFORMS

The analytical challenge of metabonomics is to provide a comprehensive measurement of small molecular metabolites in the biometrics, both qualitatively and quantitatively. The two principal technologies or platforms used to generate metabolic profiles are NMR spectroscopy and mass spectrometry (MS). Both NMR and MS platforms are capable of profiling the full complement of metabolites in a hypothesis-generating (“open”) manner to provide an all-inclusive spectrum that can be mined for further information on targeted components. However, MS is more amenable to be used in a

TABLE 9.1 Comparison of NMR Spectroscopy Versus Mass Spectrometry for Metabonomics Applications

	NMR Spectroscopy	Mass Spectrometry
Maintenance	Easy to maintain	Not as easy
Instrument run time	Fast, 2–3 min per sample	Slow, 20–30 min per sample
Sensitivity	Less sensitive; detection limits of 10^{-5} – $10^{-4}M$	Superb sensitivity; detection limits of $\sim 10^{-12}M$
Reproducibility	Very reproducible	Not as reproducible
Quantitation	Inherently quantitative	Not quantitative; requires metabolite standards
Sample capacity (samples per day)	Runs up to ~ 300 samples per day with flow probes and automation	Less sample capacity due to requirement for separation
Sample preparation requirement	Requires larger (0.5 mL) samples although microprobes can handle as little as $10\mu\text{L}$; compatible with both liquids and solids (e.g., HR-MAS NMR)	Requires much less sample volume ($1\mu\text{L}$); handle only liquids, and not compatible with solids; requires derivatization for GC
Sample recovery	Nondestructive; samples can be fully recovered	Destructive; samples are not recoverable
Sample analysis automation	Option available to be automated with flow probes and robotic handling instrumentation	Mature technology with fully automated GC- or LC-MS settings
Sample separation	No sample separation required	Requires sample separation
Sample coverage	Excellent; detects all classes of organic species, but not for inorganic ions and nonprotonated species	Good; may requires different ionization sources to get full coverage of metabolites
Resolvable metabolites	Yes; requires large body of software and database for metabolite identification	Yes; excellent for structural elucidation with high resolution MS
Identification of unknowns	Yes; allows identification of novel metabolites	Mature technology for identification of unknown metabolites
Data processing automation	Robust data processing	Mature data analysis tools; large body of MS databases

hypothesis-driven (“closed”) manner, allowing specific analysis of known biochemical entities and chemical classes such as lipids or amino acids [34–36]. The selection of an “open” or “closed” metabolic profiling platform will largely depend on *a priori* knowledge of the biochemical pathways of the associated investigation. Table 9.1 provides a brief comparison of the two analytical platforms one may find useful when considering their potential for metabonomics analysis. A complete discussion of all the technology platforms, methodological details, specific protocols, and potential pitfalls associated with these technologies is beyond the scope of this chapter. Several reviews on these subjects have been recently published [37–40].

9.2.1 NMR Spectroscopy

NMR spectroscopy is an inherently quantitative and nondestructive technique that provides detailed information for hundreds of compounds in a single measurement [41]. During early development of metabonomics, the majority of work in this field was generated using NMR as the method of choice [42–50]. Relative quantitation of small molecule metabolites by performing NMR is intrinsically accurate and precise as demonstrated by the Consortium on Metabonomic Toxicology (COMET) project [51], but caution should be taken in the areas of high spectral overlaps (e.g., sugar area) [52]. Individual signals are dispersed depending on the chemical environment of the source nuclei; hence, NMR spectra are very information-rich with regard to molecular structure. This also means that sample conditions, such as pH and ionic strength, are critically important and will affect the observed spectra [52]. Recent arrangements have included custom-designed automated liquid handlers to minimize sample variations, and sample insertion systems to standardize timing and preparation [53]. Because pH can have a significant impact on chemical shifts of molecules, an automated pH measurement station integrated with a liquid handler will be a very useful module. Although pH is not the only parameter that affects the spectra, its measurement provides an additional level of quality control, as the subsequent multivariate data analysis (MDA) requires spectra to be acquired in the same physicochemical conditions, particular at the same pH [54].

The ubiquitous presence and abundance of hydrogen in biological samples as well as magnetic sensitivity and favorable isotope distributions make ^1H NMR the obvious choice for generating spectrum profiles. There are other NMR active nuclei to study biological samples including ^{13}C , ^{15}N , and ^{31}P . Phosphorus-31 is 100% abundant and is very useful for studying phospholipids and metabolites involved in energy metabolism (e.g., AMP, ADP, and ATP). Low natural abundance and poor magnetic sensitivity of ^{13}C and ^{15}N can also be, in part, overcome by “inverse” methods of detection that typically generate two (2D) or more dimensional data sets combining information from different nuclei on the same molecule to increase the dispersion of resonance [55,56]. These 2D methodologies fall into two categories: protein–protein and proton–carbon correlation experiments. The most active development area is the $^1\text{H}^{13}\text{C}$ heteronuclear single quantum coherence (HSQC) experiment where carbon is used to spread out and resolve peaks in the second dimension [57–61]. A number of investigators have opted for 2D ^1H – ^1H methodologies such as homonuclear correlation spectroscopy (COSY) [62], total correlation spectroscopy (TOCSY) [63], 2D J-resolved experiments [56,64], and DQF-COSY/IDOSY [65] for better sensitivity and compromised on the limited spectral dispersion of proton.

The primary limitation of NMR spectroscopy is the relatively low sensitivity as compared to MS methods. Detection limitations of NMR spectroscopy are typically $\sim 10^{-5} M$ in biofluids or $\sim 10^{-4} M$ in tissue extracts, compared to $\sim 10^{-12} M$ typically achieved by MS [37,39]. Increasing field strength has resulted in considerable enhancements in sensitivity and resolution. Development of cryogenically cooled probes and capillary probes has provided significant improvements in spectral signal-to-noise ratio, and pushed the limits of detection to nanomolar levels or sample volumes as low as 1.5 mL [39]. This enables metabonomics to be applied to a number of volume or mass limited experiments, such as the use of microdialysates and regular blood sampling from small animals without termination.

The development of high resolution magic angle spinning (HR-MAS) NMR allows intact tissues and cells measured directly with little or no sample preparation, affording spectral resolution comparable to that of biofluid NMR [16,66]. Rapid spinning of the sample at an angle of 54.7° relative to the applied magnetic field serves to reduce the line-broadening effects often observed in nonliquid samples such as tissues [16]. These broadening effects are caused by sample heterogeneity and residual anisotropy that are normally averaged out in solution samples such as biofluids. NMR spectroscopy on a tissue sample obtained using HR-MAS NMR is the same as biofluids NMR, and all common pulse techniques can be applied to studying metabolic changes and performing structural elucidation as well as molecular dynamic studies. Profiles generated via HR-MAS NMR have been used to reveal the effects of toxin treatment on the metabolite composition within different cellular environments either via diffusion and relaxation measurements in intact tissues [67–71] or by comparing NMR profiles from various tissue isolates [72,73]. The ability to localize biochemical changes to a specific tissue, cell type, or organelle provides valuable information with regard to investigation of mechanisms of toxicity and can aid the interpretation of biofluid analysis. While the HR-MAS NMR will not be a high throughput procedure in the near future, it provides an alternative means to validate *in vitro* systems in terms of *in vivo* metabolic responses of a specific target tissue to exogenous challenges [24]. This is particularly important when products of intermediate metabolism dominate the observed changes in biofluids and are difficult, if not possible, to link to any specific target organ [31,74]. Several excellent examples of application of HR-MAS NMR technology integrated with traditional biofluid NMR-based metabonomics have been published [75–81]. It is clear that the use of HR-MAS NMR is synergistic with biofluid NMR with regard to understanding drug metabolism and mechanisms of toxicology.

9.2.2 Mass Spectrometry

MS has been widely used for years in metabolic identification and metabolic profiling in the field of drug metabolism. To date, most of MS-based metabonomics have been used on plant metabonomics [27], however, its application to mammalian studies is rapidly increasing [82–84]. The development of multiple soft ionization sources, namely, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption ionization (MALDI), provides the metabonomics researcher with multiple tools to cover the chemical diversity of the metabolome. Yet, the primary limitation of the MS-based metabonomics is that not all metabolites can be ionized with one particular ionization source. As a result, multiple analyses are often needed to provide a complete coverage of different classes of metabolites. The extent of ionization can also be impaired by ionization suppression parameters, such as high salt content, lipids, and other polar moieties [7], underscoring the need for chromatographic separations to minimize or eliminate suppression effects [34,39].

MS as a means of metabonomics research has one major advantage in comparison with NMR, namely, the vastly greater sensitivity. Detection limitations of MS can typically reach up to $\sim 10^{-12} M$ when operating in a full scan mode [82]. This enables MS-based metabonomics to detect small chemical moieties such as sulfate, amine, and carboxyl groups. In addition, the ability to acquire exact masses of metabolites is critical for molecular formula determination and structure elucidation. Fourier transform mass spectrometry (FTMS) and quadrupole-time-of-flight (QTOF) are two major platforms

capable of delivering accurate and reproducible mass information. For example, FTMS can provide extremely high mass resolution ($R = 500,000 - 1 \times 10^6$) and high mass accuracy (<1 ppm) that allows for mass separation and differentiation of complex mixtures without a prior separation by chromatography [85]. Structure elucidation can be further assisted by increasing the collision energies to produce fragment ions.

In general, a prior separation of the complex mixture sample using chromatography is required for MS-based metabonomics, namely, gas chromatography (GC), liquid chromatography (LC), and more recently, capillary electrophoresis (CE) [39]. The resolution provided by multidimensional separations significantly minimizes the problematic effects of ion suppression and “matrix effects,” while at the same time providing further physicochemical discrimination among metabolites. The recently introduced ultra performance liquid chromatography (UPLC) is a combination of a $1.7\text{ }\mu\text{m}$ reversed-phase separation column and a chromatographic system which operates at $\sim 12,000$ psi. With much improved peak resolution and increased speed and sensitivity, the problem of ion suppression from coeluting peaks is greatly reduced [86–90]. UPLC-MS has been used for metabolite profiling of urines from phenotypically normal mouse strains and a nude mouse strain [91]. Its application in the field of metabonomics is rapidly increasing [92,93]. On the other hand, CE, in all its formats, is a high resolution separation technique that is suitable for the analysis of different groups of compounds: charged, neutral, polar, and hydrophobic. Recently, CE-MS has also been explored in the metabonomics research [94–99]. Metabolites are first separated by CE based on their charge and size, and then selectively detected by MS scans. This method has been used to measure 352 metabolic standards and then employed for the analysis of 1692 metabolites from *Bacillus subtilis* extracts, revealing changes in metabolic levels during the bacterial growth [100].

Direct and physical combination of multiple analytical technologies gives the opportunity to obtain structural information from different sources in parallel, and to offer the most comprehensive view of metabolic composition [101,102]. It has already been noted that NMR and LC-MS analysis of the same biological samples from toxicology studies have shown a similar time course of biological events, even if the compared biomarkers are quite different [103]. The most general of the “hyphenated” approaches is the LC-NMR-MS [104], in which the eluting LC peak is split, with parallel analysis by directly coupled NMR and MS techniques. This integrated system can be operated in on-flow, stopped-flow, and loop-storage modes, providing the full array of NMR- and MS-based metabonomics tools [104]. These include 2D NMR spectroscopy, FTMS, or QTOF accurate mass measurement, as well as MS/MS fragmentation for full molecular characterization. Clearly, NMR- and MS-based platforms are highly complementary in the metabonomics field.

9.2.3 Data Processing

Both NMR- and MS-based techniques described above generate large amounts of data per sample, making it impossible to manually organize, analyze, and interpret the multi-dimensional datasets. Chemometrics support is as important as the analytical techniques in the metabonomics research. A number of accessible and useful databases to aid in the identification of unknown metabolites have been listed in Table 9.2. In general, appropriate data preprocessing is required for MDA [105–108]. General procedures include data condensation and reduction, chromatographic (MS) or spectral (NMR)

TABLE 9.2 Mass Spectrometry and NMR Spectroscopy Databases that Aid in Metabolite Identification

Name	Website Address	Database
MassBank	www.massbank.jp	MS
METLIN	metlin.scripps.edu	MS
ExPASy-GlycoMod tool	www.expasy.org/tools/glycomod	MS
NIST Chemistry WebBook	webbook.nist.gov/chemistry	MS
Lipid Maps	www.lipidmaps.org	MS
Biological Magnetic Resonance Data Bank	www.bmr.b.wisc.edu	NMR
NMRShiftDB	nmrshiftdb.ice.mpg.de	NMR
The Magnetic Resonance Metabolomics Database	www.liu.se/hu/mdl/main	NMR
Human Metabolome Database	www.hmdb.ca	MS and NMR
Madison Metabolomic Consortium Database	mmcd.nmr.fam.wisc.edu	MS and NMR
SpecInfo	cds.dl.ac.uk/cds/datasets/spec/specinfo/specinfo.html	MS and NMR
Spectral Database for Organic Compounds	riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi	MS and NMR

peak alignment, background filtering, and variable scaling and normalization. To identify principle components (PCs) in a complex dataset, pretreated data are projected onto a new coordinate system based on pattern recognition (PR) algorithm or MDA. There are two categories of MDA methods, namely, unsupervised and supervised MDA, which both have widely been used in metabonomics data analysis [109]. In unsupervised MDA, sample classification is unknown or intentionally blinded to the analysis, while this information is provided to the analytical software in supervised MDA for the purpose of model construction. Both unsupervised and supervised MDA have been used in metabonomics depending on the stage and purpose of the investigation.

Principal component analysis (PCA) has become the most popular unsupervised MDA method that reduces a great number of variables into a smaller number of uncorrelated or independent variables which are called PCs [109]. The first PC explains the greatest variability in the dataset, the second PC is independent of and orthogonal to the first PC and second best explains the variability of the data and so on. In short, PCA is a decomposition of the raw dataset into “scores” that indicate the correlations between the variables. Each point on a metabonomics PCA scores plot represents the whole dataset contained in one spectrum. Thus, PCA plots are very robust for rapid screening of inherent clusters suggesting a comment effect or mechanism, assessment of dose- or time-dependent changes, and identification of individual outliers. Because of its indiscriminate nature, the biomarkers identified in a PCA model can usually be validated. However, the metabolic variations between classes are often very subtle and the inherent complexity of an organism requires utilization of more sophisticated supervised algorithms.

Supervised MDA include partial least squares (PLS), orthogonal partial least squares (OPLS), soft-independent modeling of class analogy (SIMCA), and partial least squares-discriminant analysis (PLS-DA) [109,110]. Supervised statistical methods present the unique advantage of yielding a model that can be readily tested,

validated and ultimately applied for predictive purposes. Indeed, predictability is the main objective for applying metabonomics in the pharmaceutical industry as it can be extremely valuable in identifying novel metabolic pathways, toxicity screening, disease monitoring and diagnoses, and investigation of mechanism of toxicity.

9.3 METABONOMICS IN UNDERSTANDING DRUG METABOLISM

The application of the metabonomics approach in the field of drug metabolism was first introduced in 2003 by Plumb and colleagues [111]. Unlike traditional metabolic profiling with focus on a particular metabolite or group of metabolites, metabonomics measures as many metabolites as possible, both endogenous and exogenous. In the past few years, metabonomics has demonstrated its great potential to accelerate the processes of (i) identifying novel metabolites; (ii) elucidating metabolic pathways; and (iii) identifying human polymorphisms responsible for drug-induced toxicities. Several excellent reviews on these subjects have been published [3,112–114].

9.3.1 Metabolite Identification

Given the low abundance of most drugs and metabolites relative to hundreds of endogenous chemical species present in serum or urine, it is often difficult to unequivocally identify xenobiotic compounds. To date, most of the *in vivo* absorption, distribution, metabolism, and excretion (ADME) studies including drug metabolism studies have been performed using radiolabeled (^{14}C or ^3H) compounds [115]. Radioactivity detection permits not only the identification of drug metabolites, but also the quantitative measurement of absorption, disposition, and routes of excretion. However, the time and costs associated with radiosynthesis, as well as safety and environmental concerns, have largely limited the use of this approach. This has led to a growing shift toward alternative nonradioactive approaches, such as LC-MS and NMR methods [115,116]. In particular, the emergence of high throughput metabonomics approaches has opened up a new vista for the field of drug metabolism [3,117].

One of the recent examples of using the metabonomics approach in metabolite identification was the *in vivo* metabolic profiling of aminoflavone in mice [118]. It was reported that aminoflavone was extensively metabolized to a number of metabolites by CYP1A1 and 1A2 in rat and human microsomes, one of which was a potentially reactive hydroxylamine [119]. CYP1A induction by aminoflavone enhance its own metabolism and toxicity [119]. Given the role of CYP1A isozymes in the metabolism and bioactivation of aminoflavone, a metabonomics study was initiated which employed wild-type, *Cyp1a2*-null, and *CYP1A2*-humanized mice. As a result, 13 metabolites of aminoflavone, of which 12 were novel, were readily detected and identified by using this metabonomics approach with the combination of UPLC-ESI-QTOF-MS and PCA data analysis. In addition, significant difference between wild-type and *Cyp1a2*-null mice, within the relative composition of urinary metabolites, demonstrated that CYP1A2-mediated regioselective oxidation was a major contributor to the metabolism of aminoflavone. Comparison between wild-type and *CYP1A2*-humanized mice further revealed interspecies differences in CYP1A2-mediated catalytic activity [118].

Similarly, 17 urinary metabolites of the potent carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), including 9 novel metabolites, were revealed and

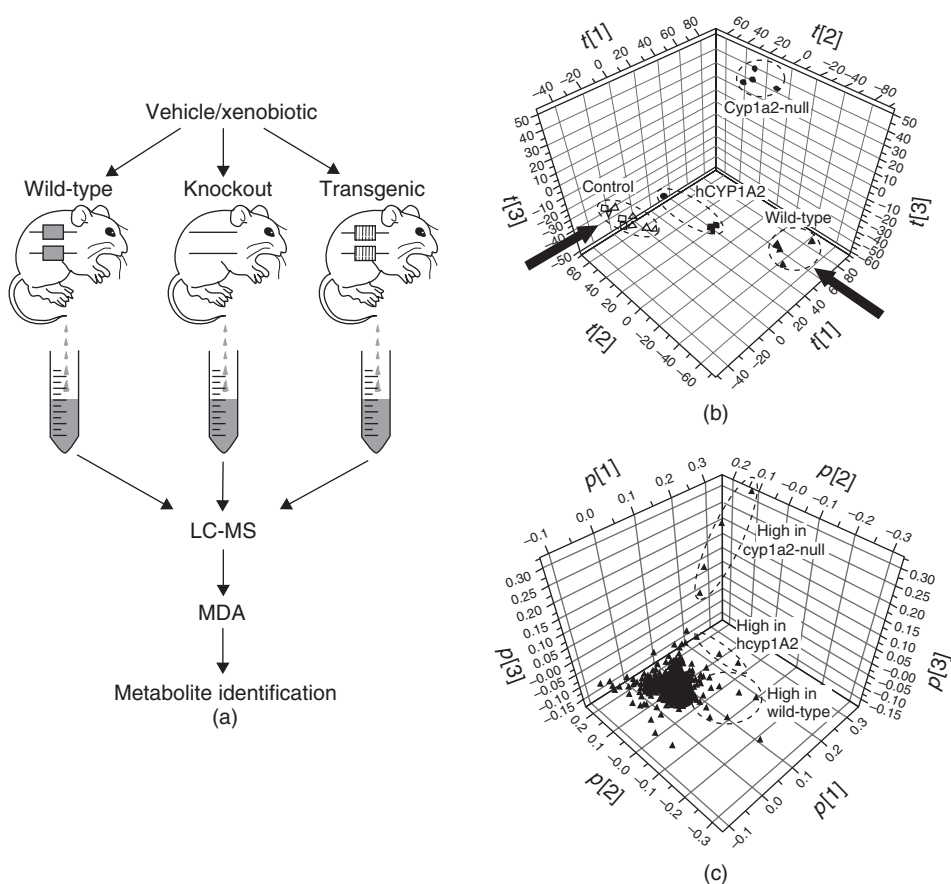


Figure 9.1 Identification of novel metabolites and metabolic pathways by the combination of genetically modified animal models and MS-based metabonomics. (a) Experimental scheme. Samples for LC-MS measurement are collected after dosing wild-type, knockout, and transgenic mice with vehicle or xenobiotic. Identification of metabolites and analysis of metabolic pathways are conducted after MDA (supervised or unsupervised) of LC-MS data. (b) Scores plot of a PCA analysis on 24h mouse urine samples from vehicle treatment (Δ , wild-type; $\diamond$, *Cyp1a2*-null; $\square$, *CYP1A2*-humanized) and 10 mg/kg PhIP treatment ($\blacktriangle$, wild-type; $\blacklozenge$, *Cyp1a2*-null; $\blacksquare$, *CYP1A2*-humanized). The $t[1]$, $t[2]$, and $t[3]$ values represent the scores of each sample in principal components 1, 2, and 3, respectively. LC-MS measurement was conducted using UPLC-QTOF-MS (Waters), data processing using MarkerLynx™ (Waters), and MDA using SIMCA-P+ software (Umetrics). (c) Loadings plot of ions in the urine samples from wild-type, *Cyp1a2*-null, and *CYP1A2*-humanized mice treated with vehicle and PhIP. The $p[1]$, $p[2]$, and $p[3]$ values represent the contributing weights of each ion to principal components 1, 2, and 3 of PCA model, respectively. Source: Adapted from Ref. 112.

identified in a single metabonomics study using a high resolution LC-MS system followed by MDA [120]. As depicted in Fig. 9.1, urine samples from the wild-type mice treated with vehicle and PhIP (indicated by arrow points) were clearly clustered and separated in the PCA scores plot (Fig. 9.1b). Accordingly, a metabolic space was created in the PCA loadings plot that defined the PhIP metabolites (Fig. 9.1c). This

study clearly demonstrates that the separation of vehicle-treated and drug-treated sample groups can be conveniently achieved in the scores plot of the multivariate model, and drug metabolites can be subsequently identified by analyzing ions contributing to the separation of drug treatment from vehicle treatment. Structures of these new metabolites were further elucidated by MS² fragmentation analysis [120].

More recently, comparative metabolism of two isomeric oxazaphosphorine cytostatic drugs cyclophosphamide (CP) and ifosfamide (IF) was systemically studied in mice using the MS-based metabonomics [121]. While CP and IF are positional isomers, only IF is known to cause nephrotoxicity and neurotoxicity. The differential metabolism of CP and IF most likely plays a central role in differentiating toxicity profiles of these two isomeric drugs. Thus, a systemic comparison of CP and IF *in vivo* metabolites in mice would potentially reveal reasons for this selective toxicity. Drug metabolites were profiled by performing UPLC-ESI-QTOF-MS and the results were subsequently analyzed by an unsupervised MDA. Of the total 23 drug metabolites identified for both CP and IF, 5 were found as novel metabolites [121]. This study also revealed that IF preferentially underwent N-dechloroethylation leading to the formation of 2-chloroacetaldehyde, while CP underwent ring-opening forming acrolein. In addition, two downstream CP and IF metabolites, namely, *S*-carboxymethylcysteine and thiodiglycolic acid, were produced similarly in CP- and IF-treated mice. These results suggested that other metabolites, perhaps precursors of thiodiglycolic acid, may contribute to IF-induced encephalopathy and nephropathy [121].

A stable isotope-based metabonomics approach has also been developed and applied to facilitate metabolite identification of acetaminophen (APAP) [122]. This is particularly important when the metabolism of xenobiotics dramatically alters or disrupts the endogenous metabolic reactions, and subsequently both xenobiotic metabolites and endogenous ions induced by the xenobiotic treatment contribute to the separation of vehicle and xenobiotic treatment in the PCA loadings plot. By dosing animals with APAP and its stable isotope-labeled form, the contributions of endogenous ions altered by xenobiotics can be phased out using the mass isotopomer analysis and a PCA model. As a result, three novel toxicity-associated APAP metabolites were revealed and identified [122]. These studies, in accordance with recent studies on the metabolism of fenofibrate [123,124], tolcapone [125], arecoline and arecaidine [126], and melatonin [127], underscore the value of MS-based metabonomics approach as a powerful tool for metabolite identification and drug metabolism.

As compared to traditional radioactivity detection methods, metabonomics-based approaches have a clear advantage for not being dependent on the costly and time-consuming preparation of the radiolabeled compounds. More importantly, this systemic approach provides a global view of the detected major and minor drug metabolites in a single metabonomics experiment. Because the existence of xenobiotics and their metabolites in vehicle control and treated samples is a “none-and-all” situation, the variables representing xenobiotic-related species in the multivariate model can be conveniently identified by an unsupervised PCA or a supervised PLS-DA based on their profound contribution to the PCs. As a result, the *in vivo* xenobiotics and their metabolites are screened and identified in a nondiscriminant manner for both common and uncommon metabolic reactions, providing the advantage over the mass defect filtering (MDF) technique that examines the differences of the measured and expected masses of metabolites generated by common biotransformation pathways [128,129].

9.3.2 Elucidation of Metabolic Pathways

Metabolic pathways and the contributions of individual human metabolic enzymes can be evaluated by using recombinant enzymes and specific inhibitors *in vitro*. The major concern associated with these *in vitro* approaches is the lack of physiological context with regard to drug concentration and a complete package of enzyme reaction systems. Genetically modified animal models, such as gene knockout and transgenic mice, provide alternative tools for determining the metabolic pathways *in vivo* [130–134]. Gene knockout models, such as *Cyp1a2*-null mice described above, are ideal for studying the endogenous function of metabolic enzymes and their roles in xenobiotic metabolism. On the other hand, transgenic models, such as *CYP1A2*-humanized mice, are suitable for identifying interspecies difference in xenobiotic metabolism and more importantly can serve as surrogate models for predicting drug metabolism in humans. Because of the systemic and global nature of the “-omics” approaches, the combination of genetically modified animal models and MS-based metabonomics can provide a powerful and efficient technical platform for identifying *in vivo* metabolic pathways.

This integrated approach [120] was applied to determine the role of CYP1A2 and interspecies difference in the metabolism of PhIP. The PCA analysis revealed three distinctive metabolic patterns among the PhIP-treated wild-type, *Cyp1a2*-null, and *CYP1A2*-humanized mice, while the vehicle controls from three mouse lines cluster together in the three-PC scores plot (Fig. 9.1). Subsequent structural elucidation based on accurate mass measurement and tandem MS/MS fragmentation was achieved for these distinctive PhIP metabolites. Taken together, this study demonstrated the role of CYP1A2 in PhIP metabolism and a human–mouse interspecies difference in the catalytic activity of CYP1A2. Mouse CYP1A2 has higher PhIP 4-hydroxylase activity while human CYP1A2 has higher PhIP N2-hydroxylase activity, which is mainly responsible for the genotoxicity of PhIP. In *Cyp1a2*-null mice, PhIP metabolism shifts from the hydroxylation reaction to the N-methylation reaction because of the absence of CYP1A2 enzyme [120]. Additionally, coupled with traditional *in vitro* approaches, this study also demonstrated that other P450s, such as CYP2Cs, are involved in the PhIP metabolism based on the observation that PhIP was still extensively metabolized in *Cyp1a2*-null mice. This finding provides an explanation that disruption of the *CYP1A2* gene in *Cyp1a2*-null mice failed to inhibit PhIP-induced carcinogenesis [120].

More recently, metabolism of melatonin was also evaluated by the combination of genetically modified mouse models and MS-based metabonomics [127]. Metabolite profiling confirmed that 6-hydroxymelatonin glucuronide was the most abundant metabolite in mouse urine. Compared to wild-type and *CYP1A2*-humanized mice, *Cyp1a2*-null mice yielded much less 6-hydroxymelatonin glucuronide (~10%) but more *N*-acetylserotonin glucuronide (~195%), and melatonin glucuronide (~220%) in urine. This study also suggested that there was no apparent interspecies difference between human and mouse with regard to CYP1A2-mediated metabolism of melatonin, but in a significant difference in phase II conjugation, namely, 6-hydroxymelatonin glucuronide in mice and 6-hydroxymelatonin sulfate in human [127].

In summary, these studies demonstrated the combination of genetically modified animal models and MS-based metabonomics as a powerful analytical platform to identify novel metabolic pathways *in vivo*. The graphic presentation of genotype-dependent grouping in the scores plot of MDA models can directly pin down the influence of a targeted gene [xenobiotic metabolizing enzymes (XMEs) or nuclear receptors] on

xenobiotic metabolism [3]. Further structural elucidation of metabolite ions yields important information for the *in vivo* metabolic pathways. On the other front, several XME knockout (CYP1A1, CYP1A2, CYP1B1, CYP2E1, and cytochrome P450 reductase) and humanized (CYP1A1, CYP1A2, CYP1B1, CYP2D6, CYP2E1, and CYP3A4) mouse lines have been established [130–132]. To study drug–drug interactions, knockout and humanized mouse models of nuclear receptors (AhR, PXR, CAR) have also become available [130]. Recently, chimeric mice with liver comprised of more than 80% human hepatocytes have been generated, which holds the promise to simulate human-specific drug metabolism in animal models [135–138]. Coupled with these genetically modified animal models, metabonomics is expected to expand further and find more new applications in the fields of drug metabolism and biochemical pharmacology.

9.3.3 Identifying Human Metabolic Polymorphism

There is a clear case for drug treatments to be selected according to the characteristics of an individual patient, in order to improve efficacy and reduce the number of severity of adverse drug reactions (ADRs) [114]. The occurrence of ADR is not uncommon for many prescription drugs, such as anticancer drugs, but it also occurs for drugs that are widely used, well tolerated, and generally considered as safe [139]. The latter type of ADR has been largely attributed to genetic polymorphisms of drug-metabolizing enzymes. Therefore, obtaining the metabolic information related to ADRs provides a promise to identify the polymorphic changes associated with these patients and in turn, serves as a prediction tools to help establish individualized drug treatment for the susceptible population. One example is the identification of a correlation between CYP2D6 polymorphism [140] and an excessive hypotensive response to debrisoquine [141]. As a result, phenotyping screening of drug candidates with the highly polymorphic P450 enzymes such as CYP2D6 has become a standardized procedure in the pharmaceutical industry to minimize the potential drug–drug interactions in clinical trials. Other examples include the correlation of warfarin responsiveness and genetic polymorphisms in CYP2C9 and VKORC1 [142], and UDP–glucuronosyltransferase 1A1 (UGT1A1) polymorphism and irinotecan [143].

Identification of ADR-related human metabolic polymorphisms still remains empirical and normally engages an extensive investigation of the pharmacokinetics and metabolite profiling of the suspect agent in clinical studies. Because of the multivariate nature and the systemic view into the “downstream products” of multiple genes, proteins, and environmental interactions, metabonomics affords a fresh promise for identifying the genetic polymorphisms associated with ADR, as compared to other “-omics” approaches. For example, over 75 variant alleles of the *CYP2D6* gene have been detected (<http://www.imm.ki.se/CYPalleles/>). Unless every variant site in the genome that affects *CYP2D6* expression is tested, it is extremely difficult, if not impossible, to conclude that a patient is a poor, intermediate, efficient, or ultra-rapid metabolizer based on a DNA test.

In fact, the feasibility of the MS-based metabonomics approach was validated in a proof-of-concept experiment, in which the urine samples from human subjects of known CYP2D6 genotype were analyzed by UPLC-ESI-QTOF-MS and supervised MDA analysis [112]. Samples from poor metabolizers (PMs) were clearly separated from those of extensive metabolizers (EMs) in the scores plot of an OPLS model.

Not surprisingly, the ions contributing most to the separation of PMs from EMs was debrisoquine, while ions responsible for the separation of EMs from PMs are 4-hydroxy-debrisoquine and two ring-open products of debrisoquine [112]. This proof-of-concept experiment clearly demonstrated that the pharmacogenetic phenotypes of CYP2D6 can be visualized and translated to metabolic phenotyping at a systemic level, which provides a rationale for the application of MS-based metabonomics approach to screen human metabolic polymorphisms.

NMR-based metabonomics also showed promise in identifying metabolic polymorphisms. A recent study [21] demonstrated that a combination of pre-dose metabolite profiling and chemometrics methods can predict the outcome for individual subjects. The hypothesis is that “the pre-dose metabolite profile of an individual contains sufficient information to enable the prediction of aspects of drug metabolism and toxicity with any prior knowledge of the subject’s genomic profile.” Major paracetamol-related metabolites included the sulfate, glucuronide, and mercapturic acid, and the nonmetabolized parent drug was also measured. The molar ratio of paracetamol glucuronide to paracetamol (G/P) best predicted the quantities of several post-dose metabolites [21]. A projection-to-latent-structure (PLS) model was constructed and validated to predict the post-dose G/P values for individual rats from pre-dose metabolite profiles. There was a positive correlation ($r = 0.48$) between G/P and the integral of a particular region of pre-dose NMR spectra, and negative correlations ($r = -0.56$ and -0.54) between G/P and integrals of two other NMR regions [21]. Moreover, the principal pre-dose metabolites underlying the mean histology score of liver damage following paracetamol administration included taurine, trimethylamine-*N*-oxide, and betaine. Clearly, these metabonomics approaches have demonstrated its potential in identifying human metabolic polymorphisms, and in turn, hold a great deal of promise to serve as a powerful prediction tool to avoid or minimize ADR in the arena of personalized drug treatment.

9.4 METABONOMICS IN UNDERSTANDING DRUG-INDUCED TOXICITY

One of the earliest applications of metabonomics was the preclinical drug safety assessment and screening of acute toxicity [144]. Metabonomics combines the techniques of high resolution NMR with PR technology to rapidly evaluate the metabolic status of an animal, such that the onset, duration, severity, and target organ localization can be determined from a peripheral sample such as urine. The diagnostic utility of any one trace molecule is limited by the commonality of the biochemical processes disrupted by toxicants due to the number of variables affecting its concentration. However, as metabonomics provides a means to simultaneously target all molecules in the metabolome, the resulting overall pattern as the response to toxic challenges become more consistent and predictive than any single marker. Not surprisingly, many of the available literature on metabonomics and drug development concentrate on its application in drug-induced toxicity and preclinical safety screening [145–149]. To date, the utility of metabonomics in measuring acute preclinical drug toxicity is quite clear. Indeed, with its robust analytical and data processing capacity, metabonomics has been used to rapidly and noninvasively perform a number of toxicological assays including (i) early toxicity screening, (ii) identification of biomarkers to monitor toxicity, (iii) identifying the target organ toxicity, and (iv) investigation of toxicity mechanisms.

9.4.1 Toxicity Screening

Early identification and characterization of a known or presumed toxicity within a pharmacological or chemical class is extremely valuable to drug discovery and development. With its fast NMR spectroscopic acquisition, automated chemometric batch data analysis, and relatively high throughput of sample processing (~300 samples per day) with flow probes and robotic systems, metabonomics provides an efficient tool that uses multivariate PR analysis to effectively perform early toxicity screening [22,23]. In an effort to optimize the process of lead optimization and decrease attrition rates in early drug discovery, metabonomics has been used to define unique spectral profiles that correspond to organ specific toxicities [23].

Toxicity screening using metabonomics is primarily based on the assumption that compounds with similar toxicological profiles will generate similar metabonomics profiles. By building a large spectral database describing hundreds of known toxins with well-characterized pathologies, metabonomics “expert systems” for the prediction of toxicity can be constructed from a series of mathematical models including PCA, neural network analysis, SIMCA, rule induction, and PLS analysis [148]. The statistical models can then be validated with an independent or “test” set of samples, where the outcome of toxicity is known, but not used in the multivariate component analysis. Once validated, the metabonomics “expert systems” can be used to assess and predict the toxicity of drug candidates in the early safety screening. Urine samples appear to be the biofluid of choice for toxicity screening because of the adequate volume available for repeated sampling and its noninvasive nature for collection in refrigerated metabolism cages [150]. Representative ^1H NMR spectra of control rat urine and blood serum samples are shown in Fig. 9.2, displaying a wide range of metabolites such as sugars, amino acids, osmolytes, as well as of larger molecules such as lipoproteins. Other target tissues, such as renal papillary, renal cortex regions (S1 and S3) [151], renal glomerula, testes, and liver [152–154] are also considered suitable for toxicity screening by metabonomics approaches.

While the initial support for the use of metabonomics as a fast and inexpensive solution to laborious toxicological and histopathological screens has cooled recently, metabonomics is still being used as a safety screening approach by pharmaceutical industry [29]. The major hurdle for practical use of metabonomics-based toxicity screening is that the data profiles generated are rarely simple and each toxin induces its characteristic lesions with its own time-dependence, recovery rate, excretion profile, and effects on other tissues. Therefore, assignment of a particular toxicity to an unknown compound, based on spectral profiles, is not always straightforward and reliable. Because the development of robust and reliable paradigms for drug safety screening requires substantial data analysis efforts to transfer the large spectral database into classification systems that are able to distinguish between toxic and nontoxic compounds, a COMET was formed between five major pharmaceutical companies (Bristol-Myers-Squibb, Elli Lilly, Hoffmann-La Roche, Novo Nordisk, and Pfizer) and the Imperial College London, UK [51]. The main objects of COMET were to assess and develop methodologies to generate a metabonomic database using ^1H NMR spectroscopy of rodent urine and blood samples for preclinical toxicity screening of drug candidates, and to build a predictive expert system for target organ toxicity [51]. The chosen COMET target compounds represented a wide range of metabolic space with diverse structures and pharmacological activities, carrying its focus on building a

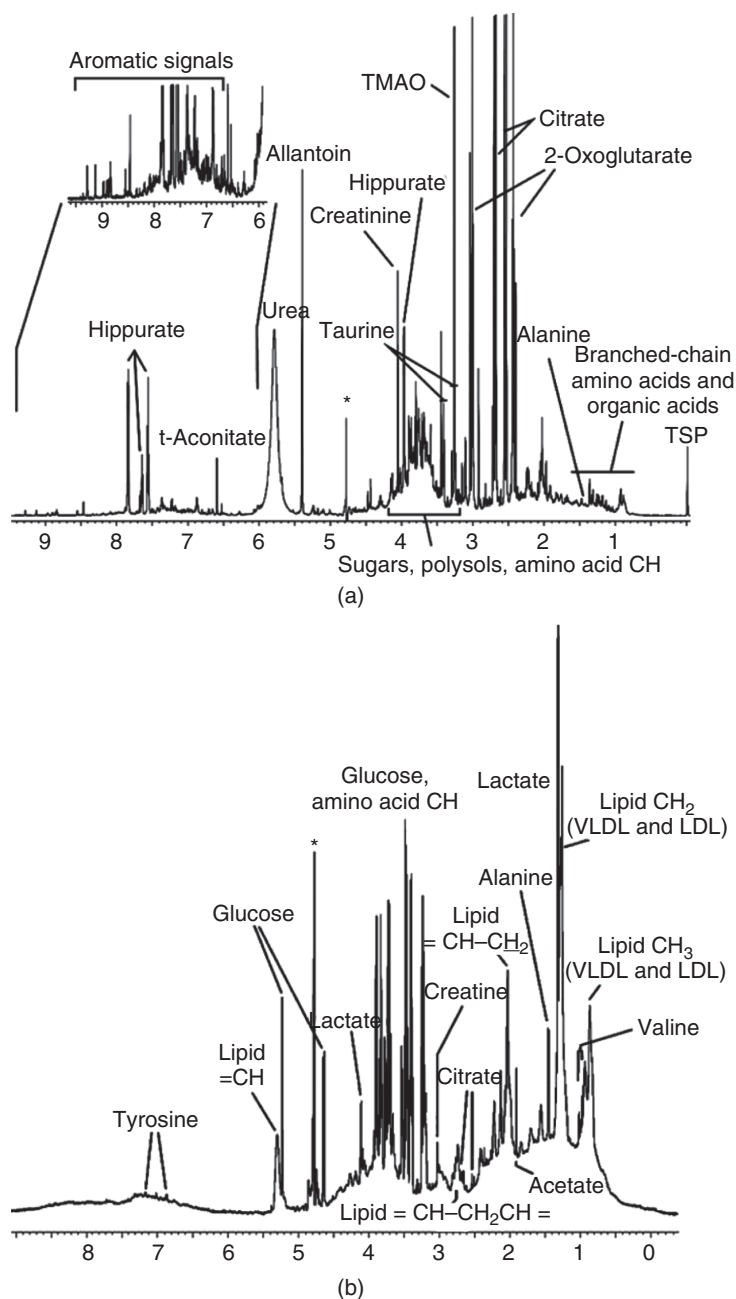


Figure 9.2 (a) Representative ^1H NMR spectrum of control rat urine displaying a wide range of metabolites such as aromatic, aliphatic compounds, sugars, amino acids, and other osmolytes. (b) Representative ^1H NMR spectrum of control rat blood serum sample showing signals of low molecular weight metabolites as well as of larger molecules such as lipoproteins. LDL, low density lipoprotein; VLDL, very low density lipoprotein. *Source*: Adapted from Ref. 66.

database for predicting kidney and liver toxicity in rat and mouse. A previous subset of the COMET project with 19 model toxins reported a predictability of 86% for the control group, 85% for the hepatotoxicity group, 91% for the nephrotoxicity group, and 88% for the other toxicity group, thereby illustrating the potential value for this application [155]. Recently, the COMET database was also used to develop an expert system for prediction and classification of drug-induced toxicity, which enabled collections of metabolic trajectories representing diverse responses to nephrotoxins and hepatotoxins to be compared, and the likely type of toxicity from a drug candidate to be predicted [156]. A subset of the COMET database representing 80 model toxins was used to build a modeling system that was capable of differentiating NMR urinary metabolic profiles from controls and treated animals. The classification of unknowns by density superposition (CLOUDS) approach [156,157] was then applied to determine similarities between different treatments, which enabled the separation of kidney and liver toxins based on their urinary metabolic profiles, providing the similarity matrix/pattern which could be used to predict the toxic outcomes of unknown treatments. The sensitivity and specificity of this expert system for liver toxins were 67% and 77%, and for kidney toxins, 41% and 100%, whereas the predictability of the system had an error rate of 8%. To date, this approach has provided the largest validation of the application of metabonomics in preclinical safety assessment and offered practical utility for *in vivo* drug toxicity screening in animal models.

To gain knowledge and confidence in the ability to screen and identify drug candidates with potential toxicological liabilities remains as the key for the toxicity screening using metabonomics approaches. Whether a preclinical animal model is indeed representative of how the drug would behave in humans remains to be monitored and validated. Another assumption is that animal models would also provide safety assessment of idiosyncratic responses in humans. Because idiosyncratic toxicity is by definition physiological response peculiar to an individual who does not respond as the majority of population, building a predictive model for idiosyncratic toxicity can be a very challenging venture that requires systemic understanding of underlying mechanisms by which idiosyncratic events may occur, such as genetic variations, genotypic diversity, disease status, environment factors, diet, and relevance to human. An alternative approach to elucidate idiosyncratic toxicity may reside in the use of metabonomics platforms to analyze the biofluid samples from individuals subject to idiosyncratic responses in a well-targeted clinical protocol [114].

9.4.2 Biomarker Identification

The quest for biomarkers in the pharmaceutical industry is imminent, and any biomarker that can either exclude patient *a priori* [21] or enable early removal of patients before significant adverse effects occur is highly desirable. As summarized in Table 9.3, metabonomics has demonstrated, to date, its enormous potential to identify novel biomarkers of drug-induced toxicity, with early work focused primarily on biomarkers of renal and hepatic toxicity [158–171]. Having established that a given compound reproducibly alters the metabolic profile of an organism, the metabolites that differentiate between biofluid samples from control and treated animal models can be elucidated. One of the examples for hepatotoxicity biomarkers was metabonomic detection of drug-induced phospholipidosis [167,168]. As an early onset of hepatotoxicity, drug-induced phospholipidosis is often difficult to be detected

TABLE 9.3 Summary of Biomarkers Determined by Metabonomics

Species	Metabonomics Biomarkers	Associated Toxicity
Rat	↑ Phenylacetyl glycine (PAG) and dimethyl glycine (DMG)	Phospholipidosis, liver necrosis
Rat	↑ Urinary taurine and creatine; bile aciduria	Liver lesions/cholestatic lesions
Rat	Urinary dicarboxylic aciduria; ↑ taurine and ↓ citrate, succinate, 2-oxoglutarate, and hippurate	Impaired fatty acid metabolism/liver toxicity
Rat	↑ Urinary taurine and creatine	Liver lesions
Rat	↑ Serum creatine	Liver (hepatocellular necrosis, steatosis, cholestasis)/testicular lesions
Rat	Altered serum/plasma lipoproteins (changes in broad alkyl features)	Liver toxicity
Rat	↑ <i>N</i> -methyl nicotinamide (NMN) and <i>N</i> -methyl-4-pyridone-3-carboxamide (4PY)	Peroxisome proliferation
Rat	↑ Urinary trimethylamine <i>N</i> -oxide (TMAO), trimethyl amine (TMA), dimethyl amine (DMA), betaine; ↓ DMG, citrate, β-hydroxy butyrate, α-ketoglutarate (ketone bodies)	Renal papillary necrosis
Rat	TMAO perturbation, ↑ DMG, DMA, and succinate	Renal papillary lesions
Rat	↑ Glucose, various amino acids; ↑ organic acids or lactate, ↓ citrate, succinate	Renal proximal tubule
Rat	Early ↑ urinary polyamines, sugars (glucose, mannose, fucose, <i>N</i> -acetylneuraminate, and gluconate); amino aciduria; ↑ urinary phosphate, 3-hydroxybutyrate, 3-hydroxyphenylacetate, monoethanolamine, and hippurate	Early kidney malfunction, nephrotoxicity
Rat	↑ Glucose, acetate, TMA, succinate; ↓ TMAO, kynuric acid, xanthurenic acid, citric acid, riboflavin	Cyclosporine A-induced nephrotoxicity
Pig	↑ Urinary TMAO, DMA, acetate	Renal medullar damage by ischemic reperfusion injury
Rat	Ketone bodies in plasma: 3-hydroxybutyrate, acetoacetate.	Fasting/reduced feeding/ketosis
Rat	↓ Alanine and lactate concomitant with ketone bodies in plasma	Gluconeogenesis
Human	Urinary salicylic acid (~75%), salicyluric acid (~20%), and gentisic acid (~5%)	Lysine acetylsalicylate poisoning
Human	Appearance of urinary valproyl- <i>O</i> -glucuronide	Valproic acid poisoning
Human	Appearance of urinary paraquat (dimethyl-bipyridylium ion)	Paraquat poisoning
Human	Appearance of tetrahydrofuran (THF) and 4-hydroxybutyric acid, and lactate in urine and plasma	THF poisoning
Human	Urinary ethylene glycol and glycolic acid	Ethylene glycol poisoning

by conventional biochemical procedures, such as histopathology. By using NMR spectroscopy and PR methods, the authors have shown that administration of two cationic amphiphilic drugs (CADs) induced foamy alveolar macrophages in rats, and resulted in urinary increase of phenylacetylglycine (PAG) with decreases in citrate and 2-oxoglutarate. The mechanism by which PAG is elevated as result of phospholipidosis is unclear. The association of increased urinary PAG levels in the event of phospholipidosis was later confirmed for three other CADs administered in rats, namely, amiodarone, chloroquine, and DMP 777. This study demonstrated that monitoring PAG in rat urine as a biomarker of phospholipidosis is a promising development [167–169].

Recently, metabonomics has also revealed two novel biomarkers for peroxisome proliferation (PP) in Wistar Han rats, namely, *N*-methylnicotinamide (NMN) and *N*-methyl-4-pyridone-3-carboxamide (4PY) [159]. Because many lipid-lowering drugs can induce PP, early biomarkers that detect PP in animal models have been pursued [159]. Although there is little evidence of significant PP in human, the assessment of human hepatic responses to drugs such as PPAR α agonists is hampered by the lack of PP biomarkers, as well as the limited understanding of the mechanisms of PP in rodents. Both NMN and 4PY are end products of the tryptophan-nicotinamide adenine dinucleotide (NAD⁺) pathway. Urinary NMN and 4PY were elevated 24- and 3-fold, respectively, with a correlation between NMN and peroxisome count of $r = 0.87$ ($r^2 = 0.76$) [159]. The metabonomics datasets were further exploited to build a PLS predictive model for PP and successfully applied to classify samples from Sprague-Dawley rats dosed with fenofibrate, a PP model drug [160].

There are also novel hepatotoxicity biomarkers identified by MS-based metabonomics approaches. It was reported that GC-MS was used to study carbon tetrachloride (CCl₄)-induced acute liver injury in mice [172]. A systemic metabolic profiling of liver tissues and plasma at various post-dose time points was completed, and the study showed elevated levels of malate and several fatty acids in the liver, as well as increased concentrations of citrate and several amino acids. The relative levels of several bile acids have been reported as better indicators of liver functional status than total bile acids. Recently, a UPLC-MS-based metabonomics method was used to investigate bile acid changes in rodent models of liver failure induced by CCl₄ and α -naphthylisothiocyanate (ANIT) [173]. Twenty-two bile acids were evaluated in the rat serum by PCA along with canonical discriminant analysis, and the results demonstrated that levels of the different bile acids varied between treated and untreated rats and the pattern could potentially serve as a more sensitive indicator of hepatotoxicity.

Recently, an increased number of metabonomics studies have employed both NMR and MS to provide a more complete understanding of the metabolic changes in response to toxic challenges, particularly in the search for the new biomarkers of nephrotoxicity [174,175]. Gentamicin is an aminoglycoside antibiotic prescribed to treat life-threatening infections but causes a 10–15% incidence of nephrotoxicity. A combined NMR and HPLC-TOF-MS study of gentamicin-induced nephrotoxicity in rats detected an increased urinary level of glucose and decreased level of trimethylamine N-oxide (TMAO) by NMR with decreases in xanthurenic and kynurenic acids and changes in sulfation patterns by MS [176]. Glucosuria has been previously shown as a potential marker of nephrotoxicity [176,177]. In a separate study that investigated the effect of age on the severity of gentamicin-induced renal toxicity in Sprague-Dawley rats, UPLC-ESI MS showed increases in urinary levels of 6-hydroxymelatonin that

directly correlated with the histopathology findings [178]. 6-Hydroxymelatonin is an oxidized by-product of melatonin, a powerful antioxidant that has been demonstrated to act as a scavenger of nitric oxide [179]. Melatonin supplementation in Sprague-Dawley rats has also been shown to protect the kidney from gentamicin and mercuric chloride induced renal toxicities [180,181]. Therefore, the elevated urinary levels of 6-hydroxymelatonin may represent the increase in melatonin as the body tries to protect against kidney damage following dosing with gentamicin. Taken together, these studies demonstrated great potentials of the combination of both MS- and NMR-based metabonomics approaches in identifying novel biomarkers, as well as elucidating the mechanism of toxicity.

9.4.3 Mechanisms of Toxicity

While mechanisms of toxicity and biomarkers are closely linked together as described above, a mechanistically elucidated biomarker should be far more predictive and therefore valuable. Metabonomics has proven to be a powerful tool for gaining insights on the mechanism of drug-induced toxicity. Most of the mechanistic metabonomics work has been focused on nephro- and hepatotoxicity, linking the temporal metabolic dysregulation with toxicity endpoints [24,31,74].

Several samples of metabonomic investigations of the mechanisms of drug-induced toxicity have been published. The neurotoxic effects of hydrazine was mechanistically linked to the markedly elevated levels of 2-aminoadipate (2AA), which is known to affect kynurenic acid levels in the brain. The results provided a plausible hypothesis for the heretofore unexplained neurotoxicity of the compound [182]. Clayton *et al.* mechanistically linked creatine to hepatotoxicity via effects on cysteine synthesis, and later on the elevated creatine levels were related with hepatotoxicity and nutritional effects [162,183]. Urinary dicarboxylic aciduria has also been associated with impaired fatty acid metabolism, which may be common to hepatotoxicity [183].

Whereas investigation of toxicity mechanisms is an ongoing subject, novel integrated metabonomics studies have also served to enhance the utility of the technology for mechanistic purposes, notably the HR-MAS NMR for metabolic profiling of intact tissues (Section 9.2.1). These efforts have been successfully employed to study the mechanisms of toxicity of acetaminophen [81], ANIT [79], Bay41-4109 (an anti-HBV compound) [184], and cadmium [185].

9.5 CONCLUSIONS AND FUTURE PERSPECTIVES

This chapter has attempted to highlight some of the key applications of metabonomics in drug discovery and development. While MS-based platforms have demonstrated clear advantage in identifying novel metabolites and metabolic pathways in the drug metabolism field, biofluid and tissue NMR play a growing role in the drug safety screening and biomarker identification. Both MS- and NMR-based platforms have shown great values toward a deeper understanding of drug metabolism and drug-induced toxicity, and an integrated approach will provide a holistic metabolic profile of biometrics. Although each technology is currently evolving in its full speed, future

developments, such as construction of merged databases of metabolome and their associated metabolic pathways containing both MS- and NMR-derived information, will greatly facilitate the identification of thousands of metabolites and metabolic networks.

Owing to its nature measuring the “downstream products” of multiple genes, proteins and environmental interactions, metabonomics best reflects a phenotypic metabolic status and therefore has great potentials to generate valuable data for a “systems biology” approach. In the studies of drug metabolism and toxicology, however, the integrated approach applying all the accessible technologies (e.g., genomics, transcriptomics, proteomics) will have the best chance of success, simply because biochemical interpretation of results from each of these “-omics” approach individually can lead to premature conclusions as exogenous challenges can perturb cellular processes at multiple molecular levels [54]. Meantime, to achieve the objective of understanding the mechanisms of drug metabolism and toxicity-related events, careful experiment design, well-controlled sample collection, high resolution data acquisition, robust data processing, and stringent data analysis are critically important and remain to be further improved.

It is clear that metabonomics will continue play an increasing role in the fields of drug metabolism and drug safety evaluation. It can also be expected that metabonomics will enter the “mainstream” activities of pharmaceutical research in the foreseeable future. As a result, successful impact of metabonomics on the pharmaceutical portfolio should lead to a reduction in the attrition rate of drug candidates in the pharmaceutical R&D process, and in turn, determine future investment, support, and sponsorship for this rapidly evolving field.

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10 Proteomics in Drug Discovery and Development

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10.1	Summary	1
10.2	Introduction	1
10.3	2DE-based protein expression analysis	3
10.4	Liquid chromatography mass spectrometry (MS)-based proteomic analysis	12
10.5	Protein microarray	26
10.6	Bioinformatics	27
10.7	Conclusion	27
	References	27

10.1 SUMMARY

Aided by major technological advances, the field of proteomics has become extremely active in the identification of drug targets that can guide drug development and treatment decisions and ultimately improve treatment outcomes. Proteomics has provided a means for molecular profiling that will assist in the development of tailored therapies. Although the implementation of genomic and proteomic testing in clinical practice is still in its infancy, the rapid development of new technologies and platforms provides hope for personalized medicine. This chapter describes these technologies and platforms, presenting many of their technical aspects along with examples of how they have been implemented in pharmaceutical applications.

10.2 INTRODUCTION

Target identification, lead identification, small-molecule or therapeutic protein drug optimization, and preclinical and clinical development are four major phases involved

in drug discovery. Advanced genomic, proteomic, and metabolomic technologies have been applied to one of the key steps in drug discovery, target identification, primarily because of their high throughput and large-scale analysis capabilities. However, many genomic study results have shown poor correlation with protein expression or mutation data. Moreover, genomic analysis lacks the ability to detect and characterize protein posttranslational modifications (PTMs). The measurement of genomic profile has proven to be inadequate in drug target identification. In recent years, global analysis of gene expression at the protein level and protein PTM analysis have taken the center stage in studying protein expression profiles and alterations under defined biological conditions. Proteomic technologies are growing at an extremely rapid rate and have revolutionized the way in which biological research is conducted. These technology-driving methods will no doubt be very valuable for diagnostics, prognostics, therapeutics, and drug discovery, leading to improved human health.

10.2.1 Proteomics Platforms

Proteomic analysis is most commonly accomplished by a combination of either two-dimensional gel electrophoresis (2DE) or liquid chromatography (LC) to separate and visualize proteins/peptides and mass spectrometry (MS) to identify, characterize, and quantify them.

The power of 2DE in proteomics lies in its ability to enable parallel processing of hundreds (or even thousands in many cases) of proteins concurrently. Although this technique is powerful, reliable, and effective, its lack of ability to characterize all the elements of a proteome is still its biggest disadvantage. 2DE fails in three areas: (i) the ability to detect and analyze low abundant proteins; (ii) the ability to separate proteins at the extremes of isoelectric point (pI), molecular weight (MW), and hydrophobicity; and (iii) the ability to analyze proteomes in a high throughput fashion. The individual extraction, digestion, and analysis of each spot from a 2DE gel are tedious and time-consuming processes that limit its ability to be a high throughput method.

One logical way to improve the 2DE technique is to prefractionate the protein samples to enrich for the less abundant proteins. Alternatively, nongel-based chromatography systems, such as the LC-MS-based shotgun proteomic method, can provide a powerful tool to resolve and identify thousands of proteins from a biological sample. In this method, proteins are first digested by a protease into a peptide mixture, which is then analyzed by LC/MS/MS and subsequently identified by database searching. This approach is rapid and more sensitive. It can be automated and has the ability for large-scale proteome analysis, but it requires significant bioinformatic resources for data analysis, and relative quantification remains very challenging without isotopic labeling.

Although these current technologies can facilitate diagnosis and drug development, they are still far from perfect. The barriers that hinder accurate and robust analysis of samples include (i) the protein dynamic range ($>10^9$), which poses a daunting analytical challenge for less abundant proteins; (ii) protein quantification; (iii) the identification of PTMs and splice variants, which remains sophisticated and time consuming; and (iv) the lack of high throughput and reproducible validation strategies for protein identification and quantification.

10.3 2DE-BASED PROTEIN EXPRESSION ANALYSIS

Despite its limitations [1], a principle core technology of protein separation and analysis still lies in the 2DE separation of the complex mixture of thousands of proteins represented in a living cell. Rather than an emerging technology, 2DE is an approach with a 36-year history. The analysis of protein expression profiles using a 2DE approach, combining separation by isoelectric focusing and denaturing by sodium dodecyl sulfate (SDS) electrophoresis, was first published in 1975 independently by Klose and Spielmann [2], O'Farrell [3], and Scheele [4]. Global protein analysis using 2DE was proposed early the next decade [5,6] and technical improvements, making it a large-scale, quantitative, and qualitative analytical tool, have continued to the present [7]. In conjunction with 2DE, enzymatic cleavage of separated proteins, tandem mass spectrometric (MS/MS) analysis of the resulting peptides, and online peptide sequence database searching for protein identification comprise a typical "gel-based proteomics" platform.

10.3.1 Principles of the 2DE-Based Approach

10.3.1.1 Two-Dimensional Electrophoresis. 2DE protein separation typically resolves 1000–2000 proteins in a single tissue sample and up to 10,000 on a large format gel [8], even when moderately sensitive stains are used. Saturation labeling of proteins with fluorescent cyanine (Cy) dyes provides even greater sensitivity, particularly when samples are very small [9]. Proteins are separated based on their charge, a function of their constituent acidic and basic amino acids and chemical modifications therein, by isoelectric focusing (IEF) in the first dimension and then by molecular mass via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE or DALI) in the second dimension. When combined in this manner, these two separation techniques produce a unique two-dimensional protein pattern representative of the molecular phenotype of the subcellular organelle, cell, or tissue. A typical 2DE protein pattern obtained from a rodent liver sample is shown in Fig. 10.1. This pattern contains nearly 3000 features (300 alone in the boxed region), providing an excellent snapshot of the most abundant portion of the hepatic proteome. The 2DE protein pattern can then be subjected to image analysis where each protein spot is assigned an x and y coordinate position, and the spots in each pattern are matched to a standard master or reference pattern. Quantification based on protein spot optical density and alterations in abundance, charge, and/or molecular mass can then be determined relative to a standard pattern using statistical analysis tools. Changes in abundance suggest upregulation or downregulation of gene expression or altered protein turnover rates. Modifications in charge and/or molecular mass indicate posttranslational protein modifications, such as phosphorylation, glycosylation, prenylation, cleavage, or genetic polymorphisms. Of particular relevance to the pharmacologist are the detectable charge modifications associated with conjugation, chemical adduct formation resulting from interactions with reactive intermediates resulting from drug metabolism, or amino acid substitutions resulting from point mutations in the genome. A brief summary of the individual components of the 2DE approach is presented below, and the overall scheme is depicted in Fig. 10.2.

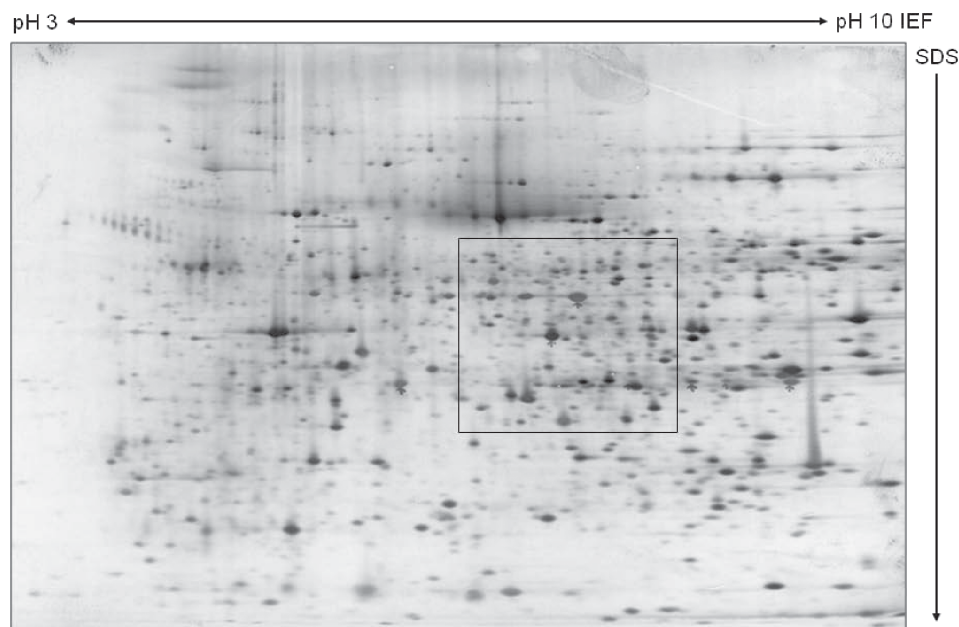


Figure 10.1 Large-format 2DE gel ($20 \times 25 \times 0.15$ cm; stained with colloidal CBB) image of whole rat liver lysate. The coordinate pattern reflects a pH gradient (IPG) of roughly 3–10 and a mass range from ~ 10 –200 kDa (bottom to top). Over 2000 protein spots are detectable in the entire pattern, and the boxed area (~ 2 pH units $\times$ 30 kDa) contains over 300 features.

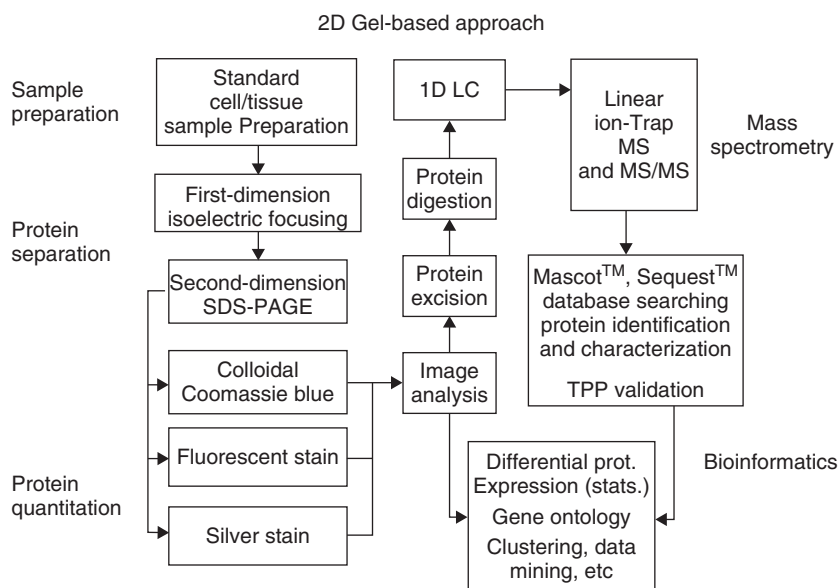


Figure 10.2 Standard platform and analytical work-flow for 2DE, including protein identification by LC/MS/MS. TPP, Trans proteomic pipeline.

10.3.1.2 Sample Preparation. In the analysis of differential protein expression resulting from toxic exposure, optimal sample solubilization is critical, and unnecessary sample handling and artifactual alteration of the isolated proteins must be avoided. Generally, one should use a one-step solubilization technique that involves physical disruption of tissues, cells, and organelles via ground-glass or Teflon homogenization, or ultrasonication in a single medium. This enables the simultaneous solubilization, denaturation, and reduction of sample proteins, breaking noncovalent and disulfide linkages, removing interfering substances such as nucleic acids, and inhibiting the actions of intracellular proteases [10]. Prepared in this way, proteins can be separated as the monomers encoded by the genome while the covalent PTMs that typify certain proteins and cellular conditions remain unaltered. A typical solubilization medium contains 7–9 M urea, 4% nonionic or zwitterionic detergent such as 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate (CHAPS) or Igepal CA-630 ([octylphenoxy] polyethoxyethanol), and 50–100 mM dithiothreitol (DTT) or tributylphosphine as a reducing agent. When immobilized pH gradient (IPG) strips are used for first-dimension separation, protein hydrophobicity becomes a problem. In this case, combining urea and thiourea in a two- to fourfold ratio during solubilization and IPG strip reswelling prevents protein adsorption to the IPG strip and loss of resolvable proteins [11].

10.3.1.3 First-Dimension Isoelectric Focusing. After solubilization (and prefractionation if desired), protein samples are separated according to their charge by IPG electrophoresis on gel strips. IPG strips consist of IPGs [12] with pI resolution up to 0.01 pH unit attainable in highly stable gradients where cathodic drift is minimal. Because each IPG strip is cast with a rigid backing, it has improved physical stability over tube gels, which simplifies handling and avoids gel breakage. Gel sample loading is simple and takes advantage of the commercially available IPG strip shipped in the dehydrated form. By uniformly rehydrating (reswelling) the entire length of the dried IPG strip with sample-containing buffer, problematic sample precipitation at a single application point is avoided, control of quantitative sample loading is improved, and, if necessary, >10 mg protein can be loaded to improve detection of low abundance proteins. Broad pH ranges and alkaline stability have produced IPG strips with functional immobilized gradients of pH 3–12.35, although narrow-range pH gradient strips are also available for analyzing proteins within a specific pI range.

10.3.1.4 Second-Dimension SDS-PAGE. After first-dimension IEF, the gel or strip is placed on a polyacrylamide gel slab for separation by molecular mass, producing a 2D pattern. The procedures involved in SDS slab gel electrophoresis vary according to the apparatus available, with most 2D systems limited to 2–12 slab gels per run. Some large-scale systems support 20 gels per run and these are particularly useful in studies where the effects of several dose groups on protein expression are being compared. A highly parallel slab gel system significantly reduces gel to gel variability and improves gel imaging and quantitative/qualitative protein analysis. SDS is an anionic detergent that denatures proteins and forms anionic complexes with constant net negative charge/mass ratio. Proteins treated with both SDS and a reducing agent, such as DTT, can be separated exclusively by MW (mass/charge). Most 2DE platforms use a tris-glycine buffer system similar to that originally described by Laemmli [13]. On the basis of its sieving properties, polyacrylamide significantly

affects protein mobility particularly when one alters acrylamide concentrations. Incorporation of acrylamide gradients optimizes resolution of heterogeneous mixtures of low, high, and intermediate MW proteins typically found in target tissues such as liver, kidney.

10.3.1.5 Protein Detection and Quantification Methods. Vital to an effective proteomics platform is the ability to quantitatively detect the resolved protein pattern in a manner that is both accurate and reproducible. A number of different methods can be used to stain and detect proteins separated by 2DE and all have their own unique limitations. The most critical properties of protein detection methods used in gel-based proteomics include high sensitivity (and a concomitant low detection limit), wide linear dynamic range (for accuracy), reproducibility, cost-efficiency, ease of use, and, perhaps most importantly, compatibility with subsequent MS protein identification and characterization techniques. Unfortunately, no single detection method meets all these requirements.

One method that is more effective than the classical “staining techniques,” in terms of sensitivity, dynamic range, and reproducibility, is the use of radiolabeled proteins (by *in vivo* metabolic incorporation of radioactive amino acids such as ^{35}S -methionine or ^{14}C -leucine into proteins), along with autoradiography and image analysis. However, the use of radiolabeled compounds is frequently impractical, particularly in routine, high capacity applications.

10.3.1.6 Protein Detection. Historically, one of the first dyes with widespread use for polyacrylamide gel-based protein staining was Coomassie brilliant blue (CBB) R-250 because its staining intensity is relatively linear (10- to 30-fold) over an acceptable range of protein concentration [14] and is thus excellent for densitometric protein quantification. CBB-stained proteins are particularly useful for proteomic studies because they are fully compatible with all forms of MS analyses of their tryptic digests. However, CBB R-250 is rather insensitive ($\sim 100 \text{ ng/mm}^2$) so that low abundance proteins are left largely undetected. By using a colloidal CBB G-250 staining protocol [15], protein detection can be greatly improved, as it approaches the sensitivity of some silver stains at $\sim 1 \text{ ng/mm}^2$. In addition, to overcome CBB R-250's lack of sensitivity, protein electrophoretic silver stains were perfected [16]. Ammoniacal (alkaline) silver staining can be very sensitive (0.1 ng/mm^2) but only has a ~ 10 -fold dynamic range. Furthermore, MS of tryptic digests from silver-stained proteins tends to be problematic [17], requiring an additional step of silver removal (destaining) with potential protein loss. Furthermore, slight variations in the reagents often caused major differences in staining intensity and background, thus creating quantification problems in large-scale experiments with very large numbers of gels.

Of significant impact was the development and 2DE application of fluorescent stains, such as the ruthenium-based SYPRO Ruby protein gel stain [18,19] with a $1\text{--}2 \text{ ng/mm}^2$ sensitivity and a dynamic range of nearly three to four orders of magnitude and Flamingo, an even more sensitive fluorescent dye [20], and the use of Cy dyes (Section 10.3.2.1) have had an even greater impact on detection sensitivity and reproducibility. Despite the great advances in sensitivity and dynamic range, the identification of proteins in gels stained in this manner is difficult and often requires a replicate gel that has been loaded with sufficient protein (overloaded in comparison to the Cy-labeled sample) and can be CBB-stained to obtain a plug for proteolytic

digestion that contains enough protein for MS detection of an accurate m/z measurement, especially in matrix-assisted laser/desorption ionization mass spectrometry (MALDI-MS) applications.

Given the interest in protein PTM status and its profound effect on biological activity, in lieu of actual gene upregulation and downregulation, numerous stains have been developed to detect prominent protein modifications such as glycosylation and phosphorylation. In 2001, a sensitive (1 ng/mm^2) green-fluorescent periodate Schiff-base, glycoprotein-specific stain referred to as *Pro-Q Emerald dye* was developed [21], which allows detection of glycoproteins directly in polyacrylamide gels or on polyvinylidene fluoride (PVDF) membranes. Similarly, Pro-Q Diamond is another fluorescent stain [22] capable of sensitive detection of phosphorylated amino acid residues in proteins separated in 1D and 2D gels, which has a sensitivity similar to the Pro-Q Emerald dye. Sequential staining and multiplexing with all three fluorescent stains is possible [22–24] and can be combined with Cy dye technology [25].

10.3.1.7 Image Analysis. Successful application of 2DE in pharmacology relies on accurate protein differential expression analysis. This necessitates precise comparison of individual protein abundances in control versus sets of treated groups to properly describe the dynamics of the experimental conditions and to distinguish the magnitude and statistical significance of various biological effects. Therefore, optimized protein separation, detection, quantification, and identification methods are central to this application and to proteomics in general [26]. There is currently no practical approach to routinely and precisely measure the absolute amounts of each protein present in a typical 2D gel. However, one can accurately quantify protein abundances relative to a reference pattern. Nevertheless, gel imaging systems are commercially available for any manner of protein stain or detection methodologies. As a result, relative protein abundance can be determined and quantitative differences in protein expression statistically compared using these analysis systems.

Most 2D gel image analysis packages commonly consist of (i) scanning a stained 2DE pattern via laser densitometry, photodiode array, or fluorescence emission detector; (ii) digitizing the image; (iii) processing the image (e.g., background and streak subtraction and mathematical morphology operations to detect and optimize each spot); and (iv) creating a reference pattern to which all other patterns are then matched to create a set of essentially superimposable 2D protein patterns. Lists of matched protein spot abundances can then be subjected to statistical analysis to search for quantitative, qualitative, and positional (charge) alterations in the proteome patterns. Despite nearly three decades of 2D gel image analysis research and development, the various strategies employed by the numerous software platforms continue to account for at least part of the variability associated with gel-based proteomics [27–29], and great care must be taken when employing this aspect of the 2D gel-based approach.

10.3.1.8 Protein Identification. After 2DE separation and image analysis, any or all the proteins in the gel pattern can be selected for identification. In large proteomic projects, the ultimate goal is to identify all expressed proteins [30]. In less ambitious, more focused studies, only those proteins altered in response to some experimental manipulation/disease state or those detected in greatest abundance are selected for identification. Regardless of project scope, protein identification is becoming less problematic as a result of technical developments in both traditional approaches that are

slow and labor intensive and contemporary techniques that are fast, less expensive, and appropriate for high throughput applications.

10.3.1.8.1 Immunoblotting. The most traditional method of protein identification involves Western blotting and immunostaining [31]. Proteins on 2DE gels are transferred electrophoretically onto a suitable membrane (e.g., nylon, nitrocellulose or PVDF), and the membrane is probed with a primary antibody that binds to the desired membrane-bound protein. If the desired protein is present, it is detected by the application of a secondary antibody incorporating either a radiolabeled molecule or an enzyme that yields a colored product on addition of an appropriate substrate. Although this approach can be extremely precise, it is labor intensive and requires significant optimization for each protein. Furthermore, nonspecific binding of either primary or secondary antibodies can be misleading. Because immunoblotting searches for known proteins with available antibodies, it is not appropriate for global protein identification. The technique's greatest utility may relate to the selective use of antibodies whose epitopes recognize posttranslationally modified proteins such as phosphoproteins [32,33] or other covalent adducts [34–36] to characterize epigenetic phenomena.

10.3.1.8.2 Mass Spectrometric Peptide Mass Fingerprinting. With vast electronic protein sequence databases containing an ever increasing fraction of the proteins coded by organismal genomes, and MS techniques that now enable the routine analysis of proteins and peptides with extremely high sensitivity and excellent precision, high throughput protein identification via peptide mass fingerprinting (PMF) is quite practical. This approach has been reviewed thoroughly [37] and is described briefly in this section. Essentially, proteins separated on a 2D gel are cut from the gels, proteolytically cleaved into their constituent peptides, the peptide ions are detected by MS, and the resulting masses are compared to theoretically derived databases of peptide masses generated by calculating all possible mass fragments (using a variety of fragmentation techniques) for all known protein sequences. Although several different approaches to generating measurable peptides are possible, the enzymatic in-gel digestion of proteins using a suitable protease such as trypsin is most common. Trypsin is an endopeptidase that cleaves peptide bonds at the C-terminal side of lysine (K) or arginine (R) residues. This protease, along with Lys-C, has been the preferred enzyme for proteomic applications mainly because it generates peptides in the 800–2500 kDa range, optimal for contemporary MS techniques. Nonenzymatic cyanogen bromide cleavage as well as other proteases such as ArgC, AspN, GluC (bicarbonate), GluC (phosphate), and chymotrypsin are also suitable. Various peptide mass databases are designed to reflect each unique cleavage point. A gel plug no larger than the stained protein spot is excised either manually or robotically. The gel piece is then washed to remove detergents and other confounding materials followed by reduction and alkylation of cysteine residues. The gel is dried and rehydrated by the addition of buffered protease, the protein is digested (often overnight), and the peptides are extracted from the gel piece(s). After extraction, the peptides can be concentrated, desalted, and eluted using microcolumn chromatography.

MALDI-MS is the most commonly used technique to perform PMF [38,39]. MALDI-MS is a rapid, reasonably robust, easy to perform, sensitive (low femtomole range), and accurate (low ppm range) identification tool. It is reasonably tolerant to contaminants, can be automated, and detects singly charged (protonated) peptide

ions. Although PMF is a useful tool for identifying relatively pure proteins, it is less effective in analyzing protein mixtures. Separation of complex protein samples by high resolution 2DE is typically well suited for PMF. However, the presence of comigrating, and thus overlapping, proteins (those with similar pI and MW) resolved at similar coordinate positions presents problems for PMF, particularly in small format slab gels, and unequivocal identification is frequently difficult. This situation is exacerbated in 1D separations where single-stained bands may contain large numbers of different proteins with similar mass.

When protein identifications obtained by PMF are ambiguous, sequence-specific peptide fragmentation and sequence searching are necessary. Significant improvements in protein identification in gel plugs have occurred with the implementation of peptide fragmentation induced by collision-induced dissociation (CID)-MALDI-MS/MS. Instruments such as MALDI-ion traps, MALDI-Q-TOF (MALDI quadrupole time of flight), and MALDI-TOF/TOF are particularly suitable for this purpose. Alternatively and more frequently, electrospray ionization tandem mass spectrometry (ESI-MS/MS) and nanoelectrospray ionization tandem mass spectrometry (nanoESI-MS/MS) are being used to achieve sequence information and unambiguous identification of 2DE gel cutouts.

10.3.1.8.3 Electrospray ionization mass spectrometry (ESI-MS). The alternative to solid-phase peptide MALDI-MS is ESI-MS of liquid-phase analytes [40]. ESI separation is based on very low flow rates of peptide eluents from high performance liquid chromatography (HPLC) columns for LC/MS or capillary electrophoresis (CE) columns for CE/MS into a nanoelectrospray ion source that evaporates the solvent and creates multiply charged ions in a droplet cloud. Various voltages are applied to the quadrupole electrodes to direct the ions toward the mass analyzer and along the way, trap and eject them according to their mass-to-charge ratios. The linear quadrupole mass analyzer thus acts as a mass filter by varying potentials on the quadrupole rods to pass only a selected mass-to-charge ratio. Ions are ejected in order of increasing mass-to-charge (m/z) ratio and detected by the ion detector resulting in a precise m/z spectrum.

One of the advantages of ESI relates to the multiply charged species it generates. Because the average charge state increases in an approximately linear fashion with MW, the true molecular mass of an ion can be calculated since more than one charge state is observed. Furthermore, multiple quadrupoles can be added in tandem (MS/MS) to make multiple ion interpretation more robust and facilitate peptide sequence analysis. For example, in a triple-quadrupole instrument, peptide ion masses are measured by the first quadrupole, a peptide of interest is sent to the second quadrupole where it is fragmented by CID, and the fragment masses are measured at femtomole levels in the third quadrupole. For peptide mass mapping, the mass information from several peptides derived from the same protein is required for protein identification by database searching. In contrast, the CID spectrum of a single peptide can contain enough information for unambiguous identification of a protein. It is a direct result of this technical development that has now made it possible to identify the components of even relatively complex protein mixtures without prior purification of individual proteins by using two-dimensional chromatography methods coupled to ESI-MS/MS. The identification (and quantification) of proteins using this technology is discussed in Sections 10.4.1.2.3 and 10.4.1.2.4.

10.3.2 Contemporary Improvements in 2DE Applications

The combination of 2DE and MS constitutes a powerful platform for protein expression analysis. Unfortunately, first-generation 2DE has several shortcomings that have spawned a number of chromatographic and MS approaches designed to overcome these deficiencies and render protein expression analysis truly global. The major weaknesses of the 2DE approach include its inability to resolve very hydrophobic proteins and those with extremes of pI (particularly basic proteins). The hydrophobicity issue is difficult to overcome [41,42], as it is in all proteomic approaches [43] using aqueous analytical systems. Another major problem that has limited 2DE's applicability is its poor dynamic range, meaning that it cannot detect simultaneously all levels of protein expression and MW. Finally, 2DE has failed to gain a broad-based, solid foothold in the pharmaceutical industry because it is so difficult to automate and generate reproducible results. Despite technical developments and attempted innovations, 2DE remains laborious, technically demanding, and low throughput. Virtually all successful developments in differential protein expression analysis, which include automation and consequent increments in throughput, have taken place through emerging gel-free, LC and MS technologies, and 2DE has been all but eliminated from the proteomic approach in large-scale, pharmaceutical applications.

10.3.2.1 Two-Dimensional Difference Gel Electrophoresis (2D-DIGE). One technical development that has successfully addressed both the sensitivity and reproducibility issues facing 2DE is a technique called *fluorescent two-dimensional difference gel electrophoresis* (2D-DIGE) [44]. This approach was developed to enable the separation and differential expression analysis of two or more samples on a single gel. This technique has been reviewed thoroughly [45–47], so a detailed description is not given here. Despite its own limitations [48], 2D-DIGE approach overcomes most gel variability issues and can be quite useful in experiments with limited numbers of samples.

In DIGE (Fig. 10.3), complex protein samples are labeled with spectrally distinct fluorescent cyanine dyes Cy2, Cy3, and Cy5 and the samples mixed, so that they can be examined in a single 2D gel with each dye giving an independent channel of measurement of the co-resolved spots. Protein samples can be labeled in two ways: “minimal” labeling of the ϵ -amino group of the lysine residues, a roughly 5% labeling efficiency, and “saturation” labeling of all available cysteines in the protein mixture. For each spot detected in the gel pattern, the intensities in the respective dye channels are compared and ratios are calculated from these intensities to indicate the extent of differential protein expression. With DIGE, the uncertainty of coordinate gel matching across two or more gels (e.g., are you correctly matching the same spot in different gels?) is overcome, for the most part. For instance, this approach enables labeling of two experimental groups with either Cy3 or Cy5 dyes with the inclusion of a Cy2-labeled internal standard composed of an equal mixture of the two experimental samples, and all three are thus present on each gel of a multigel experiment. Because the individual samples (labeled with Cy3 or Cy5) are multiplexed with an equal amount of the same Cy2 internal standard, proteins missing from one or the other sample are present and detectable in each analytical gel. Consequently, Cy3: Cy2 and Cy5: Cy2 ratios can then be calculated in each individual gel pattern, obviating the gel to gel variability inherent in one-sample-per-gel, first-generation 2DE systems. This affords the DIGE approach very low technical (analytical) noise and high statistical power [49].

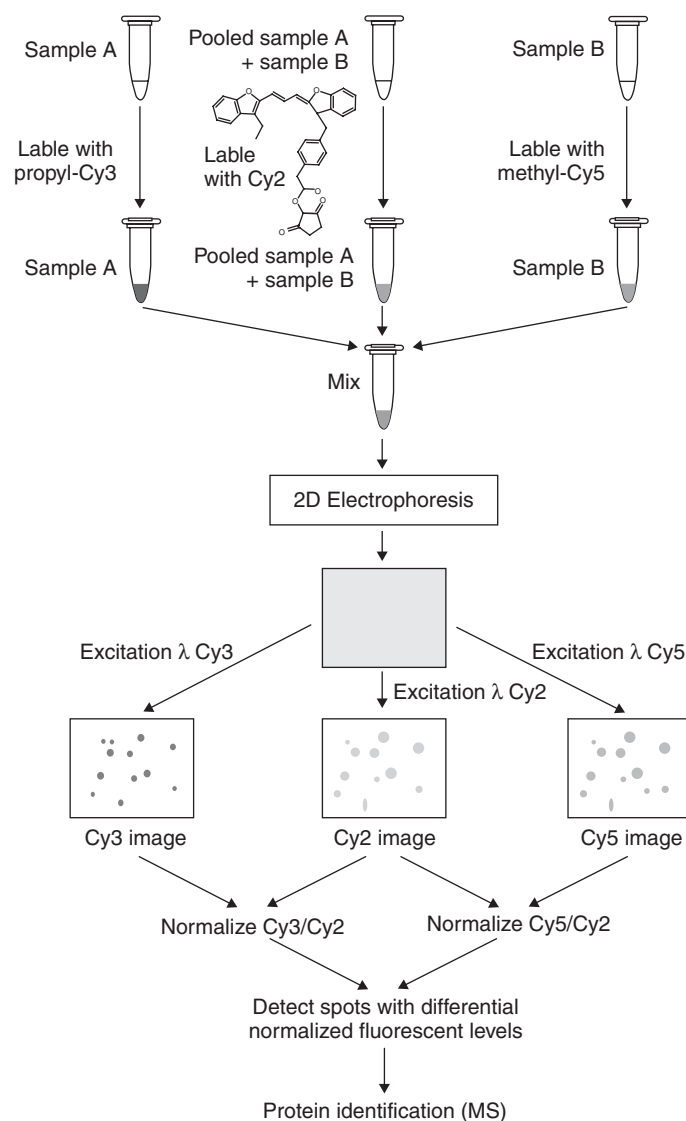


Figure 10.3 2-D DIGE experiment with three fluorescent dyes. Samples A and B are labeled with either Cy3 or Cy5, but a pooled internal standard is also constructed from equal amounts of all the samples in the experiment and labeled with Cy2. After mixing these protein samples and performing 2DE, the respective protein spot patterns can again be visualized by illuminating the gel with the specific excitation wavelengths. The protein spot intensities of samples A and B can now be normalized by dividing each spot intensity by the corresponding spot intensity of the pooled internal standard. Analyzing these normalized spot intensities enables the detection of more subtle differences in protein expression levels with a higher statistical confidence. *Source:* With permission from the Thomson Reuters publication: Witzmann FA, Bai F, Hong D. *et al.* Gels and more gels: probing toxicity. *Curr Opin Mol Therapeut* 2004;6:608–615. (See color insert.)

10.3.3 2DE Applications in Drug Discovery, Development, and Toxicity

Given 2DE's fundamental limitations and its relative ineffectiveness as a high throughput application, its once widespread use in drug discovery and pharmacoproteomics has been augmented and in many cases supplanted by LC-MS-based technologies. For the most part, 2DE remains a useful tool, albeit in more focused applications. Nonetheless, gel-based proteome profiling has been applied to the analysis of preclinical drug effects on gene expression as well as the discovery of potential protein targets, thus making significant contributions to drug discovery. The approach involves proteome profiling to identify and validate disease-specific proteins, identification and candidate target assignment, lead-compound identification and evaluation, mode of action assessment, and formal drug toxicology studies [50,51].

For instance, 2DE has been instrumental in the discovery of early markers of drug toxicity [50], including the development of a chimeric mouse model for prediction of idiosyncratic drug toxicity *in vivo* in preclinical studies [52]. This approach has facilitated the development of surrogate markers to determine the activity of SMTKIs (small-molecule tyrosine kinase inhibitors) designed to produce an antitumor effect [53]. Owing to its ability to resolve charge-modified proteins, 2DE has been integral in the development of efficient and sensitive analytical methods for the separation, identification, and quantification of drug–protein adducts that have important clinical and toxicological implications [54].

2D-DIGE has been used in a variety of ways, including the characterization of antifungal drug resistance [55]; assessment of antineovascular therapy with liposome drug delivery systems targeted to specific vascular proteins [56]; discovery of potential biomarkers and drug targets for sepsis-induced acute renal failure [57]; characterization of the antineoplastic activity, drug resistance, and unexpected toxicity in nonHodgkin lymphoma (NHL) Raji cells of the antitumor drug adriamycin at the mitochondrial proteomic level [58]; and the identification of early changes in protein expression associated with cisplatin ototoxicity [59]. This approach has also led to the identification of various potential cancer biomarkers for the development of diagnostic tests and therapeutic targets [60]. In addition, proteome profiling including gel-based approaches has been used to determine protein markers indicative of therapeutic efficacy or toxicity for lead-compound prioritization in the development of effect antibacterial agents [61]. Such studies specifically investigated adaptation networks that are crucial for bacterial survival and emphasized the characterization of the stress response to antibiotic treatment.

10.4 LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (MS)-BASED PROTEOMIC ANALYSIS

MS-based proteomic technologies have become widely applied analytical tools for protein identification and quantification in complex biological samples. Although some successes using this approach for protein quantification have been reported, it remains technically difficult to comprehensively characterize the global proteome. The main stream of this approach is to use stable-isotope-labeled tags to derivatize proteins and then analyze tryptic digests by MS/MS. However, owing to the high costs of the labeling reagents and the nature of the methodology [e.g., in isotope-code affinity tag (ICAT)

approach, proteins without cysteine residues cannot be labeled and thus cannot be measured], this platform has gradually lost its popularity in recent years. Furthermore, chromatographic shifts can make quantitative analysis of differentially labeled peptides computationally difficult and error prone, and simultaneous quantification of proteins from multiple sample sets is problematic due to the limitation of available labeling reagents. In recent years, the ion-intensity-based label-free quantitative approach has gained more attention as mass spectrometer performance has improved significantly. It provides a powerful tool to identify and quantify thousands of proteins from a complex biological sample in a single assay.

10.4.1 LC/MS-Based Quantitative Proteomics Platforms

10.4.1.1 Stable-Isotope-Labeled Protein Quantification Approaches. Quantification of protein expression and determination of differences between two or more physiological conditions, such as diseased or healthy, are challenging tasks in proteomics. Several MS-based techniques for quantitative protein expression analysis have been developed and applied successfully in recent years [62–69]. Since MS is not intrinsically quantitative, strategies have been developed that enable detection of protein abundance changes using mass tags. ICAT (for relative quantification) [70] and AQUA (for Absolute QUAntification) are two good examples of these methods [71].

ICAT is a widely used quantitative method for large-scale protein quantification. In this method, sulfhydryl groups of cysteine residues are derivatized by either “light” or “heavy” isotope tags (Fig. 10.4) after protein extraction, with control and experimental samples being derivatized separately (Fig. 10.5). Chemical incorporation of isotope tags allows for both identification and quantification of ICAT peptide pairs [70]. While there are many advantages of using ICAT for global quantitative protein analysis, including enrichment of cysteine-containing peptides through biotin–avidin affinity selection that reduces sample complexity for MS analysis, it is important to realize that this technology is dependent on the presence of cysteine residues, as their sulfhydryl groups are chemically modified in proteins. Proteins that lack cysteines will not be

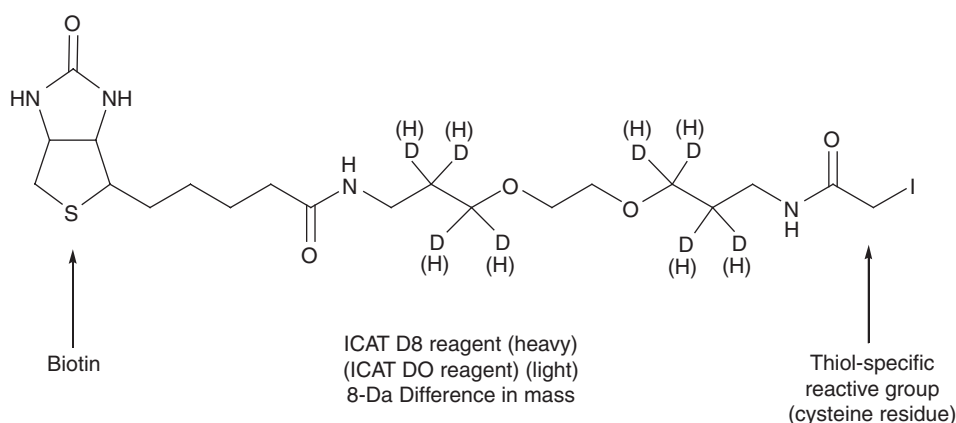


Figure 10.4 Chemical structure of ICAT reagents.

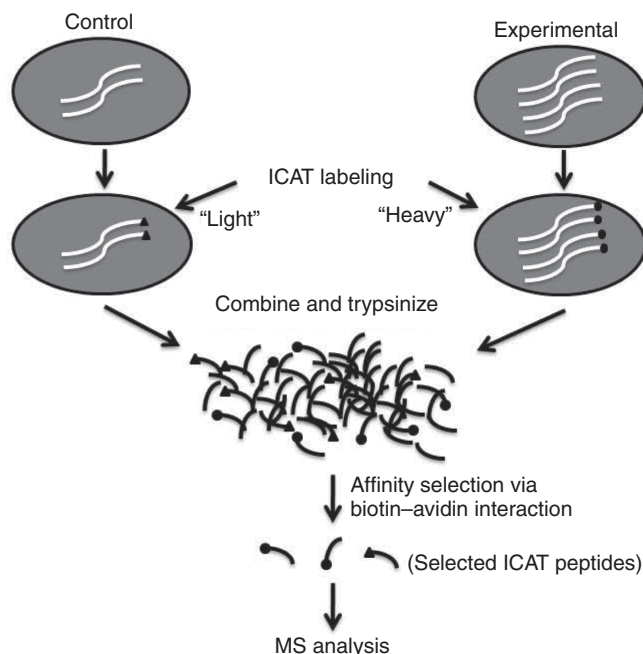


Figure 10.5 Schematic illustration of ICAT method. Proteins in both control and experimental samples are first separately labeled by “light” and “heavy” ICAT reagents. The labeled samples are then mixed and digested by trypsin. Tryptic peptides are affinity-selected using an avidin column followed by mass spectrometric analysis to determine the protein identity and relative quantity.

derivatized by ICAT reagents, thus this technology is not suitable for proteins lacking Cys residues (~10% of a proteome). Other stable isotope labeling technologies include SILAC (labels amino acids in cell culture) [72], iTRAQ (uses isobaric tags), and ^{18}O labeling [73].

AQUA, also known as *Absolute QUAntification of proteins*, is achieved by spiking internal standards (e.g., a peptide of interest) of known quantity to a protein digest to be analyzed, where stable-isotope-labeled “heavy” synthetic peptide(s) serves as internal standard(s) for peptide quantitation (Fig. 10.6). Quantification is carried out by calculating the intensity ratio between the endogenous peptide and the reference “heavy” peptide, which share the same chemical and physical properties including chromatographic retention time, ionization efficiency, and fragmentation pattern, but differ in mass (depending on the design of the quantification standards) [74]. While this approach is attractive for absolute quantification of proteins, a number of limitations still exist, such as (i) a very limited number of proteins can be quantified in a single experiment; (ii) amount of labeled standard needs to be accurately measured before spiking; (iii) tryptic digestion efficiency is not taken into account, leading to inaccurate quantitative measurement; and (iv) ambiguity in peptide identification and quantification because of the presence of multiple isobaric peptides in a tryptic digest. Some of these issues can be resolved by selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) (Section 10.4.3) in combination with a stable-isotope-labeled intact

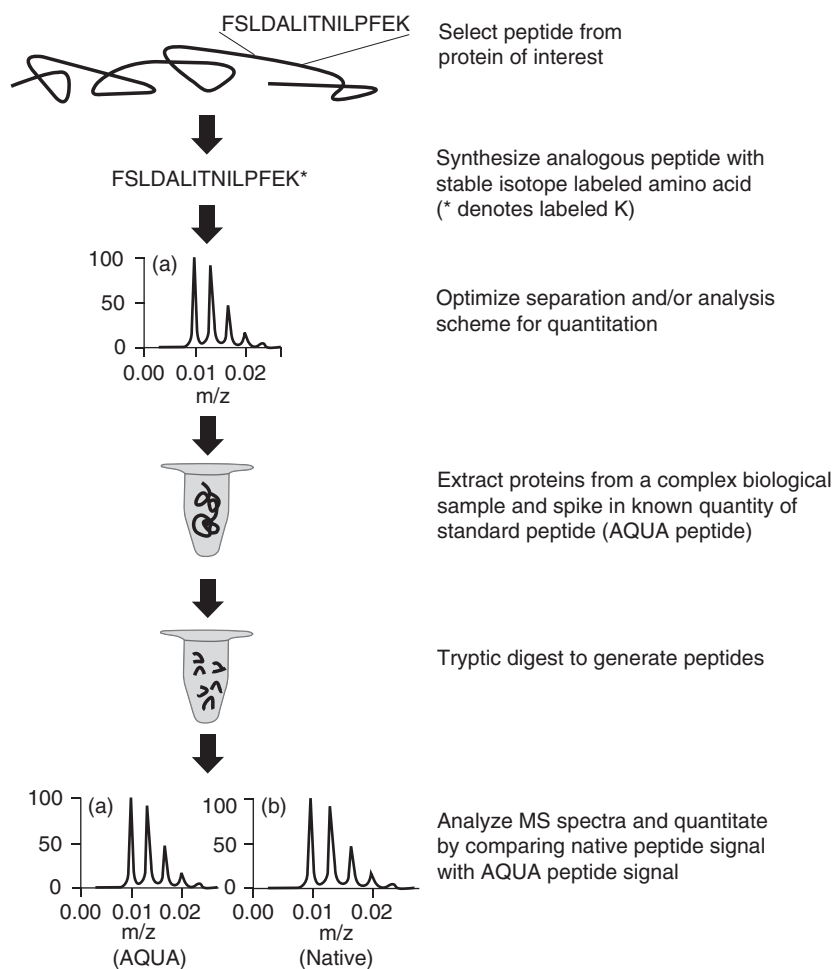


Figure 10.6 AQUA strategy for absolute protein quantification. After initial MS/MS run to determine the peptide of interest, an isotope-labeled analogous peptide is synthesized and a known quantity of this peptide is spiked into the protein mixture followed by tryptic digestion. Absolute quantity of the endogenous peptide is determined by comparing native peptide MS signal to "heavy" AQUA peptide signal.

protein [75–78]. QCAT [79] and PSAQ [77] are other two commonly used methods for absolute quantification.

10.4.1.2 Label-Free Protein Quantification Approaches. Label-free and signal-intensity-based relative protein quantification shotgun proteomics approaches are promising alternatives to stable isotope labeling approaches and have been applied to quantify differentially expressed proteins from complex biological samples [63,80–82]. One approach is to directly compare peptide peak areas between LC/MS runs. This approach is simple and cost-effective and has demonstrated high reproducibility and linearity comparing peptide level or protein abundance. Inclusion of statistical analysis with this method allows detection of small but statistically

significant changes that are biologically important. Several studies have demonstrated that extracted ion chromatograms (XICs) of selected peptide ions correlate well with protein abundances in complex biological samples [63,80–82]. Another label-free method, referred to as *spectral counting*, compares the number of MS/MS spectra assigned to each protein [83]. One advantage of the spectral counting method lies in its ability to measure relative abundances of different proteins in the same biological system as shown by significant correlations between spectral counts and independent estimates of protein copy number in yeast [84]. However, the application of these methods, especially to mammalian systems, requires more robust computing power and algorithms capable of handling chromatographic peak alignment and peptide ion intensity measurements for analyzing changes in protein abundances in complex biological samples.

All label-free LC/MS-based protein quantification methods include four fundamental steps as depicted in Fig. 10.7: (i) sample preparation—protein extraction, reduction, alkylation, and digestion; (ii) sample separation and analysis—(LC and MS/MS); (iii) data analysis—identification, quantification, and statistical analysis; and (iv) data interpretation—protein–protein interaction network and pathway analysis.

10.4.1.2.1 Sample Preparation. For MS analysis, in general, proteins are extracted from tissues, cultured cells, or biofluids in freshly made lysis buffer containing 8 *M* urea and a reducing agent (e.g., 10 mM DTT). For tissue culture cells grown in six-well plates, culture media are first removed by aspiration. Then 100 μ L of lysis buffer is added to each well and cells are lysed by pipetting. Lysed cells are transferred to microcentrifuge tubes and incubated at room temperature with gentle agitation. Unlysed cells and insoluble particles are removed by centrifugation (12,000 *g* for 5 min) before protein assay. To analyze the same amount of proteins from each sample, protein concentrations from all samples must be measured by Bradford assay [85]. The same lysis buffer should be used as the background reference for the protein assay to obtain a relatively accurate measurement among all samples (because of the presence of urea in lysis buffer).

Resulting protein extracts are subsequently reduced and alkylated with DTT and iodoacetamide to block protein sulfhydryl groups. However, in certain instances, volatile reagents such as triethylphosphine and iodoethanol are used [86]. This volatile reduction and alkylation protocol enable all sample preparation steps to be carried out in one tube without washing, filtering, or sample transfer, which minimizes sample preparation variations. Protein mixtures are then digested by trypsin. Dried pellets

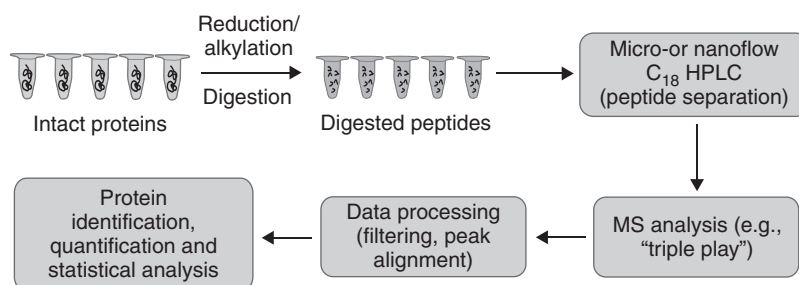


Figure 10.7 Label-free protein quantification technology workflow.

are resuspended in 500 μL of 100 mM ammonium bicarbonate buffer containing 2 μg of trypsin and incubated at 37°C overnight. Protein to trypsin ratio is 50:1, and urea concentration should be below 1.6 M (higher urea concentration inhibits trypsin activity). Tryptic digests are filtered with 0.45 μm spin filters to avoid micro- or nanocolumn clogging.

10.4.1.2.2 Mass Spectrometric Analysis. All digested samples should be randomized for injection in order to remove systematic bias from data acquisition. Typically, up to 20 μg of the tryptic peptides is required for injection onto a C18 microbore column (i.d. = 1 mm, length = 5 cm) and $\sim 2 \mu\text{g}$ for a nanoflow column (i.d. = 75 μm , length = 5 cm). Peptides are eluted with a linear gradient from 5% to 45% acetonitrile developed over 120 min at a flow rate of 50–200 $\mu\text{L}/\text{min}$ (microflow) or $\sim 300 \text{ nL}/\text{min}$ (nanoflow), and effluent is electrosprayed into an LTQ linear ion-trap mass spectrometer (Thermo-Fisher Scientific). The ESI source is operated with 4.8 kV potential, a sheath gas flow of 20 arbitrary units, and a capillary temperature of 225°C. Maximum ion time is set to 50 ms for parent ion scan and 500 ms for zoom scan and MS/MS scan. This method requires that all the MS data is collected in the data-dependent mode (e.g., triple-play mode) as shown in Fig. 10.8 (MS scan, zoom scan, and MS/MS scan). Parent ion scans and MS/MS scans are collected in centroid mode and zoom scans in profile mode. Dynamic exclusion is set to a repeat count one, exclusion duration of 120 s, and rejection widths of $-0.75m/z$ and $+2.0m/z$. The acquired data are filtered and analyzed by special algorithms such as the one developed by Higgs *et al.* [87]. Database searches against protein databases (e.g., the International Protein Index and/or the NCBI nonredundant databases) are carried out using database search engines such as SEQUEST™ and X!Tandem algorithms. Protein quantification is also often carried out using special algorithms [87]. Briefly, once the raw files are acquired from the LTQ, all XICs are aligned by retention time (Fig. 10.9). To be used in the protein quantification procedure, each aligned peak must match parent ion, charge state, daughter ions (MS/MS data), and retention time. After alignment, the area under the curves (AUCs) for individually aligned peaks from identified peptides from each sample are computed; the AUCs are then compared for relative protein abundance (Fig. 10.10).

10.4.1.2.3 Protein Identification. Proteins identified by SEQUEST and X!Tandem are generally categorized into priority groups on the basis of the quality of the protein identification. The Peptide ID Confidence classifies a protein as “HIGH” or “MODERATE” based on the peptide identification confidence. Proteins with at least one peptide having a confidence between 90% and 100% are assigned to the HIGH category regardless of whether there are other peptides having low confidence. Proteins with at least one peptide having a confidence between 75% and 89% are assigned to the MODERATE category. All peptides with confidence less than 75% are filtered out by the software before further analysis. SEQUEST and X!Tandem database search algorithms are used for peptide sequence identification. Each algorithm compares the observed peptide MS/MS spectrum and a theoretically derived spectra from the database to assign quality scores (*XCorr* in SEQUEST and *E-Score* in X!Tandem). These quality scores and other important predictors are combined in the algorithm that assigns an overall score, %ID Confidence, to each peptide. The assignment is based on a random forest recursive partition supervised learning algorithm [88]. The %ID

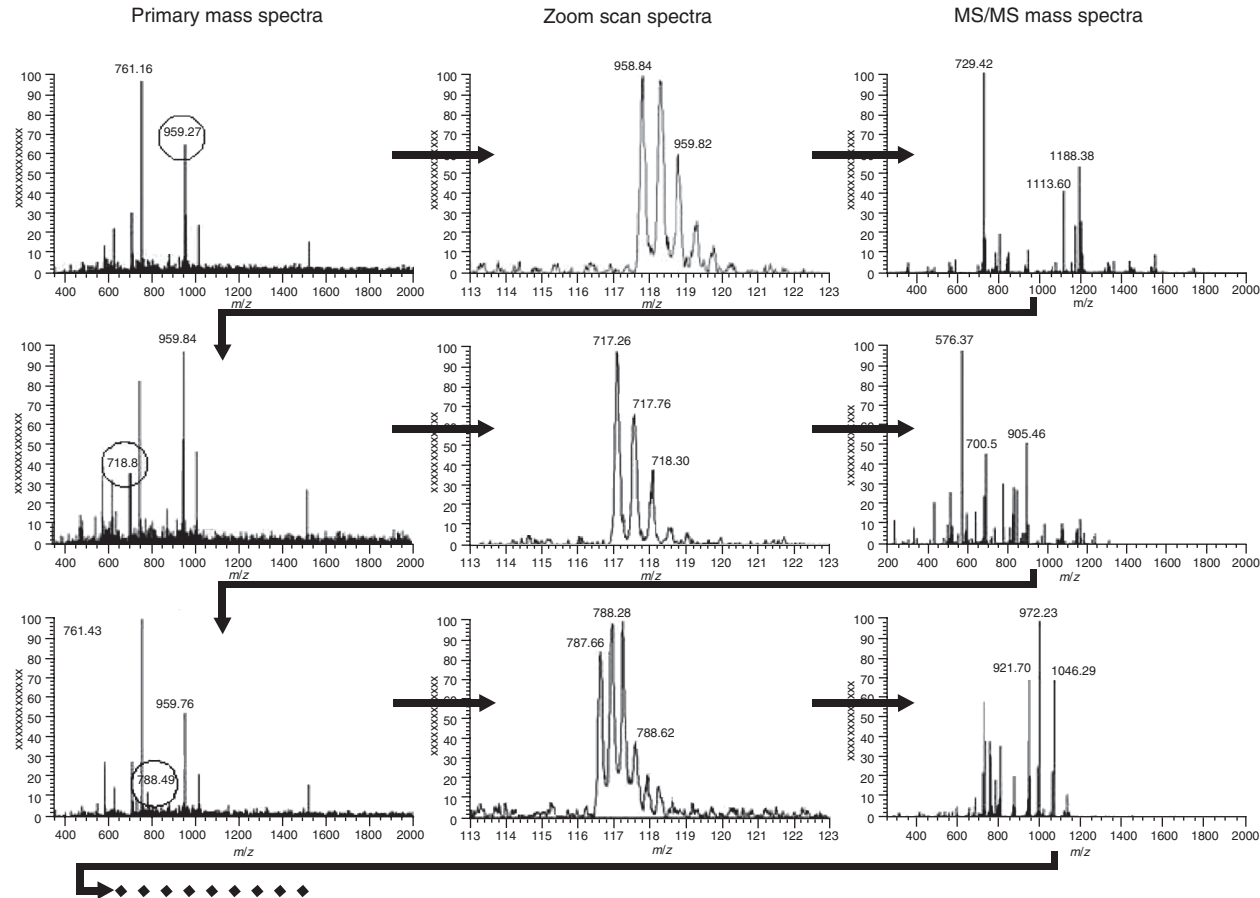


Figure 10.8 “Triple-play” MS method. After the effluent is electro-sprayed into a mass spectrometer, primary MS scans are acquired for peptide mass determination, followed by zoom scans for charge state determination, MS/MS scans for peptide sequence determination, and database searching for protein identification. Every peak above the ion intensity threshold will be selected for “triple-play.” *Source:* Adapted with permission from Wang M, You J, Bemis KG, *et al.* Label-free mass spectrometry-based protein quantification technologies in proteomic analysis. *Brief Funct Genomic Proteomic* 2008;7:329–339.

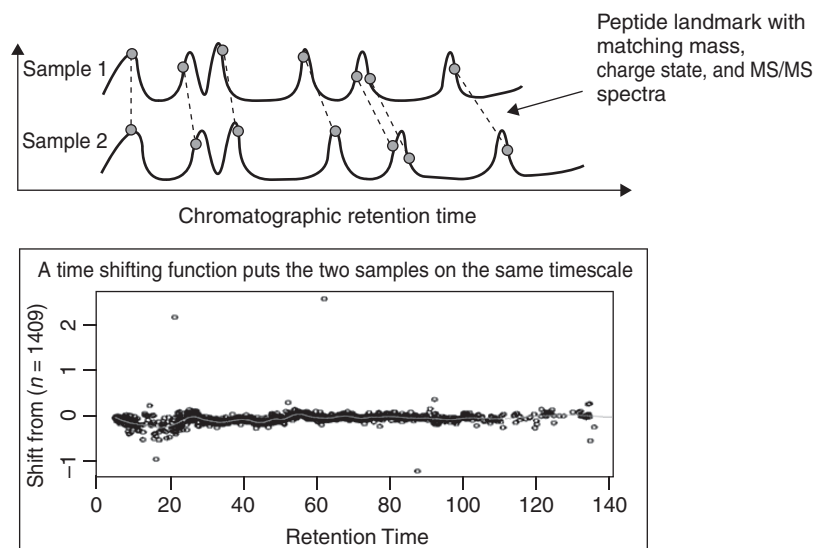


Figure 10.9 Chromatographic peak alignment. This process requires all landmark peaks matching peptide mass, charge state, MS/MS spectra, and retention time within a pre-determined window (1-min in most cases) crossing all analyzed samples. The bottom figure shows how well all the peaks are aligned after this process.

Confidence score is calibrated so that approximately $X\%$ of the peptides with %ID Confidence greater than $X\%$ are correctly identified.

The confidence in protein identification is increased with the number of distinct amino acid sequences identified. Therefore, proteins are also categorized depending on whether they have only one or multiple unique sequences of the required confidence. In general, priority assignments reflect the level of confidence in the protein identification. Priority 1 proteins would have the highest likelihood of correct identification and priority 4 proteins the lowest. This priority system is based on the quality of the amino acid sequence identification (Peptide ID Confidence) and whether one or more sequences are identified (Multiple Sequences). Many would view any protein identification outside priority 1 as questionable [89]. A highly parallel processing computing cluster and data qualification and filtering software are required for data processing and analysis.

10.4.1.2.4 Protein Quantification. One of the keys for protein quantification in label-free methods is chromatographic peak alignment because multiple injections of the samples onto the same HPLC column in large biomarker studies can produce chromatographic shifts. Unaligned peak comparison will result in larger variability and inaccuracy in peptide quantification [87]. A graphical example of a comparison of peptide quantities across a complex biological sample is shown in Fig. 10.10. All peak intensities are transformed to a $\log_2$ scale before quantile normalization [90]. Quantile normalization is a method of normalization that essentially ensures that every sample has a peptide intensity histogram of the same scale, location, and shape. This normalization procedure removes trends introduced by sample handling, sample preparation, total protein differences, and changes in instrument sensitivity while running multiple samples. If multiple peptides have the same protein identification, then their quantile

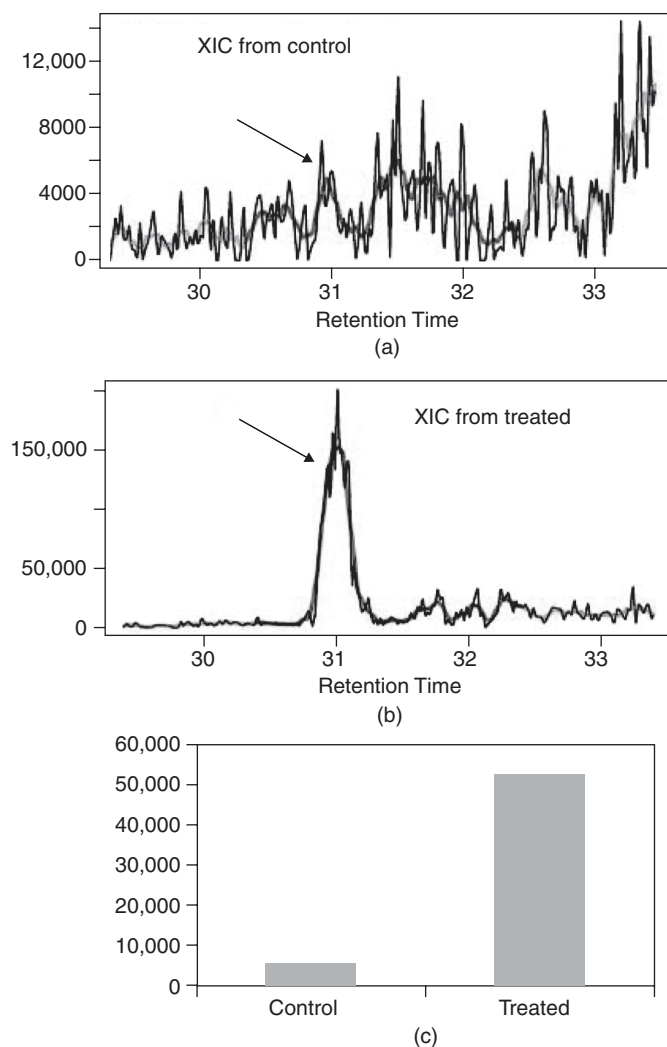


Figure 10.10 Peptide quantification by extracted ion chromatograms (XIC). XIC from (a) control and (b) treated samples: black lines are the raw XICs, from the experiment, whereas light and dark gray lines are smoothed XICs, with dark gray lines being the 2-min window of chromatographic alignment. (c) The relative quantity of the peptide of interest (as indicated by arrows in (a) and (b)) can be calculated and compared by integrating the area-under-the-curve (AUC). *Source:* Adapted with permission from Wang M, You J, Bemis K, *et al.* Label-free mass spectrometry-based protein quantification technologies in proteomic analysis. *Brief Funct Genomic Proteomic* 2008;7:329–339. (See color insert.)

normalized $\log_2$ intensities are averaged to obtain $\log_2$ protein intensities. The average of the normalized log peptide intensities is a weighted average. A peptide is weighted proportionally to the Peptide ID Confidence for its protein category and receives a weight of zero if it is outside that category. For example, peptides with less than 90% confidence contribute zero quantitative weight to a HIGH category protein. The log

transformation serves two purposes. First, relative changes in protein expression are best described by ratios. However, ratios are difficult to statistically model and the log transformation converts a ratio to a difference, which is easier to model. Second, as is frequently the case in biology, the data better approximate the normal distribution on a log scale [91]. This is important because normality is an assumption of the ANOVA (Analysis of Variance) statistical model used to analyze the data. The base of the log transform is arbitrary, with base 2 being the most common with genomic data. Base 2 is popular because a twofold change (or doubling, or 100% increase) yielding an expression ratio of 2 is transformed to 1 on a log base 2 scale (i.e., a twofold change is a unit change on the log base 2 scale). The $\log_2$ protein intensity is the final quantity that is fit by a separate ANOVA model for each protein shown below:

$$\log_2(\text{intensity}) = \text{overall mean} + \text{group effect (fixed)} + \text{sample effect (random)} \\ + \text{replicate effect (random)}$$

In this model, group effect refers to the effect caused by the experimental conditions or treatments being evaluated. Sample effect represents the random effects from individual biological samples. It also includes random effects from sample preparation. The replicate effect refers to the random effects from replicate injections of the same sample. Therefore, all the injections should be randomized and the instrument operated by the same operator for a particular study. The inverse $\log_2$ of each sample mean is calculated to determine the fold change (FC) between samples.

10.4.1.2.5 Statistical Analysis. The number of significant changes between groups, the FCs, and the variability (coefficient of variation) for each protein can be determined from ANOVA. The threshold for significance is set to control the false discovery rate (FDR) for each comparison at an investigator-desired percentile, normally 5% [92]. The FDR is estimated by the q -value, which is an adjusted p -value. The FDR is the proportion of significant changes that are false positives. If proteins with a q -value ≤ 0.05 are declared significant, it is expected that 5% of the declared changes will be false positives. In this method, the p -value to q -value adjustment is done separately for priority 1, priority 2, and the LOW confidence categories.

FC is computed from the means on the AUC scale (antilog) as follows:

$$\text{FC} = \frac{\text{Mean treated group}}{\text{Mean control group}} \\ \text{When mean treated group} \geq \text{mean control group (upregulation)} \\ \text{FC} = \frac{\text{Mean control group}}{\text{Mean treated group}} \\ \text{When mean control group} > \text{mean treated group (downregulation)}$$

Absolute FC = $|\text{FC}|$ = absolute or positive value of the FC

An FC of 1 means there is no change. Also, the median % coefficient of variation (%CV) for each priority level is determined. The %CV is the standard deviation divided by mean on a percentage scale. The %CV is given for both the replicate variation and the combined replicate plus sample variation.

10.4.1.2.6 Quality Assurance and Quality Control. To assess the stability of the HPLC system and MS instrument, a known purified standard protein or stable-isotope-labeled synthetic peptide is commonly spiked into every sample at a constant amount as an internal reference for assessment of technical variations before tryptic digestion. Several considerations should be given for the selection of the standard: (i) the protein/peptide must not come from the same species as the sample of interest, (ii) a series of signature peptides should be easily detected and identified by the instrument, and (iii) the amount of the standard spiked into each sample should be comparable to the amount of median abundant proteins in the sample. After global protein identification and quantification, these peptides and their relative quantities should be inspected for QA/QC purposes.

10.4.1.3 LC/MS-Based Targeted Proteomic Analysis. Although the global quantitative expression proteomics is used extensively as the principal platform for biomarker discovery, it is costly in terms of lengthy MS data acquisition and analysis time. To facilitate more rapid quantitative analysis of larger, clinically relevant sample sets ($n > 100$), the MS-based targeted proteomics technologies such as multiple-reaction-monitoring (MRM) are becoming the more popular platforms for verification and validation of potential biomarkers [93]. MRM is not a new technology; it has been used widely for over 30 years in small-molecule drug metabolism studies [94–98]. Only recently, the MRM assay approach with new-generation linear ion-trap mass spectrometers has been developed to measure specific peptides in complex biological mixtures such as human plasma or serum for targeted proteomic analysis (Fig. 10.11) [93,99–104]. This approach is based on the selection of specific peptide(s) to be used as stoichiometric representatives of a protein [93]. Protein content can be quantified by comparing the peptide peak area with a stable-isotope-labeled internal standard [101]. MRM is an MS/MS scan mode unique to triple-quadrupole mass spectrometers that are capable of rapid, sensitive, and specific quantification of a set of targeted molecules in highly complex samples [93]. When combined with the use of stable-isotope-labeled synthetic peptide standards, MRM analysis is capable of sensitive and absolute quantification of peptides of interest across a wide concentration range (10^3 – 10^4).

10.4.1.3.1 Selection of Peptide of Interest. Protein quantitative assay development based on proteolytic peptide surrogates requires that the peptides of interest selected are both easily detectable in every sample and are reproducible in MS measurements. Selection of a peptide to represent a protein in an MRM assay is a crucial step that can dramatically affect the ultimate sensitivity and specificity of an assay. When selecting a target peptide, the following factors must be considered: (i) the peptide amino acid sequence must be unique to the target protein and should not contain missed cleavage sites, (ii) the peptide should be reproducibly observed in proteolytic digests of every sample, (iii) the peptide should not contain amino acids that are susceptible to PTMs (e.g., Cys, Met) unless the purpose of the assay is to analyze PTMs, and (iv) the peptide length should be in the range of 7–30 amino acids to accommodate the m/z range of the triple-quadrupole instrument.

10.4.1.3.2 MRM Transition Determination. Design and validation of hundreds of MRM transitions for the quantitative analysis of the target peptides in complex biological samples are two major determining factors for the fate of the assay. When working

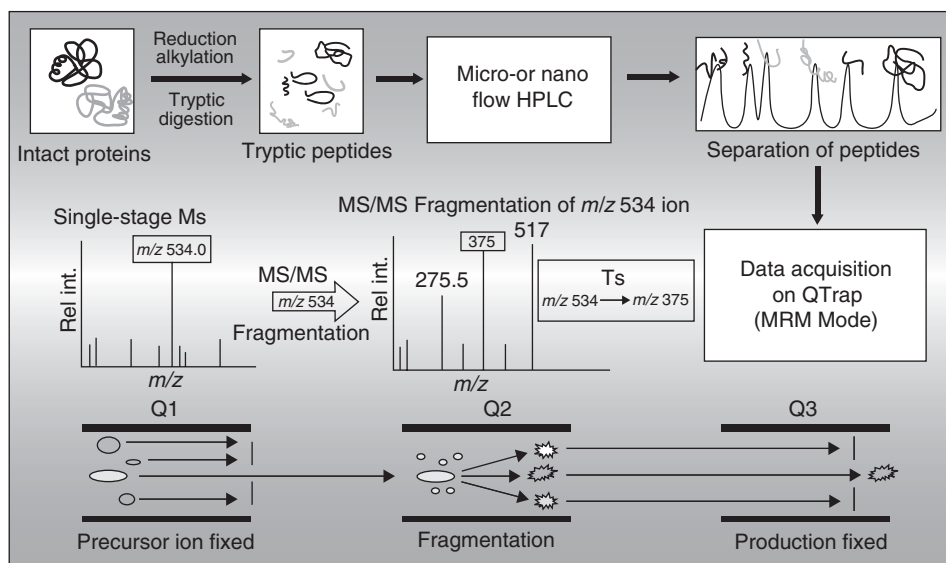


Figure 10.11 Schematic of the MRM scanning on a triple quadrupole mass spectrometer. The targeted precursor ion is selected in the first quadrupole (Q1) and enters the second quadrupole (Q2) where it undergoes collision-induced dissociation (CID) (fragmentation). One or more diagnostic fragment ions (product ion) are then selected in the third quadrupole (Q3) according to the predefined MRM transitions (TS) (e.g., m/z 534 precursor ion $\rightarrow$ m/z 375 product ion). Quantification is achieved by integrating area-under-the-curve (AUC) from extracted ion chromatogram (XIC) of the MRM ion-pair. For absolute quantification, a synthetic isotopically labeled peptide standard is required to optimize MS parameters and to generate a standard curve.

with a species where the genome has been completely sequenced (e.g., *Homo sapiens*), publicly available MS/MS spectra databases (e.g., Global Proteome Machine & Peptide Atlas) are very useful for determining which tryptic peptides and their fragment ions should be selected and are experimentally frequently observed with relatively high ionization efficiency [105]. However, when such databases are not available, *in silico* methods such as MRMAid and Skyline can be used to assist the determination of the MRM transitions [106,107]. Commercially available software packages capable of creating the most appropriate MRM transitions include MRMPilot (Applied Biosystems), QuanOptimise (Waters), SRM Workflow (Thermo-Fisher), and MassHunter Optimizer (Agilent). These methods generally employ fairly complicated algorithms based on a combination of theoretical rules and empirical observation for optimal peptide MS/MS. However, to date there is no computer algorithm-based method that can accurately and reliably generate MRM transitions. All MRM transitions generated by *in silico* methods still need to be experimentally validated. Optimization often is required to facilitate the development of sensitive and accurate MRM assays that are capable of detecting endogenous levels of proteins in “real” biological samples, essentially eliminating false positives. This optimization stage involves the use of standard protein(s) or peptide(s) for the establishment of the peptide eluting time window and the standard curve for quantification.

10.4.1.3.3 MS Analysis. For MRM assays, an autosampler (e.g., Agilent 1100 autosampler), an HPLC system (e.g., Agilent 1100 LC system), and a triple-quadrupole mass spectrometer (e.g., AB/MDS SCIEX 4000 QTrap) are required. If possible, three unique peptide analytes per protein and three MRM transitions per peptide should be monitored in order to obtain confirmatory and/or qualitative information in addition to the quantitative data. A triple-quadrupole instrument can monitor a large number of transitions simultaneously (~ 80 different MRM transitions) and still achieve the reasonable number of points per chromatographic peak for accurate quantitation. Typically, 10 points per LC peak are required to achieve reasonable %CV's ($< 10\%$). When an instrument is operated in MRM mode, predetermined MRM transitions (~ 20 ms dwell time per transition) trigger data-dependent scans. MS/MS spectra are first acquired and used to confirm all the specific MRM ion pairs (to minimize false positives). For QA/QC purpose, a constant known amount of standard (e.g., chicken lysozyme digest) should be spiked into each sample before the digestion step.

10.4.1.3.4 Quantification and Data Analysis. MRM-based analysis provides significant improvement in sensitivity and specificity compared to the IDA (information-dependent acquisition) method for analyzing a specific set of target proteins. However, designing and running MRM assays for relative or absolute protein quantitation require considerable instrument time and sample replicates for improved reproducibility, especially for low abundance proteins. Specific software such as MultiQuant 1.0 (Applied Biosystems) with the MQL algorithm is required to determine AUC for MRM ion pairs for peptide quantitation. A standard curve is needed to assess LOD (limit of detection) and LOQ (limit of quantification) [108,109]. Stable-isotope-labeled (^{15}N and/or ^{13}C) internal standard peptides (iSTDs) and standard calibration curves are normally used for absolute quantification of selected peptides in a complex mixture (Fig. 10.12). A three-day spike-recovery experiment to estimate the working range of the assay is often performed. Serial dilutions of synthetic standard peptides are made in a matrix

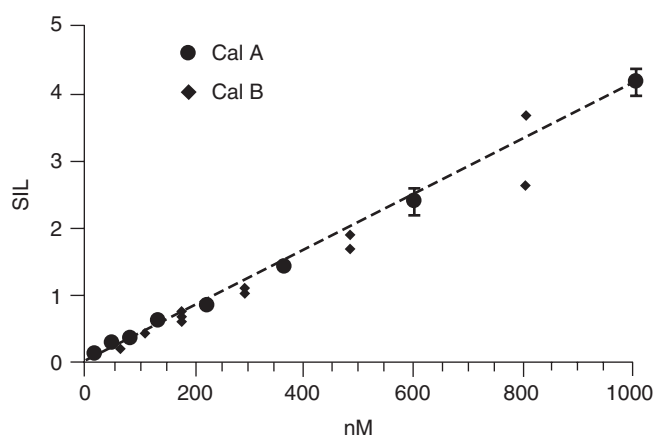


Figure 10.12 An Example of a statistically validated standard curve. Serial dilutions of the SIL (stable isotope labeled) peptide is monitored by MRM; Concentration points (black circles and dark gray diamonds) represent two calibration series (with known peptide concentrations). When Cal A is used to establish the calibration curve, Cal B can be used for validation, and *vice versa*.

(e.g., horse plasma), which lacks the target peptide(s). The purpose of using a matrix is to mimic the complex biological environment that can lead to a chromatographic shift (in comparison with purified standard peptide mixture). Overall working range (e.g., lowest concentration to highest concentration with %CV < 10%), assay accuracy (absolute value of % relative error < 10% for lowest limit), and the overall assay performance (overall %CV) can be determined from this curve. Once the standard calibration curves are established, stable-isotope-labeled iSTDs are synthesized and used for normalization of sample-to-sample variations within an assay during the course of sample preparation and handling and are used for statistical validation of the standard curves. All the standard peptides (labeled or unlabeled) are designed, so they will have at least five additional amino acids flanking each N- and C-terminal (the so-called extended standard peptides). This is to minimize the effects of trypsin digestion efficiency, which can lead to inaccurate quantitation of the peptides [75]. A different matrix (e.g., *E. coli* extracts) can be used to test the matrix effect if necessary.

As mentioned earlier, because the data has multiple sources of random variation, a Linear Mixed Model (a generalization of ANOVA) is used for biostatistics analysis in MRM studies. This model, especially when applied to complex experimental designs, cannot be handled by introductory methods such as *t*-tests. Also, the exact scale of protein expression used in the model can affect the sensitivity. In general, there is usually a large technical variation introduced by the act of “measurement” in many “omics” studies. Randomization of measurement order and normalization of the data eliminate these technical biases.

10.4.2 Data Interpretation

To understand the biological significance of the protein expression or modification changes, the results including protein IDs and FCs from the LC/MS analysis can be analyzed using protein classification, protein–protein interaction, and/or biological pathway analysis software. These software packages enable the creation of protein–protein interaction networks, biological pathways, and gene regulation networks from a data set, which in turn enable one to understand specific biological processes that are involved in a particular study and identify biomarker candidates. Correlation analyses of genomic, proteomic, metabolomic, and phenotypic data are also often conducted to elucidate specific molecular signatures for certain biological conditions.

10.4.3 MS-Based Quantitative Proteomics Applications in Drug Discovery and Development

In recent years, the MS-based proteomics technologies have become major focuses in drug target identification and quantitative proteomics [110–112]. The ability to accurately identify, quantify, and characterize protein expression levels and modifications under different biological conditions in order to diagnose a disease has enabled the identification of new pharmacological targets for therapeutic intervention. For example, Herceptin is used to treat patients with metastatic breast cancer. This monoclonal antibody binds HER2. Patients with over expressed HER2 in their tumors can be effectively treated by Herceptin, while breast cancer patients with normal HER2 expression will not benefit from this treatment [113,114]. Another example is gefitinib (IRESSA) treatment of nonsmall-cell lung cancer (NSCLC). Even though a large phase III ISEL

(IRESSA Survival Evaluation in Lung Cancer) trial demonstrated some improvement in survival with gefitinib, it failed to reach statistical significance compared with placebo in the overall population and in patients with adenocarcinoma. Interestingly, in subgroup analyses, a significant increase in survival was observed with gefitinib treatment in patients of Asian origin and in nonsmokers [115]. Biomarker data analysis from a subset of patients in this trial suggested that patients with epidermal growth factor receptor (EGFR) mutations have a higher chance of survival when treated with gefitinib. Thus, it is important to classify patients with NSCLC based on EGFR mutation status in order to benefit those who will be more responsive to the treatment with gefitinib.

MRM-based applications in diagnostics and drug development include the measurement of C-reactive protein in serum [101], prostate-specific antigen (PSA) in serum [104], fibulin-2 for breast cancer in a mouse model [116], and carcinoembryonic antigen in lung cancer [117]. One early example of the use of MRM for absolute quantification of protein expression is a study carried out on cytochrome P450 (CYPs) enzymes in human liver [118]. CYPs are responsible for the metabolism of most therapeutic drugs, and while they are not classified as biomarkers of diseases, their expression levels can be highly induced by a wide range of drugs [119]. Thus, they are routinely monitored during preclinical safety assessment within the pharmaceutical industry due to the risk of initiating adverse drug reactions (ADRs) [120]. MRM assays have also been used as tools for protein modification profiling [121–123].

The key point in the development of protein biomarkers is to ensure accurate and reproducible data collection and analysis processes with the use of techniques such as peak alignment and subsequent statistical modeling. The most important aspect is the integration of clinical and proteomic data to facilitate the search for protein biomarkers that allow for the prediction of treatment outcomes in clinical applications and drug discovery. Hopefully, this will bring us a step closer to practical medicine.

10.5 PROTEIN MICROARRAY

Protein microarray is a new platform that has become more accessible to researchers for quantitative identification of protein signatures to discriminate between normal and disease samples. The value of this platform in drug discovery is highlighted through the identification of ligands that offer not only excellent potency but also good selectivity. It can also be used in high throughput fashion for drug screening [124]. Protein microarrays offer the potential for more sensitive, specific, and high throughput analyses of the proteome [125,126]. Using specific antibodies, targeted antigens can be quantitatively mapped simultaneously in small, clinically relevant tissue or body fluid samples [126]. For example, tissue microarrays (TMAs) can be used to analyze hundreds of tissue samples simultaneously, thus represent attractive methods for high throughput detection of antigens of interest, especially in high throughput immunohistochemical studies [127–130]. Their applications include identification and validation of novel biomarkers for diagnostic and prognostic profiles [127,128,131,132]. Other array-based platforms are forward-phase protein microarrays (FPAs) and reverse-phase protein microarrays (RPAs) [133–137]. In FPAs, specific antibodies or bait molecules are fixed on a surface, which can then bind specific antigens from a complex protein mixture such as serum or tissue lysates [134]. In RPAs, analytes (e.g., cellular lysates) are immobilized

and probed with specific antibodies [135,136]. Since many analytes are present in low abundance, RPAs are particularly useful for the amplification of signals for detection by fluorescent-labeled secondary antibodies [136,137]. Both these arrays have been used widely in biomedical research, particularly for the identification of protein biomarkers [138–141]. However, the specificity of antibodies and quantification accuracy remain as two major obstacles in array-based proteomics [126].

10.6 BIOINFORMATICS

The development of powerful computational tools and bioinformatics resources has grown exponentially over the last decade, driven primarily by the need to convert information from biological discoveries into clinical applications and to identify significant correlations between a clinical phenotype and potential protein biomarker(s). With advances in bioinformatics, it is now possible to establish predictive models based on genomic, proteomic, and metabolomic data obtained from a defined set of clinical samples, allowing for the identification of drug target and/or protein biomarkers for diagnostics and prognostics. However, significant scientific and technical bottlenecks remain in data integration and in the analysis of complex biological systems. The lack of an intellectual framework that enables a better linkage of biological and clinical data to experimental outcomes has slowed our efforts on building predictive models that can diagnose and prognose disease and disease progression, and thus the ability to provide more precise biomarker(s) or drug target for biomedical research.

10.7 CONCLUSION

The goal of genomic and proteomic profiling is to obtain differential expression knowledge related to the patient samples being analyzed. The results from such an analysis uncover information about protein expression changes in the diseased state, providing the basis for biomarker discovery. Many believe that differentially expressed proteins can be used as molecular signatures for accurate predictive modeling. However, recent studies suggest that other factors such as environment and lifestyles can also play important roles in an individual's health [142]. Thus, integration of a great diversity of data sources from clinical, genomic, and proteomic to environmental and lifestyle must be established before accurate predictive modeling can be successful. Today, a comprehensive scientific method to interpret and understand clinical information and sophisticated molecular measurement data from a set of testing samples has not yet been fully developed. Our hope is that the integration of clinical data, biological data including pathways, cellular locations, and interacting partners, and better informatics tools will eventually make biomarker discovery more useful in building predictive models and supporting better health care.

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11 Stereochemical Elements Associated with Drug Metabolism and Toxicity

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11.1	Summary	1
11.2	Introduction	1
11.3	Stereoselectivity in drug metabolism	7
11.4	Stereoselectivity in drug toxicity	18
11.5	Regulatory and development trends	22
11.6	Conclusion	22
	References	23

11.1 SUMMARY

Many drugs used in clinical practice today are administered as a racemic mixture. As a consequence, these drugs can often be associated with complex pharmacokinetics and observed toxicities compared to drugs that are administered as a single enantiomer. It is well established that two enantiomers of a chiral drug commonly possess different pharmacological properties. In addition to variable pharmacodynamic properties, numerous studies have shown that the individual enantiomers of a chiral drug often display stereoselectivity in pharmacokinetics, toxicity, and drug disposition, particularly in the area of drug metabolism. This chapter attempts to provide a representative appraisal of the various stereochemical aspects associated with the metabolism and toxicity of chiral drugs.

11.2 INTRODUCTION

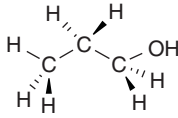
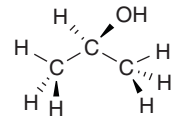
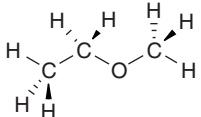
The study of the stereochemical aspects of chemicals began over 150 years ago when Louis Pasteur (1822–1895) discovered the optical isomerism of tartaric acid. In these

experiments, Pasteur observed that a sodium ammonium salt preparation of tartaric acid resulted in two distinguishable types of crystals [1]. When Pasteur separated the two types of crystals, dissolved each in water, and focused a beam of polarized light through the solution, the rotation of the polarized light was different for the two crystal specimens, where one solution rotated the light clockwise and the other counterclockwise. On the basis of these observations, Pasteur concluded that the two separated crystal forms of tartaric acid were either spinning to the right or to the left and neither could be superimposed on the other.

In chemistry, isomers (Greek *isos* = “equal”, *méros* = “part”) are defined as compounds with the same molecular formula but different structural formula. There are two types of isomers, constitutional isomers and stereoisomers. Constitutional isomers are molecules that differ from one another due to unique molecular connectivity and as a consequence, oftentimes do not share similar physical–chemical properties (Table 11.1). In contrast to constitutional isomers, stereoisomers are molecules that share the same molecular connectivity but differ in their spatial orientation. The principal structural basis of stereochemistry for organic molecules is associated with a tetrahedral atom, most commonly carbon. In this instance, a carbon atom with four different groups bonded to it is described as having a chiral center. The term *chiral* is derived from the Greek, *chiros* or handed, and refers to an object that cannot be superimposed on its mirror image (Fig. 11.1).

Molecules that contain a single chiral center and are nonsuperimposable mirror images of one another are described as enantiomers. Enantiomers cannot be separated using traditional separation methods such as distillation, crystallization, or chromatography on columns packed with achiral materials. Their physical properties (e.g., dielectric constant, melting point, boiling point) are also the same. However, enantiomers (as described earlier) do differ in their ability to rotate plane polarized light. Compounds that rotate light clockwise are called *dextrorotatory*; those that rotate light counterclockwise are termed *levorotatory*. Dextrorotatory compounds are

TABLE 11.1 Constitutional Isomers That Exist with Molecular Formula C_3H_8O Each with Different Bond Connectivities and Physical–Chemical Attributes

Compound	Structure	Specific Density (g/mL)	Boiling Point ($^{\circ}C$)
1-Propanol		0.804	97.8
2-Propanol		0.789	82.5
Ethyl methyl ether		0.725	10.6

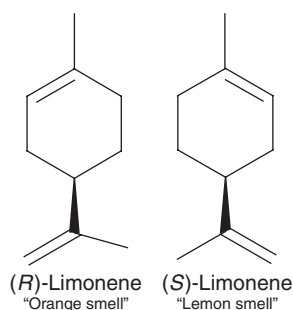


Figure 11.1 Example of the differing biological activity between enantiomers.

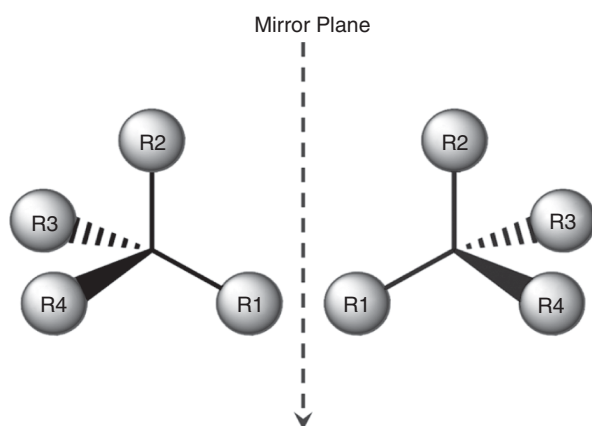


Figure 11.2 A chiral molecule possesses at least one chiral atom in its structure. This is most often an atom of carbon with four different substituents. If all four substituents are different, then two stereoisomers (enantiomers) exist, one of which is the mirror image of the other.

designated with the (+) descriptor in their chemical names; levorotatory compounds are designated with the (−) symbol (e.g., (+)-dobutamine and (−)-dobutamine). In addition, the configuration of enantiomers may also be described in terms of its stereochemistry using the Cahn–Ingold–Prelog (CIP) convention [2]. The CIP naming system assigns a priority to each substituent on the chiral center (Fig. 11.2) and ranks atoms attached to a chiral carbon according to atomic number. Specifically, the higher the atomic number, the higher the priority. If two atoms attached to the central carbon have the same atomic number (e.g., two carbon atoms), one moves along the chain until there is a difference between the atoms; the group in which the higher atomic number atom is found closest to the chiral carbon receives the higher priority. For example, an ethyl group receives a higher priority than a methyl group. Once the various substituents have been ranked, the molecule is visualized in an orientation that places the lowest priority substituent, often a hydrogen atom, directly behind the chiral center. If the remaining substituents decrease in priority around the chiral center in a clockwise direction, the substance is described as the R (rectus) enantiomer. Alternatively, if the direction is counterclockwise, the substance is declared to be the S (sinister) enantiomer (Fig. 11.3). In this fashion, a chiral substance is typically

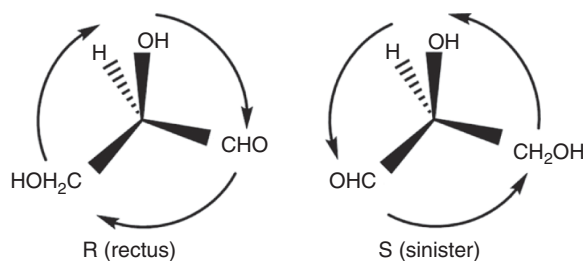


Figure 11.3 The stereochemical assignment for glyceraldehyde according to the Cahn–Ingold–Prelog convention. The chiral center is oriented with the lowest priority substituent (in this example hydrogen) pointing away from eye. In the case of glyceraldehyde, priority decreases in the following order: OH, -CHO, and CH₂OH. If these three substituents in decreasing order are placed clockwise, the chiral center is described as being in the R (rectus) configuration. Conversely, if the substituents result in an anticlockwise ordering, the chiral center is in the S (sinister) configuration.

described by its stereochemical descriptor (R or S), optical properties (+ or –), and chemical name (e.g., (+) (*S*)-mephénytoin).

Emil Fisher first described the significance of stereoselectivity in biological systems. In his “key and lock” hypothesis, Fisher attempted to account for the achiral nature of biological systems, where only one of the two possible enantiomers of amino acids and sugars (specifically, L-amino acids and D-sugars) make up living systems. Further refinement of the “key and lock” hypothesis led to the assumption that there existed a stereochemical relationship between the achiral protein and enantiomeric substrates (Fig. 11.4). The notion of a stereochemical influence governing the dynamic relationship between enzyme and substrate was further refined by Pauling and Delbruck, who

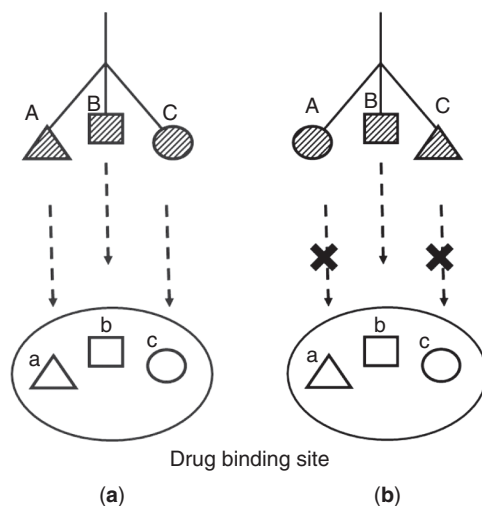


Figure 11.4 (a) The active enantiomer has a three-dimensional structure that allows drug moiety A to interact with binding site domain a, B to interact with b, and C to interact with c. In contrast, (b) the inactive enantiomer cannot align in a manner which allows the three sites to bind simultaneously. The difference in three-dimensional structure allows the active enantiomer to bind and elicit a biological effect, while the inactive enantiomer cannot.

proposed that the catalytic activity of an enzyme derives from the steric distortion of the substrate when it binds toward the structure of the transition state for the reaction [3].

A general theory that holds for many interactions of enantiomers with biological systems was proposed by Carl Pfeiffer who, in 1956, observed that potent chiral molecules display large differences in potency between enantiomers, whereas weakly active compounds have relatively little difference between enantiomers [4]. This observation can best be illustrated by consideration of how molecules interact with the environment (e.g., a receptor or enzyme active site). For any ligand, the possible noncovalent interactions can be separated into subsets of van der Waals, hydrogen bonding, charge transfer, and electrostatic interactions. Collectively, all these interactions types combine to produce a three-dimensional property cloud [5]. Any of these forces may predominate in a particular receptor interaction, but for molecules that possess high affinities, each force should approach maximal effect. This is only possible when the molecular envelopes around the ligand and receptor are complementary. For instance, in the case of directly acting adrenergic agonists, the Easson–Stedman hypothesis proposes a three-point attachment of the active enantiomer (–)-(R)-adrenaline to the adrenergic receptor as opposed to only a two-point attachment for (+)-(S)-adrenaline, which has a lower affinity. Thus, binding of a drug molecule to a receptor, enzyme, or other protein depends on certain structural features [6]. The active drug molecule has several points of attachment to corresponding areas within the receptor site and, in addition, for drugs with an asymmetric carbon atom require a certain spatial configuration of the functional groups for optimal binding to the receptor (Fig. 11.5). Obviously, there can be different affinities for each enantiomer in its interaction with a particular receptor or enzyme (Table 11.2). Under these conditions, the enantiomer with the highest affinity is termed the *eutomer* and the one with the lower affinity is the *distomer*. The eutomer:distomer ratio is called the *eudismic ratio*, and the logarithm of the eudismic ratio is described as the *eudismic index* [7].

In nature, the number of chiral natural molecules is very large, and they represent immense structural variability. Among these substances, an overwhelming majority

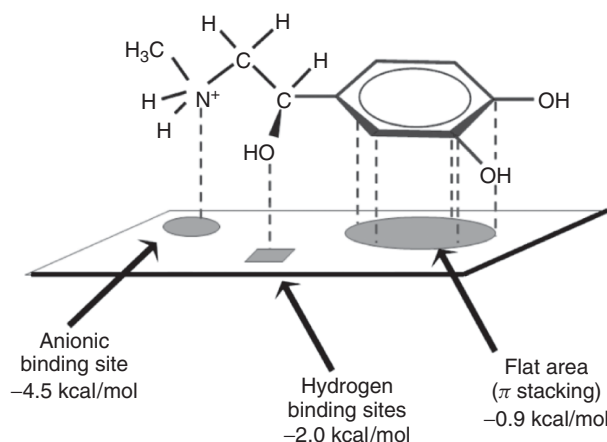


Figure 11.5 A cartoon of a receptor ligand complementary interaction using a three-point receptor model with (–)-(R)-adrenaline as a representative ligand.

TABLE 11.2 Examples of Chiral Drugs Across Various Therapeutic Classes That Exhibit Stereoselective Potencies

Therapeutic Class	Isomer Potency
<i>β-Blockers</i>	L > D
Propranolol, metoprolol, labetalol	Example: (<i>S</i>)-propranolol > (<i>R</i>)-propranolol
<i>Anesthetics</i>	L > D
Isoflurane, ketamine	Example: (<i>S</i>)-ketamine > (<i>R</i>)-ketamine
<i>Analgesics</i>	D > L
Ketoprofen, ibuprofen	Example: (<i>S</i>)-ibuprofen > (<i>R</i>)-ibuprofen
<i>Tranquilizers</i>	D > L
Oxazepam, temazepam, lorazepam	Example (<i>S</i>)-oxazepam > (<i>R</i>)-oxazepam
<i>Opiate Analgesics</i>	D > L
Methadone, pentazocine	Example: (<i>R</i>)-methadone > (<i>S</i>)-methadone
<i>Calcium Channel Blockers</i>	L > D
Verapamil, nimodipine, felodipine	Example: (<i>S</i>)-verapamil > (<i>R</i>)-verapamil
<i>Sedatives</i>	L > D
Secobarbital, hexobarbital, mephobarbital	Example: (<i>S</i>)-secobarbital > (<i>R</i>)-secobarbital
<i>Bronchodilators</i>	L > D
Albuterol, salmeterol, terbutaline	Example: (<i>S</i>)-albuterol > (<i>R</i>)-albuterol

occur in unichiral form, reflecting a single enantiomer. For example, chiral α -amino acids, and the peptides and proteins containing them, sugars and their polysaccharides, steroids, antibiotics, and many other compounds from nature are generally unichiral. A second interesting aspect of many naturally occurring chiral molecules is their homochirality. This term suggests that related chiral molecules in the same chemical class usually have the same sense of chirality. For example, with rare exceptions, α -amino acids occurring in nature consistently possess an L-configuration; similarly, monosaccharides are typically found to exist in a D-configuration. With respect to other molecules derived from natural sources, chirality is also the norm.

As mentioned above, stereoselective interactions between a ligand and receptor are observed across a wide range of pharmacological classes; this is particularly true for the vast majority of early drugs that are derived from natural sources [8]. For example, opium powder obtained from the dried juice from the unripe seed capsule of the poppy *Papaver somniferum* was recognized for its medicinal properties by ancient civilizations in Egypt and Mesopotamia. However, it was not until 1805 before the active ingredient in opium powder was isolated and purified by Friedrich Wilhelm Sertürner, a German pharmacist who named the molecule morphium after Morpheus, the Latin god of dreams. The morphine molecule is a pentacyclic tertiary amine with five chiral centers and exists as the levorotatory enantiomer [9].

Quinine is another example of a chiral drug which has some historical interest. Early civilizations of South America used the bark of the *cinchona* tree to treat fevers. At the beginning of the seventeenth century, Spanish conquerors learned of the bark's medicinal uses, and by the middle of 1600s, the extract of cinchona was being used in Europe for the treatment of malaria. In 1820, quinine, the main antimalarial ingredient from cinchona bark was isolated. The quinine molecule contains four chiral carbon centers [10] and like morphine also exists as the levorotatory enantiomer.

By the mid-twentieth century, a shift from molecules from plant origins to synthetic drugs was forming the basis of contemporary drug therapy. Interestingly, unlike the earlier molecules derived from biological sources, the vast majority of synthetic drugs introduced were racemic, and by the late 1980s, roughly a quarter of the drugs on the market were chiral and racemic [11]. Among the earliest entirely synthetic racemic drugs class was the anticonvulsant or sedative barbituric acid derivatives (e.g., phenobarbital). The significance of this class of molecule with respect to the scope of the current chapter is that the first report of the synthesis of the enantiomers of a barbituric acid and a comparison of the efficacy and toxicity for each enantiomer was released in 1927 [12]. However, the FDA did not explicitly request the inclusion of information on the enantiomer composition of chiral substances in new drug applications until 1992, when they issued their guidelines governing the development of new chiral drugs [13].

The role of stereochemistry in the pharmacology of drug action is well documented. An equally important aspect of stereoselectivity of drug action is the role chirality plays in drug metabolism and toxicity. To this end, the remainder of this chapter is divided into two sections: the role of stereoselectivity in drug metabolism and the implications of stereoselectivity in drug toxicity.

11.3 STEREOSELECTIVITY IN DRUG METABOLISM

Drug metabolism represents an integral contributor to the many physiological processes that govern the dispositional fate of most therapeutic agents. In rudimentary terms, drug metabolism can be described as the biological transformation of lipophilic, nonpolar molecules (e.g., drugs) to more hydrophilic, polar molecules (e.g., metabolites), which are in turn more readily eliminated from the body. The chemical routes by which drugs are susceptible to drug-metabolizing enzymes are large and varied. In fact, one particular feature that distinguishes drug-metabolizing enzymes from other “classical” enzymes is the wide variety of reactions that these enzymes can carry out. In 1959, Williams classified the drug metabolism reactions that are catalyzed by this group of enzymes into two general phases: phase I and phase II reactions [14]. Phase I (oxidative) reactions typically involve the addition or removal of a functional group to increase a compounds polarity, and phase II (conjugative) reactions involve the addition of a hydrophilic moiety to the lipophilic molecule to make it more water soluble.

As with all enzyme-mediated reactions, drug-metabolizing enzymes are capable of accelerating a chemical reaction as compared to the rate of reactions carried out on a laboratory bench top under the mild conditions of aqueous solution, room temperature, and neutral pH. In terms of the concepts describing chemical thermodynamics, enzyme catalysis is essentially a lowering of the free energy function (G) associated with a given chemical reaction [5]. A change in free energy for a reaction, ΔG , determines if a reaction is spontaneous or not. Equilibrium conditions are governed by the standard free energy change ΔG° , and there are several means by which enzymes are able to decrease activation energy. The catalytic power and specificity of an enzyme is a reflection of the steric and electrostatic features associated with the enzyme active site [6]. The most important of these involves the enzyme initially binding the substrate and allows the molecule to exist in close proximity to the catalytic groups found within the active enzyme complex [15]. When the substrate is bound to the enzyme, the enzyme

changes conformation and forces the substrate into a strained or distorted structure that mimics the reaction transition state [3], thus reducing the energy of activation. In this conformation, the substrate is forced into a reactive state and, due to the loss of the substrate's translational and rotational entropy, is shifted toward the total activation energy [16].

Although small in relative numbers, drug-metabolizing enzymes are able to catalyze a large variety of reactions because of their structural and chemical diversity. However, similar to receptor interactions, biotransformation reactions carried out by the drug-metabolizing enzymes also exhibit a wide degree of stereoselectivity [17,18].

Stereoselective drug metabolism can be further refined in terms of substrate and product stereoselectivity. Substrate stereoselectivity applies to a condition in which two enantiomers (or other optical isomers) are metabolized at different rates, without the introduction of a new asymmetric center. When a (new) chiral center is formed during biotransformation, and the resulting products are formed at different rates (selective generation of stereoisomers from a single substrate), this is called *product stereoselectivity*. Reactions are defined as stereospecific when they are completely stereoselective, that is, when only one of the stereoisomers participates in the reaction while the other does not.

11.3.1 Substrate Stereoselective Metabolism

As mentioned earlier in the chapter, the cornerstone of chiral protein–ligand interactions is based on the fact that proteins are chiral in nature, as they are exclusively composed of L-amino acids. In this light, all enzyme reactions are, in theory, stereoselective, and thus, interactions with other chiral molecules have the potential to reflect stereoselectivity. As a consequence of, mechanisms of stereoselective drug metabolism can occur at the substrate binding step and/or at the catalytic step, which may result in different affinities and/or reactivity's of the two enantiomers. With respect to the drug metabolism, the recognition of enantiomeric substrates is assessed in terms of substrate enantioselectivity (i.e., the differential metabolism of two enantiomers under identical conditions). This condition is in contrast with product enantioselectivity, which describes the differential formation of two enantiomeric metabolites from a single prochiral substrate.

In the case of substrate stereoselectivity, the two enantiomers are metabolized either at different rates or by different metabolic routes leading to the differential disposition of one enantiomer compared to the other. The differences in metabolism of the stereoisomers of chiral drugs are a result of different spatial orientations between the two enantiomers and the manner in which they interact with the active site of the enzyme. If one enantiomer aligns in a more productive orientation of functional groups within a particular enzyme active site than the other, the rate of reaction for a particular metabolite will be enantioselective. For example, the oxidative metabolism of the racemic anticonvulsant drug, mephenytoin, is enantioselective [19]. In this instance, the (*S*)-enantiomer is metabolized by oxidation to 4-hydroxymephenytoin, a reaction catalyzed by the polymorphically expressed enzyme cytochrome P450 2C19 (CYP2C19) [20]. In contrast, (*R*)-mephenytoin is metabolized principally by N-dealkylation to phenylethylhydantoin by several other cytochrome P450 (CYP) enzymes [21]. *In vivo* oxidation of (*S*)-mephenytoin is more rapid than N-demethylation of (*R*)-mephenytoin, and as a consequence, there are large differences between the pharmacokinetics of

(*R*)- and (*S*)-mephenytoin [22]. A second example of stereoselective drug oxidation associated with CYP2C19 is found with the metabolism of omeprazole. Interestingly, unlike the enantioselective oxidation observed between (*R*)- and (*S*)-mephenytoin, CYP2C19 is able to bind and oxidize both the enantiomers of omeprazole. However, in this instance, the enzyme favors the 5-hydroxylation of (*R*)-omeprazole and the 5-*O*-demethylation of the (*S*)-omeprazole benimidazole group [23,24].

CYP2C19 is not the only P450 isoform involved in stereoselective substrate metabolism. For instance, (*S*)-methadone is preferentially metabolized by CYP2B6 compared to (*R*)-methadone [25]. In the case of CYP3A4, the enzyme is capable of carrying out N-dealkylation reactions on both (–) and (+)-cisapride; however, the enzyme selectively oxidizes (+)-cisapride to form 4-fluoro-2-hydroxycisapride, while the (–)-enantiomer is metabolized to yield 3-fluoro-4-hydroxycisapride [26]. In fact, stereoselective drug oxidation is observed for select substrates for all known drug-metabolizing P450 enzymes.

As stated above, stereoselectivity in drug metabolism is a manifestation of the interaction between the chiral drug and the achiral environment, which makes up the drug-metabolizing enzyme active site and, as a consequence, is not limited to CYP-mediated oxidation reactions. For example, propranolol, a nonselective β -adrenergic blocker used as a racemic mixture in the treatment of hypertension, cardiac arrhythmias, and angina pectoris is cleared (~20%) by direct glucuronidation [27]. Two of the primary UDP-Glucuronosyltransferase (UGT) enzymes responsible for the conjugation of propranolol in humans are UGT1A9 and UGT1A10 [28]. Stereoselective analysis of the rates of conjugation of the enantiomers of propranolol revealed a high degree of stereoselectivity in the glucuronidation of each enantiomer. In this instance, UGT1A9 was able to glucuronidate (*S*)-propranolol much faster than (*R*)-propranolol, whereas UGT1A10 possesses the opposite enantiomeric preference [29]. In light of the fact that the expression of UGT1A10 is limited to intestine [30] compared to UGT1A9, which is expressed in liver [31], experiments that examined the tissue selectivity of propranolol glucuronidation revealed that human liver microsomes glucuronidated (*S*)-propranolol faster than (*R*)-propranolol, while human intestine microsomes glucuronidated (*R*)-propranolol faster than (*S*)-propranolol [29].

In fact, it is hardly surprising that there are examples of stereoselective substrate recognition observed for majority of the drug metabolism enzyme systems: sulfotransferases (e.g., salbutamol) [32], esterases (e.g., pyrethroid insecticides) [33], glutathione transferases (e.g., 4-hydroxynonenal) [34], flavin-containing monooxygenases (FMOs) (e.g., sulindac) [35].

11.3.2 Metabolite Stereoselectivity

While the stereoselectivity associated with the favored recognition of one enantiomer over its antipode by a particular enzyme is impressive, perhaps a more subtle example of stereoselective drug metabolism is seen with the oxidation of a prochiral substrate. The term *prochirality* also applies to an achiral molecule or entity that contains a trigonal system, which can be made chiral by the replacement of an existing atom (or achiral group) by a different one [36]. In this instance, the observed difference in recognition or the rates of formation of two enantiomers derived from a prochiral substrate is a direct reflection of the topography of the active site of the enzyme since

the architecture of the active site is the only physical feature in the system that can elicit this effect.

In light of the topographical constraints an enzyme active site may exert onto a chiral drug molecule it is possible that metabolism of an achiral compound can introduce a new center of chirality. There are several types of enzymatic (reductive and oxidative) pathways of drug metabolism that can produce new chiral centers in substrate molecules (Fig. 11.6). These reactions include (i) ketone reduction leading to a secondary alcohol; (ii) reduction of a carbon–carbon double bond; (iii) oxidation of a sulfide to a chiral, configurationally stable sulfoxide; (iv) oxidation of a tertiary amine to an *N*-oxide; and (v) oxidation of an enantiotopic or a prochiral moiety leading to a chiral metabolite.

11.3.2.1 Ketone Reduction Leading to a Secondary Alcohol. Stereoselective reduction of a ketone is reflected in the metabolism of the achiral neuroleptic drug, haloperidol. The major pathway of haloperidol metabolism in humans is reduction of the ketone to a secondary alcohol [37,38]. This enzyme reaction is catalyzed by cytosolic ketone reductases and is stereospecific, producing primarily the (S)-enantiomer of haloperidol [39,40]. The mechanism of chiral metabolite formation by carbonyl-reducing enzymes is via two-electron reduction, involving hydride transfer from the cofactor to the carbonyl carbon, which is facilitated by general acid chemistry through a catalytic tetrad (Tyr, Asp, Lys, and His), which forms an oxyanion binding site with the nicotinamide ring of the cofactor [41]. In this reaction, nonsymmetrical ketones generate chiral centers on reduction to alcohols, where the stereoselectivity of carbonyl-reductase-catalyzed ketone reduction is dependent on the steric situation of the substrate, and may be predicted with “Prelog’s rule” [42]. Prelog’s rule describes the reduction of ketone to yield optically active secondary alcohols. In this instance, the chirality of an alcohol arising from nucleophilic addition to a ketone depends on which prochiral face

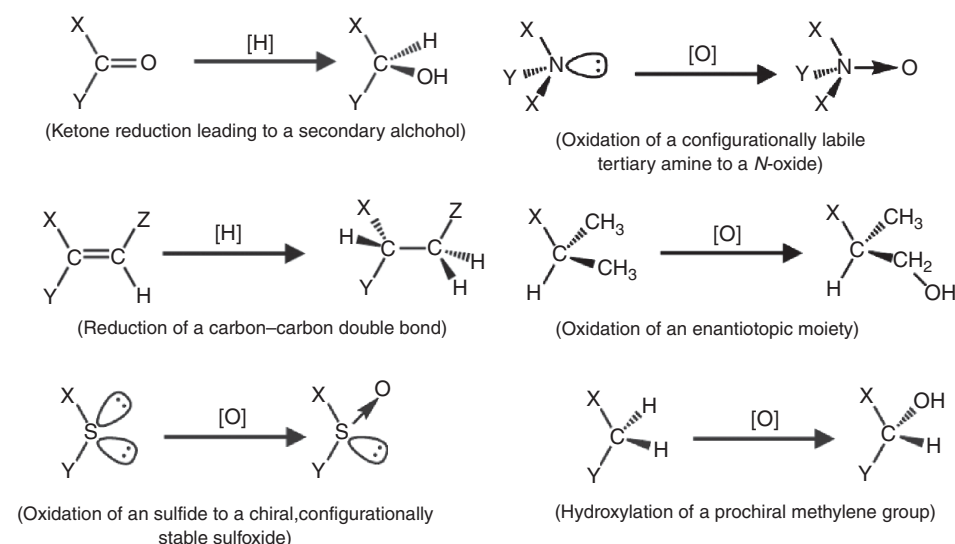


Figure 11.6 Selected examples of various reductive and oxidative metabolism reactions that are capable of introducing new centers of chirality in molecules.

accepts the nucleophile and the sequence priority of the nucleophile relative to other substituents of the carbonyl function [43]. In hydride transfer reactions, the hydride nucleophile will always be the lowest priority group; thus, the *re* face addition of the hydride to a ketone will always generate an (S)-configuration alcohol. The stereoselective product specificity can be represented using a simple scheme (Fig. 11.7). In this scheme, the S (small) and L (large) represent the relative bulk of the groups adjacent to the carbonyl.

11.3.2.2 Chiral Reduction of a Carbon–Carbon Double Bond. Reduction is defined in chemistry terms as loss of oxygen, gain of hydrogen, or gain of electrons. With respect to a molecule's double bond, reactions tend to be addition reactions, where the unsaturated carbon atoms become saturated. When the reaction involves the addition of hydrogen to a carbon–carbon double bond, this process is called *hydrogenation*. The overall effect of such an addition is the reductive removal of the double bond functional group, and in most cases, the stereoselectivity of the reaction is not controlled. In contrast, reductions of double bonds carried out in biological systems are typically stereoselective in nature [44]. An example of stereoselective reduction of a drug which contains a carbon–carbon double bond is reflected in the metabolism of tirilazad. Tirilazad, a 21-aminosteroid, is a potent inhibitor of membrane lipid peroxidation *in vitro* [45] and has shown efficacy in the clinical treatment of subarachnoid hemorrhage [46]. From *in vitro* experiments, the major pathway of tirilazad metabolism was determined to be mediated by CYP3A [47]. The role of CYP3A was confirmed on coadministration of tirilazad with ketoconazole, a specific CYP3A inhibitor, which dramatically reduced the clearance of tirilazad

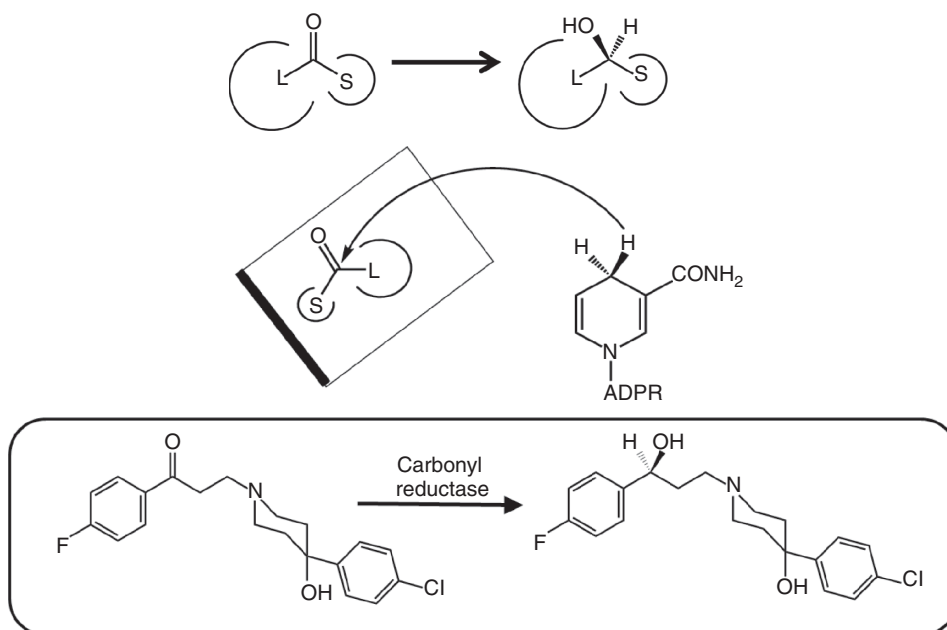


Figure 11.7 Schematic of steric constraints associated with carbonyl reduction reactions. Inset: The reduction of haloperidol to (*S*)-reduced haloperidol.

in vivo [48]. However, despite the contribution of CYP3A to drug clearance, the major metabolite of tirilazad found in circulation was formed via reduction [49]. In human liver microsomes, 5 α -reductase was found to catalyze the formation of the reduced metabolite [50]. Under these conditions, 5 α -reductase catalyze the bisubstrate reaction between tirilazad and NADPH to stereoselectively produce the 5 α -dihydrotirilazad metabolite and NADP⁺ [51]. Mechanistic studies have demonstrated that the 4-ene steroid 5 α -reductase reaction proceeds through the direct regioselective transfer of protons from NADPH to steroid in a random bireactant process [52].

11.3.2.3 Oxidation of a Sulfide to a Chiral Sulfoxide. Thioethers are a common chemical moiety for several drug molecules across a range of therapeutic classes. These molecules can exist as either diaryl-dialkyl or arylalkylthioethers (Fig. 11.8), and because the sulfur group can exist in a variety of oxidation states (including -2, +4, and +6), it is not surprising that thioethers are susceptible to single oxidation reactions leading to a sulfoxide formation. The most common chemical oxidation protocols utilize peroxides or peracids as oxidants in solution and proceed with no stereochemical preference generating racemic sulfoxides.

In contrast, sulfide oxidation via drug-metabolizing enzymes can proceed in a stereoselective manner because of the potential constraints associated with active site topography associated with these reactions. Perhaps the greatest influence of active site topography on the stereoselectivity of drug oxidation is observed with FMO oxidation reactions [53,54]. In the 1980s, Walsh and coworkers examined the stereochemistry of sulfoxidation of ethyl *p*-tolyl sulfide by hepatic microsomal FMO and found that the enzyme catalyzed formation of the (R)-enantiomer of ethyl *p*-tolyl sulfoxide in great enantiomeric excess [55,56]. In this fashion, unlike the ketone reductase mechanism, the topography of the enzyme active site not the substrate dictates the final chirality

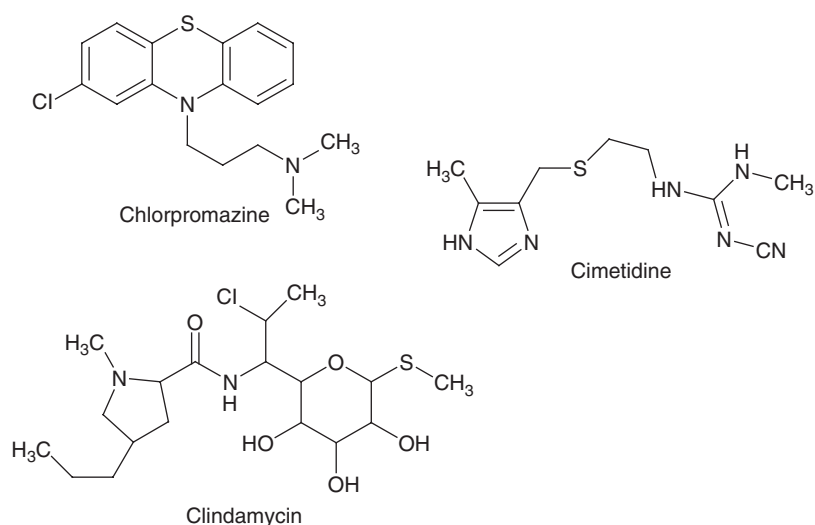


Figure 11.8 Examples of drug molecules that exist as either diaryl-dialkyl or arylalkyl-thioethers.

of the product, as the enantiotopic electron pairs on the sulfur atom of an asymmetrically substituted sulfide are equivalent [57]. However, in the chiral environment of the enzyme active site, the electron pairs are not equivalent and stereoselective sulfoxidation can occur. For example, albendazole is a broad-spectrum anthelmintic agent used to treat tropical parasitic disease [58]. Albendazole contains a sulfur group that is stereoselectively oxidized by FMO to form an active metabolite, (*S*)-sulfoxide albendazole [59].

11.3.2.4 Oxidation of a Tertiary Amine to an *N*-Oxide. The oxidation of drugs possessing a tertiary amine frequently yields *N*-oxides as major metabolic end products [60,61]. In this circumstance, a tricoordinate nitrogen atom with three different substituents is not chiral due to rapid inversion of the two possible antipodes via a planar transition state [62]; however, the corresponding quaternary *N*-oxide is chiral. Thus, the metabolic formation of an *N*-oxide of this type may show product stereoselectivity or stereospecificity, which may have implications if the metabolite is either pharmacologically or toxicologically active and such activity is selective for one enantiomer. Many of the chiral *N*-oxide metabolites observed are the product of FMO oxidation, where the sp^3 -hybridized electron-rich nitrogen atom serves as a suitable reactant toward the weak oxidation potential of the 4 α -hydroxyflavin of the enzyme [63].

The subtlety of the stereoselectivity of *N*-oxide formation by FMOs is a consequence of the enzyme active site and the mechanism of oxidation by the FMO enzyme. For FMO enzymes, in the first step of the catalytic cycle, FAD undergoes two-electron reduction by NADPH [64]. Chemistry of NADPH requires that reduction of the flavin cofactor is facilitated through a hydride transfer from the C4 atom of the nicotinamide ring to the flavin N5 atom [65]. The reduced flavin reacts rapidly with molecular oxygen to form the stable peroxyflavin intermediate, which is able to wait for a suitable nucleophile (in this case, a tertiary amine) with which to react [66]. Stabilization of the intermediate requires the presence of $NADP^+$ [67], which remains tightly bound to the enzyme throughout the catalytic cycle. It has been proposed through computer simulation of the FMO active site that $NADP^+$ is able to shield the enzyme active site and can provide a possible H-bonding environment for stereospecific oxidations [68]. Finally, the binding of oxygen is followed by the nucleophilic attack by the tertiary amines on the peroxyflavin, which results in one atom of molecular oxygen being transferred to the substrate.

One molecule that undergoes stereospecific *N*-oxide formation is nicotine. In humans, there are large interindividual differences in nicotine disposition, and it has been proposed that genetic polymorphisms in nicotine metabolism may be a major determinant of an individual's smoking behavior [69]. Hepatic cytochrome P450 2A6 (CYP2A6) catalyses the major route of nicotine metabolism: oxidation to cotinine, followed by hydroxylation to *trans*-3-hydroxycotinine [70]. Nicotine also undergoes *N*-oxidation to form *trans*-*N*-1-oxide a reaction catalyzed by hepatic flavin-containing monooxygenase form 3 (FMO3) [71].

11.3.2.5 Oxidation of an Enantiotopic Moiety Leading to a Chiral Metabolite. There are several examples of achiral molecules that on enzyme metabolism yield a chiral product. Perhaps the most cited achiral drug that undergoes stereospecific metabolite formation is observed with the epileptic drug, phenytoin. Phenytoin (5,5-diphenylhydantoin, Dilantin[®]) is a hydantoin anticonvulsant that is widely used for the

treatment of complex partial seizures, secondarily generalized tonic–clonic seizures and acute seizures [72]. The primary metabolic pathway for phenytoin is aromatic oxidation to form 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) [73]. From *in vitro* studies, it has been established that the major enzyme responsible for phenytoin metabolism is CYP2C9 [74], with a minor contribution arising from CYP2C19 [75]. For the CYP2C9-mediated pathway, ~95% of the *p*-HPPH formed by this enzyme results in the (S)-configuration of the metabolite [76]. Interestingly, the second enzyme, CYP2C19, also oxidizes phenytoin to the phenolic metabolite; however, in this reaction, the (R)-*p*-HPPH is primarily formed [77]. The dominance of CYP2C9 stereoselective metabolism of phenytoin is evident from the urinary (S)/(R) ratio of *p*-HPPH, which is about 24:1 [78].

A second example of an enantiotopic drug being metabolized to a chiral product is observed with the 4-hydroxylation of the antihypertensive debrisoquine, a reaction that is principally catalyzed by CYP2D6 [79]. In humans, CYP2D6 exhibits a genetic polymorphism [80] such that for extensive metabolizers, more than 99% of the 4-hydroxydebrisoquine metabolite possesses the (S)-configuration [81]. In contrast, poor metabolizers of debrisoquine not only have a severely impaired capacity to form 4-hydroxydebrisoquine but also show less stereoselectivity, with 5–36% of the 4-hydroxydebrisoquine possessing (R)-configuration [82,83].

11.3.3 Chiral Inversion

As previously mentioned, enantiomers may have similar or different pharmacological effects, and these effects may be related to stereoselective pharmacokinetics or pharmacodynamics. In the latter case, the terms *eutomer* for the more potent isomer and *distomer* for the less potent one are applied. However, independent of the qualitative or quantitative differences in pharmacological activity between isomers, the definition of a racemate being a 50:50 mixture of each isomer remains constant. While this is typically the case, there are some circumstances where there is an interconversion (chiral inversion) between one enantiomer and the other [84].

There are two kinds of drug chiral inversion: unidirectional and bidirectional inversion.

Bidirectional chiral inversion or racemization of enantiomers is thermodynamic in nature and is governed via an equilibrium process. An example of racemization of enantiomers is observed with 3-hydroxy-benzodiazepines (e.g., oxazepam) in which the (R)- and (S)-enantiomers are able to racemize in aqueous solutions [85]. In contrast, enzyme-mediated inversion is not a thermodynamic phenomena but rather occurs through an enzyme-mediated process where there is stereoselective recognition of one enantiomer over another, which results in a unidirectional racemization.

Unidirectional inversion from (R)- to (S)-configuration is the most common feature for 2-arylpropionate nonsteroidal anti-inflammatory drugs (NSAIDs) exemplified by the common over-the-counter (OTC) analgesic, ibuprofen [86,87]. This is of particular importance, given that for this class of molecule, the (S)-enantiomer is the more active enantiomer (i.e., has an analgesic and anti-inflammatory effects). In fact, (S)-ibuprofen is over 100 times more potent as an inhibitor of cyclooxygenase I than (R)-ibuprofen [88].

The inversion of a chiral center is, from a chemical reaction perspective, an energetically demanding reaction where a number of possible reaction sequences can be

hypothesized. From a first principal perspective, C2 (i.e., the carbon atom adjacent to the carboxyl group) of ibuprofen must become planar for epimerization to occur. For ibuprofen as well as other arylpropionic acids, this planar structure can be achieved *via* β -oxidation to form a dibond between C2 and C3, or alternatively, by carbanion formation at C2 resulting from proton dissociation. In this fashion, formation of a planar intermediate is similar to the mechanism of acyl-CoA synthetases formation of acyl-thioesters from fatty acids in the presence of ATP, CoA, and Mg^{2+} , as part of a critical step in fatty acid metabolism.

The stereoselective introduction of CoA into (*R*)-ibuprofen to form the thioester bond increases the acidity of the proton on the C2. The dissociation of the α -methine proton of (*R*)-ibuprofen CoA ester is facilitated by both the aromatic ring and the thioester bond during racemization step. This step of reaction does not occur spontaneously and requires the presence of an epimerase [89], as the predicted pK_a value of the α -methine is greater than 10 and therefore is incapable of dissociation under physiological pH conditions [90]. On proton abstraction, an unstable carbanion intermediate is formed, which undergoes tautomerism to form a double bond between C2 and C1. The planar structure is now able to accept a solvent proton, from either the upper or the lower side, in a nonstereoselective manner to yield (*S*)-ibuprofen CoA ester. The last step of the unidirectional inversion of (*R*)-ibuprofen is simply the hydrolysis of CoA ester by the nonstereoselective catalysis of acyl-CoA thioesterase [91]. The complete scheme of the unidirectional inversion of (*R*)-ibuprofen is described in Fig. 11.9.

11.3.4 Stereoselective Inhibition of Drug-Metabolizing Enzymes

The stereoselective metabolism of chiral drugs has been evaluated thoroughly in the first section of this chapter; however, the notion of stereoselective enzyme inhibition is also an important component to understanding potential liabilities associated with a particular medicine.

For many therapies, it is quite common for two or more drugs to be coadministered to a patient, and as a consequence, the patient has an increased risk for incurring an adverse drug–drug interaction (DDI) [92,93]. DDIs are often caused by one of the administered drugs inhibiting the metabolism of the second drug. If the drug whose metabolism is inhibited has a narrow therapeutic index, the increase in systemic exposure can lead to adverse reactions.

Of the various drug-metabolizing enzymes, by far the CYP enzymes are responsible for the clearance of drugs in humans [94]. As a consequence, CYP enzymes are particularly prone to inhibition because of their broad substrate specificity [95], which makes them amenable to competitive inhibition arising from different types of structurally diverse drugs that can be metabolized by the same enzyme.

As one would predict, a chiral drug can often inhibit P450-mediated metabolism in a stereoselective manner. For example, dramatic stereoselective inhibitory interactions have been observed between the individual enantiomers of a chiral drug and P450 enzymes [96]. The degree of stereoselective inhibitory potency of a chiral molecule toward P450 enzymes is oftentimes a reflection of the topography of the enzyme active site [97]. In fact, the restricted active site of CYP2D6 has been extensively studied and modeled in an effort to understand the important structural features and biochemical interactions of this polymorphic enzyme [98].

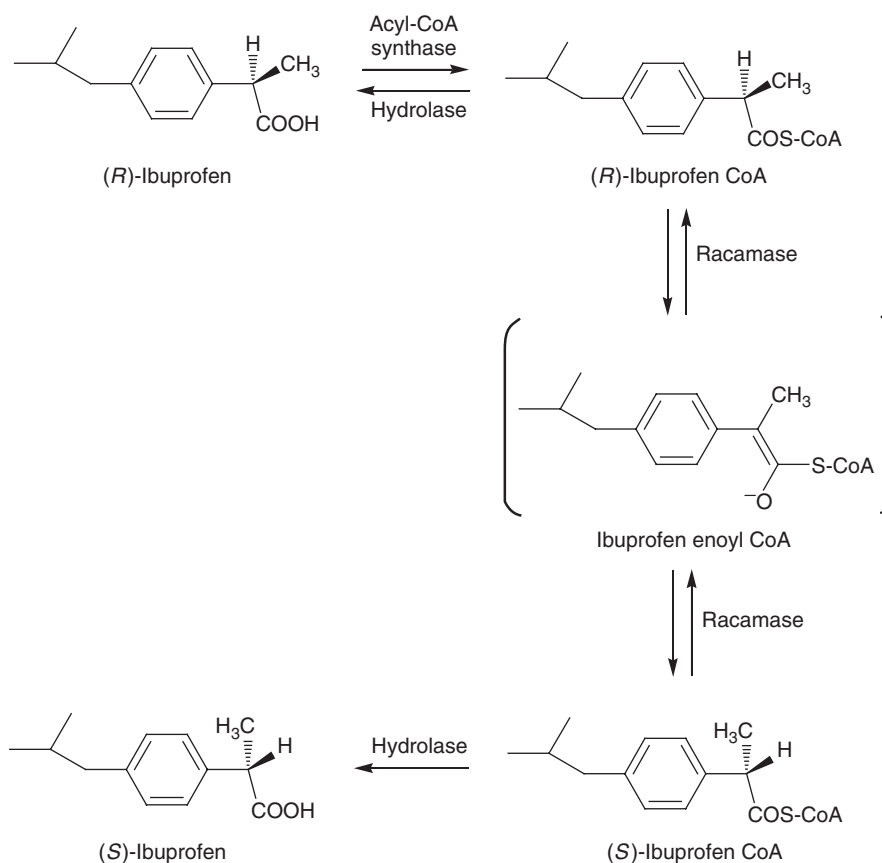


Figure 11.9 Proposed scheme depicting the unidirectional chiral inversion of ibuprofen.

Of all known chemical inhibitors of CYP2D6, quinidine is one of the most potent and selective inhibitors of the enzyme and is often used as a chemical inhibitor in P450 reaction phenotyping studies and DDI screening assays [99]. In contrast, quinine, the stereoisomer of quinidine, is about two orders of magnitude less potent as an inhibitor of CYP2D6 [100], which illustrates the significance that stereochemistry can play in binding and inhibition of this enzyme. Mechanistic studies suggest that in addition to different enzyme affinities, quinidine and quinine also possess distinct binding orientations within the CYP2D6 active site. For these enantiomers, quinidine produces a type I binding spectrum on binding with CYP2D6 [100], which indicates that quinidine binding causes a low to high spin state transition resulting from displacement of water as the sixth ligand to the heme iron [101]. In contrast, the binding of quinine to CYP2D6 results in a type II spectrum, which suggests that the sp^2 nitrogen in the quinoline ring, likely to be uncharged at neutral pH is complexed with the heme iron [102], which leads to a more definitive binding orientation, one which is clearly distinct from that of quinidine. Moreover, molecular modeling of the quinidine CYP2D6 interaction suggests that the enzyme bound quinidine blocks the entrance of the active pocket of CYP2D6 more effectively than quinine does [103], which provides

additional rationale for why the inhibitory activity of quinidine toward CYP2D6 is so much greater than quinine.

11.3.5 Metabolic Interactions Between Enantiomers and a Second Drug

As described previously in this chapter, a racemic drug can be thought of as mixture of two xenobiotics each displaying its own pharmacokinetic and pharmacodynamic profile. Under this circumstance, the introduction of a second drug in combination with the chiral drug has the potential to greatly complicate the situation since under these conditions, there are now effectively three possible interacting species as there is no anticipation that the second drug will interact with the two enantiomers in same way. As a consequence, the metabolism of a racemic drug may be stereoselectively modified, which can result in a variety of possible pharmacokinetic and pharmacodynamic alterations.

For example, warfarin is administered as a racemic mixture and undergoes extensive oxidative metabolism [104]. In humans, essentially all the anticoagulant effect of the drug resides with (*S*)-warfarin [105], which is primarily cleared by a single P450 enzyme, CYP2C9, to form two inactive metabolites, (*S*)-6- and (*S*)-7-hydroxywarfarin [106]. In contrast, the less potent warfarin enantiomer, (*R*)-warfarin, is metabolized by several enzymes: CYP3A4, CYP1A2, and CYP2C19 [20,107].

In clinical studies, it has been reported that total warfarin plasma concentrations were increased when the drug was coadministered with drugs such as cimetidine or sulfinpyrazone. Interestingly, while both drugs increased total warfarin plasma levels, only coadministration of sulfinpyrazone had an increase in the drug's anticoagulant effect [108]. This observation can be explained by the enantioselective metabolism of warfarin isomers. In this case, concomitant administration of warfarin with cimetidine resulted in a decreased clearance of the (*R*)-enantiomer but had no impact on the clearance of the pharmacologically active enantiomer, (*S*)-warfarin [109]. In contrast, coadministration of sulfinpyrazone and warfarin resulted in a decreased clearance of (*S*)-warfarin and, therefore, an increased effect of warfarin but relatively little impact on (*R*)-warfarin disposition [108]. Interestingly, the warfarin situation is further confounded by the fact that while (*R*)-warfarin is not metabolized by CYP2C9, it is an inhibitor of the enzyme. *In vitro* inhibition studies that examined the effect of (*R*)-warfarin on the metabolism of (*S*)-warfarin revealed that the production of both (*S*)-6- and (*S*)-7-hydroxywarfarin were competitively inhibited by (*R*)-warfarin [110]. Therefore, it is possible for a second drug, which is an inhibitor of CYP1A2 but lacks any affinity for CYP2C9, to decrease the clearance of (*R*)-warfarin as well as to a smaller extent (*S*)-warfarin through this complex interaction scheme.

11.3.6 Enantioselective Pharmacokinetics and Drug Effect

The impact of stereoselective drug metabolism and pharmacokinetics is dependent on whether individual enantiomers demonstrate qualitative and quantitative differences in pharmacological or toxicological properties. If both enantiomers demonstrate equivalent biological activity then stereoselective metabolism is likely of no practical consequence. In contrast, if enantiomers differ in their biochemical and physiological effect profile then stereoselective pharmacokinetic differences can attenuate or potentiate both therapeutic and toxicological effects. Verapamil is an excellent case

example of this later scenario and has been extensively studied over the last twenty plus years [111]. Verapamil is a racemic calcium channel antagonist for the treatment of cardiac arrhythmias, angina, and hypertension by both intravenous and oral routes of administration. Both verapamil enantiomers possess vasodilatory activity; however, the (*S*)-verapamil enantiomer has an order of magnitude more potent negative chronotropic, dromotropic, and ionotropic effects than the (*R*)-enantiomer [112,113]. Vasodilator activity is required for angina and hypertension indications while the negative tropic properties are necessary for the treatment of arrhythmia. Obfuscating the relative contribution of the individual enantiomers to the overall pharmacological effect are their complex stereoselective ADME (absorption, distribution, metabolism, and excretion) and pharmacokinetic properties. Both clearance and steady state volume of distribution were higher for (*S*)-verapamil than those for the (*R*)-enantiomer while both enantiomers demonstrated comparable terminal plasma half-life [114]. After intravenous administration, (*R*)-verapamil concentrations are approximately twofold higher than that of the (*S*)-enantiomer. Verapamil is a high hepatic extraction drug that undergoes extensive first pass metabolism further exacerbating the stereoselective pharmacokinetic profiles for the two component isomers. After oral administration, (*R*)-verapamil concentrations are approximately fourfold higher than that of the (*S*)-verapamil [115]. This administration-route-dependent enantiomer pharmacokinetic profile is believed to be the primary explanation for pharmacodynamic differences observed for racemic verapamil when dosed intravenously and orally. Verapamil is metabolized by members of several different CYP subfamilies: CYP3A, CYP1A, and CYP2C, yielding *N*-dealkyl, *O*-demethyl, and *N*-demethyl metabolites [116–118]. Results from *in vitro* human liver microsomal experiments were generally consistent with the observed *in vivo* results, which indicates a preferential metabolism of the (*S*)-enantiomer. Cytochrome P450C8 has been reported to demonstrate preferential metabolism of (*S*)-verapamil and the (*S*)-enantiomer of its *N*-demethyl metabolite norverapamil using recombinant enzyme preparations [119]. The two enantiomers of verapamil also demonstrate differences in the extent of plasma protein binding [120] and interaction with p-glycoprotein transporters [121], which may also contribute to the pharmacokinetic differences between the two enantiomers.

11.4 STEREOSELECTIVITY IN DRUG TOXICITY

Despite several decades of increased regulatory and scientific focus, there is a paucity of information in the open literature on the comparative toxicity between individual enantiomers and their respective racemate or between individual enantiomers. While there is no doubt a wealth of comparative stereoisomer toxicity data from evaluations conducted within the pharmaceutical industry, very little of this information has subsequently been formally documented in published articles. Moreover, while there are numerous examples of racemic drugs that have been withdrawn from the market because of safety concerns and adverse drug effects, the literature exploring the mechanistic basis of these adverse events and the underlying role of stereochemistry is limited and fragmented at best. Nevertheless, there are selected instances that can be extracted from the open literature that can serve as representative examples of the impact stereochemistry can have on animal and clinical safety assessment.

Comparative evaluation of the safety and efficacy of individual enantiomer compounds form the foundation of the development decision tree when considering racemic drugs versus individual enantiomers as well as the comparative evaluation of enantiomers [122].

This information has lead to the acknowledgment that the chemical and pharmaceutical characterization of potential new drug candidates are pivotal to the drug development assessment process. From a nonclinical and clinical perspective, the decision to develop a single enantiomer does not solely depend on the comparative evaluation of efficacy and safety. The stereoselective interaction of potential drugs with the enzymes, receptors, and proteins that influence a molecule's pharmacokinetic profile can often enhance or mask the inherent molecular interaction that gives rise to an enantiomer's pharmacologic and/or toxicologic effect [123].

Many of these interactions fall into two categories: (i) one enantiomer is responsible for both the therapeutic and adverse effects, while the opposing enantiomer is inactive at normal doses (e.g., albuterol) or (ii) one enantiomer is responsible for the desired pharmacological effect but its antipode is responsible for the adverse reactions (e.g., thalidomide). As noted in earlier sections, stereoselective toxicities associated native enantiomer activity, toxicities may also arise from metabolic or dispositional features, which differ between enantiomers. In this instance, stereoselective toxicity is manifested through difference in detoxification pathways for each enantiomer or in stereoselective metabolic activation leading to adverse effects.

11.4.1 Pharmacology and Toxicity Reside in a Single Enantiomer

Albuterol is a short-acting β_2 -adrenergic receptor agonist used as a bronchodilator for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease. Albuterol is the racemate of 4-[2-(*tert*-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol. The bronchodilator effects are primarily attributable to the (*R*)-albuterol isomer, with little bronchodilatory activity demonstrated by the (*S*)-albuterol. The albuterol enantiomers also differ in their adverse effect profile. The (*S*)-albuterol causes hyperkalemia and proinflammatory properties, which some believe to moderate the beneficial effects of (*R*)-albuterol when administered as a racemate. The (*R*)-albuterol has also been demonstrated to cause hyperkalemia in addition to hyperglycemia, QT prolongation, and tachycardia [124,125]. Furthermore, the albuterol stereoisomers demonstrate differences in their pharmacokinetic profiles. The (*S*)-albuterol isomer demonstrates a slower systemic clearance and preferential accumulation versus therapeutically active (*R*)-enantiomer [126,127]. Originally introduced as the racemate (Proventil®), the (*R*)-albuterol enantiomer (Xopenex®) was subsequently registered in the United States on a rationale of a superior pharmacokinetic–pharmacodynamic profile.

11.4.2 One Enantiomer is Pharmacologically Active, while its Antipode is Toxic

Perhaps the most well-known case of purported stereospecific toxicity is that of thalidomide. Thalidomide, a racemic sedative–hypnotic, was prescribed to pregnant women in the late 1950s and early 1960s to ameliorate symptoms of morning sickness. The drug exhibited neurotoxicity and teratogenic effects resulting in newborns with significant physical deformities and was accordingly withdrawn in the early 1960s [128].

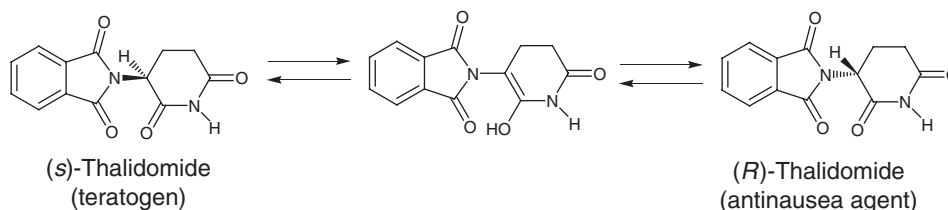


Figure 11.10 Stereochemical structures of (*R*)- and (*S*)-thalidomide and the interplay of chiral inversion.

Early preclinical studies in rabbits indicated that both enantiomers carried the teratogenic liability [129]. However, subsequent studies conducted in animals and human have reported that the (*R*)-thalidomide was responsible for the sedative effects [130], while the opposing (*S*)-thalidomide isomer was responsible for the teratogenic effects [131]. Unfortunately, the majority of these studies failed to fully consider the effects of *in vivo* biotransformation in the interpretation of the pharmacological and toxicological results. Multiple studies have now shown that both thalidomide isomers are unstable in solution and readily interconvert (Fig. 11.10) to form the racemic mixture in biologically relevant media [132]. It is now thought that phocomelia (birth defects) results from the inhibition of angiogenesis and vasculogenesis perhaps mediated by metabolites arising from the *in vivo* hydroxylation and/or hydrolysis of the two thalidomide enantiomers [133]. Meyring and others demonstrated that the hydroxylation of the two thalidomide enantiomers is stereospecific [134]. The chiral center of the thalidomide enantiomers is unaffected by this hydroxylation. The (*S*)-enantiomer is preferentially metabolized on the phthalimide moiety to the (*S*)-5-hydroxythalidomide. In contrast, the (*R*)-enantiomer is metabolized to (3'*R*, 5'*R*)-*trans*-5'-hydroxythalidomide, which rapidly epimerizes to yield the more stable (3'*S*, 5'*R*)-*cis* isomer further clouding the role of stereochemistry factors in the pharmacology of thalidomide [135]. Despite the long held public perception of thalidomide stereospecific toxicity, given the complexities of the solution chemical stability and biotransformation properties of the two thalidomide enantiomers, it is impossible to conclusively ascribe either the hypnotic and teratogenic effects exclusively to one or the other stereoisomer of this drug.

A second example of a case where one enantiomer is responsible for the desired pharmacological effect but its antipode is responsible for the adverse reactions seen with MK-0571. MK-0571 is a racemic leukotriene antagonist, which demonstrated protection against bronchoconstriction due to inhaled allergen challenge, exercise-induced bronchoconstriction in early clinical trials [136,137]. In safety studies in mice, MK-0571 was found to induce peroxisome proliferation precluding further clinical development. In subsequent studies, when the individual enantiomers of MK-0571 were administered to mice, a pronounced enantioselective induction of peroxisome proliferation was observed [138]. The Merck researchers demonstrated that the antiasthmatic activity resided in both (*S*)- and (*R*)-enantiomers (L-668,018; L-668,019), while the peroxisomal proliferative activity was a property of the (*S*)-enantiomer (L-668,018). Importantly, the toxicological assessment conducted by the Merck researchers ruled out pharmacokinetic and drug metabolism explanations for the observed stereoselective biological response profile in mice. In 1993, Boie and others subsequently demonstrated that the (*S*)-enantiomer preferentially activated the mouse peroxisome

proliferator-activated receptor (PPAR) versus the (R)-enantiomer [139]. On the basis of this information, the (R)-enantiomer (L-688-019; MK-0679; verlukast) was advanced to clinical evaluation contributing important data and insight to the ultimate development and registration of montelukast (Singular®).

11.4.3 Stereoselectivity in Detoxification Pathways

In a manner quite similar to proteins, the unique three-dimensional structure of DNA may also interact with certain compounds in a stereospecific manner. For most drugs, these types of interactions require a metabolic activation step, often times mediated by the CYPs [140]. Of the various types of CYP-mediated reaction products, epoxide transformations provide the opportunity for observed stereospecific carcinogenicity or mutagenicity [141]. For example, several P450 enzymes have been demonstrated to produce an electrophilic, chiral epoxide metabolite: styrene-7,8-oxide [142]. An important aspect of styrene-7,8-oxide is the difference in toxicity of the two enantiomers. In this instance, the (R)-enantiomer of styrene-7,8-oxide has been found to be four times as mutagenic to *Salmonella typhimurium* TA 100 in the Ames test than the (S)-enantiomer [143]. Moreover, in mice, the (R)-enantiomer is also more toxic to lung and liver than the (S)-enantiomer [144]. Thus, toxicity resulting from styrene exposure does not only depend on the amount of (racemic) styrene-7,8-oxide in the target tissue but also depend on the enantiomeric composition of the compound.

In vivo, detoxication of styrene-7,8-oxide has the potential to proceed via two pathways. One is via hydrolysis by microsomal epoxide hydrolase (mEH), which would result in the formation of styrene glycol. The other is conjugation of styrene-7,8-oxide with glutathione mediated by glutathione-S-transferase. Interestingly, in humans, detoxication of styrene-7,8-oxide by glutathione-S-transferase plays a minor role [145], and therefore, mEH appears to be the key enzyme in the detoxication of styrene-7,8-oxide in humans.

The catalytic mechanism of mEH is a two-step mechanism involving an initial attack of an active-site carboxylate on the oxirane followed by hydrolysis of the ester intermediate in the second step [146]. The rate-limiting step in the reaction mechanism of mEH is the hydrolysis of the intermediate [147]. Hydrolysis of the styrene-7,8-oxide enantiomers is governed in an enantioselective manner, with the (S)-enantiomer having an approximately six times higher K_m and five times higher V_{max} than the (R)-enantiomer. In this fashion, since the toxic potential (R)-styrene-7,8-oxide is higher than that of the (S)-enantiomer, stereoselective rates of mEH-catalyzed detoxication between the enantiomers is a major determinate in the risk of styrene-7,8-oxide toxicity.

11.4.4 Stereoselective Metabolic Activation Leading to Adverse Effects

Mianserin is a racemic tetracyclic antidepressant that demonstrates reduced cardiotoxicity and has a lower overdose potential than tricyclic antidepressants of its period [148]. Mianserin is an antagonist of H1, several 5HT subtypes, as well as $\alpha 1$ and $\alpha 2$ adrenergic receptors. While the overall cumulative clinical safety of mianserin has been quite favorable, there have been reports of rare but serious toxicologic side effects including agranulocytosis (~1 in 10,000), skin rashes, hepatotoxicity, and other blood dyscrasias [149–151]. On the basis of the effect on measures of serotonin receptor and noradrenaline uptake, it has been reported that (S)-mianserin is primarily

responsible for antidepressant effect [152] likely reflecting its higher affinity for the 5-HT₂ receptor [153]. The (*R*)-mianserin enantiomer has cytotoxic properties and has been reported to be toxic to human white blood cells after stereoselective metabolic activation [154]. DDI data and *in vitro* experiments indicated that CYP3A4 is primarily responsible for the metabolism of (*S*)-mianserin to 8-hydroxy-mianserin, whereas CYP3A4 and CYP2D6 have been demonstrated to preferentially *N*-demethylate (*R*)-mianserin as the primary metabolic pathway for this enantiomer [155,156]. At low drug concentrations, Riley and others demonstrated human mononuclear leukocyte cytotoxicity was more pronounced with (*R*)-mianserin than with (*S*)-mianserin and that the observed cytotoxicity exhibited a strong correlation with *N*-demethylation [154]. Riley and colleagues also demonstrated by measurement of irreversible protein binding that the generation of chemically reactive metabolites was significantly greater for the (*S*)-enantiomer than the (*R*)-enantiomer, which adds further evidence of the role of stereoselective metabolic activation as likely the underlying cause of adverse clinical effects of mianserin.

11.5 REGULATORY AND DEVELOPMENT TRENDS

As previously noted, a result of the increasing scientific body of evidence indicating the impact of molecular stereochemistry to drug safety and efficacy throughout the 1970s and 1980s, the FDA issued an initial guidance on the registration of stereoisomeric drugs in 1992 [13]. The guidance covers the chemical and biological factors that need to be described and justified if a racemic mixture is proposed for registration. In addition to pharmacological activity this guidance addresses chemical points to consider on drug substance identity purity, quality, and strength. From a biological perspective, pharmacology, toxicology, and ADME and pharmacokinetic properties must also be adequately characterized.

The increased burden of scientific and regulatory characterization of two or more molecular entities for racemic drugs as well as significant advances in stereospecific synthesis [157], preparative scale resolution of chiral entities [158], and quantitative analysis methodologies [159] have resulted in single enantiomers or achiral drugs as the primary focus of drug development efforts over the last 20 years. In the last reported survey [11] of worldwide registrations of new molecular (chemical) entities (NMEs) over the period of 1991–2002, only 14% represented racemic mixtures, 42% were achiral molecules, and 44% were single enantiomers [160,161]. In addition to these trends, there has been a pattern of converting registered racemic drugs to single-enantiomer products [162]. One of the most publicly visible examples of this strategy is the switch of racemic omeprazole (Prilosec®), a proton pump inhibitor, to (*S*)-(–)-omeprazole (Nexium®) for gastroesophageal acid reflux (GERD) and gastric and duodenal ulcers [163].

11.6 CONCLUSION

Since the discovery of chirality by Pasteur, stereochemistry has emerged as one of the most challenging and important areas of research within the discipline of pharmaceutical sciences. Stereochemistry embraces a broad variety of closely intertwined

static and dynamic aspects that are all related to the three-dimensional structure of molecules and how they relate to their achiral environment. The few examples highlighted in this chapter are hoped to reflect both the significance of chirality and the fundamental role that dynamic stereochemistry plays in the drug discovery/development continuum. Evaluation of chiral compounds must take into account three-dimensional structure–activity relationships beyond receptor–ligand interactions. In this fashion, the principles of stereodynamic chemistry of chiral compounds are discussed in terms of the complexity surrounding the toxicological and dispositional features associated with chiral molecules. The stereoselective metabolism of the chiral drugs is an interesting and a demanding field. It is anticipated that the increased knowledge of the stereoselective metabolism, as it pertains to enantioselective toxicity and carcinogenesis of the chiral molecules may be useful for the safer treatment of diseases.

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12 Genetics of Drug Disposition

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12.1	Summary	1
12.2	Introduction	2
12.3	Assessing genetic variants in ADME-related genes	4
12.4	Nomenclature of allelic variants—star nomenclature	4
12.5	Cytochrome P450s	4
12.6	Flavin-containing monooxygenases	10
12.7	Arylamine <i>N</i> -acetyltransferases	10
12.8	UDP-glucuronosyltransferases	11
12.9	Sulfotransferases	12
12.10	Drug transport proteins	13
12.11	Discussion	17
	References	17

12.1 SUMMARY

Drug disposition is a complex process culminating from the interplay of numerous drug-metabolizing enzymes, drug transport proteins, serum-binding proteins, and transcription factors and is affected by multiple environmental and endogenous factors. Knowledge (and applications resulting from the knowledge) about the factors that affect exposure can enable more safe and effective drug dosing. Genetic variations in drug-metabolizing enzymes (including the cytochrome P450s, glucuronosyltransferases, and sulfotransferases) and the drug transport proteins affect the function of these proteins and thereby contribute toward variability in drug disposition. Our knowledge of these functional genetic variants and the tools to interrogate them are well developed. These tools have found utility in drug development within the pharmaceutical industry, and for limited applications, making clinical treatment decisions. This chapter discusses the genetic variations in drug-metabolizing enzymes and transporters and their relevance for clinical pharmacokinetics.

12.2 INTRODUCTION

Drug disposition is a complex process affected by many intrinsic and extrinsic factors including age, body mass, renal and liver function, concomitant medication, and dietary intake. This chapter discusses how an additional intrinsic factor, genetics, affects drug absorption, distribution, metabolism, and excretion (ADME).

The concept of a heritable component to drug disposition was postulated over 100 years ago, long before Friedrich Vogel coined the term *pharmacogenetics* and defined it as the “study of the role of genetics in drug response” [1]. The early years of pharmacogenetics witnessed the identification of several, now well characterized, genetic associations with drug response, a few seminal examples of which are reported below:

1956 Succinylcholine was introduced as a muscle relaxant in the early 1950s. Soon after, it was noticed that some patients experienced much longer than the expected duration of action (h compared to min) and required mechanical ventilators for support. In the mid-1950s, Werner Kalow described an atypical cholinesterase with significantly decreased affinity for succinylcholine and showed the trait to be heritable [2,3]. Since that early seminal discovery, multiple genetic polymorphisms within cholinesterase have been identified and associated with response to multiple drugs ([4] and references therein).

1950s Isoniazid was introduced as an extremely effective treatment of tuberculosis in the early 1950s but was associated with peripheral nerve damage in a subset of individuals. Larger studies conducted to determine the cause of this toxicity led to the observation of a bimodal population distribution of isoniazid plasma levels following a standard dose of the drug and an association between the amount excreted unchanged and toxicity. Familial studies revealed that the half-life of isoniazid was an inherited trait and ultimately led to the identification of *N*-acetyltransferase (NAT) polymorphisms affecting acetylation phenotype [5–9].

1970s Adverse events in some subjects exposed to the antihypertensive drug debrisoquine or the antiarrhythmic drug sparteine were attributed to a deficiency in metabolic ability. This observation led to the characterization of an inherited, autosomal recessive trait and ultimately to the identification of CYP2D6 genetic variants ([10,11] and references therein).

As apparent from the examples above, sequence variations in the genes encoding drug-metabolizing enzymes can have a major effect on drug disposition; in recent years, similar observations have been made for genetic variants in drug transport proteins or transcription factors associated with drug-metabolizing enzymes or drug transporter genes. Such sequence variations include single nucleotide polymorphisms (SNPs), indels (short insertions or deletions), and copy number variations (CNVs; large deletions or duplications). Understanding the functional genetic variability in enzymes or transporters related to drug disposition is critical for the safe and effective use of drugs.

Since the early studies described above, the progress in this field has continued at a rapid pace—the genetic contributors to drug disposition are well studied and understood, for the most part. In the last 30 years or so, the drug-metabolizing enzymes have been cloned and genetic variants characterized; similarly, the last 10

years or so have seen much progress in the understanding and characterization of drug transporters.

Over 170 gene products are identified to have a role in drug disposition, and more than half of these are known to be polymorphic [12]. A systematic assessment of the literature showed that 59% of the drugs cited in adverse drug reaction (ADR) literature are metabolized by at least one enzyme, which is polymorphic and known to have an allele resulting in a poor metabolizer (PM) phenotype, whereas only 22% of randomly selected drugs sold in the United States and 7% of randomly selected top-selling US drugs are metabolized by enzymes with such polymorphic variability [13]. Approximately 10% of drugs approved by the US FDA between 1945 and 2005 contain pharmacogenomic information within the label, and a significant portion of these are drug disposition related [14]. In most cases, the drug labels provide pharmacogenetic (PG) information without specific recommendations or specific actions for individuals within particular genetic subgroups. An exception to this is the drug thioridazine, which is contraindicated in particular subsets of individuals, including one which is genetically identifiable (however, this may be of limited utility, given the low importance of thioridazine in routine medical practice).

Drug-metabolizing enzymes categorized by the US FDA as “valid genomic biomarkers in the context of approved drug labels” are listed in Table 12.1. These valid biomarkers are well characterized in terms of genotype–phenotype associations and have a high level of clinical validation attached to them. Other CYPs and drug transport proteins that have not attained this burden of proof yet are referred to as *exploratory* biomarkers. Only by including these exploratory biomarkers in routine PG-PK (pharmacogenetic-pharmacokinetic) analyses and generating data on genotype–phenotype correlations can they ultimately transition to “known valid” biomarker status.

PG information pertaining to drug pharmacokinetics is routinely generated by most pharmaceutical companies during the drug development process (in phase I–III clinical trials) in attempts to better understand the pathways for disposition of the compound, understand the basis of interindividual variability, and use the information for improving population PK models. After a drug is marketed, larger PG studies can be carried out given the availability of a large population of drug-exposed individuals for study.

TABLE 12.1 Valid Genomic Biomarkers in the Context of Approved Drug Labels

Context-Specific Biomarker	Prototypic Drug Associated with the Label Information Defining the Biomarker Context
CYP2C19 variants	Clopidogrel, voriconazole, prasugrel
CYP2C9 variants	Celecoxib, warfarin
CYP2D6 variants	Atomoxetine, fluoxetine HCl, codeine sulfate
DPD deficiency	Capecitabine
NAT variants	Rifampin, isoniazid, pyrazinamide
TPMT variants	Azathioprine
UGT1A1 variants	Irinotecan, nilotinib

Abbreviations: DPD, dihydropyrimidine dehydrogenase; TPMT, thiopurine methyltransferase.

Source: Adapted from <http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm>.

12.3 ASSESSING GENETIC VARIANTS IN ADME-RELATED GENES

Historically, the search for PG-PK associations has been performed with a candidate gene approach, wherein polymorphisms within one or a few genes are assessed for their contribution toward observed variability in the measured PK parameters of a drug. More recently, high throughput and high multiplex, microarray-based technologies have become available, whereby multiple SNPs in multiple genes can be assessed simultaneously [15,16]. Such ADME panels allow for a comprehensive and simultaneous analysis of the known genes (and SNPs) for assessing PG-related PK variability for a drug and have already yielded interesting and clinically significant associations for drugs including warfarin and clopidogrel [17,18].

12.4 NOMENCLATURE OF ALLELIC VARIANTS—STAR NOMENCLATURE

The cloning and characterization of multiple highly homologous genes encoding the drug-metabolizing enzymes and transporters necessitated the development and adoption of systematic, controlled nomenclature systems for the superfamilies of CYPs, UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), NATs, and drug transporters [19–28]. Similarly, increased knowledge about genetic variations necessitated the development of a nomenclature system for allelic variants of individual genes. Genetic variants within the drug-metabolizing enzymes and drug transporters are classified by the star nomenclature. The star nomenclature has been adopted by scientists as a system to organize, annotate, and, most importantly, standardize the global explosion of genetic information [29]. In the star nomenclature system, *1 is designated the reference allele against which all other sequences are compared. The *1 designation is typically given to the first sequenced clone of the gene, and subsequent unique numbers are assigned to novel functional alleles. The tremendous explosion of identified sequence polymorphisms in the postgenome era, resulting from improvements in sequencing technologies, are bringing to the forefront some of the limitations of the star nomenclature system to handle this immense wealth of data; there are ongoing efforts to organize the star nomenclature system for current practice to maximize utility [30].

12.5 CYTOCHROME P450s

The cytochrome P450s are a superfamily of drug-metabolizing enzymes, which catalyze the majority of phase I drug-metabolizing activity in humans. On the basis of amino acid sequence homology, the human CYPs comprise 18 families (>40% sequence identity) and 44 subfamilies (>55% sequence identity). Completion of sequencing of the human genome provided confirmation of the presence of 57 CYP genes and 58 pseudogenes (<http://drnelson.uthsc.edu/CytochromeP450.html>; [23]). The CYPs in families 1–3 are responsible for approximately 70–80% of all phase I drug metabolism activity of clinically used drugs, whereas the CYP enzymes in other families are primarily involved in metabolism of endogenous compounds such as fatty acids, steroids, and bile acids. This section focuses on the CYPs relevant to drug metabolism.

The drug-metabolizing CYPs are a highly polymorphic group of enzymes. A comprehensive database of CYP allelic variants with links to the primary literature is maintained at <http://www.cypalleles.ki.se/>. The major relevant CYP enzymes are discussed below in order of their PG clinical relevance, and the reader is referred to <http://www.cypalleles.ki.se/> for more information on polymorphisms within these or other CYPs.

12.5.1 CYP2

The human CYP2 family is a major contributor toward drug disposition, and the various subfamilies and members comprising this family (in order of PG importance) are discussed below.

12.5.1.1 CYP2D. The human CYP2D family comprises three genes, *CYP2D6*, *CYP2D7*, and *CYP2D8*. *CYP2D7* and *CYP2D8* are pseudogenes, but *CYP2D6* is an extremely well-characterized gene and an important enzyme for drug metabolism. CYP2D6 metabolizes many commonly prescribed drugs including codeine, dextromethorphan, tamoxifen, and most tricyclic antidepressants. Of the CYPs, *CYP2D6* genomic information is the most frequently mentioned in drug labels. CYP2D6 is highly polymorphic with 78 alleles characterized to date (<http://www.cypalleles.ki.se/cyp2d6.htm>). Some of these variants result in complete lack of CYP2D6 activity, whereas others result in reduced enzymatic activity (and in certain cases, the reduction in activity is substrate specific). Genetic polymorphisms within CYP2D6 have been well characterized for their contribution toward the interindividual variability observed in the PK of CYP2D6 substrates, and clinically relevant (based on frequency or function) CYP2D6 alleles are shown in Table 12.2, but the reader is referred to <http://www.cypalleles.ki.se/cyp2d6.htm> and [31,32] and references therein for a comprehensive listing and description of alleles.

The combination of alleles carried by an individual determines the CYP2D6 activity contributing to drug metabolism. Multiple methods have been used to predict CYP2D6 phenotype (or activity) from genotype. One method for doing so is based on the number of fully functional, low function, and nonfunctional alleles carried by an individual, resulting in PM, IM (intermediate metabolizer), EM (extensive metabolizer), or UM

TABLE 12.2 Clinically Relevant CYP2D6 Alleles

Allele	Effect	Functional Outcome
*3	Frameshift	No activity
*4	Alteration of splice site	No activity
*5	Gene deletion	No activity
*6	Frameshift	No activity
*9	Amino acid deletion	Decreased activity
*10	Amino acid substitutions	Decreased activity
*17	Amino acid substitutions	Substrate-specific decreased activity
*29	Amino acid substitutions	Substrate-specific decreased activity
*41	Alteration of splice site	Decreased activity
*1/*2xN	Gene amplification	Increased activity

(ultrarapid metabolizer) phenotype. The number of CYP2D6 alleles and the range of activity of each of these alleles make the inference of phenotype from genotype quite challenging and, perhaps, equivocal. More recently, innovative methods have been employed to improve the accuracy of phenotype prediction from genotype [33,34]. Extensive amount of data have been generated on the metabolism of CYP2D6 substrates in the different CYP2D6 phenotype classes and are briefly discussed below. The reader is encouraged to review the references in this section for additional materials.

CYP2D6 PMs have low CYP2D6 metabolic capacity, exhibit higher maximum concentrations and AUCs of drugs metabolized primarily via the CYP2D6 route, and potentially result in safety issues (especially in drugs with narrow therapeutic indices). Also, the efficacy of prodrugs that require activation by CYP2D6 (e.g., codeine, tramadol, and tamoxifen) may be reduced in CYP2D6 PMs ([35–37] and reviewed in Ref. 38). On the basis of extensive research on the effect of *CYP2D6* polymorphisms on the metabolism of antipsychotic and antidepressant drugs, dose adjustments for both CYP2D6 PMs and UMs have been suggested [39], although these are generally not employed in the clinic and mostly not reflected in antipsychotic and antidepressant drug labeling. Individuals with CYP2D6 IM or EM phenotypes exhibit a wide and overlapping range of activity, and as such, distinguishing IM from EM may not be clinically relevant. At the other end of the activity spectrum, compared with PMs, the UMs exhibit high metabolic activity as a result of amplification of functional CYP2D6 alleles, and these individuals may not achieve therapeutic efficacy at normal doses of drugs metabolized by CYP2D6. For example, the UM phenotype is 10-fold more common in nonresponders than in responders to antidepressant therapy [40], and association is reported between maternal codeine use and CNS depression in breast-fed neonates [41,42].

Given the extensive amount of information available for CYP2D6 genotype–phenotype associations, it is of no surprise that CYP2D6 phenotype information and effect on metabolism is found in the labels for several drugs including aripiprazole, thioridazine, atomoxetine, protriptyline (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm). Even though CYP2D6 metabolizer status is known to affect the PK of several drugs, genotyping before start of therapy is not required and is provided for informational purposes only; the one exception to this being thioridazine, which is contraindicated in CYP2D6-PMs (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm).

As with most of the drug-metabolizing enzymes, there are interethnic differences in CYP2D6 allele frequencies (and the resulting PM/EM/UM phenotypes), making genotype selection in PG studies a critical factor [43,44].

12.5.1.2 CYP2C. The CYP2C subfamily of CYPs comprises CYP2C8, CYP2C9, CYP2C18, and CYP2C19, with CYP2C9 and CYP2C19 being the most clinically relevant members, although CYP2C8 also has a role in drug metabolism.

CYP2C9 is one of the principal CYPs contributing to the metabolism of commonly used drugs such as warfarin, phenytoin, tolbutamide, diclofenac, and celecoxib. Thirty-four alleles have been identified and characterized within the *CYP2C9* gene (<http://www.cypalleles.ki.se/cyp2c9.htm>), but *CYP2C9**2 and *CYP2C9**3 primarily cause the PM phenotype in individuals. The *CYP2C9**2 and *3 alleles both contain a point mutation resulting in a single amino acid change and decreased enzymatic activity. Association between *CYP2C9**2 or *CYP2C9**3 genotype and PK parameters of

at least 17 drugs has been reported [45]. Other impaired *CYP2C9* alleles such as *CYP2C9*6* and *CYP2C9*25* consist of a frameshift that results in absence of enzyme activity, but these alleles are rare. The importance of *CYP2C9* variability is reflected in the drug label information for celecoxib and warfarin (http://www.accessdata.fda.gov/drugsatfda_docs/label/2005/020998s018,0191bl.pdf and http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/009218s1051blv2.pdf). In both cases, subjects who are *CYP2C9* PMs have decreased ability to metabolize the drugs and therefore have increased plasma levels given the same dose of the drug as *CYP2C9* EMs.

CYP2C19 is clinically important due to its role in the metabolism of commonly used drugs including proton pump inhibitors (e.g., omeprazole and lansoprazole), fluoxetine, sertraline, clopidogrel, and nelfinavir. There are currently 26 allelic variants of *CYP2C19* described and characterized. Several genetic variants result in decreased function, but the majority of PM phenotypes can be attributed to *CYP2C19*2* and *CYP2C19*3*. The *CYP2C19*2* allele contains a single point mutation that results in aberrant splicing, an altered reading frame, and a prematurely truncated protein with no activity; the *CYP2C19*3* allele contains a point mutation resulting in a premature stop codon and an inactive protein. Variability in *CYP2C19* activity is clinically relevant. For example, the AUCs of omeprazole, lansoprazole, and pantoprazole are approximately fivefold higher in *CYP2C19* PMs than in *CYP2C19* EMs, and at least for omeprazole, this PK association is further reflected by differences in efficacy—*CYP2C19* PMs have higher exposures and higher cure rates ([46,47] and references therein). The clinical relevance of genetic variability in *CYP2C19* is reflected in the drug labels for several *CYP2C19* substrates including those for clopidogrel and prasugrel. *CYP2C19* is a critical enzyme for conversion of clopidogrel to its active metabolite, and therefore, *CYP2C19* PMs have reduced levels of the active metabolite and potentially poorer outcomes with clopidogrel therapy [48–50]. Issues related to the *CYP2C19* genotype-guided usage of clopidogrel have been addressed extensively in the literature by researchers and medical practitioners [51–55].

12.5.1.3 CYP2A. The human *CYP2A* family comprises *CYP2A6*, *CYP2A7*, *CYP2A13*, and *CYP2A18P*. The functional members of this family, *CYP2A6* and *CYP2A13*, are expressed predominantly in the liver and respiratory tract, respectively. On the basis of its expression in the liver, *CYP2A6* is the primary contributor toward drug disposition in this family, and this gene has been extensively studied for its role in nicotine metabolism.

In addition to being the principal CYP involved in nicotine metabolism, *CYP2A6* is also involved in the metabolism of drugs such as coumarin, halothane, tegafur, and losigamone [56–59]. The *CYP2A6* gene is highly polymorphic, with 37 alleles, including a gene deletion and duplication, currently identified and named (<http://www.cypalleles.ki.se/cyp2a6.htm>). Inactive alleles of *CYP2A6* include *CYP2A6*2* (does not incorporate heme), *CYP2A6*4* (gene deletion), *CYP2A6*5* (unstable protein), and *CYP2A6*20* (truncated protein) [60–66]. Alleles with decreased activity include *CYP2A6*6*, **7*, **9*, **10*, **11*, **12*, **17*, and **19* [67–72]. As with other drug-metabolizing enzymes, there is considerable interethnic variability in the frequency of *CYP2A6* alleles. The combined frequencies of low activity (impaired or absent) alleles (*CYP2A6*2*, **4*, **5*, **7*, **9*, **10*, **11*, **17*, **19*, and **20*) are 9.1%, 21.9%, 42.9%, and 50.5% in white, black, Korean, and Japanese subjects, respectively [73],

and these interethnic differences in allele frequency are reflected in the interindividual and interethnic variability in the metabolism of nicotine and coumarin [64,74–76].

12.5.1.4 CYP2B. The human CYP2B subfamily comprises one functional gene, *CYP2B6*, and one pseudogene, *CYP2B7*. CYP2B6 metabolizes several drugs including efavirenz, nevirapine, bupropion, and propofol. The *CYP2B6* gene is highly polymorphic, and 29 alleles are currently defined (<http://www.cypalleles.ki.se/cyp2b6.htm>). Polymorphisms within CYP2B6 affect the activity and expression of the enzyme, resulting in variable pharmacokinetics of CYP2B6 substrates. For example, considerable interindividual variability in efavirenz levels and half-life is reported following oral administration and is consistently associated with CYP2B6 activity. CYP2B6 polymorphisms that affect enzyme activity are associated with higher plasma levels and the occurrence of CNS side effects; furthermore, the increased efavirenz exposure is associated with the development of resistance after treatment discontinuation [77–83]. Specifically, the 516G>T polymorphism (resulting in change from glutamine to histidine at amino acid position 172), which is part of multiple alleles (including *CYP2B6**6, *CYP2B6**7, and *CYP2B6**9), is associated with increased exposure to efavirenz. However, the metabolic phenotypes assigned to CYP2B6 (e.g., PM or EM) are not as clear as they are for other CYPs, and the effects of certain polymorphisms may be substrate specific [84]. In addition to the variability conferred by polymorphisms, CYP2B6 is also inducible by various drugs including phenobarbital, cyclophosphamide, rifampin, efavirenz, carbamazepine, and others [84]. Induction of CYP2B6 by nuclear receptors, constitutive androstane receptor (CAR), and pregnane X receptor (PXR) also affects the pharmacokinetics of CYP2B6 substrates and may confound genotype–phenotype studies.

12.5.2 CYP3

12.5.2.1 CYP3A. The human CYP3A subfamily comprises four members, CYP3A4, CYP3A5, CYP3A7, and CYP3A43. The developmental timing and expression patterns of the four isozymes are differentially regulated. CYP3A7 is present in the fetal liver and intestine but its expression decreases after birth; CYP3A43 is expressed at low levels throughout the body, but its contribution to drug metabolism is not well understood. CYP3A4 and CYP3A5 are the most relevant members of the subfamily for drug metabolism in adults. CYP3A4 is involved in the metabolism of approximately 50% of currently used therapeutic agents and, as such, has been extensively studied [85–87]. Interindividual variability in CYP3A activity is high. *In vivo* studies have shown 5- to 10-fold variability in the metabolism of CYP3A substrates, and *in vitro* studies show even greater variability (>30-fold) [88–91]. Furthermore, well-characterized drug interactions with CYP3A can increase the range of variability to approximately 400-fold ([92] and references therein). An early study that analyzed the existing literature and used a repeated drug administration measure to calculate the genetic versus environmental components of high CYP3A interindividual variability suggested that 70–90% of the variability in CYP3A activity was attributable to genetic factors [93]. However, the specific genetic factors or polymorphisms contributing to this variability remain elusive to date. The SNPs identified within the *CYP3A4* gene are generally of low frequency and do not account for the large variability observed. The continuous and unimodal distribution of CYP3A activity suggests that multiple

genes are involved in its regulation and that individual genetic factors can play only a small role [92]. Twenty CYP3A4 alleles have been reported and characterized. The *CYP3A4*1B* allele is the most extensively characterized of the CYP3A4 alleles and has been associated with multiple disease states but does not appear to affect the *in vivo* metabolism of endogenous compounds or drugs ([94,95] and references therein). Current weight of evidence suggests that interindividual variability in CYP3A4 activity may be more related to differential regulation than to genetic polymorphisms. Induction of CYP3A4 by a variety of compounds (including rifampicin, phenobarbital, dexamethasone, and hyperforin) is mediated via the PXR pathway and can increase the metabolism of other drugs that are CYP3A substrates, making them ineffective. For example, St. John's wort, which is a potent inducer of CYP3A, can cause therapeutic failure of cyclosporine, oral contraceptives, or HIV-protease inhibitors (also CYP3A substrates), if taken concurrently ([96–99] and references therein). Conversely, CYP3A inhibition, caused by drugs such as nitroimidazole antifungals, diltiazem, and verapamil, as well as dietary components such as grapefruit juice, can increase the exposure to CYP3A substrates taken concurrently and lead to adverse events [89,100,101].

In contrast to *CYP3A4*, *CYP3A5* is polymorphically expressed—CYP3A5 expression is detected in the livers of only 10–30% of Caucasians and Asians but in more than 50% of African-Americans [102–104]. The most common CYP3A5 allele in a number of ethnic populations is *CYP3A5*3*, in which a polymorphism creates a cryptic splice site resulting in an improperly spliced mRNA and a truncated protein [103,104]. Interestingly, the original CYP3A5 cDNA sequence came from a liver sample expressing CYP3A5, thus making it one of the few CYPs in which the *1 nomenclature is not designated to the most common allele. The clinical relevance of polymorphic expression of *CYP3A5* is complicated by the substrate overlap between CYPs 3A4 and 3A5 but is well characterized in the field of transplant pharmacogenetics where CYP3A5 polymorphisms are associated with elevated tacrolimus levels and dosing adjustments [105–107].

12.5.3 CYP1

The human CYP1 family comprises CYP1A1, CYP1A2, and CYP1B1. These enzymes have a major role in the bioactivation of procarcinogens, including polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, and aminoazo dyes, and in the metabolism of estrogens, fatty acids, and xenobiotics. The CYP1 family of proteins is upregulated by environmental pollutants (including dioxins and polycyclic aromatic hydrocarbons) and certain drugs via an aromatic hydrocarbon receptor (AHR)-mediated pathway, and interindividual variability in expression is observed [108,109]. Currently, 11 CYP1A1 alleles (CYP1A1*1–*11), 16 CYP1A2 alleles (CYP1A2*1–*16), and 26 CYP1B1 alleles (CYP1B1*1–*26) have been characterized (<http://www.cypalleles.ki.se/>). To date, there are no reports of clinically relevant associations between CYP1A genetic polymorphisms and variability in drug disposition. Polymorphisms within the CYP1 family are not expected to affect drug metabolism in as appreciable a manner as interindividual variability in expression due to enzyme induction. Inducibility of these enzymes by a variety of drugs and environmental pollutants does affect their activity, potentially leading to undesirable outcomes. Induction of CYP1A in humans is highly variable, and high expression can potentially increase the risk of lung cancer in smokers because of the increased bioactivation of polycyclic aromatic hydrocarbons

[110,111]. Similarly, overexpression of CYP1B1 can result in increased conversion of steroid hormones or exogenous substances into toxic metabolites resulting in neoplastic progression (e.g., the conversion of 17 β -estradiol to 4-OHE₂ or activation of procarcinogens such as polycyclic aromatic hydrocarbons); [112,113] the CYP1B1 locus is also linked to primary congenital glaucoma, and the mechanisms underlying this replicated association are being extensively studied [114,115]. Since AHR-mediated activation of CYP1 genes results in unwanted physiologic consequences (such as the activation of procarcinogens) and is an unwanted drug effect, pharmaceutical companies routinely screen new chemical entities for CYP1A induction potential (a detrimental property).

12.6 FLAVIN-CONTAINING MONOOXYGENASES

The mammalian flavin-containing monooxygenases (FMO) are a family of microsomal flavoproteins that catalyze the oxidation of a wide range of nucleophilic heteroatom-containing compounds. There are five functional FMO genes and several pseudogenes in humans. The FMO genes are expressed in a tissue- and temporal-selective manner. FMO1 is the major fetal isoform, and FMO3 is the major adult isoform [116]. FMO3 is the most abundantly expressed FMO in adult human liver, with levels comparable to some of the abundant CYPs and, as such, is the most relevant for drug metabolism ([117,118] and references therein). Similar to the other FMO, FMO3 has a broad substrate specificity and is involved in the metabolism of commonly used drugs including cimetidine, ranitidine, clozapine, methimazole, itopride, ketoconazole, tamoxifen, and sulindac sulfide [119–123]. Numerous polymorphisms have been reported in the *FMO3* gene (http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3/Tables/tableall.html). Genetic variants resulting in FMO3 deficiency are responsible for the phenotype of trimethylaminuria, otherwise known as *fish-odor syndrome*. Allelic variants that result in FMO3 deficiency are associated with the inability to metabolize the tertiary amine, trimethylamine (TMA), that is derived *in vivo* from the bacterial degradation of dietary choline, carnitine, and lecithin and found in various dietary sources including marine fish, beetroot, and legumes. Accumulation of TMA in the body results in a malodor resembling decaying fish. Genetic variants of FMO3 are also associated with altered metabolism of various substrates, including sulindac, ranitidine, and benzydamine [124–127].

12.7 ARYLAMINE *N*-ACETYLTRANSFERASES

The arylamine NATs are a family of drug-metabolizing enzymes that catalyze the transfer of an acetyl group from acetyl-CoA to the terminal nitrogen of substrates including isoniazid, some sulfonamides, dapsone, phenelzine, hydralazine, and procainamide ([128,129] and references therein). There are two functional NATs in humans, NAT1 and NAT2, and both genes contain sequence polymorphisms affecting activity. As mentioned in Section 12.2, the identification of the polymorphic inactivation of the antitubercular drug isoniazid is one of the early success stories of pharmacogenetics, and as such, this immense body of work has been extensively reported and reviewed [5–9]. The molecular genetic bases of the acetylation

polymorphism are well characterized, and individuals can be classified as “slow” or “rapid” acetylators based on the genotype. Slow acetylators often experience adverse reactions in response to drugs such as isoniazid, sulfonamides, procainamide, and hydralazine, whereas rapid acetylators may not respond to isoniazid and hydralazine [5,6,130–132]. Information related to acetylator status is included in several drug labels (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm).

12.8 UDP-GLUCURONOSYLTRANSFERASES

The UGTs superfamily of phase II drug-metabolizing enzymes catalyzes the transfer of the glucuronic group from uridine diphosphoglucuronic acid to a substrate, thereby increasing polarity and facilitating excretion of the substrate in bile or urine. UGTs are responsible for the conjugation of endogenous compounds such as bilirubin, steroid and thyroid hormones, and bile acids and exogenous compounds including drugs (e.g., irinotecan, clozapine, and buprenorphine), dietary components, environmental contaminants, and chemical carcinogens [133–135]. The 18 functional UGT proteins are encoded by 2 gene families, *UGT1* and *UGT2*, which comprise 3 subfamilies—*UGT1A*, *UGT2A*, and *UGT2B*. The major members of these families are described below.

12.8.1 *UGT1A*

The *UGT1A* subfamily is most interesting in that the entire *UGT1A* subfamily is derived from a single gene locus comprising 17 exons that encode 9 functional proteins (*UGT1A1* and *UGT1A3–1A10*). Each *UGT1A* isoform is encoded by a unique exon 1 and common exons 2, 3, 4, and 5 downstream, which means that all the *UGT1A* proteins have a unique N-terminal substrate-binding region but a common C-terminal cosubstrate-binding domain. Recent studies suggest that there may be additional genetic diversity at the *UGT1A* locus resulting from alternative splicing but requires additional characterization [136,137].

UGT1A1 and *UGT1A6* are expressed in the liver and are the major contributors toward xenobiotic metabolism; the other *UGT1A* members are expressed extrahepatically and therefore may play less roles in overall xenobiotic metabolism.

UGT1A1 is the most extensively studied member of the UGT superfamily because of its level and location of expression and its importance in the metabolism of endogenous and exogenous compounds. It is the primary enzyme responsible for the conjugation of bilirubin and also has an important role in conjugation of xenobiotics such as irinotecan and nilotinib. Genetic alterations that affect *UGT1A1* expression or function are associated with severe familial forms or milder, more common, usually asymptomatic forms of unconjugated hyperbilirubinemia (Crigler–Najjar type I and II disorders and Gilbert’s syndrome, respectively) [138–143]. A common dinucleotide (TA) repeat polymorphism within the TATA-box region of the *UGT1A1* promoter affects *UGT1A1* expression level and glucuronidation capacity. On the basis of the number of dinucleotide TA repeats [A(TA)_{*n*}TAA...], four different alleles are identified—*UGT1A1**1 (*n* = 6), *UGT1A1**33 (*n* = 5), *UGT1A1**28 (*n* = 7), and *UGT1A1**34 (*n* = 8). In general, there is an inverse correlation between the number of TA dinucleotide repeats and level of *UGT1A1* expression [141,144,145]. The most common (or wild type)

allele has six TA repeats, while the most common variant allele (*UGT1A1**28) has seven repeats and is associated with decreased glucuronidation capacity for *UGT1A1* substrates. While the promoter polymorphism is highly prevalent (and most relevant from a drug disposition perspective) in the Caucasian and African population, another polymorphism (*UGT1A1**6) that comprises an amino acid change (Gly71Arg) affecting *UGT1A1* activity is predominant in the Japanese population and is associated with hyperbilirubinemia in this population [133,146,147].

The *UGT1A1**28 polymorphism was extensively characterized during studies of the anticancer drug, irinotecan, and information related to this polymorphism is included in the drug label for its association with adverse events (see also labeling information for nilotinib) (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm). Irinotecan is a camptothecin derivative that shows antitumor activity via topoisomerase inhibition [148]. The active metabolite of irinotecan, SN-38, is formed by carboxylesterase enzymes in the liver. *UGT1A1* is the primary enzyme responsible for the subsequent conjugation and elimination of SN-38 in bile and urine [149]. The dose-limiting toxicities of irinotecan (severe diarrhea and neutropenia) are attributed to the increased levels of the active metabolite SN-38 resulting from decreased glucuronidation and elimination. Reduced expression of *UGT1A1*, due to polymorphisms within the gene, is associated with lower SN-38 glucuronidation and irinotecan toxicity [141,150–152].

Approximately 10% of the North American population is homozygous for the *UGT1A1**28 allele. These patients have lower *UGT1A1* activity, reduced capacity for eliminating SN-38, and consequently are at increased risk for neutropenia. A reduced initial dose of irinotecan is recommended for these patients. Results for patients who are heterozygous for the *UGT1A1**28 allele are mixed, but, in general, these individuals are thought to tolerate normal doses of irinotecan. Other polymorphisms affecting *UGT1A1* activity will theoretically have similar implications for irinotecan toxicity, but the associations are not as yet well characterized.

The other important *UGT1A* subfamily member, *UGT1A6*, is involved in the metabolism of acetaminophen, aspirin, and other nonsteroidal anti-inflammatory agents. Missense mutations within the *UGT1A6* gene result in reduced catalytic activity toward at least some *UGT1A6* substrates [153]. The reports of high linkage disequilibrium between *UGT1A6* missense polymorphisms and *UGT1A1**28 alleles suggest an importance of haplotype structure and the need for caution when interpreting association results.

UGT2B7 and *UGT2B15* are important drug-metabolizing enzymes (substrates include morphine, epirubicin, zidovudine, gemfibrozil, and oxazepam) and are also involved in the metabolism of endogenous substances including steroid hormones. Common missense polymorphisms in these genes may affect glucuronidation activity in a substrate-specific manner, but the clinical relevance of drug metabolism is yet unclear (see Refs 133,135, and 154 and references therein).

12.9 SULFOTRANSFERASES

There are two classes of sulfotransferases in vertebrates—the membrane-bound sulfotransferases found in the Golgi apparatus and the soluble, cytosolic forms [155]. The membrane-bound sulfotransferases act on endogenous macromolecules and are not

discussed further since they are not known to metabolize xenobiotics. The cytosolic sulfotransferases metabolize hormones and xenobiotics. The soluble sulfotransferases catalyze the transfer of the sulfonyl group from 3'-phosphoadenosine 5'-phosphosulfate to the hydroxyl, sulfhydryl, amino, or N-oxide group of a variety of substrates.

The human cytosolic SULT enzymes are a superfamily comprising 12 isoforms. Of all SULT family members, SULT1A1 is the most relevant for drug metabolism because of its abundance in the liver and its broad substrate specificity. SULT1A1 is involved in the metabolism of various hormones and xenobiotics (e.g., acetaminophen, minoxidil, and the active metabolites of tamoxifen) (see Ref. 156 and references therein). Considerable interindividual variability, largely attributed to inherited factors, is observed in platelet SULT1A1 activity and thermal stability [157,158]. The most common variant allele, *SULT1A1**2 (with a nonsynonymous SNP, Arg213His), is associated with decreased SULT1A1 activity and thermal stability [159,160]. Additionally, two polymorphisms within the 5'-flanking region of the *SULT1A1* gene are also associated with variability in SULT1A1 activity, but these polymorphisms are in linkage disequilibrium with the *SULT1A1**2 polymorphism, and therefore, the effect of these and the *SULT1A1**2 polymorphism on activity may be attributed to the haplotype [161]. However, the SULT1A1*2 polymorphism (and 5'-flanking region polymorphisms) only account for a portion of the inherited variability in SULT1A1 activity. The more recent identification of relatively common copy number variability of the *SULT1A1* gene explains a greater portion of the variability in activity. Similar to the copy number variability observed for CYP2D6, a *SULT1A1* gene deletion/duplication event results in individuals being "slow sulfators" or "rapid sulfators" [162]. Therefore, any genotype–phenotype correlations for *SULT1A1* must include an assessment of both copy number and SNPs. The contribution of both CNVs and SNPs to variability in SULT1A1 activity is comprehensively described by Hebring *et al.* [163].

12.10 DRUG TRANSPORT PROTEINS

Drug transport proteins are important determinants of drug pharmacokinetics and pharmacodynamics. Drug transporters affect all aspects of ADME—drug absorption, distribution, and elimination are affected by transport across tissues, and metabolism is affected by controlling access of the drug to the site of metabolism. Transporters also affect drug pharmacodynamics by their role in allowing access to the site of action [e.g., P-glycoprotein (P-gp) expression at the blood–brain barrier]. Variability in the expression or function of drug transport proteins can affect the PK or pharmacodynamic profiles of a drug and hence efficacy, adverse event, or drug–drug interaction profiles. Drug uptake and efflux from tissue are carried out by over 400 transporters in 2 major superfamilies—the solute carrier (SLC) transporters and the ATP-binding cassette (ABC) transporters, respectively [164,165]. The SLC transporter family comprises uptake transporters, which mediate the passage of small molecules across biological membranes along concentration gradients in a process known as *facilitated diffusion*. The ABC transporters utilize energy from ATP hydrolysis to transport substrates across biological membranes, either against or along concentration gradients, in a process known as *active transport*. The combined, concerted action of uptake and efflux transporters within the different membranes is a critical determinant of the extent and net direction of drug movement in or out of organs.

As with the drug-metabolizing enzymes, most drug transport proteins contain polymorphisms, but genotype–phenotype relationships are not as well characterized as those of drug-metabolizing enzymes. Comprehensive discussion of the pharmacogenetics of drug transporters is outside the scope of this chapter, but the topic is well reviewed in the literature [166–168]. The International Transporter Consortium, comprising industry, regulatory, and academic scientists, identified transporters that are currently known to be important determinants of drug pharmacokinetics [169]. This list of transporters is provided in Tables 12.3 and 12.4 and annotated for PG relevance. Only the most clinically relevant transporters are described below.

12.10.1 OATP

The organic anion transporting polypeptides (OATPs) (gene family—*SLCO*), belong to the SLC family of transporters, have broad substrate specificity and are expressed in multiple tissues including liver, gut, and blood–brain barrier. OATP1B1, which is predominantly expressed in the liver and has a major role in the transport of several drugs, including rifampin, the statins (atorvastatin, rosuvastatin, pravastatin, and more), and repaglinide, is probably the best-characterized member of the OATP family. OATP1B1 is encoded by the *SLCO1B1* gene. Allelic variants of *SLCO1B1* are associated with decreased transporter activity (e.g., *SLCO1B1**1b, *2, *3, *4, *5, *6, *9, *13, *15, and *17) [170–172] and are clinically relevant since they affect the disposition or effect of several drugs including pravastatin, fexofenadine, atrasentan, and repaglinide [173–180].

12.10.2 ABC Transporters

The ABC transporters are encoded by 49 different genes classified into 7 subfamilies based on sequence homology (ABCA–ABCG; nutrigene.4t.com/humanabc.htm). The ABC transporters are responsible for the transport of various substrates across membranes against a concentration gradient, using energy provided by ATP. Many members of this family are known to confer multidrug resistance to cancer chemotherapy.

P-gp is encoded by the multidrug resistance 1 gene (*MDR1/ABCB1*), and because of clinical relevance, it is the best-characterized member of the ABC transporter family. The *MDR1* gene was aptly named as such for its ability to transport various anticancer agents such as docetaxel, etoposide, paclitaxel, topotecan, and vinblastine out of the cell, thereby conferring the phenotype of multidrug resistance. However, we now know P-gp has a broad substrate specificity that extends across a wide range of therapeutic agents including antiarrhythmics (e.g., digoxin, verapamil), antibiotics (e.g., erythromycin), and immunosuppressants (cyclosporine, tacrolimus) [168]. P-gp is expressed in multiple tissues including the brain, small and large intestines, kidney, liver, and testes and is an important mediator of drug efflux. Over 105 variants have been detected within the *ABCB1* gene [167,181–185], and many studies have been performed to characterize the functional consequences of these variations. Much of the research on *ABCB1* pharmacogenetics has focused on three SNPs that are in linkage disequilibrium—two synonymous SNPs (1236 C>T in exon 12, 3435 C>T in exon 26) and a nonsynonymous SNP (2677G>T, resulting in an amino acid change at position 893, A893S). PG studies investigating the relationship between these (and other) *ABCB1* polymorphisms and the pharmacokinetics of well-characterized P-gp

TABLE 12.3 Clinically Relevant SLC Transporters and Associated Pharmacogenetic Information

Transporter/Alias (Gene)	Organs/Cells	Pharmacogenetic Relevance	References
OATP1B1/OATP-C, OATP2, LST-1 (<i>SLCO1B1</i>)	Hepatocytes (sinusoidal)	Consistently replicated associations between genotype and clinical pharmacokinetics	12,169,180
OATP1B3/OATP-8 (<i>SLCO1B3</i>)	Hepatocytes (sinusoidal)	<i>In vitro</i> evidence of common functional variants, but not yet associated with clinical pharmacokinetics	12,169,180
OAT1 (<i>SLC22A6</i>)	Kidney proximal tubule, placenta	Functional genetic variants exist but are uncommon	12,169
OAT3 (<i>SLC22A8</i>)	Kidney proximal tubule, choroid plexus, blood–brain barrier	Functional genetic variants exist but are uncommon	12,169
OCT2 (<i>SLC22A2</i>)	Kidney proximal tubule, neurons	Association between genotype and clinical pharmacokinetics of at least one drug	12,169,188
OATP1A2/OATP-A (<i>SLCO1A2</i>)	Brain capillaries endothelia, cholangiocytes, distal nephron	<i>In vitro</i> evidence of common functional variants, but not yet associated with clinical pharmacokinetics	12,169,180
OATP2B1/OATP-B (<i>SLCO2B1</i>)	Hepatocytes (sinusoidal), endothelia	<i>In vitro</i> evidence of common functional variants, but not yet associated with clinical pharmacokinetics	12,169,180
OCT1 (<i>SLC22A1</i>)	Hepatocytes (sinusoidal), intestinal enterocytes	Association between genotype and clinical pharmacokinetics of at least one drug	12,169,189–191
PEPT1 (<i>SLC15A1</i>)	Intestinal enterocytes, kidney proximal tubule	Functional genetic variants exist but are uncommon	12,169,191
PEPT2 (<i>SLC15A2</i>)	Kidney proximal tubule, choroid plexus, lung	<i>In vitro</i> evidence of common functional variants, but not yet associated with clinical pharmacokinetics	12,169
MATE1 (<i>SLC47A1</i>)	Kidney proximal tubule, liver (canalicular membrane), skeletal muscle	Functional genetic variants exist but are uncommon	12,169,192,193
MATE2-K (<i>SLC47A2</i>)	Kidney proximal tubule	<i>In vitro</i> evidence of functional variants, but not yet associated with clinical pharmacokinetics	12,169,193

Source: Adapted from Refs 12 and 169.

TABLE 12.4 Clinically Relevant ABC Transporters and Associated Pharmacogenetic Information

Transporter/Alias (Gene)	Organs/Cells	Pharmacogenetic Relevance	References
MDR1/P-gp, ABCB1 (<i>ABCB1</i>)	Intestinal enterocytes, kidney proximal tubule, hepatocytes (canalicular), brain endothelia	Inconsistently replicated associations between common polymorphisms and the disposition of various drugs	12,164,169
BCRP/MXR (<i>ABCG2</i>)	Intestinal enterocytes, hepatocytes (canalicular), kidney proximal tubule, brain endothelia, placenta, stem cells, mammary glands (lactating)	Association between genotype and clinical pharmacokinetics of at least one drug	12,169,187
BSEP/SPGP, cBAT, ABCB11 (<i>ABCB11</i>)	Hepatocytes (canalicular)	Common genetic variants exist, but functional effects of these are not yet demonstrated	12,169
MRP2/ABCC2, cMOAT (<i>ABCC2</i>)	Hepatocytes (canalicular), kidney (proximal tubule, luminal), enterocytes (luminal)	Association between genotype and clinical pharmacokinetics of at least one drug	12,169
MRP3/ABCC3 (<i>ABCC3</i>)	Hepatocytes (sinusoidal), intestinal enterocytes (basolateral)	<i>In vitro</i> evidence of common functional variants, but not yet associated with clinical PK	12,169
MRP4/ABCC4 (<i>ABCC4</i>)	Kidney proximal tubule (luminal), choroid plexus, hepatocytes (sinusoidal), platelets	Common genetic variants exist, but functional effects of these are not yet demonstrated	12,169
MDR3/ABCB4 (<i>ABCB4</i>)	Hepatocytes (canalicular)	Common genetic variants exist, but functional effects of these are not yet demonstrated	12,169

Source: Adapted from Refs 12 and 169.

substrates, such as digoxin, fexofenadine, and cyclosporine A, have yielded inconsistent or even contradictory results ([186] and references therein). Therefore, while the importance of P-gp for drug disposition is indisputable, the clinical consequences of *ABCB1* polymorphisms (specifically, association between genetic variants and drug pharmacokinetics) remain unclear, and the utility of *ABCB1* genetic testing remains to be determined.

The ABCG family comprises several members that are thought to form homo- or heterodimers for transporter function. The most clinically relevant member of this family is ABCG2 [also known as *breast cancer resistance protein* (BCRP)] that was initially identified by and named for its role in conferring multidrug resistance to cancer cell lines. Polymorphisms within the *ABCG2* gene are associated with gefitinib-induced diarrhea and with altered pharmacokinetics of several drugs including irinotecan, rosuvastatin, sulfasalazine, topotecan, diflomotecan, and 9-aminocamptothecin, but contradictory results have been reported for other substrates [187].

12.11 DISCUSSION

Genetic factors are important contributors toward variability in drug pharmacokinetics. Understanding the genetic bases for PK variability can allow for the safer and more efficacious use of drugs. Increased understanding of the genetic bases for PK variability in a drug is being encouraged by regulatory agencies including the US FDA, European Medicines Agency (EMA), and the Pharmaceuticals and Medical Devices Agency, Japan (PMDA), such that the pharmacogenetics of new drugs are better characterized before market launch. Pharmaceutical companies now routinely dedicate effort toward determining whether PG factors contribute to observed PK variability. Such efforts include routine collection of DNA samples in clinical PK studies and appropriate analyses of these samples for functional variants in the enzymes and transporters for which the drug is a substrate. Early generation of this information has been used to inform dose selection in early trials, provide information for population PK modeling, and contribute toward safer clinical trials.

The immense amount of information on the genetics of drug disposition, the tremendous advances in sequencing and genotyping technologies, and the decreasing costs of these technologies may serve further to the stratified use of drugs, such that all factors, including age, weight, medical history, and genetics are used in the treatment decision-making process and further “personalizing” therapy.

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13 Drug–Drug Interactions 1: Inhibition

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13.1	Introduction	1
13.2	Basic inhibition mechanisms	2
13.3	<i>In vitro</i> – <i>in vivo</i> correlations for drug–drug interactions: experimental and theoretical factors	9
13.4	Conclusions	21
	References	21

13.1 INTRODUCTION

Nearly two million adverse drug reactions are reported annually, of which 26% can be attributed to avoidable drug–drug interactions (DDIs) [1]. Predicting potential DDIs is one of the most important activities that a pharmaceutical company undertakes before the approval of any new drug. DDI predictions are primarily governed by elucidation of the metabolism of any new entity. Seventy-three percent of the top 200 drugs are cleared primarily by metabolism, of which 75% of the metabolism occurs due to cytochrome P450 enzymes, a superfamily of oxidative enzymes found mainly in the liver and also distributed in the intestine, kidneys, adrenal glands, lungs, and other organs. Of all the P450 enzymes, CYP3A4, CYP2C9, CYP2C19, and CYP2D6 are responsible for more than 80% of the oxidative metabolism of the top 200 drugs and hence are of special interest to the drug metabolism scientist [1]. Of late, other P450 enzymes such as CYP1A2, CYP2B6, and CYP2C8 have been recognized to be of increasing importance because of an expansion of drugs metabolized by these enzymes, their role in formation of toxic metabolites, and the occurrence of genetic polymorphisms affecting activity.

Although known about for many years, DDIs were generally mild to moderate in severity and could be managed clinically. However, the clinical importance of DDIs truly became front and center with the occurrence of the life-threatening and sometimes fatal interaction of combinations such as terfenadine–ketoconazole and terfenadine–erythromycin, for example, Refs 2–4. In these cases, the potentially fatal arrhythmic condition, Torsades de Pointes, was induced by the coadministration of

these agents. It was later learned that this interaction was due to a reduced metabolism of the parent drug, terfenadine, which normally undergoes rapid metabolism to a non-cardiotoxic metabolite, fexofenadine. These and similar interactions that have been identified subsequently clearly demonstrated that as medications become more potent, the potential clinical ramifications of a DDI can also become more pronounced.

When more than 25% of the clearance of a given drug is carried out by a single P450 enzyme, there is cause for concern from a DDI perspective [5]. However, a confounding factor in predicting P450-mediated metabolism of a compound is the promiscuous nature of these enzymes, allowing them to catalyze the metabolism of a wide variety of substrates with sometimes overlapping substrate specificity. A practical implication of this is that some drugs may be a substrate to a particular P450 but an inhibitor of another P450. In addition, P450 enzymes tend to be allosteric enzymes and in fact may have two or three catalytic sites within their active site, sometimes necessitating the use of multiple probe substrates for a single enzyme such as CYP3A4. Finally, the interplay of P450 enzymes with other metabolizing enzymes such as uridine diphospho glucuronosyltransferase (UGT) enzymes and glutathione S-transferase (GST) enzymes, and transporters such as P-glycoprotein (P-gp) also serve to confound the prediction of DDIs. Indeed, the US Food and Drug Administration (FDA) has released guidance documents for conducting experiments and clinical studies related to DDIs [6,7]. The focus of this chapter is to elucidate the basic concepts of the various kinds of inhibition of drug-metabolizing enzymes, the equations that can be used to describe the various types of inhibition and the prediction of the *in vivo* inhibition DDIs utilizing *in vitro* data.

13.2 BASIC INHIBITION MECHANISMS

The interaction of two drugs to produce inhibition of single or multiple enzymes can lead to DDIs with clinical consequences. Hence, the determination of a candidate drug's ability to inhibit metabolizing enzymes forms a critical part of drug discovery screens. *In vitro* studies can also help determine a drug's potency to inhibit drug-metabolizing enzymes, typically through determination of the K_i (inhibition constant) or IC_{50} (concentration producing 50% inhibition). Kinetically, enzyme inhibition can be categorized into four main types—competitive, noncompetitive, uncompetitive, and mixed. In addition, irreversible inhibition, also termed *mechanism-based inhibition*, is increasingly being observed (but will be dealt with in another chapter of this series).

The first step in the determination of the ability of a compound to inhibit an enzyme is typically the determination of its IC_{50} toward a particular enzyme–substrate combination. This involves measurement of the turnover of the substrate in the absence and presence of the purported inhibitor. Either fluorescent or luminescent assays have been used in the past for high throughput determinations of IC_{50} values for candidate compounds. Both technologies involve the use of prefluorescent (or preluminescent) substrates that are incubated with recombinant enzymes with and without inhibitor, with the fluorescence or luminescence occurring following metabolism [5] and any decrease in signal as compared to incubations absent, the inhibitor being used as a measure of inhibition. However, lack of isoform specificity (thus, requiring use of expressed enzymes) and sometimes unusually high K_m values for the reaction and frequent occurrence of atypical kinetics with these compounds has greatly limited their

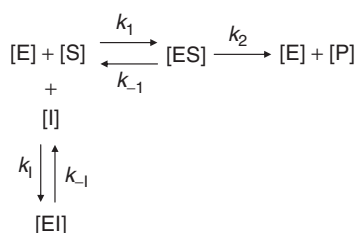
utility. Thus, because of advances in LC-MS/MS technology, use of specific probe substrates and monitoring of metabolite production by mass spectrometric methods has become the standard for inhibition kinetics determinations. Particularly with the speed and resolution of ultra performance liquid chromatography (UPLC) and the selectivity of an LC-MS/MS, minimal method development is required and highly specific substrates can be used to measure turnover. Cassette analysis wherein multiple compounds are dosed together can further enhance throughput, and utilizing robotics for sample processing can automate most of the process. However, again one must be cautious when using cassette methods because of isoform cross-reactivity of compounds.

Commonly, early in the drug discovery process, only IC_{50} values are determined since this requires just one substrate concentration (typically at or near K_m) and incubation of inhibitor at multiple levels with an enzyme source. Compounds exhibiting IC_{50} values less than $1\ \mu M$ are generally considered high risk to cause *in vivo* DDIs, while those exhibiting IC_{50} values greater than $30\ \mu M$ are generally considered to be low risk [8]. Compounds with IC_{50} values between these extremes are of concern and require additional consideration. When conducting *in vitro* inhibition analyses, solubility of inhibitor and substrate are concerns due to the increasing propensity for compounds to be very lipophilic. In this case, nephelometric methods can be used to assess whether precipitation of compound has occurred [9]. For promising drug candidates that also exhibit a low IC_{50} value toward a particular P450, it may be instructive to conduct additional experiments to determine K_i values (which are independent of substrate concentration) and the mechanism of inhibition. When determining K_i values, initial substrate conditions (turnover less than 10%), substrate not at saturation conditions (which would diminish inhibitory effects), and the possibility of atypical kinetics need to be taken into account.

The FDA recommended list of *in vivo* substrates and inhibitors (as positive controls) include the following substrate and inhibitor pairs, respectively, caffeine and fluvoxamine for CYP1A2, efavirenz and ticlopidine for CYP2B6, rosiglitazone and phenelzine for CYP2C8, *S*-warfarin and fluconazole for CYP2C9, omeprazole and fluvoxamine for CYP2C19, dextromethorphan and quinidine for CYP2D6, chlorzoxazone and disulfiram for CYP2E1, and midazolam and ketoconazole for CYP3A4/3A5 [10]. To accurately determine the type of inhibition and the K_i of the inhibitor require incubations of at least 3–4 concentrations of the substrate (bracketing the K_m) and at least 3–4 concentrations of the inhibitor. In contrast to IC_{50} values, K_i values are independent of substrate and protein concentration. Diagnostic plots, such as the Eadie–Hofstee and Dixon plots, can help determine the nature of the inhibition and give an approximate estimation of the various parameters involved [11]. The various types of *in vitro* systems that can be used have been reviewed in another chapter, but briefly, liver microsomes are considered the gold standard for *in vitro* studies and provide an excellent representation of all the enzymes present in the system. However, it should be recognized that they do not include cytosolic enzymes and transporters as well as several conjugation enzymes. If one suspects these other enzymes or transporters are involved in a compound's metabolism, use of human hepatocytes (fresh or cryopreserved) is likely a more appropriate system [12].

13.2.1 Competitive Inhibition

In competitive inhibition, the substrate and inhibitor directly compete for the catalytic site of the enzyme (Scheme 13.1). Commonly, both the inhibitor and substrate are catalyzed by the enzyme; hence, any two substrates for a single enzyme would be in competitive inhibition. Competitive inhibition (Fig. 13.1) is the most commonly observed type of inhibition and the kinetic scheme for this phenomenon is given in Scheme 13.1.



Scheme 13.1

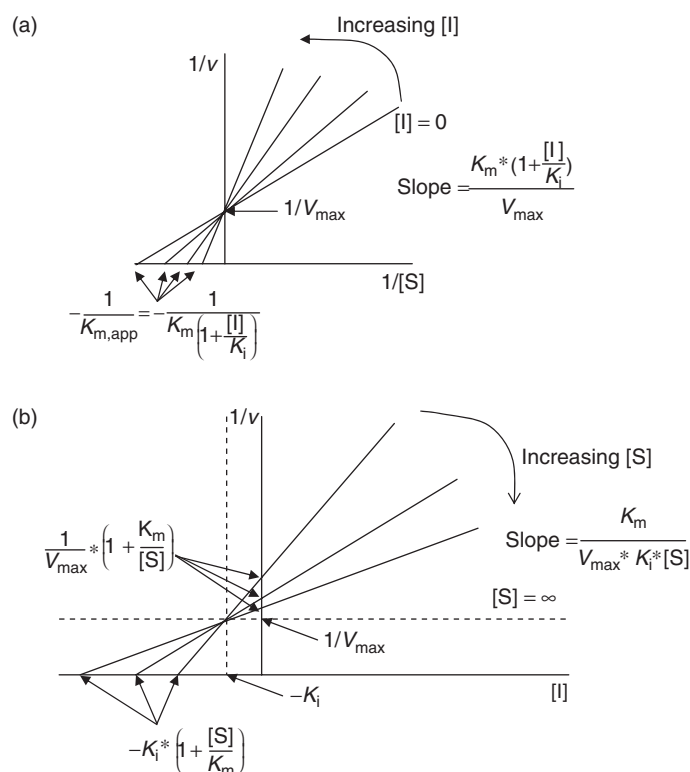


Figure 13.1 Competitive inhibition diagnostics. (a) The EH plot and (b) the Dixon plot. Both the plots can be used to diagnose competitive inhibition and calculate $V_{\max}$, K_m , and K_i .

For the purposes of this chapter, $V_{\max}$ and K_m are the Michaelis–Menten kinetic parameters for the substrate in the absence of inhibitor, while $V_{\max, \text{app}}$ and $K_{m, \text{app}}$ are the parameters in the presence of inhibitor and K_i is the first-order inhibition rate constant (k_{-1}/k_1).

In the case of competitive inhibition, in addition to the usually formed [ES] species, an [EI] species is also reversibly formed. Using a subsaturating concentration of inhibitor and high enough concentration of substrate, maximum ($V_{\max}$) velocities can be obtained. Essentially, at high enough substrate concentrations, the complex shifts away from [EI] and toward [ES], hence $V_{\max}$ is unaltered. However, to achieve this shift requires high enough substrate concentrations to offset the inhibitor concentrations; the result being increased K_m . The diagnostic plots for this kind of inhibition are given in Fig. 13.1 [11]. As seen in the Lineweaver–Burk reciprocal plot (Fig. 13.1a), the various fitted lines intersect at the y-axis at $1/V_{\max}$, while the x-axis intercept is equal to $1/K_{m, \text{app}}$. The various slopes and intercepts can be solved to obtain $V_{\max}$, K_m , and K_i . The Dixon plot (Fig. 13.1b) can also be used to diagnose competitive inhibition and to solve for all the kinetic parameters.

From the scheme above, one can derive the kinetic equation for competitive inhibition (Eq. 13.1), which is used to estimate the relevant variables:

$$v = \frac{V_{\max}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (13.1)$$

where [S] is the substrate concentration and [I] the inhibitor concentration and the other parameters are as described previously.

It is worthy to note that IC_{50} can be directly related to K_i by application of the Cheng–Prusoff Equation [13] (Eq. 13.2):

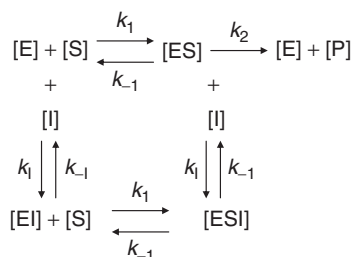
$$IC_{50} = K_i \left(1 + \frac{[S]}{K_m}\right) \quad (13.2)$$

In this case, if $[S] = K_m$ in the *in vitro* incubations, then $IC_{50} = 2 \times K_i$. Likewise, if $[S] \ll K_m$ (as in *in vivo* situations) then $IC_{50} \approx K_i$.

13.2.2 Noncompetitive Inhibition

In noncompetitive inhibition, the inhibitor can complex with either the free enzyme [E] or an enzyme–substrate complex [ES] to form an unproductive enzyme–substrate–inhibitor [ESI] complex (Scheme 13.2). In addition, the substrate can bind to the enzyme active site even when the inhibitor is already bound to the enzyme. Noncompetitive inhibition occurs when the inhibitor binds to an allosteric location on the enzyme or occupies a spot in the active site exclusive of the catalytic site. This could result in conformational changes in the enzyme or altered substrate binding. For example, curcumin noncompetitively inhibits CYP2C9 and CYP2D6, while olanzapine noncompetitively inhibits CYP2C9 and CYP3A4 [14,15]. The kinetic scheme for noncompetitive inhibition is given in Scheme 13.2.

Since [I] can complex with both [E] and [ES], forming either [EI] or [ESI], increasing the concentration of [S] is inadequate to drive the equilibrium of the reaction all the way toward the [ES] complex. The presence of the unproductive complex, [ESI],



Scheme 13.2

reduces the effective concentration of [ES] and hence reduces the maximum achievable velocity, $V_{\max}$. Since [S] and [I] bind at different sites on the enzyme, the presence of one ligand (either substrate or inhibitor) does not affect the binding affinity of the other, meaning that [S] can bind to both [E] and [EI] with equal affinity. In addition, the [ESI] complex can dissociate to form [ES] plus [I], of which the [ES] complex is then productive. For these reasons, in noncompetitive inhibition, K_m is unchanged [11]. The diagnostic plots for noncompetitive inhibition are shown in Fig. 13.2 [11]. The reciprocal Lineweaver-Burk plot (Fig. 13.2a) has a y-intercept of $1/V_{\max, \text{app}}$, while the x-intercept is $-1/K_m$. All kinetic parameters can be determined by solving for the intercepts and slope. The K_i can also be obtained from the Dixon plot (Fig. 13.2b).

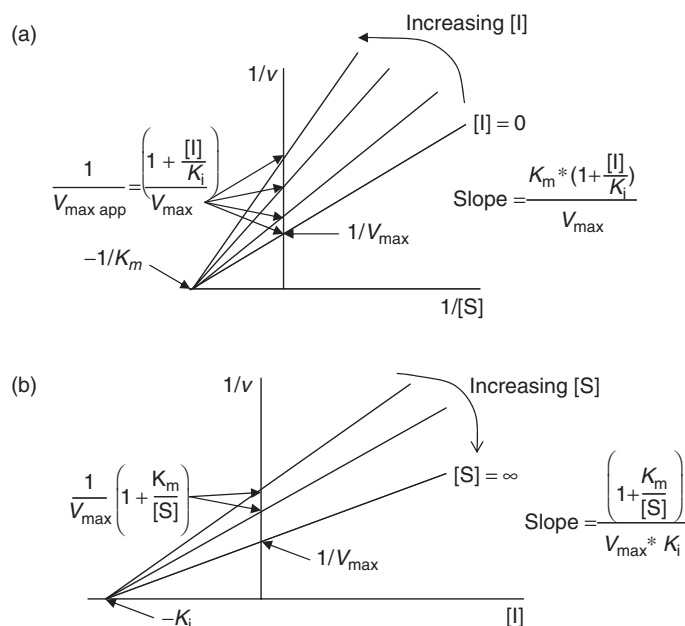


Figure 13.2 Noncompetitive inhibition diagnostics. (a) The EH plot and (b) the Dixon plot. Both the plots can be used to diagnose competitive inhibition and calculate $V_{\max}$, K_m , and K_i .

The equation for noncompetitive inhibition is given below [11]:

$$v = \frac{V_{\max}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad (13.3)$$

In this case, IC_{50} is related to K_i by the following equation:

$$IC_{50} = K_i \quad (13.4)$$

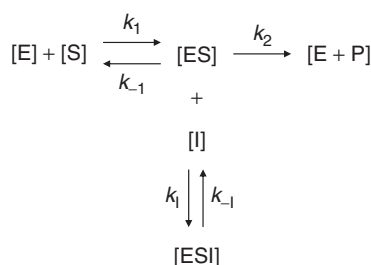
13.2.3 Uncompetitive Inhibition

In uncompetitive inhibition, the inhibitor binds only to the [ES] complex resulting in the formation of a catalytically inactive [ESI] complex (Scheme 13.3). Since substrate-bound enzyme is required to make the enzyme amenable to inhibitor binding, the inhibitor cannot directly bind to the enzyme itself without substrate being present [11]. Uncompetitive inhibition is rarely observed exclusively and when observed, usually occurs in association with other types of inhibition. However, examples of this kind of inhibition have been observed with the drug-metabolizing enzymes. For example, meloxicam uncompetitively inhibits quinidine metabolism by CYP3A4 [16]. The kinetic scheme for uncompetitive inhibition is given in Scheme 13.3.

Since [I] binds to [ES] forming an unproductive [ESI] complex, the addition of excess substrate is insufficient to convert all the [ESI] complexes to [ES] complexes, resulting in a reduction in $V_{\max}$. However, since [I] reacts with [ES] forming [ESI], thereby depleting the [ES] pool, the forward reaction of $[E] + [S]$ is favored and thus, likewise, K_m values are also decreased [11]. The diagnostic plots for uncompetitive inhibition are presented in Fig. 13.3 [11]. The Lineweaver–Burk reciprocal plot is (Fig. 13.3a) characterized by parallel lines with y -intercepts of $1/V_{\max, app}$ and x -intercepts of $-1/K_{m, app}$. The Dixon plot (Fig. 13.3b) can be used to directly obtain K_i .

The equation for uncompetitive inhibition (Eq. 13.5) is given below:

$$v = \frac{V_{\max}[S]}{K_m + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad (13.5)$$



Scheme 13.3

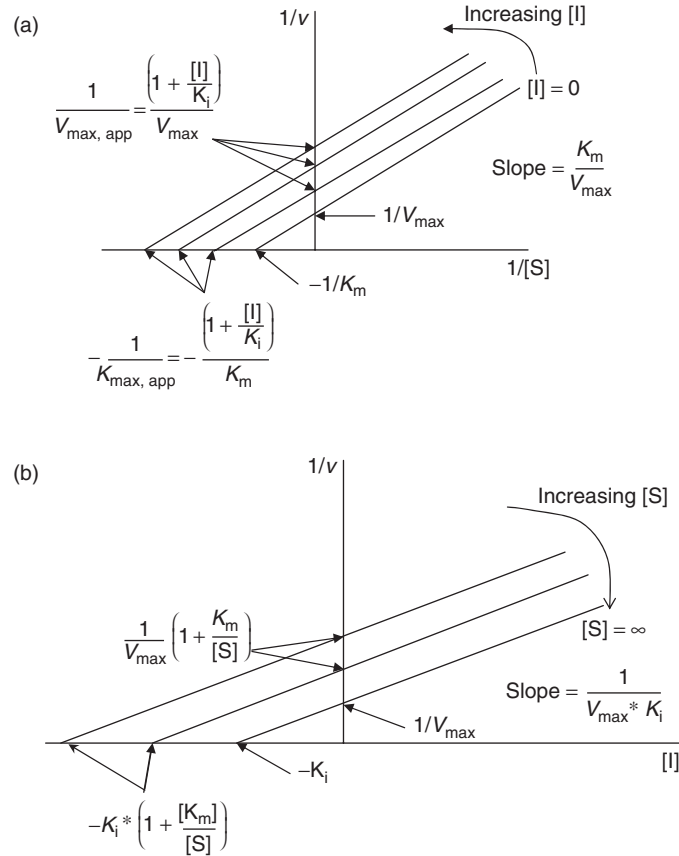


Figure 13.3 Uncompetitive inhibition diagnostics. (a) The EH plot and (b) the Dixon plot. Both the plots can be used to diagnose competitive inhibition and calculate $V_{\max}$, K_m , and K_i .

For uncompetitive inhibition, IC_{50} is related to K_i by the following equation:

$$IC_{50} = K_i \left(1 + \frac{K_m}{[S]}\right) \quad (13.6)$$

In this case, if $[S] = K_m$ then $IC_{50} = 2 * K_i$ and if $[S] \ll K_m$ (as is typical of *in vivo* situations) then $IC_{50} \gg K_i$, meaning that *in vivo*, even if the inhibitor exhibits a low K_i , achieving 50% inhibition is unlikely because of low $[S]$ concentrations [13].

With competitive inhibition, IC_{50} values are dependent on the substrate concentration employed and are usually higher than K_i values (and thus, the higher the substrate concentration, the higher the IC_{50} since the inhibitor has to compete with substrate for the active site). In the case of uncompetitive inhibition, the scenario is reversed. Since the inhibitor can only bind the enzyme after the substrate has bound to the enzyme, increasing the substrate concentrations leads to lowering of the IC_{50} . For noncompetitive inhibition, the inhibitor binds to the enzyme at a site different from the substrate-binding site, and this binding is independent of whether the substrate is

bound to the enzyme. Hence, for noncompetitive inhibition, IC_{50} is independent of substrate concentrations [13].

13.2.4 Mixed Inhibition

Mixed inhibition is for the most part a special case of noncompetitive inhibition, wherein the dissociation constants for the formation of [ES], [EI], [ESI] (formed from [ES] + [I] and [EI] + [S]), and [IE] (in cases wherein the formation of [IE] precludes further [S] binding) are different. The discussions of these various kinds of inhibition are beyond the scope of this chapter.

13.3 IN VITRO–IN VIVO CORRELATIONS FOR DRUG–DRUG INTERACTIONS: EXPERIMENTAL AND THEORETICAL FACTORS

13.3.1 In Vitro–In Vivo Correlations for Drug–Drug Interactions

Being able to predict DDIs on the basis of *in vitro* IC_{50} and K_i data is extremely important to pharmaceutical companies developing drugs. The ability to accurately accomplish this would allow for identification of DDI-based liabilities at an early stage. To this end, many groups have proposed *in vitro*–*in vivo* correlations based on drug metabolism kinetic theory and tested the equations for success in predicting clinical DDIs [17–26]. Success is usually measured by comparing predicted DDIs with values obtained from clinical trials. While the basic principles of predicting DDIs using *in vitro*–*in vivo* extrapolations (IVIVES) has remained unchanged, the methodology has constantly been refreshed. Incorporation of various sources of data (microsomes, hepatocytes, *in vivo* data), allowances for the critical inhibitor concentration, inclusion of protein binding, evaluating the role of the intestine in mediating DDIs and other factors have greatly expanded the utility of this work. This section discusses highlights of the vast literature in this area with a view toward the basic tenets underlying each approach. While these approaches were first developed for P450 enzymes, they can be applied to any drug-metabolizing enzyme, such as UGT enzymes [27].

Most drugs are either competitive or noncompetitive inhibitors of P450 enzymes; hence, the vast majority of literature uses Equations 13.1 and 13.3 to assess the effect of an inhibitor on a drug's disposition when calculating intrinsic clearance of the drug when given with an inhibitor. In the following derivations, it is assumed that the inhibitor does not affect the fraction absorbed from the intestine and the protein binding of the drug (i.e., free fraction stays the same). In addition, the well-stirred model is usually applied to model systemic clearance and is assumed below, as well. Finally, it is also assumed that Michaelis–Menten kinetics is also exhibited by the drug of interest, that is, a V_{max} and K_m can be obtained, and when $[S] \ll K_m$, $[S] + K_m \cong [S]$. When Michaelis–Menten kinetics is not applicable, such as in the case of atypical kinetics that can be observed with most P450 enzymes (although most commonly with CYP3A4 and CYP2C9), one has to make additional allowances. In this case (atypical kinetics), the K_m and V_{max} values of the relevant catalytic site (usually the higher affinity or smaller K_m) as determined by estimation using the appropriate atypical kinetic equation should be applied.

Assuming a drug is cleared by two P450 enzymes, with a fraction metabolized by the primary enzyme (f_{m1}), the intrinsic clearance of the drug from *in vitro* data, Cl_{int} is estimated as in the following Equation 17:

$$Cl_{int} = \frac{V_{max1}}{K_{m1}} + \frac{V_{max2}}{K_{m2}} = f_{m1}Cl_{int} + (1 - f_{m1})Cl_{int} \quad (13.7)$$

where V_{max1} and V_{max2} and K_{m1} and K_{m2} are the Michaelis-Menten parameters for enzymes 1 and 2, respectively. The clearance in the presence of the inhibitor (which inhibits only the primary enzyme), $Cl_{int,i}$, at concentration $[I]$ and using the inhibition rate constant K_i is then given by the following equation:

$$Cl_{int,i} = \frac{V_{max1}}{K_{m1} \left(1 + \frac{[I]}{K_i}\right)} + \frac{V_{max2}}{K_{m2}} = \frac{f_{m1}Cl_{int}}{1 + \frac{[I]}{K_i}} + (1 - f_{m1})Cl_{int} \quad (13.8)$$

The ratio of intrinsic clearances (Eq. 13.9) and the ratio of the areas under the curve (AUC, measure of exposure) (Eq. 13.10) are then given by

$$\frac{Cl_{int,i}}{Cl_{int}} = \frac{f_{m1}}{1 + \frac{[I]}{K_i}} + (1 - f_{m1}) \quad (13.9)$$

and

$$\frac{AUC_i}{AUC} = \frac{1}{\frac{f_{m1}}{1 + \frac{[I]}{K_i}} + (1 - f_{m1})} \quad (13.10)$$

where AUC and AUC_i are the areas under the curve in the absence and presence of inhibitor. Equation 13.10 is frequently referred to as the *Rowland-Martin equation* [28]. When only one enzyme is responsible for the metabolism of a drug, that is, $f_m = 1$, the equation reduces to (for competitive and noncompetitive inhibition) the following equation:

$$\frac{AUC_i}{AUC} = 1 + \frac{[I]}{K_i} \quad (13.11)$$

When two P450 enzymes are inhibited by an inhibitor, with inhibition rate constants of K_{i1} and K_{i2} for inhibitors 1 and 2 respectively, then the equation becomes

$$\frac{AUC_i}{AUC} = \frac{1}{\frac{f_{m1}}{1 + \frac{[I]}{K_{i1}}} + \frac{(1-f_{m1})}{1 + \frac{[I]}{K_{i2}}}} \quad (13.12)$$

For uncompetitive inhibition, Equation 13.11 becomes

$$\frac{AUC_i}{AUC} = 1 + \left(\frac{[S]}{K_m}\right) \left(1 + \frac{[I]}{K_i}\right) \quad (13.13)$$

A close examination of Equation 13.10 reveals that successful prediction of DDIs requires confident estimates of $[I]$, K_i , and f_m . While K_i can be reasonably well

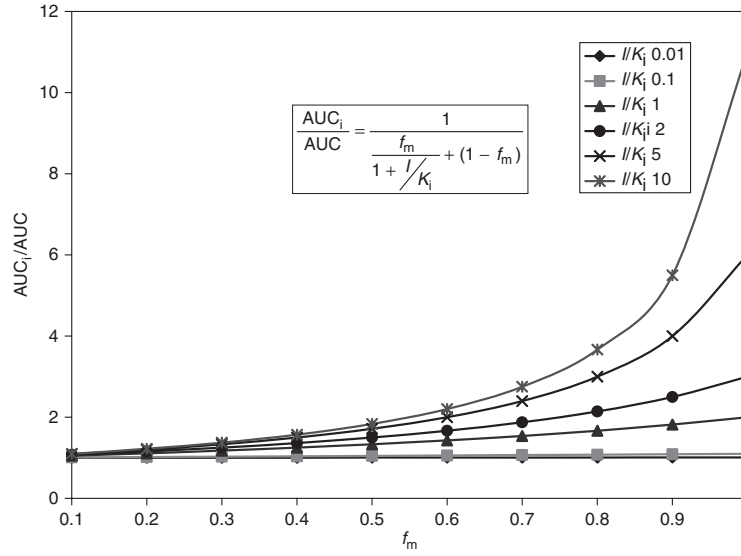


Figure 13.4 Role of f_m and I/K_i in AUC changes on inhibition. As seen, when $f_m < 0.5$, the AUC change will not be greater than twofold, hence will not have clinical significance. Also, when I/K_i is less than 2, the AUC ratio changes are not significant.

estimated from *in vitro* studies, and f_m can be determined with some measure of confidence from *in vitro* systems, considerable uncertainty exists about the value of the appropriate inhibitor concentration that will be relevant at the enzyme active site *in vivo*. Figure 13.4 gives a graphical representation of the role of f_m and the ratio $[I]/K_i$ in the AUC change. As seen, if $f_m < 0.5$ then the increase in AUC at even an infinite concentration of inhibitor will never be greater than twofold. Looking at this relationship from a different perspective, if $[I]/K_i$ is less than 2 then even a twofold increase in AUC is only observed when f_m is greater than 0.75. Hence, if $f_m < 0.5$, or if $[I]/K_i < 2$, then the possibility of a twofold AUC change is minimal.

Using the above methodology, generated *in vitro* data were compared to reports of *in vivo* DDIs culled from 193 studies published in the literature involving CYP2C9, CYP2D6, and CYP3A4/5 [18]. Three inhibitor concentrations were evaluated for their degree of contribution to the accuracy of predications: systemic average inhibitor plasma concentration ($[I]_{\text{avg}}$) after repeated oral administration, maximum inhibitor hepatic input concentration ($[I]_{\text{in}}$), and maximum inhibitor systemic plasma concentration after repeated oral administration ($[I]_{\text{max}}$). The equations used in estimating these three inhibitor concentrations are listed below as derived by Ref. 29:

$$[I]_{\text{avg}} = \frac{\left(\frac{D}{\tau}\right)}{\left(\frac{Cl}{F}\right)} \quad (13.14)$$

$$[I]_{\text{max}} = \frac{[I]_{\text{avg}} k \tau}{1 - \exp(-k \tau)} \quad (13.15)$$

$$[I]_{\text{in}} = [I]_{\text{avg}} + \frac{k_a F_a D}{Q_h} \quad (13.16)$$

where D and τ are the dose and the dosing interval, respectively, of inhibitor used in the *in vivo* interaction study, k is the elimination rate constant, k_a is the absorption rate constant, F_a is the fraction absorbed from gut into the portal vein, Q_h is the hepatic blood flow rate, and Cl/F is the oral clearance of the victim drug. The values of k_a , F_a , Q_h , and RB (blood-to-plasma concentration ratio) were assumed by these authors to be 0.1 min^{-1} , 1, 1610 mL/min, and 1, respectively. Unbound $[I]$ concentrations were also calculated by multiplying $[I]$ by the free fraction of inhibitor (f_u). The fraction metabolized (f_m) term was not included in these analyses, hence, the underlying assumption is that $f_m = 1$.

It is generally assumed that only free drug or inhibitor is available to exert its effect. However, in these simulations, the use of unbound inhibitor concentration did not always result in the best predictions. In this case, the use of unbound $[I]$ resulted in a number of false negatives. It was observed that $[I]_{\max}$ and $[I]_{\text{avg}}$ provided similar degrees of accuracy in the predictions, while use of $[I]_{\text{in}}$ resulted in the most accurate predictions. When $[I]_{\text{avg}}$ was used, accuracy of the predictions was 74%, with 7% false positives and 19% false negatives. When $[I]_{\text{avg},u}$ was used, accuracy of the predictions was 59%, with 5% false positives and 36% false negatives. In these latter two cases, fewer accurate predictions, and a higher degree of false negatives were observed, mainly because the $[I]$ values were lower. When $[I]_{\max}$ was used, accuracy of the predictions was 70%, with 9% false positives and 21% false negatives. When $[I]_{\text{in}}$ was used, accuracy of the predictions was 69%, with 29% false positives (mainly from CYP2D6 interactions) 2% false negatives. Hence, false negatives were almost completely eliminated when $[I]_{\text{in}}$ was used; however, the number of false positives was increased.

In a subsequent analysis, it was observed that the use of hepatic input concentration ($[I]_{\text{in}}$) resulted in the best balance between positive and negative interactions and, most importantly, eliminated false negatives [17]. It was also noted that there was a high incidence of false positive predictions, and many true positives were substantially overpredicted when multiple pathways were present and f_m was not included in the AUC equation. Subsequent inclusion of f_m helped reduce the number of false positives, and increased the success in predicting positive or negative interactions increased from 54% to 84%.

Another model for prediction of inhibition DDIs reported that use of $[I]_{\text{avg}}$ provided the most accurate predictions [20] and resulted in the highest number of predictions within twofold of *in vivo* AUC_i/AUC . This work also reported a corresponding reduction in bias and an increase in precision when $[I]_{\text{avg}}$ was used compared with either $[I]_{\text{in}}$ or $[I]_{\text{in},u}$ for a subset of 45 *in vivo* studies. In this case, predictions of AUC ratios for only 9% of the studies were outside twofold of actual when $[I]_{\text{avg}}$ was included in the predictive model, compared with 24% and 60% outliers for predictions in which either $[I]_{\text{in}}$ or $[I]_{\text{in},u}$ were, respectively, included. Overall, the use of the refined data significantly improved the prediction accuracy by 16% and 35% for predictions based on $[I]_{\text{in}}$ and $[I]_{\text{avg}}$, respectively, but use of $[I]_{\text{in},u}$ or $[I]_{\text{avg},u}$ worsened predictions. The use of $K_{i,u}$ was also found to improve predictions. It seems unusual that unbound K_i improves predictions, whereas total $[I]$ are associated with improved predictions. It is unclear why this paradox is observed.

Obach and coworkers also published a comprehensive evaluation of the correlation of *in vitro* predictions and *in vivo* data [21]. In this examination, estimations of K_i and IC_{50} values for drug-substrate combinations conducted in human liver

microsomes were made for CYP1A2, CYP2C9, CYP2D6, CYP3A4, and CYP2C19 enzymes, which together account for most of the observed P450 metabolism *in vivo*. The IC_{50} values of inhibitors toward all the P450 enzymes were rank ordered to determine the propensity of an inhibitor for causing inhibition *in vivo*. This *in vitro* rank order was then compared against similarly ranked *in vivo* data. In only 3 of 21 cases, the *in vitro* IC_{50} rank ordering did not correlate with the rank ordering of the *in vivo* data. An initial comparison of IC_{50} data versus *in vivo* DDIs found that inhibitors with an *in vitro* IC_{50} of less than $1\ \mu M$ were more likely to cause a twofold increase in AUC [21]. Among inhibitors with an *in vitro* IC_{50} of more than $1\ \mu M$, only 3 of 44 inhibitors resulted in a twofold or higher AUC change *in vivo*. Conversely, only two drugs that exhibited an *in vitro* IC_{50} of less than $1\ \mu M$ did not result in at least twofold AUC change *in vivo*. However, the IC_{50} requirement of $1\ \mu M$ was found not to apply in the case of mechanism-based inhibitors. These same authors also reported that unbound hepatic inlet inhibitor concentration, in general, provided the best estimates of change in AUC.

More recently, a new approach to predict DDIs utilizing *in vivo* data has been recently proposed [26]. Potential uncertainty introduced by atypical kinetics, mechanism-based inhibitors, and inability to accurately assess the contribution of intestinal metabolism from *in vitro* data are frequently reasons that *in vitro*–*in vivo* correlations of DDI predictions are not always successful. In an attempt to overcome these shortcomings, in the work of Ohno *et al.*, an apparent inhibition ratio (IR) and contribution ratio (CR), analogous to K_i and f_m , respectively, were defined. Since the largest amount of *in vivo* data were available for CYP3A4, this study examined the feasibility of this approach for that isoform only.

In this work, the CR was determined by the following equation:

$$CR_{CYP3A4} = \frac{Cl_{int,CYP3A4}}{Cl_{int}} \quad (13.17)$$

where $Cl_{int,CYP3A4}$ is the *in vivo* intrinsic clearance mediated by CYP3A4 and Cl_{int} the total *in vivo* intrinsic hepatic clearance. Compounds with a CR of 1 are fully metabolized by CYP3A4, while those with lower values of CR are metabolized by other isoforms (lower the CR, the greater the contribution by other isoforms).

An IR was also calculated from *in vivo* data using the following equation:

$$IR_{CYP3A4} = \frac{[I_{app}]}{[I_{app}] + K_i} \quad (13.18)$$

where $[I_{app}]$ is the apparent time-average unbound liver concentration. Under these conditions, an IR of 1 represents the lowest possible value of K_i and hence the most potent CYP3A4 inhibitor. Values of IR less than 1 signify less potent CYP3A4 inhibitors (lower the IR, the less potent the inhibitor).

The AUC change (for reversible inhibition) is then given by the above equation:

$$\begin{aligned} \frac{AUC_i}{AUC} &= \frac{Cl_{oral} \cdot F_a}{Cl_{oral,i} \cdot F_a} = \frac{Cl_{int}}{Cl_{int,i}} = \frac{1}{1 - CR_{CYP3A4} \cdot \frac{[I_{app}]}{[I_{app}] + K_i}} \\ &= \frac{1}{1 - CR_{CYP3A4} \cdot IR_{CYP3A4}} \end{aligned} \quad (13.19)$$

This equation essentially resembles Equation 13.10 correlating AUC change with f_m and $[I]/K_i$.

In this study, the IR and CR were calculated from 53 *in vivo* data sets, beginning with the assumption that simvastatin exhibited a CR of 1. From the *in vivo* AUC change for simvastatin interactions, the IR for the various inhibitors was calculated. This sequential approach was subsequently applied to 60 data sets to determine the CR and IR for 14 substrates and 18 inhibitors, respectively.

A portion of the data was then used as the training set to estimate CR and IR, while the remaining data set was used to check the accuracy of the predictions. The AUC ratio change was predicted within 67–150% for 50 of the studies (83%) and within 50–200% (twofold) for 57 of the studies (90%) [26]. However, this approach only works if the IR and CR of the inhibitor and the substrate are within the calculated list, meaning that *in vivo* clinical data are needed for this calculation. Thus, only the interaction potential of combinations of substrates and inhibitors from the list can be predicted. However, since the list includes 14 substrates and 18 inhibitors, a total of 252 DDIs can be predicted. However, to use this approach for new chemical entities, additional work and testing will be needed since no *in vivo* data for these compounds would exist.

An expanded review of the above work was conducted to correlate IR with K_i and CR with f_m [24]. *In vivo* and *in vitro* CR (f_m) were highly correlated and 29 of 38 combinations sets were within 50–200% when making the *in vitro* to *in vivo* CR comparison. Because of this correlation, once an *in vivo* CR is determined for a new chemical entity, then its potential for DDIs with the original set of 18 inhibitors described by Ohno *et al.* [26] can be predicted with some degree of confidence. However, the potential for DDIs with an inhibitor from outside of this list would be outside the scope of this method's predictive capabilities. IR values, however, were not well correlated with *in vitro* K_i values. Similarly, the $[I]$ concentration used (systemic unbound, systemic total, systemic maximum, or portal vein unbound concentration) also did not make a significant difference in the predictive abilities of the model. IR was correlated with dose/K_i . The authors did propose that if one used a substrate with a known CR, then a clinical trial could be conducted, wherein the new chemical entity (NCE) is the inhibitor. From this trial, IR values for the NCE can be determined and compounds with high IR values then would be flagged for additional studies.

13.3.2 Role of Intestinal Metabolism

When a drug is taken orally, it may be subject to presystemic metabolism in the intestine. While CYP3A4 is the most prevalent P450 in the gut, other important enzymes such as CYP2C9 and the UGT enzymes are also present. In addition, transporters such as P-gp are also present in the intestine. P-gp is present in the apical (luminal) side of mature intestinal enterocytes, while CYP3A4 is present in the cytoplasm of the intestinal mucosal enterocytes. Drugs that escape the efflux action of P-gp and the metabolic action of CYP3A4 enter the portal vein, wherein they can undergo metabolism by the liver CYPs and other drug-metabolizing enzymes. For some drugs, this degree of presystemic metabolism or efflux in the gut can comprise a substantial fraction of the overall metabolism, thus preventing their systemic uptake. For example, 57% of orally dosed midazolam is lost due to intestinal metabolism [30], and the liver extraction ratio and intestinal extraction ratio are almost the same (0.44 vs 0.43) [31]. Coadministration

of grapefruit juice with compounds is a convenient experiment that can be performed to determine the contribution of the intestine to clearance of a drug, since grapefruit juice reversibly and irreversibly inhibits CYP3A4 in the intestine [32].

Another indicator of the occurrence of gut metabolism is when the fold change in AUC_{oral} /fold change in AUC_{IV} with and without the inhibitor is greater than 1 [33]. In intravenous (IV) dosing with and without inhibitor, only hepatic clearance is affected by the inhibitor, while during oral dosing with and without inhibitor, both hepatic and first pass (absorption and intestinal metabolism) are affected. Hence, the ratio of oral AUC to IV AUC provides a measure of the effect of the inhibitor on first pass clearance. Ratios of up to 2.8 have been observed, indicating significant gut metabolism. However, in addition to inhibiting P450 enzymes, some inhibitors also inhibit P-gp or other transporters, thereby altering absorption [33]. Hence, the fraction absorbed of the drug also needs to be factored into gut-mediated clearance considerations.

Developing correlations for intestinal *in vitro*–*in vivo* correlations for DDIs have been an area of substantial study. Since CYP3A4 is the main enzyme responsible for metabolism in the gut, most correlation attempts have focused on inhibition of this important isoform. Since the principles of inhibition and intrinsic clearance remain the same (V_{max}/K_m is constant for CYP3A4 irrespective of its location), the basic equation governing the ratio of intestinal clearances is analogous to the equation for hepatic clearance (Eq. 13.20) [21]:

$$\frac{Cl_{int,g,i}}{Cl_{int,g}} = \frac{1}{1 + \frac{[I]_g}{K_i}} \quad (13.20)$$

where $[I]_g$ is the estimated concentration of the inhibitor in the intestinal wall during absorption and is estimated from Equation 13.21. $Cl_{int,g,i}$ and $Cl_{int,g}$ are the intestinal intrinsic clearances in the presence and absence of inhibitor, respectively. K_i is the inhibition rate constant in the gut.

The inhibitor concentration is estimated by application of the following equation [21]:

$$[I]_g = \frac{Dk_a F_a}{Q_g} \quad (13.21)$$

where F_a is the fraction absorbed, k_a the absorption rate, D the dose, and Q_g the enterocytic blood flow rate (0.3 L/min average). F_g , the intestinal bioavailability, is a function of the intestinal clearance [34] (Eq. 13.22):

$$F_g = \frac{A}{A + Cl_{int,g}} \quad (13.22)$$

where A is the absorption constant, assumed to be unaffected by the presence of inhibitors.

The F_g ratio is then determined by the following equation [34]:

$$\frac{F_{g,i}}{F_g} = \frac{A + Cl_{int,g}}{A + Cl_{int,g,i}} = \frac{1}{F_g + (1 - F_g) \left(\frac{Cl_{int,g,i}}{Cl_{int,g}} \right)} \quad (13.23)$$

where $F_{g,i}$ and F_g are the intestinal bioavailability in the presence and absence of inhibitor.

Combining these equations, estimation of the change in AUC due to inhibition, incorporating both intestinal and hepatic effects is given by the following equation [21]:

$$\begin{aligned} \frac{AUC_i}{AUC} &= \frac{\left(\frac{Cl_{int}}{F_g}\right)}{\frac{Cl_{int,i}}{F_{g,i}}} = \frac{F_{g,i}}{F_g} \frac{Cl_{int}}{Cl_{int,i}} \\ &= \frac{F_{g,i}}{F_g} \frac{1}{\left(\frac{f_{mCYP3A4}}{1 + \left(\frac{[I]_{in\ vivo}}{K_i}\right)}\right) + (1 - f_{mCYP3A4})} \end{aligned} \quad (13.24)$$

where AUC_i and AUC are the area under curves in the presence and absence of inhibitor and $Cl_{int,i}$ and Cl_{int} are the intrinsic hepatic clearances in the presence and absence of inhibitor. $[I]_{in\ vivo}$ is the systemic or hepatic inhibitor concentration and $f_{mCYP3A4}$ is the fraction metabolized by CYP3A4, since CYP3A4 is the predominant enzyme causing intestinal metabolism.

The ratio of $F_{g,i}/F_g$ must be determined from *in vivo* studies using both IV and oral dosing, in the presence and absence of inhibitors [33]. Such data sets are rare. However, the ratio can also be determined by substituting Equation 13.22 into Equation 13.24. F_g and F_h have to be determined from *in vivo* studies in anhepatic patients or from a combination of *in vitro* and *in vivo* studies [33,34]. Galetin and coworkers have compiled F_g values for 25 drugs [33]. The requirement of having *in vivo* data makes predicting intestinal DDIs more challenging than predicting hepatic DDIs.

With all of this said, the actual value of including intestinal metabolism in DDI predictions remains unclear. One study found that when the physiologically relevant unbound hepatic inlet concentration was used, ignoring the intestinal component of metabolism increased the error [21]. However, another study involving cases of time-dependent inhibition observed that the percentage of cases where predictions were within twofold accuracy actually declined from 90% to 70% when an intestinal component was considered [33]. Another study found that utilization of the intestinal component did not improve or in some cases, worsened the predictions [20]. Predictions were found to be the most accurate (with and without incorporation of intestinal component) for compounds with high intestinal bioavailability (low first pass metabolism, high F_g).

13.3.3 Role of Fraction Metabolized (f_m)

The fraction of the substrate metabolized by a given enzyme (f_m) is another key parameter included in the successful prediction of DDIs from *in vitro* data (Eq. 13.10). Hence, accurate estimation of f_m is critical to the quality of the prediction. Fortunately, f_m can generally be estimated from *in vitro* reaction phenotyping studies, wherein the substrate is incubated with individual recombinant P450 enzymes and the relative contribution of each P450 to the metabolism of the substrate is determined. A more realistic determination of f_m can be obtained from HLMs with and without specific P450 inhibitors. In addition, studies have also used *in vivo* metabolism

data to determine f_m . For example, the role of CYP2D6 in the metabolism of a substrate can be obtained by comparing poor metabolizers with extensive metabolizers [19]. Comparing the formation clearance of metabolites measured in the urine also provides the contribution of an enzyme in formation of a metabolite, provided that metabolite is formed by a single enzyme. Obviously, if multiple enzymes produce a common metabolite, then formation clearances from urine data will be of limited value. The change in f_m in the presence of the inhibitor can also be determined by this technique.

One of the common assumptions made in determining AUC changes due to enzyme inhibition is that f_m remains essentially constant for a certain drug–enzyme combination. However, f_m may vary depending on substrate concentration if K_m is substantially exceeded. At higher substrate concentrations, enzymes with larger K_m values may make a greater contribution to the drug's metabolism than would be the case at lower substrate concentrations. In addition, interindividual variation in terms of individual polymorphisms and variability in quantity of enzymes may also affect f_m values. Hence, an additional complicating factor in DDI predictions is that f_m can vary through the course of the experiment.

Several studies have examined the utility of including an f_m term in their DDI predictions from *in vitro* data [17,19]. Brown *et al.* observed that the number of predictions within twofold of actual increased from 54% to 84% when f_m was incorporated [19]. The percentage of estimations overpredicting changes in AUC decreased from 46% to 15% and the percentage of underpredictions changed from 0% to 1%. Hence, the improvement was due to the reduction in overpredictions which makes intuitive sense, because when f_m is not incorporated in the equation, the underlying assumption is that $f_m = 1$. This generally overestimates the contribution of an individual enzyme, leading to overprediction of DDI potential, when multiple enzymes may in fact be involved [19].

13.3.4 Role of Genotype

A small number of clinical trials have examined the role of genotype in DDIs. Since *in vitro* studies have demonstrated that various polymorphic variants of drug-metabolizing enzymes can be inhibited to different extents, even while employing the same substrate and inhibitor, clinical trials were performed to determine if this effect persisted *in vivo*. For example, when the CYP2C9 inhibitor fluconazole was administered to steady state (400 mg q.d. for 7 days) and then flurbiprofen (a CYP2C9 substrate) was coadministered, individuals with the CYP2C9*1/*1, CYP2C9*1/*3, and CYP2C9*3/*3 genotypes exhibited different responses to the inhibitor [35]. CYP2C9*1/*1 individuals exhibited almost a threefold decrease in flurbiprofen clearance, CYP2C9*1/*3 individuals exhibited a twofold reduction in flurbiprofen clearance, and CYP2C9*3/*3 individuals did not exhibit any change in flurbiprofen clearance in response to the inhibitor. The authors pointed out that although the formation clearance of 4'-hydroxy-flurbiprofen was reduced in all genotypes, since f_m was 10% for *3/*3, the effect on flurbiprofen clearance was minimal [35]. Hence, f_m also played an important role, and since f_m changes from CYP2C9*1 through CYP2C9*3, an interplay existed between f_m and genotype, the end result of which is to protect the lower activity variants from DDIs. In addition to CYP2C9, similar results have been obtained for CYP2D6 and CYP2C19, although in those cases, the

resulting polymorphic protein is nonfunctional resulting in an inactive protein [36,37]. Hence, for polymorphic enzymes, genotype of the individuals can affect the degree of DDI observed.

13.3.5 Role of Protein Binding

The binding of drug or inhibitor to plasma proteins or nonspecific binding to microsomal or hepatocyte proteins results in a decrease in the effective drug concentration and the potential to falsely overestimate K_i values. In the case of nonspecific binding to microsomal or hepatocyte proteins, one must carry out extensive experiments to estimate the true unbound concentration so that appropriate corrections can be made. With respect to binding to plasma proteins, some researchers have proposed carrying out incubations with microsomes or hepatocytes suspended in serum [22,38]. Incubations with freshly isolated rat hepatocytes were performed with and without serum to determine if serum incubations were an appropriate system to predict *in vivo* clearance [38]. Sixteen compounds that were cleared mainly by the liver were tested. When incubations were performed in the absence of serum, a poor correlation ($r^2 = 0.55$) of *in vitro* clearance with *in vivo* clearance was obtained. If fraction unbound in serum was determined separately and multiplied by the clearance values, then the r^2 increased to 0.86, thus accounting for the binding of the drug to the serum. This method was then extended to verify DDIs of four combinations of inhibitor and substrate by performing incubations with cryopreserved hepatocytes suspended in 100% serum [22]. First pass hepatic clearance and systemic hepatic clearance were separately predicted using this system. Ketoconazole–terfenadine, ketoconazole–indinavir, and desipramine–quinidine interactions were well correlated with *in vivo* predictions. The accuracy of the predictions was within 0.46–1.5 of the actual *in vivo* values. In this case, no corrections for microsomal binding or serum protein binding were required and since hepatocytes were used, the role of transporters and conjugation enzymes were also represented. These results are contingent on the proper choice of inhibitor concentration.

Since both K_i and $[I]$ values are affected by protein binding, the inclusion of protein binding corrections has been evaluated in a few studies [20]. Forty-five *in vivo* studies were compared with *in vitro* results generated in the investigator's laboratory. In general, use of $K_{i,u}$ improved DDI prediction twofold accuracy by at least 15%. The number of predictions within twofold increased from 56% to 91%; however, $[I]_{avg}$ was total concentration, not a free concentration. While this is a highly successful prediction, the paradox of using $K_{i,u}$ but $[I]_{total}$ is noteworthy. Use of $[I]_{avg,u}$ and $K_{i,u}$ resulted only in a 42% twofold accuracy with mainly underpredictions being responsible for the error. Use of $K_{i,u}$ was particularly important for lipophilic inhibitors with less than 0.06% free fraction (e.g., miconazole and itraconazole). Hence, while unbound concentrations are important, it is still unclear why using both $K_{i,u}$ and $[I]_u$ results in significant underpredictions.

13.3.6 Non-P450 Metabolism and Drug–Drug Interactions

While the principles of *in vitro*–*in vivo* correlations are the same for non-P450 enzymes as P450 enzymes, the methodology of obtaining the data may be different. The choice of an enzyme system is crucial to the success of the predictions. A recent

review of three test compounds, gemfibrozil, bupropion, and ezetimibe, revealed how the incorrect choice of test system can lead to wrong conclusions [12]. These authors demonstrated that HLMS underpredicted the ability of gemfibrozil to inhibit CYP2C8 in a mechanism-based manner. Since the acyl glucuronide of gemfibrozil was responsible for the interaction, use of human liver microsomes (without uridine 5'-diphospho-glucuronic acid (UDGPA)) prevented the successful prediction of this interaction, whereas use of hepatocytes would have been appropriate for prediction of this DDI [12]. Another drug, bupropion, a CYP2B6 substrate is also a CYP2D6 mechanism-based inhibitor *in vivo*, although *in vitro* human liver microsomal experiments demonstrated a high K_i (low inhibitory potency) for bupropion against CYP2D6. Once again, non-P450 reductases converted the parent to erythro- and threo-hydrobupropion, both of which are potent mechanism-based inhibitors of CYP2D6. In this instance too, use of hepatocytes would have better predicted the DDI than human liver microsomes [12]. In contrast, ezetimibe is a potent mechanism-based inhibitor of CYP3A4 in human liver microsomes, but when administered to humans, it is extensively directly glucuronidated which prevents the mechanism-based inhibition. Hence, choice of *in vitro* system is crucial in the predictions of non-P450-mediated DDIs.

Some DDIs involving the glucuronosyl transferases (UGT enzymes) and altered drug pharmacokinetics have been reported for compounds such as acetaminophen, codeine, zidovudine, carbamazepine, lorazepam, and propafenone [39]. However, in many cases, these pharmacokinetic changes are of minimal to moderate clinical significance. The reasons underlying the relatively infrequent observation of UGT DDIs have been recently reviewed [40]. The relatively high K_m values of UGT enzymes toward common drugs, which are about 10-fold higher than the typical therapeutic drug concentrations, implies that the velocity is far from being saturated, and hence, a competitive inhibitor that increases the K_m will have minimal impact on the enzyme kinetics. Hence, the continuation of linear kinetics will diminish any impact of the inhibitor on AUC. In addition, due to the high K_m values, the affinity of the UGT to the drug is poor at low substrate concentrations; hence, the hepatic extraction ratios of UGT enzymes are typically lower than observed with the P450 enzymes. A lower hepatic extraction ratio also implies a reduced chance for an adverse DDI reaction. Additionally, the key ratio of $[I]/K_i$ is typically lower for UGT enzymes than P450 enzymes, and multiple UGT enzymes usually catalyze a single substrate. For these reasons, UGT-mediated DDIs are lesser in number than P450-mediated DDIs [40].

Adding to the complexity of predicting UGT DDIs is the lack of specific substrates and inhibitors for these enzymes, although lately selective index reactions appear to have simplified the reaction phenotyping problem. The most commonly employed index substrates/reactions for some of the UGT enzymes are β -estradiol (UGT1A1, 3-glucuronide), 1-naphthol (UGT1A6), propofol (UGT1A9), and naloxone (UGT2B7) [41]. A more comprehensive review of index reactions and substrates for UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 substrates has been presented, but specific inhibitors were not identified [27].

13.3.7 Role of Drug Pharmacokinetics in Predicting DDIs

As previously alluded to, highly extracted drugs are more susceptible to DDIs, since metabolism can be assumed to be a predominant clearance pathway. This assumption

is not true if biliary excretion of the parent is also a significant clearance pathway. Hence, inhibition of metabolism can cause significant decreases in intrinsic clearance, hepatic clearance, and therefore systemic clearance for highly extracted drugs. However, if a drug has a low extraction ratio to start with then metabolism contributes a minimal amount to its clearance, and therefore further inhibition does not change the pharmacokinetic parameters significantly. For highly extracted drugs, inhibition will predominantly cause $C_{\max}$ increases and increased systemic availability, whereas for poorly extracted drugs, inhibition will cause an increase in elimination half-life with no effect on $C_{\max}$ [8].

13.3.8 Role of Circulating Metabolites

While most analyses of DDIs restrict themselves to the concentration of the inhibitor, there are situations in which the metabolites of the inhibitors can also possess inhibitory properties. The oft-cited example is that of norfluoxetine, a metabolite of fluoxetine, which is a more potent inhibitor than the parent [8]. In the case of racemic drugs such as fluoxetine and warfarin, the differential inhibitory capacities of S and R enantiomers of parent and metabolites may also impact DDIs. If the circulating concentration of the metabolite is high enough, and the metabolite exhibits a low enough K_i , significant DDIs can result from contributions of the metabolite. The new FDA MIST (Metabolites in Safety Testing) guidance requires the analysis of metabolites greater than 10% of overall exposure (sum of all peak areas) for safety and efficacy. A recent analysis found greater than 94% of drugs have circulating metabolites or are extensively metabolized, hence with potential for DDIs [42]. The role of circulating metabolites may play a factor in the underprediction of DDIs.

13.3.9 Role of Multiple Binding Sites (Allosterism)

Certain P450 enzymes, notably, CYP3A4 and CYP2C9 have multiple catalytic sites within their active sites. In addition, some UGT enzymes also exhibit this property. A number of reviews have provided detailed descriptions of the mechanisms and kinetics governing atypical kinetics [43–46]. Hence, this section only briefly describes the role of atypical kinetics in DDIs.

A direct implication of enzyme allosterism in DDI predictions is the characteristic of substrate-dependent inhibition, wherein the same inhibitor can exhibit differing K_i values depending on the substrate. Thus, reliance on probe substrate results with the compound of interest as the potential perpetrator inhibitor may be tenuous. Substrate-dependent inhibition has been observed for CYP2C9, CYP3A4, and CYP2D6, where K_i values have been found to differ by orders of magnitude depending on the substrate [35,47–49]. One method of addressing this has been to employ multiple substrates for determination of K_i values, as has been done for CYP3A4, since this enzyme is thought to have up to three catalytic sites within its active site [21,50]. Similar considerations may be true for CYP2C9 as well [51]. Substrate-dependent inhibition has also been observed *in vivo* in a randomized, double-blind crossover three phase clinical trial [52]. Nine subjects were orally administered 50-mg mibefradil or 5-mg isradipine or placebo for 3 days followed by the administration of a single dose of 0.25-mg triazolam. Mibefradil, but not isradipine, was found to increase the AUC, plasma concentration, and elimination half-life of triazolam compared to placebo. Hence, although mibefradil,

isradipine, and triazolam are all substrates of CYP3A4, only mibefradil had an effect on triazolam disposition, demonstrating the *in vivo* relevance of substrate-dependent inhibition.

Other types of atypical kinetics may also confound *in vitro*–*in vivo* correlations and prediction of DDIs, particularly as it relates to the choice of substrate concentrations. For example, in the case of sigmoidal kinetics, use of very low substrate concentrations will lead to the inhibition of the high affinity binding site, while slightly higher concentrations will lead to the inhibition of the lower affinity binding site [51]. In the case of biphasic kinetics, the K_m of interest is usually the higher affinity, lower capacity site; hence, substrate concentrations around the K_{m1} are most appropriate for *in vitro* testing.

In some cases, where competitive inhibition between two substrates is expected heteroactivation can instead be observed. In heteroactivation, the purported inhibitor is actually an activator and increases the turnover of the substrate, while simultaneously undergoing metabolism, for example, dapsone activating flurbiprofen metabolism by CYP2C9 [53]. Thus, determination of whether an interaction actually will occur is confounded by this activation, rather than the expected inhibition.

13.4 CONCLUSIONS

Predicting DDIs is important to the discovery and development of a drug. Unexpected toxicities due to DDIs can lead to adverse patient outcomes, sometimes fatal, causing the withdrawal of a drug from the market. Considering the cost of discovering and bringing to market a new drug, even isolated incidents of DDIs can have potentially serious consequences for both the patient and the manufacturer. Hence, being able to predict (and subsequently cease) development of compounds that have the potential to cause DDIs is crucial to the success of a drug. While the science of predicting DDIs from *in vitro* data has advanced substantially, and the basic principles elaborately elucidated, a 100% prediction success rate has not yet been achieved. Thus, additional research is needed to further refine our capabilities to predict DDIs from *in vitro* data and enhance the drug discovery and development process.

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14 Clinical Implications of CYP Induction-Mediated Drug–Drug Interactions

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14.1	Introduction	1
14.2	Effects of CYP induction on pharmacokinetics	2
14.3	Effects of CYP induction on pharmacodynamics	11
14.4	Conclusions	16
	References	17

14.1 INTRODUCTION

There are two major concerns with cytochrome P450 (CYP) induction-mediated drug interaction in clinical practice. First, induction may cause a reduction in therapeutic efficacy of comedications. For drugs whose effect is produced primarily by the parent drug, induction would increase the drug's elimination, resulting in lower drug concentrations and decrease the drug's pharmacological effect. Second, induction may create an undesirable imbalance between detoxification and activation as a result of increased formation of reactive metabolites, leading to an increase in the risk of metabolite-induced toxicity. Unlike CYP inhibition, which is an almost immediate response, CYP induction is a slow regulatory process involving gene transcription, replication of RNA, and biosynthesis of protein. Enzyme induction occurs when a drug stimulates the synthesis of more enzyme protein. During CYP induction, it takes time to reach a new steady state level of CYP enzymes as a result of the balance between the rate of biosynthesis and degradation. On the other hand, the induced CYP enzymes return gradually to basal levels via protein degradation after the inducer is discontinued. The purpose of this chapter is to review the effects of CYP induction on pharmacokinetics of drugs and its clinical implications.

14.2 EFFECTS OF CYP INDUCTION ON PHARMACOKINETICS

14.2.1 Theoretical Considerations

Although CYP induction can be readily evidenced by direct measurement of the enzyme amount and activity in the liver in animal studies, it is difficult to perform direct assessment of CYP induction *in vivo* in humans by measuring enzyme amount and activity in the liver, because of ethical consideration and practical limitations. A common but indirect way of assessing the effect of CYP induction in clinical settings is the comparison of plasma AUC of a drug before and after coadministration of an inducer. For this reason, it is important to understand the kinetic relationship between enzyme induction and plasma AUC.

If a drug is metabolized exclusively by the liver, the total clearance (CL_{total}) of the drug can be considered to be equal to the hepatic clearance (CL_H), which can be expressed as Equation 14.1 or Equation 14.2, according to the well-stirred model or parallel-tube model [1]:

$$CL_{\text{total}} = CL_H = Q_H \cdot E = (Q_H \cdot f_u \cdot CL_{\text{int}}) / (Q_H + f_u \cdot CL_{\text{int}}) \quad (14.1)$$

$$CL_{\text{total}} = CL_H = Q_H \cdot E = Q_H \cdot (1 - e^{-f_u \cdot CL_{\text{int}} / Q_H}) \quad (14.2)$$

where Q_H is the hepatic blood flow, E is the hepatic extraction ratio, CL_{int} is the intrinsic clearance, and f_u is the unbound fraction of drug in the blood. Drugs can be further classified as low or high clearance compounds, depending on whether their elimination clearance is enzyme limited or flow limited [1]. Elimination clearance of a drug, which is much less than hepatic blood flow, is termed *enzyme limited*. In contrast, elimination clearance of a drug is termed flow limited when it is equal to or approaching hepatic blood flow.

Following an oral absorption, hepatic bioavailability (F_H) can be expressed as Equation 14.3 or Equation 14.4, depending on the well-stirred model or parallel-tube model.

$$F_H = 1 - E = \frac{Q_H}{(Q_H + f_u \cdot CL_{\text{int}})} \quad (14.3)$$

$$F_H = 1 - E = e^{-f_u \cdot CL_{\text{int}} / Q_H} \quad (14.4)$$

As shown in Equations 14.3 and 14.4, an increase in the CL_{int} as a result of enzyme induction will cause an increase in first-pass metabolism resulting in a decrease in bioavailability. Following an oral administration, the oral AUC_{po} can be expressed as Equation 14.5 or Equation 14.6, according to the well-stirred model or parallel-tube model.

$$AUC_{\text{po}} = \frac{F_H \cdot f_a \cdot \text{Dose}}{CL_H} = \frac{f_a \cdot \text{Dose}}{f_u \cdot CL_{\text{int}}} \quad (14.5)$$

$$AUC_{\text{po}} = \frac{F_H \cdot f_a \cdot \text{Dose}}{CL_H} = f_a \text{Dose} \frac{(e^{-f_u \cdot CL_{\text{int}} / Q_H})}{Q_H(1 - e^{-f_u \cdot CL_{\text{int}} / Q_H})} \quad (14.6)$$

where f_a is the fraction of dose absorbed from the gastrointestinal lumen.

On the other hand, the AUC_{iv} following intravenous administration can be expressed as Equation 14.7 or Equation 14.8.

$$AUC_{iv} = \frac{\text{Dose}}{CL_H} = \frac{\text{Dose}}{\left[\frac{Q_H \cdot f_u \cdot CL_{int}}{Q_H + f_u \cdot CL_{int}} \right]} \quad (14.7)$$

$$AUC_{iv} = \frac{\text{Dose}}{CL_H} = \frac{\text{Dose}}{Q_H(1 - e^{-f_u \cdot CL_{int}/Q_H})} \quad (14.8)$$

To illustrate the effect of enzyme induction on the AUCs of drugs after oral and intravenous administration, computer simulations have been carried out for drugs using Equations 14.5–14.8. For simplicity, the fraction (f_a) of absorption of drug from intestinal lumen and the unbound fraction of drug in plasma are assumed to remain unchanged during enzyme induction. The hepatic blood flow is assumed to be 1500 mL/min for the simulations. As shown in Figs. 14.1 and 14.2, an increase in CL_{int} of a drug caused by enzyme induction always results in a decrease in AUC of the drug, with the exception of high extraction ratio drugs ($E > 0.9$) after intravenous administration.

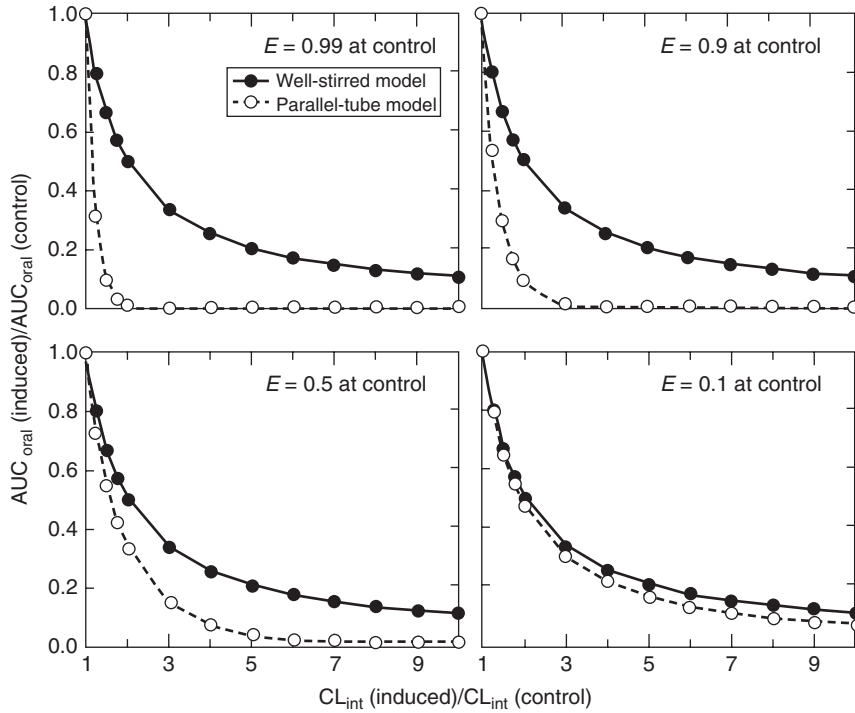


Figure 14.1 Simulations of effect of enzyme induction on the AUC of drugs after oral administration conducted using Equations 14.5 and 14.6. On the basis of their extraction ratio (E), drugs are divided into high clearance ($E > 0.9$), moderate clearance ($E = 0.5$), and low clearance ($E < 0.1$) drugs. For simplicity, the f_a and f_u are assumed to be unchanged during enzyme induction. The hepatic blood flow is assumed to be 1500 mL/min for the simulations.

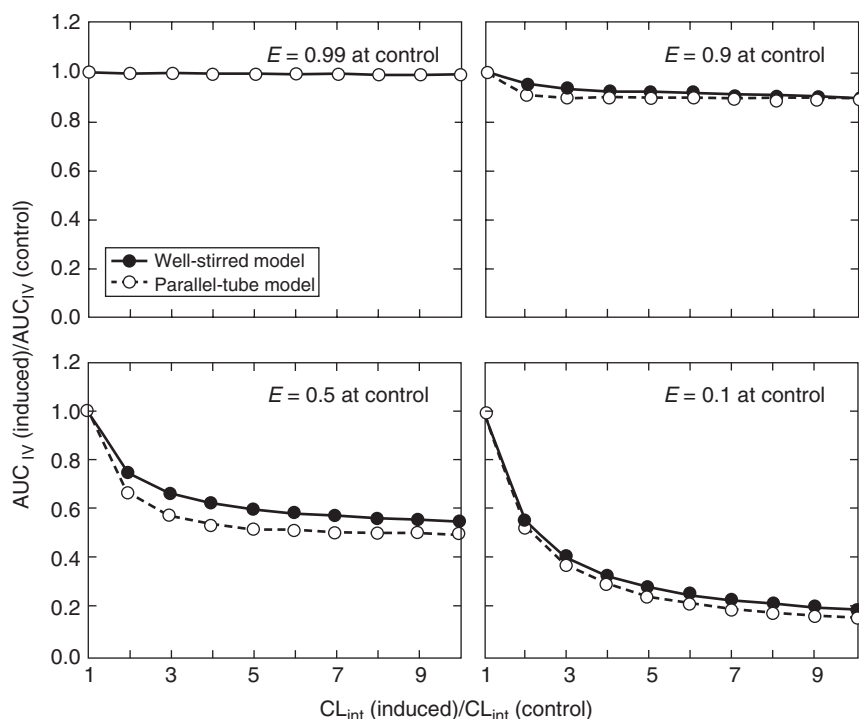


Figure 14.2 Simulations of effect of enzyme induction on the AUC of drugs after intravenous administration conducted using Equations 14.7 and 14.8. On the basis of their extraction ratio (E), drugs are divided into high clearance ($E > 0.9$), moderate clearance ($E = 0.5$), and low clearance ($E < 0.1$) drugs. For simplicity, the f_a and f_u are assumed to be unchanged during enzyme induction. The hepatic blood flow is assumed to be 1500 mL/min for the simulations.

As shown in Fig. 14.1, for low clearance drugs ($E < 0.1$), there is no significant difference in the simulations by either using hepatic well-stirred model or using parallel-tube model. For example, for low clearance drugs ($E < 0.1$), a twofold increase in the CL_{int} resulted in a twofold decrease in AUC, independent of hepatic model (well-stirred or parallel-tube model). However, for moderate and high clearance drugs ($E > 0.5$), enzyme induction appeared to have more profound effect on the AUC of drug after oral administration when the simulations were conducted using parallel-tube model compared to that using well-stirred model. For example, for high clearance drugs ($E = 0.9$), a 2-fold increase in the CL_{int} resulted in a 10-fold decrease in AUC when hepatic parallel-tube model was used for simulation, whereas a 2-fold increase in the CL_{int} resulted in a 2-fold decrease in AUC when hepatic well-stirred model was used. As discussed later, clinical data of CYP induction-mediated drug interactions appear to support the notion that hepatic parallel-tube model is more appropriate for prediction of CYP induction-mediated drug interaction, particularly for moderate and high clearance compound, following oral administration. This is consistent with the general belief that parallel-tube model is better than well-stirred hepatic model for describing the disposition of moderate and high clearance drugs [1,2].

Following intravenous administration, an increase in the CL_{int} has little effect on the AUC_{iv} of high clearance drugs ($E > 0.9$) (Fig. 14.2). As shown in Equations 14.1

and 14.2, when the CL_{int} of a drug is much greater than hepatic blood flow (Q_H), the hepatic extraction and clearance are insensitive to changes in CL_{int} of the drug. This is because hepatic blood flow is the rate-limiting step for drug delivery to the liver. Since the elimination clearance of high clearance drugs is hepatic blood flow limited, it is not surprising to see that enzyme induction has little effect on the AUC of high clearance drugs after intravenous administration. On the other hand, the clearance of low clearance ($E = 0.1$) drugs is highly dependent on the enzyme activity. Thus, the clearance of low clearance drug is enzyme limited and sensitive to enzyme induction. As shown in Fig. 14.2, a twofold increase in the CL_{int} resulted in a twofold decrease in AUC of low clearance drugs after intravenous administration. The effect of enzyme induction on the AUC of low clearance drugs after oral dosing is quite similar to that following intravenous administration (Fig. 14.1).

From the above simulations, it becomes evident that the magnitude of AUC reduction caused by enzyme induction is not only dependent on the drug's characteristics, being a low or high clearance compound, but also dependent on the route of administration of the drug.

14.2.2 Route-Dependent CYP Induction

The notion that the magnitude of AUC changes caused by CYP induction is highly dependent on the route of drug administration is best exemplified by the case of pentobarbital–alprenolol interaction. Alprenolol, a high hepatic clearance drug with a clearance of 1200 mL/min which is approaching to the hepatic blood flow of 1500 mL/min in humans, is known to be induced by pentobarbital in humans. In a clinical study, alprenolol was administered orally or intravenously to 5 healthy subjects on 2 different occasions before and after 10–14 daily doses of 100 mg pentobarbital [3]. The AUC_{po} of alprenolol after a 200 mg oral dose decreased significantly from an average of 706–154 ng h/mL with barbiturate treatment. In contrast, the AUC_{iv} of alprenolol following an intravenous administration of 5 mg dose only slightly decreased from 67 to 58 ng h/mL with pentobarbital treatment. There was a 458% decrease in the AUC_{po} of alprenolol after oral dosing but only a 15% decrease in the AUC_{iv} of alprenolol after intravenous administration.

The differential effect of route administration has also been reported for another high clearance drug, verapamil, which has a clearance of > 1000 mL/min in humans. Treatment with rifampicin had little on the AUC_{iv} of (*S*)- and (*R*)-verapamil after intravenous administration of verapamil, whereas it caused a substantial decrease in the AUC_{po} of (*S*) and (*R*)-verapamil after oral administration of verapamil in humans [4]. After rifampicin treatment for 16 days (600 mg/day), the AUC_{po} of active (*S*)-verapamil decreased from an average of 151 to 5 ng h/mL after oral administration (120 mg), while the AUC_{iv} of (*S*)-verapamil decreased from 77 to 61 ng h/mL following intravenous dosing (10 mg). There is a 3000% decrease in the AUC_{po} of active (*S*)-verapamil after oral administration, while only a 20% decrease in the AUC_{iv} of (*S*)-verapamil following intravenous administration. Similar observations of rifampicin's effects were observed for (*R*)-verapamil after oral and intravenous administration.

Rifampicin also had small effect on the AUC_{iv} of nifedipine, a drug with a clearance of 700 mL/min in humans, after intravenous administration, whereas it greatly reduced the AUC_{po} of nifedipine following oral administration [5]. After seven days of rifampicin treatment (600 mg/day), the AUC_{po} of nifedipine decreased from 280 to

18 ng h/mL when a 20 mg oral dose of nifedipine was given. In contrast, the AUC_{iv} of nifedipine only slightly reduced from 38 to 27 ng h/mL, when the drug was dosed intravenously at 20 μ g/kg body weight. There was a 1555% decrease in the AUC_{po} of nifedipin after oral administration, while only a 30% decrease in the AUC_{iv} after intravenous dosing.

From the above examples, it is evident that CYP induction has a little effect on the AUC_{iv} of high clearance drugs after intravenous administration, while it causes a marked decrease in the AUC_{po} of high clearance drugs following oral administration. Kinetically, the AUC_{iv} of a drug after intravenous administration is determined solely by the systemic clearance, as indicated in Equations 14.7 and 14.8. Because the systemic clearance of high clearance drugs is limited by hepatic blood flow, it is insensitive to the changes in enzyme activity. Therefore, for high clearance drugs, an increase of CL_{int} caused by CYP induction will have little effect on the AUC_{iv} after intravenous administration. In contrast, the AUC after oral administration (AUC_{po}) is determined by both systemic clearance and bioavailability, as shown in Equations 14.5 and 14.6. Although the systemic clearance is not sensitive to the changes in enzyme activity for high hepatic clearance drugs, their hepatic first-pass metabolism and bioavailability are very sensitive to the changes in enzyme activity.

Unlike high clearance drugs, enzyme induction yields significant effects on the systemic clearance and thereby AUC for low clearance drugs, independent of the route of administration. The systemic clearance of low clearance drugs is enzyme limited and sensitive to the changes in enzyme activity. Because low hepatic clearance drugs are generally not subject to significant first-pass metabolism, both the AUC_{iv} and AUC_{po} are determined mainly by the systemic clearance. Therefore, an increase in the intrinsic clearance caused by CYP induction will have similar magnitude of inductive effect on both the AUC_{iv} and AUC_{po} of the low hepatic clearance drugs. As shown in Figs. 14.1 and 14.2, it is expected that the magnitude of the changes in the AUC of the low clearance drugs will be quantitatively similar between oral and intravenous administration.

The methadone–rifampicin interaction is a good example that both the AUC_{iv} and AUC_{po} of the low clearance drugs are sensitive to the changes of enzyme activity caused by CYP induction. In a study, the effect of rifampicin (600 mg/day for 5 days) on the pharmacokinetics of methadone has been studied with 12 healthy volunteers [6]. Methadone, a low clearance drug (140 mL/min), is used to treat opiate addiction by preventing opiate withdrawal syndrome. After an intravenous dose (4.5 mg), the AUC_{iv} of methadone decreased from an average of 816 ng h/mL before rifampicin treatment to 259 ng h/mL after rifampicin treatment. Similarly, the AUC_{po} of methadone decreased from an average of 1128 ng h/mL before rifampicin treatment to 282 ng h/mL after oral administration of methadone (10 mg). The rifampicin treatment caused a similar magnitude of reduction of methadone AUC after both oral and intravenous administration. There was a 3.2-fold decrease in the AUC_{iv} of methadone after intravenous administration and a 4-fold decrease in the AUC_{po} of methadone after oral administration.

14.2.3 Clearance-Dependent CYP Induction

Another intriguing aspect of the effect of CYP induction on the pharmacokinetics of drugs is that the magnitude of AUC reduction caused by enzyme induction is dependent on the drug's characteristics, being a low or high clearance compound.

As shown in Fig. 14.1, the magnitude of AUC_{po} reduction after oral administration appears to be greater for high clearance drugs compared to that for low clearance drugs. Cyclosporine, tacrolimus, methadone, alprazolam, diazepam, zolpidem, and zolpiclone are metabolized mainly by CYP3A4 and are categorized as low clearance drugs because their clearance (<200 mL/min) is much smaller than hepatic blood flow (1500 mL/min). On the other hand, nifedipine, midazolam, triazolam, and indinavir are good substrate for CYP3A4 and are considered as moderate clearance drugs as their clearance is about half of hepatic blood flow, ranging from 400 to 700 mL/min. Verapamil, a good substrate of CYP3A4, has a clearance of 1000 mL/min and is categorized as high clearance drugs. Clinical studies have shown that treatment with rifampicin (a potent CYP3A4 inducer) caused significant but different magnitude of reduction in the AUC_{po} of these drugs. As shown in Table 14.1, the magnitude of the reduction in the AUC_{po} appears to be smaller for the low clearance drugs (3- to 8-fold) than that for moderate clearance drugs (9- to 24-fold) or high clearance drugs (30- to 50-fold) [4–16]. As shown in Table 14.1, high clearance drugs are more sensitive to CYP induction as compared to low clearance drugs. These observations are very similar to the simulations when parallel-tube model, but not well-stirred model, was employed (Fig. 14.1). In other words, simulations by parallel hepatic model, rather than well-stirred model, more accurately reflect the *in vivo* CYP induction-mediated drug–drug interactions.

CYP2C8 and CYP2C9 can also be induced by rifampicin. However, the effect of rifampicin on drugs that are predominately metabolized by CYP2C8 or CYP2C9 appears to be less significant compared to that on drugs metabolized by CYP3A4 (Table 14.2). Rifampicin treatment only caused three- to fourfold changes in the AUC_{po}

TABLE 14.1 Effect of Rifampicin on the Oral AUC of Drugs that are Metabolized Predominately by CYP3A4

Drug	Type of Clearance (CL) ^a	Rifampicin (mg/d)	AUC (ng h/mL)		Fold of Induction ^b	References
			Before RIF	After RIF		
Cyclosporine	Low	600 × 11 d	8986	2399	3.7	7
Tacrolimus	Low	600 × 18 d	351	112	3.1	8
Methadone	Low	600 × 5 d	1128	262	4.3	6
Alprazolam	Low	450 × 4 d	224	28	8.0	9
Diazepam	Low	600 × 7 d	4430	1040	4.2	10
Zolpidem	Low	600 × 5 d	1202	336	3.6	11
Zolpiclone	Low	600 × 5 d	473	86	5.5	12
Quinidine	Moderate	600 × 7 d	8000	910	8.8	13
Midazolam	Moderate	600 × 5 d	612	25	24.0	14
Triazolam	Moderate	600 × 5 d	14.8	0.74	20.0	15
Nifedipine	High	600 × 7 d	280	18	15.5	4
Indinavir	High	600 × 8 d	18.8 ^c	1.2 ^c	16.0	16
S-Verapamil	High	600 × 12 d	152	5	30.0	4
R-Verapamil	High	600 × 12 d	724	14	52.0	4

Abbreviation: RIF, rifampicin.

^aType of clearance: low clearance <200 mL/min; moderate clearance >500 mL/min; and high clearance >1000 mL/min.

^bFold induction: the ratio of AUC before and after rifampicin treatment.

^cμg h/mL.

of warfarin, which is metabolized predominately by CYP2C9 [17]. After the treatment with rifampicin at 600 mg twice daily for 4 days, the AUC_{po} of (*R*)-warfarin decreased from an average of 159–47 µg h/mL, while the AUC_{po} of (*S*)-warfarin decreased from 232 to 60 µg h/mL in healthy volunteers receiving an oral dose of racemic warfarin.

Rosiglitazone, an insulin-sensitizing agent, is eliminated mainly by CYP2C8 [22]. After the treatment of rifampicin 600 mg once daily for 6 days in healthy volunteers, the AUC_{po} of rosiglitazone decreased from an average of 2676–988 ng h/mL after an oral administration [18]. Glimepiride is a new sulphonylurea antidiabetic agent, which is eliminated mainly by CYP2C9 [23]. There was only a 1.5-fold decrease in the AUC_{po} of glimepiride after the treatment of rifampicin 600 mg once daily for 5 days [19]. Following an oral administration, the AUC_{po} of glimepiride decreased from 287 ng h/mL before the rifampicin treatment to 190 ng h/mL after the rifampicin treatment. Similarly, rifampicin has less profound effect on the AUC_{po} of gliclazide, glyburide, and glipizide, which are eliminated predominately via metabolism by CYP2C9 [20,21]. The magnitude of reduction in the AUC_{po} after the rifampicin treatment ranged from 1.3- to 2.9-fold for gliclazide, glyburide, and glipizide (Table 14.2).

All of the CYP2C8/CYP2C9 drugs listed in Table 14.2 can be classified as low hepatic clearance drug because their clearance value is less than 200 mL/min in humans. The relatively small changes in the AUC_{po} of these CYP2C8/CYP2C9 drugs after rifampicin treatment are consistent with the notion that the magnitude of reduction in the AUC_{po} after CYP induction tends to be lower for low hepatic clearance drugs compared to that for high hepatic clearance drugs. However, even for low hepatic clearance drugs, the effect of rifampicin appears to be less significant for CYP2C8/CYP2C9 drugs compared to CYP3A4 drugs. As shown in Tables 14.1 and 14.2, the changes in AUC_{po} were generally smaller for CYP2C8/CYP2C9 drugs (1.3- to 3.5-fold) than that for low clearance drugs of CYP3A4 (3.5- to 8-fold). The differences in the magnitude of AUC_{po} reduction between CYP3A4 drugs and CYP2C8/CYP2C9 drugs may be because of differential induction between different CYP genes by rifampicin, suggesting that the *CYP2C8* and *CYP2C9* genes are less sensitive to rifampicin as compared with *CYP3A4* gene.

TABLE 14.2 Effect of Rifampicin on the Oral AUC of Drugs that are Metabolized Predominately by CYP2C8 or CYP2C9 in Humans

Drug	Type of Clearance (CL) ^a	Rifampicin (mg/d)	AUC (ng h/mL)		Fold of Induction ^b	References
			Before RIF	After RIF		
Rosiglitazone	Low	600 × 6 d	2676	988	2.7	18
Glimepiride	Low	600 × 5 d	287	190	1.5	19
Gliclazide	Low	600 × 6 d	44 ^c	15 ^c	2.9	20
Glyburide	Low	600 × 5 d	324	198	1.6	21
Glipizide	Low	600 × 5 d	801	621	1.3	21
<i>S</i> -Warfarin	Low	600 × 4 d	220 ^c	59 ^c	3.7	17
<i>R</i> -Warfarin	Low	600 × 4 d	159 ^c	48 ^c	3.3	17

Abbreviation: RIF, rifampicin.

^aType of clearance: low clearance <200 mL/min.

^bFold induction: the ratio of oral AUC before and after rifampicin treatment.

^cµg h/mL.

The argument that *CYP2C8* and *CYP2C9* genes are less sensitive to rifampicin is supported by data from *in vitro* studies. In a study with human hepatocytes, the *CYP3A4* mRNA increased about 25-fold, while the *CYP2C8* and *CYP2C9* mRNA only increased 3- to 4-fold after incubation for 24 h with rifampicin [24]. In another study with cultured human hepatocytes, protein and activity of *CYP2C8* and *CYP2C9* were induced by 3- to 5-fold after rifampicin treatment, while the protein and activity of *CYP3A4* was induced by 10-fold after rifampicin treatment [25]. Similar observations of differential induction of CYP genes by rifampicin have been reported by other investigators [26,27].

14.2.4 Time- and Dose-Dependent CYP Induction

Enzyme induction is a slow regulatory process, involving gene transcription, replication of RNA, and biosynthesis of protein. Therefore, the CYP induction is expected to be a time- and concentration (dose)-dependent process. The time- and concentration-dependent induction of *CYP1A1* and *CYP1A2* by tetrachlorodibenzo-*p*-dioxin, (TCDD) has been demonstrated in studies using human splenic lymphocytes and colon carcinoma cell line LS180 [28,29]. In the human splenic lymphocytes and LS180, TCDD markedly induced mRNA, protein, and enzyme activity in a time- and concentration-dependent manner. In the LS180 cell cultures, maximal mRNA induction of *CYP1A1* and *CYP1A2* occurred between 6 and 24 h after the treatment with TCDD. *CYP1A1* protein reached to its maximum at 48 h, while the *CYP1A2* protein reached its maximum between 48 and 72 h. These results clearly suggest that there is a lag time between the mRNA translation and protein synthesis, reflecting an extra time required for protein synthesis.

Consistent with *in vitro* observations, time- and dose-dependent TCDD-mediated *CYP1A1/2* induction has also been demonstrated in rats *in vivo* [30]. The protein expression of *CYP1A1* and *CYP1A2* was induced in a dose-dependent manner in rats over a dose range of TCDD (0.01–30 µg/kg). The ED₅₀ for TCDD-induced *CYP1A1* and *CYP1A2* protein expression was estimated to be 0.22 and 0.4 µg/kg, respectively. Although a significant increase in the expression of TCDD-induced *CYP1A1* and *CYP1A2* protein was observed at 24 h, both *CYP1A1* and *CYP1A2* reached their maximal levels at 7 days following a single oral dose of TCDD (10 µg/kg). After reaching the peak, the protein level declined slowly via protein degradation. Even at 35 days after a single dose of TCDD administration, *CYP1A1* and *CYP1A2* protein still did not return to the basal levels. The persistence of the expression of TCDD-induced *CYP1A1* and *CYP1A2* protein may be related to the slow hepatic elimination of TCDD. Hepatic concentration of TCDD reached a maximum about 8 h after TCDD administration followed by a slow elimination with a half-life of approximately 10 days. A physiologically based pharmacokinetic model incorporated tissue retention of TCDD and its affinity binding to arylhydrocarbon receptor (AhR) has been successfully developed to describe the persistence of *CYP1A* induction in rats and mice for TCDD and its analogs [31–33].

In addition to TCDD, the time- and dose-dependent induction of *CYP1A1* and *CYP1A2* by omeprazole has been demonstrated in human hepatoma cell line HepG2 [34,35]. Consistent with *in vitro* observations, dose-dependent induction of *CYP1A1/2* caused by omeprazole has also been observed in humans *in vivo*. Induction of intestinal *CYP1A* by omeprazole was studied in six healthy volunteers [36]. In this study,

endoscopic tissue specimens were analyzed for mRNA and enzyme activity measured by deethylation of ethoxyresorufin before and after the treatment with omeprazole 20 mg/day for 1 week. Large interindividual variations of CYP1A induction were observed. The extent of CYP1A induction ranged from no change in one subject to a sixfold increase in another subject. The individual who did not initially respond (20 mg/day) had a marked increase in both mRNA and enzyme activity after receiving 60 mg of omeprazole daily for 1 week, suggesting that CYP1A induction is dose dependent. Similarly, dose-dependent induction of CYP1A2 by omeprazole has been demonstrated between 40 and 120 mg doses, as measured by ^{13}C -[N-3-methyl]-caffeine breath test [37]. In another clinical study, CYP1A2 was induced in poor metabolizers (PMs) of CYP2C19 but not in extensive metabolizers (EMs) after 7 days treatment with omeprazole at 40 mg/day [38]. Because that omeprazole is eliminated predominately by CYP2C19-mediated metabolism, the plasma AUC_{po} of omeprazole in PMs of CYP2C19 was approximately 5-fold higher than that in EMs after an oral dose of 40 mg. Therefore, the differential effect of omeprazole on CYP1A induction between PMs and EMs of CYP2C19 can be explained by concentration-dependent induction. Collectively, these results clearly demonstrate that omeprazole induces *CYP1A1* and *CYP1A2* genes in a concentration (dose)-dependent manner.

Cigarette smoking is known to induce CYP1A2 enzyme, and the smoking-induced CYP1A2 gradually returns to the basal level via protein degradation after cessation of smoking. In a clinical study, the degradation half-life of the smoking-induced CYP1A2 has been determined in heavy cigarette smokers after cessation of smoking [39]. This study was conducted with 8 men and 4 women who smoked 20 cigarettes or more per day. Subjects were phenotyped for CYP1A2 activity at 6, 4, and 1 days before smoking cessation and at 0, 1, 2, 3, 6, 8, 10, and 13 days thereafter by measuring caffeine clearance. The CYP1A2 activity decreased as a function of time, and a maximal decrease was observed at six or eight days after cessation. The degradation half-life of CYP1A2 activity was estimated to be about 36 h. Similar to the degradation half-life of CYP1A2 enzyme in humans, the degradation half-life of the Aroclor-induced CYP1A1 and CYP1A2 has been estimated to be 20 to 37 h, respectively, in rats [40,41].

Rifampicin is one of the most potent inducers of human *CYP3A4* gene. The time- and dose-dependent induction of CYP3A4 by rifampicin has also been demonstrated in primary cultures of human hepatocytes [42]. In human hepatocytes treated with rifampicin, the induction of CYP3A4 activity (measured by testosterone 6 β -hydroxylation) was concentration dependent, and the EC_{50} for rifampicin was estimated to be 0.3–0.5 μM [43]. The dose of rifampicin used in the treatment of tuberculosis is usually between 450 and 600 mg once daily [44]. In a clinical study, the effect of rifampicin on the pharmacokinetics of diazepam (a low hepatic clearance drug) was not significantly different between 600 and 1200 mg oral dose [45]. The AUC_{po} of diazepam decreased from an average of 4430–1040 ng h/mL after the treatment with rifampicin at 600 mg, while from an average of 4170–1130 ng h/mL after 1200 mg rifampicin. Similarly, the effect of rifampicin daily dose of 600, 900, and 1200 mg on the disposition of propranolol (a high hepatic clearance drug) was also not significantly different [46]. Together, these results suggest that the inductive effect of rifampicin at the dosage of 450–600 mg is probably near maximal. The notion of maximal inductive effect at 450–600 mg is further supported by the comparison of the EC_{50} and the systemic exposure of rifampicin at clinical dose. In a clinical study, the peak plasma concentration and AUC_{po} of rifampicin were 20 $\mu\text{g/mL}$ and 100 $\mu\text{g h/mL}$,

respectively, following an oral dose of 600 mg [47]. The peak concentration ($\sim 20 \mu\text{M}$) and average systemic exposure ($4\text{--}5 \mu\text{M}$; measured by $\text{AUC}/24\text{h}$) are much greater than the EC_{50} for rifampicin ($0.3\text{--}0.5 \mu\text{M}$).

Time-dependent CYP3A4 induction by rifampicin has also been demonstrated in humans. In a clinical study, the time course of the CYP3A4 induction was evaluated by measuring the steady state trough concentrations of verapamil before, during, and after a 12-day treatment with rifampicin 600 mg once daily [5]. The maximal effect of rifampicin on the CYP3A4 activity was observed at eight days after starting the rifampicin treatment and returned to the baseline activity about two weeks after discontinuing rifampicin treatment. The half-life for increase in CYP3A4 activity was estimated to be about 0.9 day for (*R*)-verapamil and 1.0 day for (*S*)-verapamil, and the half-life for decrease in CYP3A4 activity was 1.5 days for (*R*)-verapamil and 2.1 days for (*S*)-verapamil. It is reasonable to assume that the increase in CYP3A4 activity reflects mainly the synthesis of CYP3A4 protein and the decrease in CYP3A4 activity reflects mainly the degradation of CYP3A4 protein. If the assumption is valid, these results suggest that the process of protein degradation is somewhat slower than the process of protein synthesis.

Consistent with the half-life of CYP3A4 induction of approximately 1 day, a significant CYP3A4 induction was observed as early as 8 h after a single dose of rifampicin [48]. Nifedipine was given orally 8 h after a single dose of rifampicin (1200 mg) in healthy volunteers. The AUC_{po} of nifedipine decreased from an average of 573 ng h/mL before rifampicin treatment to 205 ng h/mL at 8 h after a single rifampicin treatment. Although the change in the AUC_{po} of nifedipine after 8 h (2.8-fold) after a single dose is much less than that after a seven days treatment (15.5-fold as shown in Table 14.1), a duration of 8 h is sufficient to induce a significant amount of CYP3A4 protein level leading to a significant increase in the activity of this enzyme.

14.3 EFFECTS OF CYP INDUCTION ON PHARMACODYNAMICS

For drugs whose elimination is cleared primarily by CYP-mediated metabolism, CYP induction will reduce the therapeutic efficacy as a result of a decrease of systemic exposure. In some cases, changes in drug dosage are required in order to attain and maintain a therapy during the initiation, maintenance, and discontinuation of the coadministration of a potent CYP inducer. In addition, CYP induction may create an undesirable imbalance between bioactivation and detoxification, leading to an increase in metabolite-induced toxicity. Although the research on the potential risk of CYP induction-mediated toxicity has received much less attention than the pharmacokinetic and pharmacological effects, there is increasing evidence that CYP induction could be a serious cause for drug-induced toxicity.

14.3.1 Effects of CYP Induction on Therapeutic Efficacy

Reduction in therapeutic efficacy caused by CYP induction is best exemplified by the rifampicin–cyclosporine interaction. Because of the treatment with immunosuppressive agents, there is a high incidence of tuberculosis among organ transplant recipients. In a retrospective study, analysis of the records of 880 renal transplant recipients in Turkey revealed that 36 patients were infected and developed tuberculosis after renal

transplantation [49]. In another study, a very high incidence of tuberculosis (42%) was reported among 305 renal transplant recipients in India [50]. Because rifampicin still remains as an effective antituberculosis agent, it is often concomitantly used with cyclosporine (or tacrolimus) in transplant recipients, and clinically significant drug interactions between rifampicin and cyclosporine have been reported. In a clinical study, treatment with rifampicin 600 mg once daily for 11 days caused a more than 3-fold decrease in the AUC_{po} of cyclosporine, presumably due to CYP3A4 induction [7]. Importantly, treatment with rifampicin had caused acute transplant rejection in patients treated with cyclosporine [51]. The acute transplant rejection is most likely because of significant reduction of cyclosporine exposure caused by rifampicin-mediated CYP3A4 induction. Similar to cyclosporine, an 18-day treatment with rifampicin 600 mg daily markedly reduced the AUC_{po} of tacrolimus (a good substrate of CYP3A4) by about 3-fold in patients [8]. Therefore, an increase in the tacrolimus dosage was required to attain the effective blood concentration of tacrolimus in order to avoid graft rejection in transplant recipients [52].

Warfarin–rifampicin interaction is also a good example for CYP induction-mediated reduction of drug efficacy. Warfarin, a prototypic CYP2C9 substrate, remains as the first line anticoagulant therapy. The unfavorable narrow range of therapeutic concentration makes warfarin prone to potentially life-threatening drug–drug interactions [53]. In a clinical study, the AUC_{po} of warfarin was decreased by about 3-fold after a 21-day treatment with rifampicin 600 mg daily [54]. The reduction of warfarin plasma concentration is believed to be due to the rifampicin-mediated CYP2C9 induction. Consistent with a threefold decrease in warfarin plasma concentration, rifampicin treatment caused a threefold decrease in the anticoagulant effect of warfarin. In a case report [55], a patient receiving concomitant rifampicin and warfarin to treat a mycobacterial infection and intraventricular thrombus, respectively, required a more than twofold increase in the dosage of warfarin to attain the therapeutic efficacy, measuring by international normalized ratio (INR). Because of temporal changes of enzyme level, the dose of warfarin should be reduced gradually after discontinuing rifampicin treatment to maintain a balance between therapeutic efficacy and adverse effect. A gradual 70% reduction in warfarin dosage over 4–5 weeks was necessary to maintain the therapeutic INR after rifampicin discontinuation [55]. In another case report, a 72-year-old patient taking warfarin was concurrently administered rifampicin for several months [56]. During this period, satisfactory anticoagulation was achieved only when a high dose of warfarin (20 mg) was given. After discontinuation of rifampicin therapy, warfarin dose was adjusted gradually, until the stabilization of the prothrombin time at warfarin 7.5 mg.

Because of narrow therapeutic index of antiarrhythmic agents, potential interaction with other drugs is of therapeutic importance in the clinical practice. Quinidine, a substrate of CYP3A4, is used for the treatment of cardiac arrhythmias. After 7 days of daily treatment with rifampicin 600 mg in 6 healthy volunteers, the AUC_{po} of quinidine decreased dramatically by 9-fold [13]. In another clinical study, a 7-day treatment with rifampicin 600 mg daily caused a 5-fold decrease in the AUC_{po} of quinidine [57]. Although no published studies or case reports have described the pharmacodynamic changes with respect to the quinidine–rifampicin interaction, it is likely that the interaction could be clinically significant. A clinically significant reduction in the QRS prolongation has been reported as a result of rifampicin–propafenone interaction. Propafenone, a sodium channel-blocking antiarrhythmic drug, is metabolized by CYP2D6 to 5-hydroxy-propafenone and by CYP3A4 to N-dealkylation metabolite

[58]. Both 5-hydroxy-propafenone and N-dealkylation-propafenone are active metabolites with activity as potent as the parent drug. The oxidative metabolites are subsequently eliminated by phase II metabolism, glucuronidation, and sulfation. The effect of rifampicin on the pharmacokinetics and pharmacodynamics of propafenone was studied in six EMs and six PMs of CYP2D6 [59]. After a 9-day rifampicin treatment (600 mg/day), the AUC_{po} of propafenone decreased from an average of $6.9\text{--}1.8\text{ }\mu\text{M h}$ in the EMs and from 54 to $16\text{ }\mu\text{M h}$ in PMs. Interestingly, rifampicin also affected the pharmacodynamics of propafenone in both EMs and PMs of CYP2D6. The maximum QRS prolongation decreased from 21% to 13% in EMs and from 15% to 9% in PMs. Since CYP2D6 is not inducible, the rifampicin's effect on the pharmacokinetic and pharmacodynamics of propafenone is probably due mainly to the CYP3A4 induction. However, interpretation of clinical consequences of propafenone by CYP induction would be somewhat complicated in this case because of the formation of active metabolites.

Similarly, the interaction between rifampicin and codeine is quite complex. Codeine is a widely used opiate analgesic agent that is converted through O-demethylation to morphine by CYP2D6 in humans [60,61]. Although the formation of morphine accounts for >10% of over all clearance of codeine, morphine is responsible for most of the analgesic effect of codeine. Unlike morphine which is eliminated mainly by UDP-glucuronosyltransferases (UGTs), codeine is metabolized by multiple enzyme systems, including CYP2D6, CYP3A4, and UGTs [62,63]. The effect of rifampicin on the pharmacokinetics and pharmacodynamics of codeine has been studied in EMs and PMs of CYP2D6 [63]. After treatment of rifampicin (600 mg/day) for 3 weeks, rifampicin had little effect on pharmacodynamics of codeine in PMs of CYP2D6. Because CYP2D6 is not inducible by rifampicin, it is expected that rifampicin will have no significant effect on the pharmacodynamics of codeine in EMs as well. Surprisingly, the codeine's pharmacodynamic effects were attenuated in EMs of CYP2D6 after rifampicin treatment. This is because that rifampicin significantly decreased the concentration of codeine through an increase in the clearance of the major elimination pathways of codeine, namely, CYP3A4-dependent N-demethylation and UGT-dependent glucuronidation, which can be induced by rifampicin. Thus, the conversion of morphine from codeine was significantly reduced in the EMs of CYP2D6 due to the decreased codeine concentration during rifampicin treatment. In addition to the decreased morphine formation, the elimination of the converted morphine can also be enhanced via glucuronidation by rifampicin-induced UGTs.

Morphine is eliminated exclusively by UGTs to form glucuronide-3-morphine and glucuronide-6-morphine in humans [64]. A significant drug interaction has been observed between morphine and rifampicin. In a clinical study, the serum concentrations of morphine, glucuronide-3-morphine, and glucuronide-6-morphine were measured before and after the treatment with rifampicin 600 mg once daily for 13 days [65]. There was only a moderate decrease in the serum concentrations of morphine after the treatment with rifampicin. The AUC_{po} and C_{max} of morphine, respectively, decreased from an average of 132 nM h and 34.5 nM before rifampicin treatment to 97 nM h and 18 nM after rifampicin treatment. Similarly, there was also a significant decrease in the AUC of glucuronide-3-morphine and glucuronide-6-morphine [65]. Although rifampicin treatment only caused a moderate decrease in concentrations of morphine and its active metabolite, a complete loss of analgesic effect of morphine was observed in these volunteers after the treatment of rifampicin.

The HIV protease inhibitors are used for the treatment of patients infected with HIV. Because of the increasing incidence of tuberculosis among patients infected with HIV, rifampicin is often concomitantly used with HIV protease inhibitors. Therefore, rifampicin-mediated CYP induction becomes a serious concern in the treatment of HIV infection [66]. After a 14-day rifampicin treatment (600 mg/day), both the AUC_{po} and C_{max} of saquinavir decreased by more than 4-fold in healthy volunteers [67]. Similarly, treatment with rifampicin (600 mg/day) for 7 days caused a 5-fold decrease in both the AUC_{po} and C_{max} of nelfinavir [68]. Significant reduction in the AUC_{po} and C_{max} of ritonavir and indinavir after the rifampicin treatment has also been reported [16,69]. Because the antiretroviral effect is highly dependent on the systemic exposures of the HIV protease inhibitors, the use of rifampicin is contraindicated to avoid treatment failure. In some cases, a dosage adjustment may be required to achieve the antiretroviral effect. For example, the dosage of efavirenz, a nonnucleotide transcriptase inhibitor, needs to be adjusted from 400 to 800 mg daily, when rifampicin is concurrently administered [70]. Interestingly, efavirenz is also a CYP3A4 inducer. In a clinical study, the AUC_{po} of indinavir was decreased by 35% when coadministered with efavirenz [71]. To compensate the efavirenz-mediated induction, the dosage of indinavir was suggested to increase from 800 mg 3 times daily to 1000 mg 3 times daily.

The effect of rifampicin on the CNS drug action has also been studied. A 5-day rifampicin treatment (600 mg/day) caused a more than 3-fold decrease in the AUC_{po} of zolpidem [11]. In parallel, a significant reduction in the effects of zolpidem was seen in all psychomotor tests (digit symbol substitution, critical flicker fusion test, and Maddox wing test). In another clinical study, rifampicin treatment at 600 mg daily for 5 days resulted in a 5.5-fold decrease in the AUC_{po} of zopiclone and a significant reduction in the hypnotic effects [12]. Similarly, a 5-day rifampicin treatment (600 mg/day) caused a 10- to 20-fold decrease in the AUC_{po} of midazolam and triazolam [14,15]. As a result of the substantial decrease in systemic exposure, both midazolam and triazolam are ineffective in patients taking rifampicin. Therefore, it is advisable to use hypnotic agents that are not predominantly metabolized by CYP3A4 during treatment with rifampicin.

14.3.2 Effects of CYP Induction on Drug-Induced Toxicity

Epidemiological and animal studies have suggested that many xenobiotics and drugs cause toxicity by generating reactive metabolites or reactive oxygen species [72–74]. CYP1A enzymes are known to be responsible for metabolic activation and detoxification of some xenobiotics, such as polycyclic aromatic hydrocarbons. Although it is highly controversial whether CYP1A induction is beneficial because of detoxification, or is detrimental because of metabolic activation forming reactive metabolites, there is increasing evidence that CYP1A induction could be a risk factor that may cause toxicity and cancer [75–77]. For example, cigarette smoking has been found to be associated with pulmonary and cardiovascular diseases and cancer via CYP1A induction [78–81]. Recently, *in vitro* and animal studies suggest that some xenobiotics exert their toxicity, not only by generating reactive metabolites but also by altering expression of specific genes through AhR activation [74,75].

Induction of CYP2E1 by ethanol is believed to be responsible for hepatotoxicity by generating oxidative stress species [82,83]. Rate of the formation of superoxide and hydrogen peroxide was significantly increased in microsomes from ethanol-treated rats, in which CYP2E1 was induced [84,85]. *In vitro* studies revealed that human CYP2E1 is

also capable of forming reactive oxygen intermediates and catalyzing lipid peroxidation in CYP2E1-expressed HepG2 cells [86]. There is an increasing body of evidence that ethanol-induced hepatotoxicity is linked to the increased production of oxidative stress species. For instance, the ethanol-induced hepatotoxicity has been shown to correlate with the expression levels of CYP2E1 as well as the elevated lipid peroxidation in rats [87]. Furthermore, treatment of diallylsulfide, an inhibitor of CYP2E1, prevented the elevation of lipid peroxidation and partially blocked the ethanol-induced hepatotoxicity. Similarly, anti-CYP2E1 IgG has been used to prevent the formation of reactive oxygen species after ethanol treatment in animals [88].

Hepatotoxicity caused by CYP2E1 induction in humans has been suggested [89]. A male subject was in the habit of consuming three glass of wine regularly with dinner. He stopped ingesting ethanol when he contracted influenza and began taking acetaminophen for treatment. Several days later, he had a complete liver failure. It is believed that the hepatotoxicity is due to an increased formation of reactive metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinone imine (NAPQI) as a result of CYP2E1 induction caused by heavy alcohol drinking. *In vitro* studies with human liver microsomes have demonstrated that CYP2E1 can modulate the formation of NAPQI [90]. Interestingly, ethanol is able not only to induce but also to inhibit CYP2E1 activity [91]. On the basis of the dual effects of ethanol, Slattey *et al.* [89] have developed a kinetic model to explain the complex ethanol–acetaminophen interaction. Through the model simulations, it becomes clear that the time interval between the last consumption of alcohol and ingestion of acetaminophen is very important in terms of the risk of hepatotoxicity. If acetaminophen is taken in the morning because of a headache as a result of the heavy drinking the night before, there is a high risk of hepatotoxicity. This is because at the next morning after heavy drinking, the low level of ethanol concentration is insufficient to inhibit the formation of NAPQI in the liver where CYP2E1 enzyme activity was induced, resulting in an increased formation of the toxic metabolite. However, if acetaminophen and ethanol are taken together at the same time, the formation of NAPQI is not expected to increase because of the opposite effects of CYP2E1 inhibition and induction by ethanol.

In addition to CYP2E1, NAPQI can also be formed by human CYP1A2 and CYP3A4 [91,92]. Therefore, it has been suggested that CYP1A2 and CYP3A4 may also contribute to the acetaminophen-induced hepatotoxicity. The role of CYP1A2 in acetaminophen-induced hepatotoxicity has been studied in wild-type, *Cyp2e1*^(-/-), and *Cyp1a2*^(-/-) mice [93–95]. Following the administration of acetaminophen at 250 mg/kg, there was no significant differences in the severity of hepatotoxicity between *Cyp1a2*^(+/+) and *Cyp1a2*^(-/-) mice, as measured by serum alanine aminotransferase. In addition, there was no difference in the urinary metabolites excreted over a 24-h period, including those derived from glutathione (GSH) conjugation of the major reactive metabolite NAPQI of acetaminophen, between the *Cyp1a2*^(+/+) and *Cyp1a2*^(-/-) mice. With these results, the investigators concluded that CYP1A2 does not play a significant role in acetaminophen hepatotoxicity in mice. Consistent with the findings in mice, induction of human CYP1A2 appears to have little effect on the formation of the reactive metabolite NAPQI of acetaminophen. In a clinical study with EMs and PMs of CYP2C19, omeprazole was administered orally at 40 mg daily for 7 days [96]. A significant CYP1A2 induction was observed only in the PMs but not in EMs of CYP2C19. Despite induction of CYP1A2 activity in the PMs, there was no significant difference in the formation of thioether conjugates,

which are terminal metabolites of NAPQI. Therefore, CYP1A2 induction may not significantly contribute to the formation of NAPQI of acetaminophen.

The role of CYP3A enzymes in acetaminophen-induced hepatotoxicity has also been studied in pregnane X receptor (PXR)-null mice [97]. Pretreatment with pregnenolone-16 α -carbonitrile (PCN), a potent inducer of mice Cyp3a11, markedly enhanced acetaminophen-induced hepatotoxicity, as revealed by increased serum concentration of liver enzymes and hepatic centrilobular necrosis in wild-type mice but not in PXR-null mice following an intraperitoneal dose of acetaminophen (350 mg/kg). Furthermore, PCN treatment significantly increased the GSH-derived metabolites of NAPQI in wild-type mice but not in PXR-null mice. These results suggest that Cyp3a11 plays an important role in acetaminophen-induced hepatotoxicity in mice.

The role of CYP3A4 in acetaminophen-induced hepatotoxicity has also been studied in humans. In a clinical study, pretreatment with rifampicin (600 mg/day) for a week showed no effect on the urine recovery of thiol metabolites formed by conjugation of NAPQI with GSH, suggesting that the contribution of CYP3A4 to the formation of NAPQI of acetaminophen may be negligible in humans [98]. On the other hand, the urine recovery of the thiol metabolites formed by conjugation of NAPQI with GSH was decreased by 69%, and the formation clearance of NAPQI was decreased by 74% by pretreatment with disulfiram, a potent CYP2E1 inhibitor [98]. These results strongly suggest that CYP2E1, rather than CYP3A4, is the major enzyme responsible for the acetaminophen-induced hepatotoxicity in humans.

14.4 CONCLUSIONS

Although our understanding of CYP induction has advanced significantly over the past 10 years through the discovery of key nuclear receptors, much still remains to be learned about the molecular mechanisms of CYP induction. One of the unsolved issues is the wide interindividual variability in CYP induction. There are a large number of factors that could contribute to the variability, including genetic and environmental variables. The relative contribution between the genetic and environmental variables is not readily assessed due in part to the complexity of CYP induction and insufficiency of proper experimental tools. Another critical issue that may complicate the prediction and interpretation of CYP induction is the interplay between efflux transporters and CYP enzymes. Although there is now an increasing evidence to suggest the interplay between CYPs and efflux transporters because of a striking overlap in substrates and inducers with CYP enzymes and efflux transporters, our knowledge about this interplay is still very limited [99–102]. This is because it is very difficult to accurately estimate the relative contribution of CYP enzymes and transporters to drug absorption and disposition. This is particularly true for drug interactions that are caused by CYP and transporter induction.

Because of the complexity of the contributing factors, quantitative prediction of CYP induction is very difficult, if not impossible. Although numerous *in vitro* systems have been developed to assess CYP induction, these systems are only useful for semiquantitative assessment whether a new drug candidate has a potential for CYP induction. Information obtained from *in vitro* induction studies is still limited in its ability to predict whether there is a “probability” of induction-based drug interactions. If the *in vitro* data suggest that a drug candidate could have a potential for CYP induction,

clinical studies should be conducted earlier to assess the degree of induction. Therefore, the induction data obtained from *in vitro* systems should not serve as a “no-gos” decision for drug development without a proper clinical assessment. Finally, a point worth mentioning is that in many cases, the CYP induction-mediated drug interaction is manageable by adjusting dosage regimen.

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15 Age-Dependent Expression of Human Drug-Metabolizing Enzymes

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15.1	Summary	1
15.2	Introduction	2
15.3	Physiological factors impacting age-dependent drug disposition	2
15.4	The cytochrome P450-dependent monooxygenases	4
15.5	Flavin-containing monooxygenases (FMO)	12
15.6	Alcohol dehydrogenase (ADH)	14
15.7	Aldehyde oxidase (AOX)	15
15.8	Carboxylesterases (CES)	16
15.9	Epoxide hydrolase (EPHX)	16
15.10	Glutathione <i>S</i> -transferase (GST)	17
15.11	Sulfotransferase (SULT)	19
15.12	UDP glucuronosyltransferase (UGT)	21
15.13	Conclusions and future perspectives	22
	Acknowledgments	24
	References	24

15.1 SUMMARY

Changes in drug-metabolizing enzyme (DME) expression during development are substantial and can impact the risk of adverse drug events in the fetus and child. Individual hepatic DME ontogeny can be categorized into one of three groups based on their developmental trajectories. Class I enzymes, for example, FMO1 or CYP3A7, are expressed at their highest level during the first trimester and either remain at high concentrations or decrease during gestation, but are silenced or expressed at low levels within one to two years after birth. SULT1A1 and CYP3A5 are examples of class II DME that

are expressed at relatively constant levels throughout gestation and minimal changes are observed postnatally. ADH1C and CYP2C9 are typical of class III DME that are not expressed or are expressed at low levels in the fetus, usually during the second or third trimester. Substantial increases in enzyme levels are observed within the first one to two years after birth. Combined with our knowledge of changes in physiological factors during early life stages, knowledge regarding DME ontogeny has permitted the development of robust physiological-based pharmacokinetic models and an improved capability to predict drug disposition in pediatric patients.

In contrast to the dramatic changes in DME expression that occur in early life stages, modest if any changes in hepatic DME expression occur in the elderly. Thus, the well-documented decreases in clearance for many drugs observed in later life stages are more likely due to physiological changes, such as a loss of renal function, changes in body composition and size, and decreases in liver volume relative to body surface area.

This chapter provides an overview of age-dependent changes in DME expression patterns and discusses some implications of the data with regard to drug therapy. Common themes emerging from our current knowledge are also discussed. Finally, the chapter presents some future perspectives and highlights gaps in knowledge that will be important to advance this field.

15.2 INTRODUCTION

Past therapeutic misadventures leading to adverse drug events have provided ample evidence that pharmacokinetic factors in children differ substantially from adults. Often cited are the incidences of gray baby syndrome resulting from the administration of chloramphenicol to neonates at doses extrapolated from those found effective and safe in midaged adults [1]. Similarly, toxicity and death in premature infants were linked to the use of benzyl alcohol as a preservative in intravenous fluids [2]. Studies subsequently demonstrated that the immature expression of critical conjugating enzymes, resulting in impaired metabolism and clearance, was primarily responsible for both toxicities [3,4]. However, increased drug sensitivity is not universal in children versus adults. Young children are more resistant to acetaminophen toxicity relative to adults largely because of an increased capacity for sulfate conjugation [5].

Less well-studied, age-dependent changes in drug disposition in the elderly also has been an issue of concern. In general, the rate of drug clearance in elderly patients, particularly over the age of 70, is decreased, although it is less clear whether this is because of corresponding decreases in the expression of specific enzymes and/or physiological factors [6]. Never the less, combined with the facts that most therapeutics utilized in pediatric patients are used off-label [7] and the increased incidence of polypharmacy and risk of adverse drug–drug interactions in the elderly [6], the above observations have been major drivers to better understand mechanisms underlying life-stage-dependent changes in drug metabolism and disposition.

15.3 PHYSIOLOGICAL FACTORS IMPACTING AGE-DEPENDENT DRUG DISPOSITION

In addition to age-dependent changes in the expression of specific DMEs, several physiological parameters undergo changes during development and aging that impact

drug disposition (for recent reviews, see Refs 6 and 8). Intragastric pH is elevated in the neonate and elderly resulting in lower bioavailability of weakly acidic drugs. Also impacting drug absorption from the gastrointestinal tract, maturation of intestinal motor activity takes place during early infancy and then decreases in the elderly. The distribution of drugs can be impacted by age-dependent changes in body composition and changes in the major drug-binding plasma proteins [6,9].

Although the above physiological changes can be important, anatomical and functional changes in the liver and kidney have a quantitatively more important influence on pharmacokinetics. Hepatic organogenesis begins during the fourth week of gestation and progresses rapidly with the fundamental components of the liver being formed by the end of the first trimester. By 12 weeks, the smooth endoplasmic reticulum is developing and is present in large amounts by midgestation (see review by Ring *et al.* [10]). However, the ratio of liver to body mass also is considerably greater in infants and young children. This difference alone accounts for some of the developmental differences in drug metabolism between middle-aged adults and children [11,12]. Scaling by normalizing to a 70-kg individual using the 3/4 power allometric rule [$\text{Activity}_{70\text{kg}} = \text{Activity}/(\text{Weight}/70\text{kg})^{0.75}$] can be used to adjust for differences in liver size relative to body mass in pediatric versus middle-aged adult individuals see Ref. 13. Liver volume relative to body surface area further decreases in the elderly. For example, in a group of 14 individuals (9 females and 5 males) with a mean ($\pm$ SD) age of 28.8 ± 8.3 years, liver volume per unit of body surface area (mL/m^2) was 948 ± 109 versus 795 ± 153 in a group of 14 individuals (7 females and 7 males) with a mean ($\pm$ SD) age of 82.5 ± 7.1 years [14]. In an even larger study of 65 individuals between the ages 24 and 91 years, Wynne *et al.* [15] documented a 30% and 43% decrease in liver volume per body weight (mL/kg) in males and females, respectively, and a decrease in liver blood flow per body weight ($\text{mL}/\text{min}/\text{kg}$) of 43% and 55% in males and females, respectively.

More recently, there has been growing appreciation for the contribution of age-dependent changes in hepatic microsomal protein content to age-dependent changes in pharmacokinetics. Although highly variable, the average microsomal protein content of a 30 years old was observed to be 40 mg/g liver with a decline to 31 mg/g liver for the average 60 years old [16]. Extending these studies using a set of pediatric samples suggested an increase in microsomal protein content from a neonatal value of 26 mg/g liver to the maximum observed at age ~ 30 years [17]. Limited data would suggest even less microsomal content in the fetal liver [18]. Importantly, age-dependent changes in microsomal content during early life stages would have an opposite effect on drug disposition relative to the changes in liver size relative to body mass. However, the decrease in microsomal content in the elderly, combined with the decrease in both liver blood flow and liver volume relative to body weight, would have at least an additive negative impact on drug disposition in the elderly.

The maturation of kidney structure and function has a profound impact on those drugs that depend on renal clearance for the elimination and/or termination of pharmacological action (reviewed in Ref. 9). Nephrogenesis begins as early as 9 and is complete by 36 weeks gestation. However, vasoconstriction and reduced renal blood flow result in a substantially diminished glomerular filtration rate (GFR) in the term infant versus the middle-aged adult. At birth, there is a decrease in vascular resistance, as well as increases in cardiac output and renal blood flow, resulting in rapid increases in GFR. Despite these parturition-associated events, GFR is more tightly correlated

with postconceptional age than postnatal age, clearly suggesting that maturation of renal structure continues to influence GFR in the postnatal period and that mature GFR levels are not attained until approximately one year of age. Tubular secretion and reabsorption can also play an important role in renal drug clearance. At birth, the renal tubules are not yet mature and secretory and reabsorption transport activity is only 20–30% of adult values. Increases in activity to mature levels of tubular secretion occur over the first seven to eight months. Information on the ontogeny of specific renal transport enzymes in the human remains deficient and would greatly aid in our understanding of early life stage differences in drug response and risk for adverse events.

Age-dependent decreases in GFR are well documented in the elderly [19]. Normal mean GFR measured by inulin clearance decreases from ~ 120 mL/min/1.73 m² at age 40 years to ~ 65 mL/min/1.73 m² in men and 80 mL/min/1.73 m² in females at age 90 years. Thus, it is important to correct for these age-dependent changes in renal function for drugs eliminated by this route and, in particular, for drugs with a narrow therapeutic window.

The development of specific antibody and/or metabolic probes for many of the enzymes involved in drug metabolism, as well as improvements in detection methods and the availability of relatively large sets of tissue samples with dense coverage of critical age brackets, has allowed for numerous *in vitro* studies focused on drug metabolism ontogeny over the last decade. Furthermore, a greater understanding of the need for pediatric clinical trials combined with the knowledge and instrumentation to carefully determine fractional metabolic clearance has provided for a limited number of *in vivo* ontogeny studies. Although less well studied, this same approach has provided substantial information on potential DME expression changes in the elderly. Thus, we have a much better understanding of the expression trajectories for many of the metabolic factors impacting drug disposition as a function of age.

15.4 THE CYTOCHROME P450-DEPENDENT MONOOXYGENASES

The human cytochrome P450 enzyme superfamily (EC 1.14.14.1) is encoded by 59 functional genes which have been classified into 18 families and 42 subfamilies based on divergent evolution. Although they may have other roles, the function of drug metabolism is carried out by 23 enzymes belonging to families one through three. Other cytochrome P450s are involved in the synthesis and degradation of important endogenous signaling molecules. Necessary for monooxygenase activity, the drug-metabolizing cytochrome P450s interact with NADPH-dependent cytochrome P450 oxidoreductase, encoded by a single gene on chromosome 7q11.2 (*POR*), which shuttles the necessary reducing equivalents from its flavin-center to the cytochrome P450 heme protein.

15.4.1 Cytochrome P450 1A2 (CYP1A2)

Cazeneuve *et al.* [20] employed caffeine as a metabolic probe and observed an age-dependent change in the CYP1A2 metabolic pattern consistent with very low to absent expression in fetal and neonatal liver and onset of expression in tissues from infants (1.5–10 months postnatal age), but at levels that were 10-fold less than those observed

in adults. These results corroborated earlier *in vivo* results [21] that measured urinary caffeine metabolites. A CYP1A2 developmental trajectory in which expression was not observed until well after birth was confirmed by Sonnier and Cresteil [22] using methoxyresorufin as a probe substrate and western blotting with an anti-rat polyclonal CYP1A1 antibody. CYP1A2 was not detectable in fetal liver, but a progressive increase in catalytic activity and protein levels was observed in postnatal samples with neonates at 4–5% of adult levels, tissue from one- to three-month-old infants at 10–15% of adult levels, samples from 3- to 12-month-old children at 20–25% of adult levels, and samples from children aged one to nine years at 50–55% of adult levels. Diet can alter the rate of CYP1A2 maturation with formula-fed infants acquiring activity faster than breast-fed infants [23], but even under these conditions, CYP1A2 exhibits the most delayed developmental trajectory of the cytochrome P450s studied to date.

Several studies have evaluated theophylline or caffeine clearance in the elderly as a measure of CYP1A2 activity (reviewed in Refs 6 and 24). Although some studies suggested little or no change with increasing age, other investigators have reported a pattern wherein clearance is reduced from 25% to 30% in individuals aged 65–90 years versus individuals aged 20–40 years. Bebia *et al.* [25] evaluated the ratio of plasma paraxanthine to caffeine in adult men and women divided into three age brackets: 18–<35 ($N = 36$), 35–50 ($N = 53$), and >50–86 ($N = 67$) years. Most importantly, because this study design employed a plasma metabolic ratio and thus minimized the impact of physiological factors on disposition, the data and conclusion more accurately reflect CYP1A2 metabolic capacity. No age-related changes were observed between the three age brackets, suggesting no significant age-dependent decrease in CYP1A2 expression in the elderly and that previous observations of decreased theophylline or caffeine clearance were largely due to decreased renal function and other physiological factors.

15.4.2 Cytochrome P450 2A Subfamily (CYP2A)

The human *CYP2A* gene family consists of three members, CYP2A6, 2A7, and 2A13. CYP2A6 catalyzes the 7-hydroxylation of coumarin [26] and the oxidation of nicotine [27], the former often used as a probe substrate for CYP2A6. CYP2A13 exhibits high activity toward the oxidation of the tobacco-specific procarcinogen, 4-(methylnitrosoamino)-1,-(3-pyridyl)-1-butanone [28]. CYP2A7 appears to be a functional pseudogene [26,29]. CYP2A6 and 2A13 exhibit tissue-specific expression patterns, largely restricted to the liver [30] and nasal mucosa [28], respectively. Developmental expression studies were consistent with the absence of CYP2A in human fetal liver, although protein expression (presumably CYP2A6) was readily detectable in adult liver [31,32] and as early as 17 weeks after birth [33]. Thus, hepatic CYP2A6 expression appears to be largely a postnatal event, although the exact developmental trajectory remains unknown.

Despite its importance in the metabolic activation and disposition of nicotine, there have been few reports on any potential CYP2A expression changes in the elderly. Sotaniemi *et al.* [34] reported that urinary 7-hydroxycoumarin as a percent of coumarin dose decreased by ~30% in individuals aged >65 years ($N = 10$) relative to individuals aged <25 years ($N = 10$), consistent with a significant decrease in CYP2A6 metabolic capacity in elderly patients. However, the sample size of the study population was insufficient to determine a more precise, age-dependent trajectory and furthermore,

these data do not allow one to differentiate between age-dependent changes in CYP2A6 expression and loss of renal function.

15.4.3 Cytochrome P450 2B6 (CYP2B6)

Croom *et al.* [35] evaluated the CYP2B6 developmental trajectory in 217 human liver samples from donors ranging in age from 10 weeks gestation to 17 years. CYP2B6 protein was detectable in 75% of the samples overall, but a progressive increase in the fraction of samples with detectable protein increased from 64% in the fetal samples to 95% in the samples from donors aged >10 years. There was a small, but significant twofold increase in protein levels immediately after the neonatal period, but otherwise, CYP2B6 expression levels changed little as a function of age. Changes in CYP2B6 expression in the elderly, if any, are unknown.

15.4.4 Cytochrome P450 2C Subfamily (CYP2C)

Encoded by four genes, *CYP2C8*, *2C9*, *2C18*, and *2C19*, clustered within an ~500 kbp locus on chromosome 10q24, the CYP2C enzymes accounts for ~18% of total adult liver cytochrome P450 [30] and the oxidative metabolism of 30% of clinically relevant drugs [36]. CYP2C9 is the highest expressed member of this family in adult liver, followed by CYP2C19 and 2C8 [37]. CYP2C9 substrates include warfarin, phenytoin, diclofenac, ibuprofen, tolbutamide, losartan [38], and indomethacin [39], while CYP2C19 substrates include omeprazole [40] and mephenytoin [41]. A single study by Tateishi *et al.* [42] is consistent with CYP2C8 expression increasing in the perinatal period, although this conclusion is based on a limited number of tissue samples. Nothing is known regarding hepatic CYP2C18 ontogeny or the ontogeny of any CYP2C enzymes in extrahepatic tissues. In contrast, the common use of several CYP2C9 substrates for pediatric indications [43–45], the activity of these enzymes in converting arachidonic acid to known signaling molecules [46,47], and an association between elevated CYP2C expression and sudden infant death syndrome [48], have stimulated several investigators to explore the ontogeny of hepatic CYP2C9 and 2C19.

Early studies on CYP2C hepatic ontogeny reported conflicting results on fetal expression. Maenpaa *et al.* [49] reported detectable protein in 11 of 17 fetal liver samples that likely was CYP2C9. In contrast, Treluyer *et al.* [50] failed to detect any CYP2C protein in 55 fetal liver samples. Total CYP2C protein levels that were 4–5% of those observed in adults were detectable in 11 liver samples from newborns aged less than one day, but rapidly increased to 22–30% of adult values in postnatal samples greater than one day, but aged less than one year. Tolbutamide hydroxylase activity generally correlated well with protein levels in these same liver samples, consistent with the majority of the measured protein representing CYP2C9. Koukouritaki *et al.* [51] more recently characterized both CYP2C9 and 2C19 developmental trajectories in a bank of 237 human liver samples using both activity assays (diclofenac 4-hydroxylase and (*S*)-mephenytoin 4'-hydroxylase, respectively) and immunoblotting. Although detectable in 80% of first and second trimester samples, CYP2C9 levels were only 1% of reported adult values [30,52]. CYP2C9 expression levels increased to 10% of adult levels in third trimester samples. Although mean CYP2C9 expression levels increased to 25% of reported adult levels in postnatal samples from birth to five months

of age, this metric is somewhat misleading. Samples from this age bracket exhibited large interindividual differences with ~50% of the samples having CYP2C9 levels that ranged in values no different than those observed in the third trimester, while the remaining 50% exhibited values as high as those observed in postpuberty samples; data that are consistent with an earlier phenytoin pharmacokinetic report [43]. Less interindividual variation was observed in tissue samples from donors ranging in age from 5 months to 18 years and mean protein levels were ~50% of adult values. In all instances, good correlation was observed between measured CYP2C9 protein levels and diclofenac 4-hydroxylase activity. Although the failure to observe CYP2C9 expression at adult levels is consistent with the study by Treluyer *et al.* [50] and suggests a late CYP2C9 maturation, this conclusion is inconsistent with pediatric pharmacokinetic studies wherein metabolic ability in children aged over one year is no different than that in adults [53,54]. This apparent discrepancy is likely explained by developmental changes in the liver size to body weight ratio, as illustrated by Takahashi *et al.* [55].

Unlike CYP2C9, CYP2C19 was detectable in all fetal liver tissue samples examined by Koukouritaki *et al.* [51] at levels that were 10–20% of those observed in adults and did not change with increasing gestational age. Furthermore, there was no apparent change in CYP2C19 levels at birth, but rather, a near linear increase over the first five months to levels that were 50–75% of those observed in adults. Thereafter, enzyme levels indistinguishable from adult levels were observed. This developmental trajectory was consistent with the (*S*)-mephenytoin 4'-hydroxylase activities observed in these same samples. Interestingly, a comparison of CYP2C9 and 2C19 levels in individual samples revealed that CYP2C19 is the dominant prenatal CYP2C enzyme in most individuals with a transition to CYP2C9 as the dominant postnatal enzyme shortly after birth.

The prevalent use of antiepileptic and antithrombosis drugs in the elderly and the importance of CYP2C enzymes in the disposition of these drug classes have been an impetus for the evaluation of age-dependent CYP2C changes in the aged. Conflicting data have been reported on the differential clearance of phenytoin, a CYP2C9 substrate, between middle-aged adults and the elderly. For example, Bach *et al.* [14] reported a significant 25% decrease in the clearance of unbound phenytoin normalized to liver volume between younger subjects (mean age of 29 years) versus elderly subjects (mean age of 83 years), while Perucca *et al.* [56] failed to find any age-dependent effects in similar populations. Consistent with the data reported by Bach *et al.* [14], estimation of an initial warfarin dose in individuals of 45 and 85 years of age, but otherwise of identical sex, genotype, body weight, and state of polypharmacy demonstrated a 25% decrease in older subjects (<http://www.warfarindosing.org>). However, none of these studies or examples differentiates between the contributions of physiological factors and actual CYP2C9 expression. Measurement of CYP2C9 (cytochrome P450₉) protein levels *in vitro* in 54 liver samples from donors ranging in age from 9 to 89 years failed to identify any age-dependent changes in expression [57], consistent with observed decreases in the clearance of CYP2C9 substrates being largely due to physiological changes, not metabolic capacity.

In contrast to CYP2C9, there is evidence for a decrease in CYP2C19 expression in the elderly relative to middle-aged adults. Early studies documented a significant 36 (female) to 50% (male) decrease in the clearance of unbound diazepam in individuals aged 61–84 years versus those aged 21–37 years [58]. Similar age-dependent decreases in clearance were reported for omeprazole [59]. Supporting a

role for decreased CYP2C19 expression contributing to the observed decrease in clearance, Bebia *et al.* [25] reported a 30–40% decrease in the ratio of urinary 4-hydroxymephenytoin to mephenytoin between individuals aged <35 and >50 years. Similar to what is observed during early life stages, the regulation of CYP2C9 and 2C19 appears substantially different in later life stages.

15.4.5 Cytochrome P450 2D6 (CYP2D6)

CYP2D6 accounts for <2% of total adult hepatic cytochrome P450 [30], yet catalyzes the oxidation of ~12% of clinically relevant drugs [36]. Substrates include propafenone [60], paroxetine [61], risperidone [62], codeine [63], and dextromethorphan [64]. These latter two reactions have been used as CYP2D6 phenotypic probes, dextromethorphan O-demethylation being particularly useful in pediatric studies. *CYP2D6* also is highly polymorphic with over 80 alleles identified to date, including several relatively common complete loss or reduced function variants, as well as copy number variants with increased activity [65].

Although fetal CYP2D6 was reported as undetectable in an early study by Ladona *et al.* [66], a much more extensive study by Treluyer *et al.* [67] reported CYP2D6 transcripts, protein, and dextromethorphan O-demethylase activity that was at least 5% of adult values in approximately one-third of the fetal samples <30 weeks of gestational age. The number of fetal samples with detectable CYP2D6 increased to nearly 80% in tissue from donors >30 weeks of gestational age, but maximum levels were not increased relative to the <30-week gestational age bracket. Beginning at one week after birth, a steady increase in CYP2D6 protein levels was observed such that neonates greater than one week of age exhibited mean CYP2D6 levels 30% of adults and infants up to five years of age, 70% of adults. There was a close correlation between CYP2D6 protein and mRNA levels in adult samples ($r^2 = 0.95$, $P < 0.005$), but not in fetal, newborn or infant samples, suggestive of a translational control mechanism overlying transcriptional regulation.

Blake *et al.* [68] employed a longitudinal study design measuring dextromethorphan O-demethylase activity in an overnight urine sample to assess CYP2D6 phenotype in 193 infants at 0.5, 1, 2, 4, 6, and 12 months after birth. Although interindividual dextromethorphan O-demethylase activities varied nearly 1000-fold, a clear correlation between activity and an assigned genotype-based CYP2D6 activity score was observed that did not change during the study period. These data are consistent with phenotype and genotype concordance by two weeks of age, which contrasts to the previous *in vitro* study [67]. A more recent *in vitro* evaluation of CYP2D6 ontogeny presented data more consistent with the above *in vivo*, longitudinal study [69]. Low levels of CYP2D6 activity and protein that did not exceed 5% of adult values were present in fetal samples and the fraction of positive samples increased with increasing gestational age. However, CYP2D6 activity and protein levels in samples greater than one week of postnatal age were no different than those observed in adults. Thus, CYP2D6 rapidly matured to an adult expression pattern shortly after birth. Consistent with this conclusion, stepwise linear regression analysis of factors contributing to CYP2D6 specific content revealed only increasing gestational age as important in fetal samples while both increasing postnatal age and haplotype were important in postnatal samples.

There is no evidence for significant changes in CYP2D6 expression in the elderly versus middle-aged adults, despite reported decreases in clearance of

CYP2D6 substrates in the elderly (reviewed in Ref. 24). Evaluating urinary 4-hydroxydebrisoquine/debrisoquine ratios in individuals <35 years of age, 35–50 years of age, and >50 years of age, no differences were observed among the different age brackets and no correlation was observed with body mass index, creatinine clearance, smoking, or alcohol intake [25]. Similarly, an evaluation of the urinary ratio of dextromethorphan to dextrorphan in a group of middle-aged (mean $\pm$ SD = 55.8 ± 5.6) versus elderly (mean $\pm$ SD = 76.2 ± 7.0) women failed to show any difference.

15.4.6 Cytochrome P450 2E1 (CYP2E1)

CYP2E1 accounts for ~7% of total hepatic cytochrome P450 in the adult liver [30] and is involved in the oxidative metabolism of about 2.5% of clinically relevant drugs [36], including acetaminophen [70], halothane [71], and chlorzoxazone [72].

The human hepatic CYP2E1 developmental trajectory was first reported by Vieira *et al.* [73], who evaluated enzyme content, chlorzoxazone hydroxylase activity, and relative transcript levels. Epigenetic regulatory mechanisms also were explored. CYP2E1 protein was undetectable in fetal samples, but was readily detectable in newborn samples at levels ~10% of adult values. Thereafter, a steady increase in CYP2E1 levels was observed such that by one year of age, protein levels and activity were essentially equivalent to adults. CYP2E1 protein levels and catalytic activity were closely associated as a function of age ($r^2 = 0.778$, $P < 0.001$), but neither correlated with transcript levels. DNA methylation at sites flanking exon 1 appeared important for controlling CYP2E1 ontogeny [74].

The inability to detect fetal hepatic CYP2E1 expression was consistent with some more limited studies [75–78], but contrasted with others [79–81]. This apparent controversy was resolved by an exhaustive study by Johnsrud *et al.* [82]. Although CYP2E1 was undetectable in first trimester fetal liver samples, it was present in 40% and 80% of second and third trimester samples, respectively. These data are consistent with nearly all previous reports. Studies reporting an absence of fetal liver CYP2E1 protein content utilized samples from the first or second trimester, while studies reporting the presence of fetal liver CYP2E1 utilized samples from the late second or third trimester period. Johnsrud *et al.* [82] also demonstrated that postnatal hepatic CYP2E1 increased slowly after birth, attaining a median level ~20% of that in the adult in the neonatal period, 50% of that in adults between 31 and 90 days after birth, and values equivalent to adults in samples from donors older than three months of age. Increasing postnatal age plus the presence of at least one copy of the *CYP2E1*1D* allele (a promoter polymorphism linked to increased gene expression in obese individuals and individuals consuming alcohol [83]), and ethnicity were all associated with greater CYP2E1 expression.

Several studies have documented minimal if any changes in the pharmacokinetics of CYP2E1 substrates in the elderly versus middle-aged adult (reviewed in Ref. 24). However, Bebia *et al.* [25] demonstrated a substantial increase in the urinary ratio of 6-hydroxychlorzoxazone to chlorzoxazone when comparing individuals <35, 35–50, and >50 years of age. In men and in comparison to the youngest group, the ratio increased by 47% in the middle-aged group and by 81% in the eldest group. In women, there was no change in the middle-aged group, but a similar 87% increase in the eldest group. Thus, these data would support a sexually dimorphic, age-dependent increase

in CYP2E1 metabolic capacity that would largely compensate for the decrease in physiological factors impacting drug clearance in the elderly.

15.4.7 Cytochrome P450 3A Subfamily (CYP3A)

The CYP3A subfamily consists of four genes, *CYP3A4*, *3A5*, *3A7*, and *3A43*, located at an ~270 kbp locus on human chromosome 7q21.1. CYP3A43 has minimal, if any, drug-metabolizing activity and is expressed at low levels where detectable. As such, this enzyme is not discussed further. In contrast, the remaining CYP3A members are collectively the most abundant members of the hepatic cytochrome P450s and account for nearly 46% of clinically relevant oxidative drug metabolism [36]. Despite sharing at least 85% sequence identity, CYP3A4, 3A5, and 3A7 exhibit considerable differences in both substrate specificity and patterns of expression. CYP3A4 comprises 10–50% of total cytochrome P450 in adult liver [30] and also exhibits high intestinal expression levels [84]. In both liver and intestine, CYP3A5 can be expressed at levels similar to CYP3A4, or even exceed them, subject to the presence of the *CYP3A5*1* allele [85,86]. CYP3A7 is the dominant enzyme in fetal liver, but can represent 10–40% of total CYP3A content in the adult liver and intestine, dependent on the presence of the *CYP3A7*1 C* allele [87].

Komori *et al.* [76] were the first to report age-dependent CYP3A expression using oligonucleotide probes specific for CYP3A7 (cytochrome P450 HFL) and CYP3A4 (cytochrome P450 NF). A strong CYP3A7 hybridization signal was observed using fetal, but not adult liver RNA, while the opposite pattern was observed with the CYP3A4 probe. In a separate study, CYP3A5 protein was shown to be expressed in a limited number of fetal and postnatal liver samples with no apparent age correlation [52]. Data supporting CYP3A-dependent fetal liver metabolic activity was obtained by evaluating the N-demethylation of codeine and dextromethorphan [66]. However, the interpretation of these data is complicated by the overlapping substrate specificities of the CYP3A enzymes. Although CYP3A4 has the greatest dextromethorphan N-demethylation activity, below a substrate concentration of 1 mM, both CYP3A7 and 3A5 exhibit activities 33% and 17%, respectively, of that exhibited by CYP3A4 (Pearce RE, Leeder JS. Children's Mercy Hospital, Kansas City, MO, personal communication). Yang *et al.* [88] demonstrated the presence of CYP3A-dependent activity and protein in early fetal hepatic tissue as early as 7–8.5 weeks gestation, but no immunoreactive protein in heart, lung, brain, or kidney. Amplification of CYP3A transcripts and sequence analysis provided strong evidence for CYP3A7 and to a lesser extent, CYP3A5, but not CYP3A4 in these fetal samples. Subsequent studies also demonstrated CYP3A7-dependent imipramine N-demethylation [89] and retinoic acid 4-hydroxylation [90] in fetal liver tissue. Other groups have demonstrated temporal-specific CYP3 expression at the transcript level consistent with these results [91,92]. These early studies clearly demonstrated a largely fetal liver-specific expression of CYP3A7 and postnatal expression of CYP3A4, while CYP3A5 appeared to be expressed in a limited number of hepatic samples during both life stages. However, the dynamics of the transition between CYP3A7 and 3A4 remained unknown.

Lacroix *et al.* [93] was the first to more fully characterize CYP3A7 and 3A4 developmental trajectories. Equal levels of total CYP3A protein expression were observed in all age brackets. In contrast, a CYP3A4-specific hybridization probe revealed fetal CYP3A4 transcripts that were 10% of adult levels in samples <30 weeks gestational

age, increasing to 20% of adult levels in fetal samples >30 weeks gestational age. Transcript levels 30–60% of adult values were present within one week after birth. Dehydroepiandrosterone (DHEA) 16 α -hydroxylation and testosterone 6 β -hydroxylation were subsequently used as semiselective phenotypic probes for CYP3A7 and 3A4, respectively. DHEA 16 α -hydroxylase activity ranged from 0.48 to 1.31 nmol/min/mg microsomal protein in fetal and neonatal samples up to 7 days after birth, but then declined steadily to 0.56 pmol/min/mg microsomal protein in neonates 8–28 days after birth, 0.34 nmol/min/mg microsomal protein in infants aged 1–3 months, 0.17 nmol/min/mg in children aged from 3 to 12 months, and finally to 0.07 nmol/min/mg microsomal protein in adult tissue samples. In contrast, testosterone 6 β -hydroxylase activity was only 0.004–0.008 nmol/min/mg microsomal protein in fetal samples. Activity rose steadily after birth to 0.012–0.015 nmol/min/mg microsomal protein in neonates aged up to seven days, 0.035–0.052 nmol/min/mg microsomal protein in neonates and infants aged from eight days to one year, and reached adult levels of 0.120–0.130 nmol/min/mg microsomal protein in children aged greater than one year. However, the possible contribution of CYP3A5 to any of these activities was not evaluated. A somewhat similar approach was taken by Stevens *et al.* [94], but using differential hydroxylation of DHEA at the 7 β - and 16 α -positions as probes for CYP3A4 and 3A7, respectively, and a multiple linear regression model developed using different ratios of recombinant CYP3A4 and 3A7 enzymes. In addition, CYP3A5 levels were determined immunologically. Consistent with earlier studies, CYP3A5 protein was observed in approximately 50% of all samples (6.0 ± 5.2 pmol/mg microsomal protein for those samples with detectable protein) with no age-dependent change. CYP3A7 was expressed at high but variable levels (289.5 ± 103.7 pmol/mg microsomal protein) in all fetal samples examined while CYP3A4 was present in <50% of the samples at levels nearly 100-fold less than CYP3A7 (3.4 ± 2.6 pmol/mg microsomal protein). After birth, there was an ~50% decline in neonatal CYP3A7 enzyme levels while little or no change was observed in CYP3A4 levels. In infants, between 30 days and 1 year of age, CYP3A7 levels again decreased by nearly fivefold while CYP3A4 levels increased modestly. In contrast to the earlier report by Lacroix *et al.* [93], CYP3A4 levels in children aged between 1 and 10 years remained well below adult levels (13.1 ± 15.5 pmol/mg microsomal protein, $n = 22$ vs 128.9 ± 99.9 pmol/mg microsomal protein, $n = 11$), although the data on CYP3A7 were consistent with an observed decrease to 3.3 ± 4.8 pmol/mg microsomal protein ($n = 22$). Thus, these data would suggest that in many individuals, CYP3A7 remains the dominant CYP3A enzyme in children up to one year after birth and that CYP3A4 expression increases steadily, but does not reach adult levels until well after one year of age. These conclusions regarding CYP3A4 postnatal ontogeny are consistent with an *in vivo*, longitudinal dextromethorphan *N*-demethylase study [68,95], as well as a metabolic study of amprenavir metabolism in fetal and postnatal liver samples [96]. Furthermore, the variable, but high levels of CYP3A7 expression in fetal liver is consistent with a report by Leeder *et al.* [97] in which mean CYP3A7 mRNA and protein levels were determined to be $14,200 \pm 15,000$ transcripts/ng total RNA and 234.8 ± 123.1 pmol/mg microsomal protein, respectively, in 54 fetal liver samples (11–32 weeks of gestational age). Protein levels correlated well with testosterone 2 α - and DHEA 16 α -hydroxylation activities. This same group also demonstrated significant correlations between NR1I2 (pregnane X receptor) and NR1I3 (constitutive androstane receptor) expression and both CYP3A7 fetal liver and CYP3A4 postnatal liver expression [98], consistent with the known role each of these nuclear receptors

have in regulating the expression of these genes. Yet the degree of association also clearly indicated the involvement of other factors.

The potential clinical implications of the CYP3A7/3A4 transition is illustrated by studies on the pharmacokinetics and pharmacodynamics of cisapride, a gastroprokinetic agent that was commonly used in pediatric patients presenting with feeding intolerance, apnea, and bradycardia associated with gastroesophageal reflux. Owing to a higher than acceptable incidence of drug-induced arrhythmia in adult patients, cisapride was removed from the market and used only through a limited access program for specific diseases, including feeding intolerance in neonates. However, a prospective study in this latter patient population demonstrated a significant increase in QT prolongation not associated with adult risk factors [99]. Given the evidence that cisapride is metabolized primarily by CYP3A4, but not by CYP3A5 or CYP3A7 [100], it was hypothesized that neonates would show deficient metabolic ability [101]. Consistent with this hypothesis, cisapride metabolism was only detectable in four of seven microsomal preparations from fetal or neonatal livers aged less than seven days and was low in those preparations that did show activity. The hypothesis was further confirmed by the *in vivo* study reported by Kearns *et al.* [102] in which the cisapride terminal elimination rate constant was shown to increase ~10-fold between 30 and 52 weeks postconceptional age. Other kinetic parameters also were consistent with compromised cisapride metabolic clearance in premature and term neonates. Thus, the differential substrate specificity of CYP3A4 and CYP3A7 combined with the developmental transition between these two enzymes can have a significant impact on the risk for adverse drug reactions in neonatal patients, particularly those born prematurely.

Because of its promiscuous drug-metabolizing activity and the potential for drug–drug interactions, there have been several studies evaluating possible changes in CYP3A4 expression in the elderly. Cotreau *et al.* [103] published an exhaustive review of numerous pharmacokinetic studies, some of which showed modest decreases in the clearance of CYP3A4 substrates. However, no age-dependent changes in midazolam clearance, a preferred CYP3A4 probe substrate, were observed between either young (mean age, 26 years) and elderly (mean age, 72 years) women or young (mean age, 27 years) and elderly (mean age, 70 years) men [104]. Similarly, there were no differences in results from an erythromycin breath test between individuals aged 20–60 years and 70–88 years [105]. Consistent with these *in vivo* results, no changes in CYP3A4 specific activity were observed in liver microsomes isolated from young versus elderly donors [106,107].

15.5 FLAVIN-CONTAINING MONOOXYGENASES (FMO)

The flavin-containing monooxygenases (FMO) family of enzymes (EC 1.14.13.8) is important for the oxidative metabolism of a wide variety of therapeutics containing nucleophilic nitrogen-, sulfur-, selenium-, or phosphorous-heteroatoms. Common substrates include tamoxifen, itopride, benzydamine, olopatidine, and xanomeline [108]. Eleven human *FMO* genes have been identified, although seven (*FMO6P-11P*) are pseudogenes. The FMO enzymes active in drug metabolism include FMO1, 2, and 3 [108].

Evidence for a developmental transition between human hepatic FMO1 and FMO3 was first reported by Dolphin *et al.* [109]. FMO1 and FMO3 mRNA levels were

quantified in individual fetal liver, kidney, lung, and brain and adult liver, kidney, and lung tissue samples from donors of unspecified ages. Although considerable interindividual variability was observed, FMO1 transcripts were the most abundant in fetal liver and kidney, but were only present at low levels in lung and brain. In the adult, FMO1 transcripts only were observed in the kidney. FMO3 transcripts were present at low levels in the fetal liver and lung, but not in kidney. In contrast, FMO3 transcripts were most abundant in the adult liver and also were expressed at low levels in the kidney and lung. The restriction of FMO1 expression to the fetal liver was confirmed by Yeung *et al.* [110] using immunological approaches to measure FMO1 protein levels in fetal and adult liver, and adult kidney and small intestine. Mean FMO1 levels of 14.4 ± 3.5 pmol/mg microsomal protein were observed in fetal liver, but expression was not detected in adult liver. FMO1 protein levels were highest in the adult kidney, 47 ± 9 pmol/mg microsomal protein, but also were present at significant levels in the adult small intestine (2.9 ± 1.9 pmol/mg microsomal protein). High levels of FMO1 expression in the adult kidney also were reported by Krause *et al.* [111]. Both of these studies are consistent with the earlier studies measuring transcript levels [109].

Although the studies by Dolphin *et al.* [109] and Yeung *et al.* [110] supported the concept of a developmental transition between human hepatic FMO1 and FMO3, several questions remained unanswered, including the time frame of the transition, whether or not coordinate regulation of both genes is involved, and whether there were interindividual differences in the timing of the FMO1/FMO3 transition? To address some of these questions, Koukouritaki *et al.* [112] quantified FMO1 and FMO3 protein levels in 240 human liver samples representing ages from 8 weeks gestation to 18 years after birth. This study revealed that hepatic FMO1 expression was largely limited to the fetus and that the highest level of expression (mean $\pm$ SD = 7.8 ± 5.3 pmol/mg microsomal protein) was observed at ages <15 weeks. FMO1 expression was detectable in 96% of the samples examined within this age bracket. FMO1 protein levels subsequently declined by ~50% in each subsequent trimester and expression was essentially silenced within three days after parturition in a birth-dependent process, rather than a gestational age-dependent process. Furthermore, the percentage of samples with detectable FMO1 decreased by 10% in the second trimester, 30% in the third trimester, and by 84% during the first three days after birth. FMO3 expression was detectable at low levels in about 15% of first trimester fetal liver samples, but expression was nondetectable throughout the remaining gestational period. Low FMO3 expression levels (1.1 ± 3.3 pmol/mg microsomal protein) also were observed in ~25% of the neonatal liver samples. However, it was only after 1 month and before 10 months of age that detectable FMO3 was observed in most samples with mean levels of 4.7 ± 5.9 pmol/mg microsomal protein. These data suggested birth is necessary, but not sufficient for the onset of FMO3 expression. FMO3 protein levels ~50% of adult values were observed in tissue samples from individuals aged between 10 months and 11 years. In postpuberty tissue samples up to 18 years, a gender-independent, near linear increase in FMO3 expression was observed to levels approaching adult values (26.9 ± 8.6 pmol/mg microsomal protein). The above studies suggest that not only is the ontogeny of hepatic FMO1 and FMO3 regulated independently, but that the ontogeny of FMO1 itself is regulated differently in different tissues. Support for this supposition has been reported by several laboratories. Zhang and Cashman [113] described FMO transcript levels in human brain as a function of age and demonstrated a decline in FMO1 mRNA levels in tissues from 18 to 21 weeks gestation donors through 15–40 years after birth. In

contrast, FMO2 and FMO3 expressions were unchanged. FMO1 transcripts also were present at relatively high levels in both fetal and adult nasal mucosa [114]. Consistent with the observed tissue-specific FMO1 developmental trajectories, Shephard *et al.* [115] demonstrated that FMO1 expression in kidney, small intestine, and fetal liver is regulated by the use of different promoters combined with alternative splicing.

Chung *et al.* [116] reported a decrease in FMO3 activity in individuals over the age of 30 years relative to 20–29 years of age using a urinary theobromine to caffeine ratio [117]. However, other investigators have been unable to corroborate the validity of using caffeine as an *in vivo* probe for FMO-metabolic capacity [118,119]. Thus, it is unclear whether or not FMO3 expression levels change in elderly relative to middle-aged adults.

15.6 ALCOHOL DEHYDROGENASE (ADH)

The alcohol dehydrogenase (ADH) (EC 1.1.1.1) family of enzymes are encoded by seven genes (*ADH1A*, *1B*, *1C*, *4*, *5*, *6*, and *7*) clustered on human chromosome 4q21–q25. Functioning as dimers, the ADH enzymes catalyze the oxidation and reduction of a wide variety of alcohols and aldehydes, respectively [120]. Smith *et al.* [121] provided definitive evidence for the progressive expression of *ADH1A*, *1B*, and *1C*, during development. Using starch gel electrophoresis, ADH1 expression in liver, lung, kidney, and intestine from 222 individuals ranging in age from 9 weeks gestation to >20 years postnatal age was examined. Only *ADH1A* was detectable in 48 fetal liver samples with a mean gestational age of 13.5 weeks. By 16 weeks both *ADH1A* and *1B* were measurable, although *ADH1A* predominated and was expressed at higher levels than observed at 13.5 weeks. By 19 weeks, products from all three loci were observed, with *ADH1A* expressed at levels modestly greater than *ADH1B*, and *ADH1C* expressed at low levels in some, but not all samples. In 30 week, prematurely born infants ($n = 10$), *ADH1A* and *1B* levels were equivalent and greater than *ADH1C* while in 36-week premature infants *ADH1B* expression that dominated the other two class 1 alleles was observed. In adult liver samples, *ADH1A* expression was low to nondetectable, whereas *ADH1B* and *1C* expression were equivalent. These studies support the conclusion that birth is more important than gestational age in triggering the activation of *ADH1C* expression. In addition to the progressive change in ADH1 subfamily expression, there was an overall increase such that adult enzyme levels were 10-fold higher than those observed in first trimester fetal samples.

Similar studies in other tissues demonstrated that the observed ADH1 developmental trajectory was tissue specific [121]. No differences between the fetal and adult samples were observed in the lung and only *ADH1C* was detectable. Relative to hepatic expression, intestinal and kidney ADH1 expression was low, was dominated by expression from the *ADH1C* locus, and did not change appreciably with age during early life stages. The observations regarding hepatic ADH1 enzyme ontogeny are largely in agreement with a study in which concentrations of the different ADH1 transcripts were determined by northern blot analysis [122]. In two different fetal liver samples of unspecified age, *ADH1A*, *1B*, and *1C* transcripts were observed in one, whereas *ADH1B* and *1C* transcripts dominated in the second. Differing from the observations by Smith *et al.* [121], *ADH1A* transcripts dominated in the lung, whereas in the fetal

kidney, only ADH1B and 1C transcripts were observed. ADH1 transcripts also were present in most other adult tissues with the exception of brain, kidney, and placenta. ADH4 and ADH7 transcripts were observed in fetal liver at concentrations comparable to those seen in the adult, but only faint signals were seen in the adult small intestine and pancreas. Similar to the class I transcripts, ADH5 transcripts were widely distributed and present at approximately the same concentrations in all fetal and adult tissues examined with the possible exception of the brain. In this tissue, the ADH5 transcripts appeared higher in the fetus than in adult. Drawing conclusions from the data on transcript levels is difficult to do with confidence as the correlation between ADH protein and transcript levels in the various tissues and whether or not the degree of correlation might change as a function of age are unknowns.

Early studies evaluating alcohol first pass metabolism in middle-aged versus elderly adults, measured as the difference in serum ethanol concentrations as a function of time after oral and intravenous ethanol administration, provided controversial results regarding late life stage changes in ADH activity. Some studies demonstrated an unexpected increase in alcohol first pass metabolism in elderly versus middle-aged subjects [123], while other studies demonstrated an age-dependent effect in the opposite direction [124]. However, an *in vitro* comparison of ADH activity in either liver [125] or gastric mucosa [126] between middle-aged and elderly subjects failed to identify any impact of age using ethanol as a substrate. Using form-specific substrates, Matsumoto *et al.* [127] were able to demonstrate a small effect of age on ADH7 activity in the gastric mucosa, but not ADH1 or 5. Thus, there appear to be no or minimal changes in ADH activity in later life stages.

15.7 ALDEHYDE OXIDASE (AOX)

The two human aldehyde oxidase (AOX) (EC 1.2.3.1) family members, AOX1 and AOX2, are encoded on chromosome 2q33. These enzymes are important for the metabolism of a wide variety of aldehyde-containing and *N*-heterocyclic compounds, including 5-fluoropyrimidine, quinine, methotrexate, 6-mercaptopurine, and zonisamide, although the relative specificity of AOX1 versus AOX2 for these compounds is poorly defined. Tayama *et al.* [128] characterized AOX ontogeny in a cohort of 101 Japanese children of both sexes and ranging in age from shortly after birth to 10 years of age. The urinary AOX-dependent oxidation products of *N*1-methylnicotinamide, *N*1-methyl-2-pyridone-5-carboxamide, and *N*1-methyl-4-pyridone-3-carboxamide were evaluated as a measure of enzyme activity. Pediatric activities were compared to those observed in 26 adults, ages 20–60 years. AOX activity in neonates was only 10–15% of that observed in adults. Between 30 days and 1 year of age, activity increased in a nearly linear manner to adult levels. No correlation was observed with donor sex. Whether or not AOX activity is present in the fetus remains unknown. AOX activity was evaluated in 13 liver samples from donors ranging in age from 29 to 73 years by evaluating the kinetics of benzaldehyde and *N*-[(2-dimethylamino)ethyl] acridine-4-carboxamide oxidation to benzoic acid and the acridone, respectively [129]. Although these data were consistent with no change in AOX expression in the elderly relative to middle-aged individuals, the sample size used for this study is small and as such the results should be considered preliminary.

15.8 CARBOXYLESTERASES (CES)

There are three functional members of the human carboxylesterases (CES) gene family, *CES1-3*, located on chromosome 16q21.1–16q21.2. Although perhaps better known for their role in pesticide metabolism [130], these microsomal enzymes also are important for the hydrolysis of numerous drugs; examples include clopidogrel [131], trandolapril [132], and oseltamivir [133] predominantly by CES1 and the hydrolysis of irinotecan predominantly by CES2 [134]. Little is known regarding the substrate specificity of CES3 [135]. CES1 is expressed predominantly in the liver, whereas CES2 is the dominant enzyme in extrahepatic tissue, particularly the gastrointestinal tract [135].

Pope *et al.* [136] was the first to provide evidence for age-dependent expression of the CES enzymes in early life stages. Although limited in available tissue samples, their studies suggested lower CES activity in liver samples from a single 2-month-old donor, whereas tissue samples from 3- to 24-month-old donors exhibited activities no different than samples from 20- to 36-year-old donors. A more recent study evaluated both CES1 and CES2 transcript, protein and activity levels in selected liver samples from 104 donors ranging in age from 12 weeks gestation to >18 years [137]. Although there was considerable interindividual variability, CES1 and CES2 expression in the fetal samples was 1% or less of that observed in the adult samples. Expression of both enzymes in the 0- to 10-year old samples was ~70% of that observed in the adult. Excellent correlation of transcript and protein levels was observed for both CES1 and CES2. Although again limited by sample size within critical windows of time, a careful analysis of the data reported by Yang *et al.* [137] is consistent with mature levels of CES1 and CES2 being achieved by two to three months of age. This CES1 and CES2 developmental trajectory also is consistent with the data reported by Zhu *et al.* [138], who also demonstrated the lack of any change in hepatic CES1 or CES2 expression in tissue samples from elderly (75–85 years, $N = 8$) versus younger (12–18 years, $N = 4$) donors.

15.9 EPOXIDE HYDROLASE (EPHX)

Although several mammalian epoxide hydrolases (EPHX) (EC 3.3.2.3) exist, two are known for their important role in detoxifying often highly reactive epoxides by the addition of water to form dihydrodiols, microsomal epoxide hydrolase (EPHX1), and soluble epoxide hydrolase (EPHX2). More recently, EPHX2 also has been recognized for its critical role in the inactivation of epoxyeicosatrienoic acid signaling molecules that exhibit potent vasodilatory, anti-inflammatory, and fibrinolytic effects and as such, EPHX2 has become a therapeutic target [139].

Pacifici and Rane [140] and Pacifici *et al.* [141] were the first to demonstrate fetal EPHX1 activity in various tissues between 14 and 25 weeks gestation. Mean ($\pm$ SD, $n = 7$) styrene EPHX1 activity (nmol/min/mg protein) was reported to be 5.9 ± 1.6 in fetal liver, 3.6 ± 1.0 in adrenals, 0.6 ± 0.1 in lung, 0.5 ± 0.4 in kidney, and 0.3 ± 0.1 in intestine. In contrast, EPHX1 activity in adult liver was approximately twofold higher. Although clear differences were noted between fetal and adult liver, there was no apparent change with gestational age. By increasing the sample size to 20 and extending the age range from 10 to 25 weeks gestation, a weak correlation between gestational age and microsomal EPHX1 activity became apparent ($r^2 = 0.6$), but this relationship was

driven heavily by one or two samples [142]. EPHX1 expression differences between fetal and adult liver were corroborated by Cresteil *et al.* [143] who measured both EPHX1 protein levels and benzo[*a*]pyrene 4,5-oxide hydrolase activity in microsomal preparations from 14 fetal liver samples, ages 17–32 weeks, and nine adult liver samples. Fetal EPHX1 activities ranged from 1.0 to 3.3 nmol/min/mg protein, while adult values ranged from 6.0 to 9.7 nmol/min/mg protein and there was an excellent correlation between activity and protein concentration ($r^2 = 0.939$). None of the above studies were able to demonstrate a change in EPHX1 activity or protein levels as a function of gestational or postnatal age, although the substantial difference between fetal and adult liver samples was highly reproducible. Age-dependent changes were demonstrated by Omiecinski *et al.* [144] who determined EPHX1 catalytic activity, protein and mRNA levels in 18 fetal liver samples, ages 7.6–21.9 weeks gestation. Activity ranged from 41 pmol/min/mg protein in the 7.6 week samples to 306 pmol/min/mg protein in the older fetal samples. A high degree of correlation was observed between age and activity ($r^2 = 0.82$), as well as between protein levels and activity ($r^2 = 0.93$). Mean adult hepatic EPHX1 activity was reported as 424 ± 236 pmol/min/mg protein ($n = 15$). No correlation was observed between activity and mRNA content in either the fetal or adult samples. Finally, analysis of the data reported by Hassett *et al.* [145] on 40 liver samples from donors ranging in age from 9 to 67 years failed to identify any changes with age, consistent with the earlier conclusion by Woodhouse *et al.* [146] that EPHX1 expression does not change in the elderly relative to middle-aged individuals.

Mean EPHX2-dependent styrene oxide hydrolase activity was determined to be 0.23 ± 0.02 nmol/min/mg protein in the fetal liver, while adult values were 0.83 ± 0.05 nmol/min/mg protein [141]. Similar results were obtained with stilbene oxide as a substrate. There was no apparent change in EPHX2 activity as a function of gestational or postnatal age, despite the substantial overall differences between the fetus and adult. Similarly, there was no apparent change in EPHX2 expression in the elderly relative to middle-aged adults. However, these conclusions are based on limited sample sets and additional studies using samples from a broader age range in the fetal, perinatal, and later life stages will be needed to better address age-dependent changes in EPHX2 expression.

15.10 GLUTATHIONE S-TRANSFERASE (GST)

The glutathione *S*-transferase (GST) enzymes (EC 2.5.1.18) catalyze the nucleophilic attack of reduced glutathione toward drugs containing electrophilic carbon, nitrogen, or sulfur atoms, thereby detoxifying and often facilitating elimination. Substrates include a wide variety of halogenonitrobenzenes, arene oxides, quinones, and unsaturated carbonyls. Although there are three families of GST enzymes, microsomal, cytosolic, and mitochondrial, this chapter focuses on the cytosolic enzymes, as these are considered most important for drug metabolism [147].

The cytosolic GST enzyme family consists of 16 genes within six subfamilies: *GSTA* (α), *GSTM* (μ), *GSTO* (ω), *GSTP* (π), *GSTT* (θ), and *GSTZ* (ζ) [148]. The encoded enzymes function as dimers with members of the *GSTA* and *GSTM* families being able to form heterodimers. The GST exhibit overlapping substrate specificities, which has made it difficult to characterize individual gene expression based on catalytic activity [147].

Pacifici and Rane [140] and Pacifici *et al.* [141] demonstrated GST activity toward styrene oxide in second trimester fetal liver, adrenal, lung, kidney, and intestine that varied little among the tissues examined and ranged from 2.6 to 5.6 nmol/min/mg protein. Activity against this same substrate increased to 9.95 ± 1.75 nmol/min/mg protein in adult liver. However, members of the GSTM, GSTT, and GSTP families also exhibit activity toward this substrate making it difficult to conclude anything regarding the expression of specific proteins from these studies. The first attempt to differentiate between GST enzymes was accomplished using starch gel electrophoresis and chromatofocusing [149]. GSTM (GST1), GSTA (GST2), and GSTP (GST3) ontogeny was examined in tissue samples that were divided into five age groups: group 1, 10–20 weeks gestation; group 2, 21–30 weeks gestation; group 3, 31–42 weeks gestation; group 4, term infants who died between 2 and 67 weeks postnatal age; and group 5, adults. A progressive increase in GSTM expression was observed as a function of age. Low GSTM expression was observed in a small number of groups 1 and 2 samples accounting for 2.5–16.5% of the total GST activity. However, readily detectable protein was present in half of the group 3 tissue samples accounting for 5.0–24.5% of total activity. The postnatal samples (group 4) exhibited GSTM activities that were no different than that observed in adults, accounting for 14.9–55.3% of total GST activity. In contrast, GSTA was expressed at relatively high levels within each of the age groups, accounting for 45–90% of the total GST activity, with no apparent change with age. The GSTP developmental trajectory was the opposite of GSTM, exhibiting the highest activity in the group 1 samples (30–50% of total GST activity), declining to 10–20% of total GST activity in the group 2 samples, and was undetectable in any of the postnatal and adult liver tissue. A subsequent study by this same group [150] further refined the enzymes being examined by distinguishing between GSTA1 and A2, as well as expanding the number of study samples. GSTA1 and A2 were expressed in fetal liver and there was a significant increase of 1.5- to 2.0-fold at birth, but no change between neonatal and adult samples. In all samples, GSTA2 was present at levels ~10% of GSTA1. GSTA1 and A2 also were present in fetal lung, but at levels 0.5–1% of that observed in the liver and no increase in expression was seen at birth. GSTM and GSTP also were detectable in fetal lung at levels similar to those observed in liver, but decreased by ~50% and 30% at birth, respectively [151]. GSTA1 and GSTA2 were both expressed in fetal kidney at similar levels (0.042 ± 0.05 and 0.03 ± 0.05 $\mu\text{g}/\text{mg}$ cytosolic protein, respectively). Levels of both enzymes increased 5- to 10-fold at birth. GSTM and GSTP were present in both fetal and postnatal kidney samples at levels similar to those observed in liver [151]. Immunohistochemistry demonstrated GSTA and GSTP in both collecting tubules and developing nephrons in fetal kidney, but after 35 weeks gestation, GSTA was restricted to the proximal tubule while GSTP was restricted to the distal and collecting tubules and the loop of Henley [152]. A similar approach demonstrated widespread expression of these same enzymes, as well as GSTO in many fetal tissues [153,154]. However, the demonstration that the GST enzymes are expressed in specific cell types within lung and kidney also suggests that the earlier-measured enzyme levels from total tissue homogenates likely underestimated expression in specific cell types.

Elucidation of GSTZ1's developmental trajectory is of interest because of its critical importance in the disposition of dichloroacetate (DCA) which classically has been used to treat lactic acidosis and more recently, solid tumors [155]. Adults are at greater risk than children for DCA-induced peripheral neuropathy [156]. Evaluation of both

GSTZ1 protein and activity levels *in vitro* demonstrated very low GSTZ1 expression prenatally. Expression increased substantially in the first two weeks after birth and then continued to rise, maturing to adult levels by one year of age. There was a good correlation between protein and activity levels which was improved substantially by the segregation of the data by haplotype [157]. These data suggest that children aged greater than one year would have higher DCA clearance rates and would be less susceptible to drug-induced side effects compared to adults because of their mature levels of GSTZ1 expression combined with their greater liver mass to body surface area.

The above studies demonstrate gene-specific developmental expression patterns for selected members of the GST enzyme family. Furthermore, the relatively high levels and ubiquitous expression would suggest important roles for these enzymes during development. Yet, our knowledge currently is limited to a select few of the 16 genes that constitute the *GST* family [148], suggesting that GST ontogeny remains an important, but understudied area. Even less is known about possible changes in GST expression in the elderly, although no differences were detected in two studies evaluating GST enzyme expression in erythrocytes [158] or lymphocytes [159], but decreased expression was reported in gastric mucosa samples from the elderly [160].

15.11 SULFOTRANSFERASE (SULT)

The family of sulfotransferase (SULT) enzymes (EC 2.8.2.1) catalyze the conjugation of a sulfonate moiety donated by 3'-phosphoadenosine-5'-phosphosulfate to a wide variety of electrophilic substrates, generally resulting in inactivation and facilitated elimination from the body. The human SULT enzymes are encoded by three gene families, the *SULT1* family, associated with phenol conjugation; the *SULT2* family, associated with hydroxysteroid conjugation; and a more recently discovered *SULT4* family expressed exclusively in the brain. The *SULT1* family consists of four subfamilies encoded by seven genes (*SULT1A1*–*1A3*; *SULT1B1*, *SULT1C2* and *1C4*; and *SULT1E1*); the *SULT2* family consists of two subfamilies each encoded by a single gene (*SULT2A1* and *2B1*). The *SULT4* family consists of a single gene, *SULT4A1* [161]. No studies have been reported on the ontogeny of *SULT1A2*, *1B1*, *1C4*, *2B1*, or *4A1*. A single study evaluated the effect of increasing age in adults on paracetamol sulfation and found no impact [162].

Early studies characterized SULT ontogeny using probe substrates with the inherent problem, perhaps not appreciated at the time, of overlapping substrate specificity. Second trimester fetal liver 2-naphthol sulfotransferase activity, most likely catalyzed by one or more members of the *SULT1A* family, was reported to be 0.18 ± 0.12 nmol/min/mg, increasing approximately threefold in adults [163]. In contrast, Duanmu *et al.* [164] described the *SULT1A1* developmental trajectory by determining protein levels, quantifying western blot data by linear regression using recombinant protein as standards. Substantial expression was observed in fetal liver that was not significantly different from liver tissue donated from individuals aged 0 to 12 months or >12 months to 18 years. Stanley *et al.* [165] also demonstrated high *SULT1A1* levels in the choroid plexus using *p*-nitrophenol as a probe substrate (45 pmol/min/mg), but much less so in other regions of the brain (<5 pmol/min/mg protein). A very different expression pattern was observed in the developing lung where a progressive

decrease in SULT1A1 protein level as a function of both prenatal and postnatal age was reported along with a significant twofold decrease in expression when comparing mean prenatal to postnatal protein levels [166].

The SULT1A3 developmental trajectory has been characterized using dopamine as a probe substrate. Enzyme activity was present in a wide variety of second trimester fetal tissue with the highest level in fetal gut (200 ± 133 pmol/min/mg) followed by fetal lung and liver (122 ± 52 pmol/min/mg and 96 ± 37 pmol/min/mg, respectively) and kidney (37 ± 30 pmol/min/mg). Dopamine sulfotransferase activity was unchanged in adult gut (188 ± 33 pmol/min/mg), but was decreased in lung (78 ± 33 pmol/min/mg), liver (30 ± 15 pmol/min/mg), and kidney (11 ± 7 pmol/min/mg) [167]. Substantially greater changes were reported by Richard *et al.* [168] and Stanley *et al.* [165] with a 10-fold decrease in hepatic SULT1A3 levels between second trimester fetal (75.6 ± 52.3) and postnatal liver (6.7 ± 7.6 pmol/min/mg) and a sixfold decrease between fetal (39.7 ± 18.8 pmol/min/mg) and postnatal lung (6.6 ± 10.0 pmol/min/mg). Low SULT1A3 activity also was detected in various regions of the fetal brain (<5 pmol/min/mg protein).

Estrone and/or ethinyl estradiol have been used commonly as probe substrates for SULT1E1. Miki *et al.* [169] detected widespread SULT1E1 expression in several fetal and adult tissues, but were unable to determine any relationship with age. Expression in most fetal tissues, including relatively high levels in the fetal choroid plexus, was confirmed by Stanley *et al.* [165]. Evidence for a decrease in SULT1E1 protein levels between fetal and adult liver also was reported. Such an inverse correlation between hepatic SULT1E1 protein levels and age was confirmed by Duanmu *et al.* [164] who described a progressive decrease in fetal liver expression from the first to third trimester, a further decrease between fetal and perinatal samples, and a final decrease between perinatal samples and tissue from older children aged up to 18 years. A sexual dimorphism also was demonstrated, with males expressing higher levels than females in fetal liver tissue, which the authors speculated might protect the male fetus from excessive estrogen levels.

Although the ontogeny of SULT1C2 has not been documented, transcripts were detected in fetal kidney and liver with the greater concentration in the former tissue [170]. In a subsequent study, the highest SULT1C2 protein levels were observed in fetal small intestine, followed by fetal kidney and liver. Low protein levels were observed in a variety of other fetal tissues. Furthermore, protein levels appeared less in adult liver. No SULT1C1 was detectable in fetal brain [165].

DHEA sulfation can be used to probe for SULT2A1 enzyme levels. Barker *et al.* [171] used this reaction to demonstrate relatively low SULT2A1 activity fetal liver, but substantially increased activity in perinatal and adult samples. Furthermore, there was a good correlation between activity and protein levels. A similar trend was observed in fetal and postnatal lung, although lung SULT2A1 levels appeared to mature somewhat earlier [166]. A subsequent study by this same group documented SULT2A1 enzyme activity in fetal liver (~ 84 pmol/min/mg protein), adrenal (~ 422 pmol/min/mg protein), kidney (~ 49 pmol/min/mg protein), small bowel (~ 56 pmol/min/mg protein), and lung (~ 7 pmol/min/mg protein), but no activity in brain. A substantial increase was observed in adult liver (~ 323 pmol/min/mg protein) [165]. This SULT2A1 developmental trajectory was confirmed by Duanmu *et al.* [164] who demonstrated readily detectable protein and activity in fetal liver which increased significantly in the perinatal period and achieved adult levels in samples older than three months.

15.12 UDP GLUCURONOSYLTRANSFERASE (UGT)

The UDP glucuronosyltransferase (UGT) enzymes (EC 2.1.4.17) catalyze the conjugation of uridine 5'-diphosphoglucuronic acid with hydrophobic compounds containing functional groups, often added during oxidative drug metabolism, to form β -D-glucopyranosiduronic acids. Generally, these products are inactive and targets for facilitated renal and biliary elimination. Glucuronidation accounts for nearly 15% of drug clearance in the adult human [36]. Two human UGT gene families exist that encode 16 proteins. Nine functional genes exist in the *UGT1* family as a single locus on chromosome 2q37, *UGT1A1*, *1A3*, *1A4*, *1A5*, *1A6*, *1A7*, *1A8*, *1A9*, and *1A10*; the *UGT2* family consists of seven genes located at chromosome 4q13–4q13.2, *UGT2A1*, *UGT2B4*, *2B7*, *2B10*, *2B11*, *2B15*, and *2B17* [172]. UGT ontogeny was reviewed by de Wildt *et al.* [173] and the reader is referred to this exhaustive review for more detail. *UGT1A1*, the major enzyme responsible for bilirubin glucuronidation, is undetectable in fetal liver. Enzyme expression increases immediately after parturition in a process independent of gestational age. Adult levels of the enzyme are attained by three to six months of age. Using estrone as a probe substrate, *UGT1A3* was demonstrated in fetal and neonatal liver at levels $\sim$ 30% of those in adult tissue, but the subsequent developmental trajectory remains unknown. *UGT1A6* was present in fetal liver at 1–10% of adult levels. At birth and independent of gestational age, expression increases slowly, achieving 50% of adult values by six months of age. However, *UGT1A6* maturation is not complete until puberty.

Because of its specificity for morphine glucuronidation, several studies have been done on *UGT2B7* ontogeny. In second trimester fetal liver samples, *UGT2B7* was detectable at levels that were 10–20% of adult values. Progressive increases in expression were observed with age such that adult expression levels were attained by two to three months after birth. *UGT2B17* also was shown to be expressed in the fetal liver at levels $<$ 10% of adults. Expression increased to 10% of adult values in the neonate, but the subsequent developmental trajectory is unknown.

Strassburg *et al.* [174] conducted an exhaustive analysis of hepatic *UGT1A1*, *1A3*, *1A4*, *1A5*, *1A6*, *1A8*, *1A9*, *1A10*, *2B4*, *2B7*, *2B10*, and *2B15* ontogeny in 16 infant liver samples, ages 6–24 months, comparing these data to that obtained from 12 adult patients. In addition, RNA from two second trimester fetal samples was available. No UGT transcripts were detected in the two 20-week gestation fetal samples. Although these data conflict with earlier reports wherein low levels of UGT activity and/or protein level were detected, all of the available data are consistent with the onset of low UGT activity during the latter half of the second trimester or the third trimester. Transcript levels in the infant samples were binned into three age brackets, 6–12 months, 13–18 months, and 19–24 months, and compared among themselves and to adult levels. No difference was observed between any of these age brackets and adult *UGT1A1* or *UGT2B7* transcript levels. In contrast, there was a progressive increase in *UGT1A9* transcripts as a function of age. For *UGT2B4*, there were no differences among the different pediatric age groups, which all exhibited protein levels 30–40% of those in adults. The catalytic activities within all of the pediatric age groups were lower than that observed in the adult samples. These data are consistent with a report by Zaya *et al.* [13] who described the maturation of *UGT2B7*-dependent epirubicin glucuronidation. A progressive increase in *UGT2B7* activity was observed across five age groups, $<$ 1 year, 1–5 years, 6–11 years, 12–17 years, and adult. However, allometric scaling

using the 3/4 power rule [175] predicted that UGT2B7-dependent clearance would be attained by two to three months of age, consistent with *in vivo* studies. The more recent study by Miyagi and Collier [176] also is consistent, providing evidence for the maturation of overall hepatic to adult levels by 20 months and more specifically, the maturation of hepatic UGT1A4 activity by 17 months of age.

Studies performed to date would suggest no change in UGT activity in the elderly. Thus, for a variety of benzodiazepines cleared primarily by glucuronidation, there were no or minimal decreases in clearance in older patients [177,178]. Similarly, no differences in paracetamol glucuronidation were observed in patients ranging in age from 40 to near 90 years [162]. Most recently, the glucuronidation of valproate by UGT1A4, 1A8, and 1A10 was evaluated in a bank of liver microsomes from young (2–56 years of age) and elderly (>65 years of age) donors and no differences were observed [179].

15.13 CONCLUSIONS AND FUTURE PERSPECTIVES

Evaluating what is currently known regarding the developmental trajectories of the human hepatic DMEs reveals a pattern consistent with the binning of the enzymes into one of three classes depending on the individual temporal-specific expression patterns (Table 15.1). Class I enzymes are expressed at relatively high levels during the first trimester. The expression of these enzymes either remains elevated or decreases during gestation. However, within a few days to as long as two years after birth, expression is either silenced or reduced to low levels. An obvious question is whether or not class I enzymes have an important endogenous function during hepatic development. Enzymes belonging to class II are expressed at relatively constant levels throughout gestation and into adulthood. Expression may or may not increase modestly within the first year after birth. Finally, class III enzymes are not detectable or are detected at low levels in the fetus. For some, the onset of expression can be seen in either the second or third trimester, but levels remain substantially less than those observed in the adult. Significant increases in expression are observed within the first one to two years after birth; however, full maturation does not occur until postpuberty for a few of the class III enzymes. Class III also appears to include the largest number of enzymes. The developmental trajectories for human hepatic FMO1, CYP2C19, and

TABLE 15.1 Classification of Drug-Metabolizing Enzymes Based on Developmental Trajectories

Class I	Class II	Class III	
ADH1A	CYP2B6	ADH1B	GSTM
CYP1A1	CYP2C19	ADH1C	GSTZ
CYP3A7	CYP3A5	AOX	FMO3
FMO1	EPHX1	CYP1A2	PON1
GSTP	EPHX2	CYP2A6	SULT2A1
SULT1E1	GSTA	CYP2C8	UGT1A1
SULT1A3	SULT1A1	CYP2C9	UGT1A6
		CYP2D6	UGT2B7
		CYP2E1	CES1
		CYP3A4	CES2

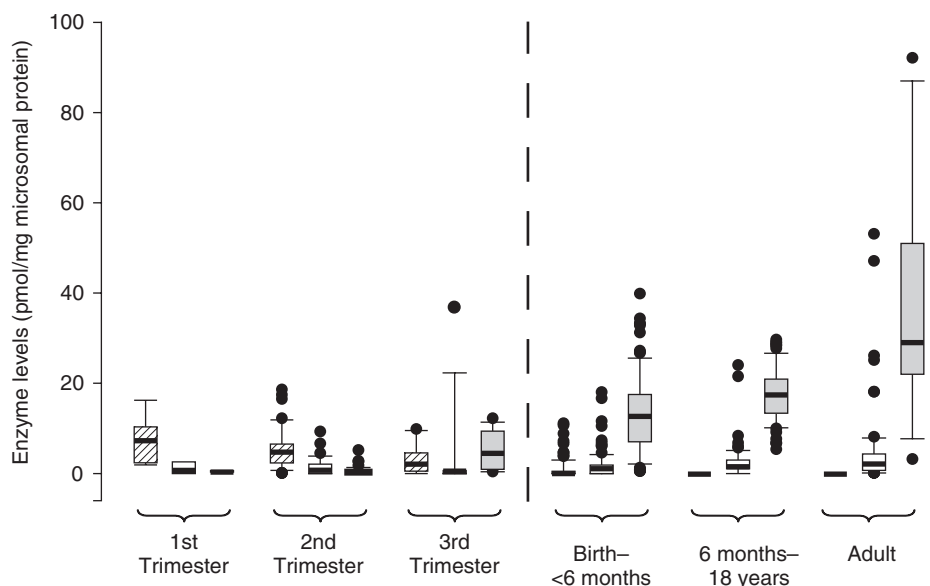


Figure 15.1 Developmental trajectories of class I, class II, and class III enzymes. Human FMO1 (hatched bars), CYP2C19 (white bars), and CYP2C9 (gray bars) hepatic protein expression levels as a function of age are depicted to illustrate the developmental trajectories typical of class I, class II, and class III enzymes, respectively. Data are plotted as medians (bars), interquartile values (boxes), and tenth to ninetieth percentiles (whiskers). Outliers were defined as 1.5 times the interquartile values. *Source:* Data on the expression of FMO1 in pediatric samples was derived from Koukouritaki *et al.* [112]. Data on the expression of CYP2C9 and CYP2C19 in pediatric samples was derived from Koukouritaki *et al.* [51]. Data on the expression of CYP2C9 and CYP2C19 in the adult were derived from Lapple *et al.* [180].

CYP2C9 as examples of class I, class II, and class III enzymes, respectively, are shown in Fig. 15.1. Whether or not this classification of enzymes also will apply to developmental trajectories in extrahepatic tissues remains to be seen.

For those DME that belong to class III, most if not all exhibit greater interindividual variability during the perinatal period compared to other age brackets. This observation is most apparent in scatter plots of the developmental expression data presented in some of the original reports on FMO3 [112], CYP2C9 [51], CYP2E1 [82], and CYP3A4 [94]. This is largely explained by what appears to be interindividual variability in the timing of the postnatal increase or onset of expression of these enzymes. As an example, both CYP2C9 and 2E1 exhibited an ~ 100 -fold range of expression in the perinatal period, which was approximately two times greater than that observed within any other age bracket. Thus, there would appear to be windows of hypervariability during drug-metabolizing enzyme ontogeny that would significantly impact risk for adverse drug reactions and/or failed efficacy and would not be predicted based on adult pharmacogenetic studies.

For multiple enzyme families with members belonging to both class I and class III, the term *developmental switch* is often used to describe the transition between the predominant fetal to the predominant adult enzyme form. However, in none of these enzyme systems is there any evidence for coordinated inverse regulation between these

“fetal” and “adult” enzymes, suggesting the term *developmental switch* is a misnomer and that developmental transition is a more appropriate description of this process. Consideration of these data also suggests that for enzyme systems such as CYP2C9 and 2C19 that appear to share some regulatory mechanisms in the adult, mechanisms regulating ontogeny are strikingly different.

Although improving, there remains a need for additional studies to define the true ontogeny of many of the enzyme systems, particularly in extrahepatic tissues. Too much of our current knowledge is based on data from tissue samples or *in vivo* studies that utilized samples or recruited patients representing narrow time windows. Conclusions drawn from such studies can be contradictory and misleading. Despite these knowledge gaps, the knowledge base is sufficiently robust to support the development of physiologically based pharmacokinetic models that provide a much improved means of predicting age-specific drug disposition [181–184]. Further advances in the field will only improve these valuable tools.

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16 Phytochemical Modulators of Human Drug Metabolism: Drug Interactions with Fruits, Vegetables, and Botanical Dietary Supplements

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16.1	Summary	1
16.2	Introduction	2
16.3	Plant–animal “warfare” and the evolution of metabolic defense mechanisms	3
16.4	Plant secondary metabolites (PSM): plant synthesis versus human metabolism	4
16.5	Food–drug interactions: cruciferous vegetables and citrus juices	10
16.6	DSHEA, dietary supplements, and the risk for herb–drug interactions	15
16.7	Uncertainty in predicting and interpreting herb–drug interactions	16
16.8	Disconnect between <i>in vitro</i> predictions and <i>in vivo</i> realities	19
16.9	Adulterants and contamination	21
16.10	Popular dietary supplements and their drug interaction potential	22
16.11	Conclusions and future perspectives	49
	References	49

16.1 SUMMARY

Plant secondary metabolites (PSMs) have been components of man’s diet for millennia and are believed to have played a significant role in steering the functional development of xenobiotic metabolizing enzymes (XMEs) and transporters within the human gastrointestinal tract. Only recently, however, PSMs have been recognized

as modulators of human drug disposition. Despite exposure to thousands of structurally diverse dietary phytochemicals, only a few appear to significantly modulate human drug-metabolizing enzymes and transporters. In some instances, these interactions may have beneficial effects, such as cancer prevention (i.e., isothiocyanates in cruciferous vegetables), whereas others may dramatically affect the pharmacokinetics of concomitantly administered drugs (i.e., furanocoumarins in grapefruit juice). In today's global economy, the opportunity for exposure to more exotic phytochemicals is significantly enhanced. Formulated as concentrated phytochemical extracts, botanical dietary supplements are vehicles for a host of PSMs rarely encountered in the normal diet. When taken with conventional medications, botanical dietary supplements may give rise to clinically significant herb–drug interactions. These interactions stem from phytochemical-mediated induction and/or inhibition of human drug-metabolizing enzymes and transporters.

In this chapter, the herb–drug interaction risks and mechanisms for several of the most popular dietary supplements are discussed. Botanicals most likely to produce clinically important herb–drug interactions are those whose phytochemicals act as mechanism-based inhibitors of cytochrome P450 enzyme (CYP) activity (e.g., *Hydrastis canadensis*, *Piper nigrum*, and *Schisandra chinensis*) or function as ligands for orphan nuclear receptors (e.g., *Hypericum perforatum*). In addition, several external factors unrelated to phytochemical pharmacology can augment the drug interaction potential of botanical supplements.

16.2 INTRODUCTION

Since the dawn of man, plants have been a rich source of food and medicine. Thus, an almost constant exposure to an enormous array of plant-derived chemicals (phytochemicals) has had a profound influence on human evolutionary and social development. Unbeknownst to many, evolutionarily ancient phytochemicals lie at the root of man's ability to metabolize modern-day drugs. This is because as humans we have, in a sense, phylogenetically “inherited” the multiplicity of XMEs and transporters necessary for detoxifying dietary and environmental chemicals. In effect, the expression and activity of human XMEs and transporters have been shaped by man's exposure to phytochemicals, either as dietary components or as natural medicines.

Modern drugs, many of which are derived from plant sources, are pharmacologically potent single-chemical entities, and XMEs and transporters are important determinants of their absorption, metabolism, distribution, and excretion. A variety of exogenous factors (e.g., diet, drugs, and environmental chemicals) can modulate XME and transporter activity. These factors, in turn, can affect the efficacy and toxicity of concomitantly administered medications. While most health care professionals and many laypersons are familiar with the concept of drug–drug interactions, as well as their adverse consequences, few recognize that many fruits and vegetables can also interact with conventional medications. Moreover, the recent upsurge of herbal dietary supplement usage and the influx of non-Western medical practices (e.g., traditional Chinese medicine (TCM), ayurvedic medicine, and Kampo medicine), that frequently incorporate a variety of botanical extracts, pose additional concerns. Together, fruits, vegetables, and especially botanical dietary supplements form the basis for this newly recognized category of herb–drug interactions.

Herb–drug interactions, while ancient in their origins, can have devastating consequences in today’s culturally diverse health care environment. In the last decade, several clinically significant herb–drug interactions have been recognized, prompting a surge of research into their cause and mechanism. Essentially two types of herb–drug interactions can be ascribed: pharmacodynamic and pharmacokinetic. Pharmacodynamic herb–drug interactions involve botanicals and conventional medications that enhance or negate each other’s effects as a result of similar or disparate pharmacologic activity, respectively. For example, weight-loss supplements formulated with *Ephedra sinica* can negate the effects of many antihypertensive medications due to the sympathomimetic effects of ephedrine alkaloids. Pharmacokinetic herb–drug interactions arise from the ability of phytochemicals to modulate the activity of XMEs and/or various drug transporters involved in the disposition of conventional drugs. This chapter focuses on pharmacokinetic herb–drug interactions, with an emphasis on their sources, causes, and clinical relevancy.

16.3 PLANT–ANIMAL “WARFARE” AND THE EVOLUTION OF METABOLIC DEFENSE MECHANISMS

Phylogenetic analyses indicate that the evolution of vertebrate XMEs, transporters, and their nuclear receptor regulators began 400 million years ago in a “war” of sorts between terrestrial plants and animals [1–5]. According to the hypothesis, as insects and higher order animals began feeding on plants; the plants, in turn, synthesized toxic phytochemicals as a means of deterring herbivory. Because plants are sessile autotrophs (organisms that synthesize organic compounds from inorganic sources of carbon and energy) incapable of avoiding predation by heterotrophs (organisms that use organic carbon for growth by consuming other organisms), they have invested heavily into chemistry for their survival. One of the principal returns on this investment is the ability to synthesize a myriad of phytochemicals known as *PSMs*. Although not part of the primary biochemical pathways of cell growth and reproduction, PSMs are involved in regulation of symbiosis, defense against herbivores and pathogens, and chemical inhibition of competing plant species [6,7]. As plants evolved and adapted to more difficult environmental stresses, so did the variety, complexity, and toxicity of their resident PSMs. These defense phytochemicals influence herbivores and microbes in a negative manner by interfering with one, or oftentimes several, molecular targets (e.g., receptors, enzymes, and other proteins). The structures of defense phytochemicals appear to have been shaped during evolution in such a way as to mimic the structures of endogenous substrates, hormones, neurotransmitters, or other ligands found in animals—a process termed *evolutionary molecular modeling* [8].

With the ingestion of myriad nonnutrient PSMs over millions of years, mammalian detoxification systems coevolved into an intricate system of XMEs, transporters, and nuclear receptors familiar to most biologists and pharmacologists today. In the case of mammals, detoxification of PSMs is accomplished through combinations of intestinal and hepatic XME-mediated oxidative, reductive, and hydrolytic biotransformations (phase I metabolism); conjugation reactions (phase II metabolism); or transporter-mediated efflux into the gut lumen or bile (phase III). Over time, continued exposure to multitudes of structurally diverse PSMs appears to have shaped the specificity, capacity, and the “promiscuity” of mammalian transporters and XMEs, rendering them

effective at recognizing, transforming, and excreting a multiplicity of xenobiotic structures. This constitutes a mammalian catabolic antidote to plant biosynthetic chemical warfare [1–5].

16.4 PLANT SECONDARY METABOLITES (PSM): PLANT SYNTHESIS VERSUS HUMAN METABOLISM

PSMs number in the tens of thousands and include such structurally diverse groups of organic compounds such as polyphenolics, alkaloids, nonprotein amino acids, polyketides, terpenes, steroid and triterpenoid saponins, cyanogenic glycosides, glucosinolates, flavonoids, phenylpropanoids, and others [6,7,9]. Central to PSM biosynthesis and accumulation are a host of plant enzymes and membrane transporters, including several whose homologous mammalian forms are familiar to most life scientists: CYPs, uridine diphosphate glycosyltransferases (UGTs), glutathione *S*-transferases (GSTs), sulfotransferases (SULTs), ATP-binding cassette transporters (ABC transporters), and solute carrier membrane transport proteins (SLCs).

CYPs, a group of heme-containing metalloenzymes, catalyze a host of crucial biological oxidation reactions in both prokaryotes and eukaryotes. In most cases, these reactions can be summarized as activation of the substrate to a reactive intermediate using electrons from nicotinamide adenine dinucleotide phosphate (NADPH) through the action of NADPH-reductase, followed by the introduction of an oxygen atom to form a more polar product. In plants, CYPs are ubiquitous. Fifty-nine families have been identified and most of them play an integral role in the synthesis of endogenous PSMs by catalyzing a wide variety of monooxygenation/hydroxylation reactions [10]. Plant CYPs also mediate the oxidative biotransformation of xenobiotics such as herbicides/pesticides into more polar, less phytotoxic compounds (phase I metabolism).

A host of UGTs, GSTs, and SULTs, catalyze the transfer of polar functional groups—sugars, sulfate, and sulfhydryl, respectively—to a wide variety of substrates as a means of increasing hydrophilicity. These polar moieties improve PSM water solubility and transportability. Of the 19 families of plant UGTs, all transfer nucleotide-diphosphate-activated sugars to low molecular weight aglycone substrates thereby increasing their water solubility and providing access to active membrane transport systems that recognize glucosides but not aglycones [11–13]. SULTs and GSTs participate in several synthetic pathways; however, the extent of their involvement in PSM production has yet to be fully elucidated [14,15]. Recently, considerable attention has been paid to plant transferases and their role in propagating resistance to man-made agrochemicals [16]. Resistance develops when plants upregulate both CYPs and transferases in response to continued herbicide exposure [17]. As phase II catalysts, UGTs, GSTs, and SULTs conjugate and further detoxify metabolites of herbicides and pollutants. These conjugated xenobiotics, in turn, are readily sequestered into plant vacuoles, where their phytotoxic threat is significantly diminished [16,17].

The ABC transporter proteins are a large and diverse superfamily of mainly membrane-bound proteins ubiquitous in all organisms, the hallmark of which is their ability to derive energy from ATP hydrolysis to actively transport molecules through membranes against a concentration gradient. Plants are a rich source of ABC transporters. More than 130 ABC protein genes have been identified in the genomes

of both monocot (*Oryza sativa*) and dicot (*Arabidopsis thaliana*) plants [18]. Among their various functions is the transport of PSMs within and between cells and cellular organelles [18]. Plants utilize ABC transporters to concentrate PSMs in those tissues vulnerable to herbivory, infection, or other stresses [18,19]. Unlike mammals, plants cannot excrete xenobiotic metabolites; therefore, ABC proteins aid in the transport of glycosylated metabolites (a phase III process) into vacuoles and the cell wall, where they are sequestered and their phytotoxicity is neutralized.

Solute carrier proteins (SLCs) promote the transmembrane movement of molecules without the hydrolysis of ATP. They can function as uniporters if a single type of molecule is transported down an electrochemical gradient, symporters if two molecules are moved simultaneously in the same direction, or antiporters if two molecules are exchanged in opposite direction. Although several SLC families (e.g., SLC7, SLC13, SLC17, SLC22, SLC36, and SLC38) have been identified as transporters of positively and negatively charged endogenous substrates essential to primary metabolism (e.g., monovalent and divalent cations, amino acids) [20], their role in the transport of PSMs remains largely unknown. Since most members of the SLC22 family in bacteria and animals are polyspecific (i.e., carry multiple substrates) and transport both endogenous and exogenous compounds including many plant-derived drugs; it is likely that several plant SLC22 members also function in the transport of charged PSMs [21]. Taken together, CYPs, UGTs, GSTs, SULTs, as well as ABCs, and perhaps SLC proteins, comprise an important assemblage of enzymes and transporters vital to the synthesis and accumulation of secondary metabolites in plants.

In order to detoxify noxious PSMs, vertebrate animals developed analogous enzymes and transporters within their gastrointestinal tracts, in addition to several heterologous transporters not found in plants [e.g., organic anion transporting polypeptides (OATPs, SLC21s)]. Unlike plants, which utilize CYPs, UGTs, and ABC transporters primarily for PSM synthesis and storage, mammals use them to metabolize and eliminate PSMs.

Of the 18 identified CYP families in humans (41 fewer than in plants), only 3 (CYP1, 2, 3) are involved in the catalytic oxidation of xenobiotics, including PSMs [22]. Within these families, nine specific isoforms constitute the bulk of human phase I reactions: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5. Of these, CYP3A4/5 is perhaps the most important, as it is involved in metabolizing almost half of all conventional medications. CYP2D6 and CYP2C9 rank second and third, respectively in numbers of drugs affected. These enzymes catalyze a variety of reactions including hydroxylations; dehalogenations; deaminations; N-, S-, and O-dealkylations; epoxidations; N-oxidations; peroxidations; and sulfoxidations, which usually render molecules more polar and easier to excrete. Present at high concentrations in intestinal enterocytes and liver hepatocytes, CYPs are the principal mediators of human phase I metabolism [22].

As for human UGTs, just 10 specific enzymes from 2 families (UGT1A and 2B) catalyze the bulk of all xenobiotic conjugations [23]. Like CYPs, UGTs are found in high concentrations in the intestine and liver. Unlike their plant homologs, however, human UGTs utilize glucuronic acid as the donor sugar. Glucuronide conjugates are more water soluble and, in many cases, less bioactive. Five classes of human GSTs catalyze the nucleophilic attack of glutathione on electrophilic substrates, which in the case of lipophilic PSMs, enhances their solubility and elimination [24]. Two families of SULTs, SULT1 and 2, which comprise 10 separate isoforms, carry out the transfer of a sulfate group to dietary PSMs [25]. As with most conjugation reactions, sulfonation usually

renders a substrate less toxic. SULTs, however, may activate certain PSMs that are pro-carcinogens (e.g., estragole and safrole) [26]. Collectively, the conjugative capabilities of UGTs, GSTs, and SULTs constitute the bulk of human phase II metabolism.

Approximately 50 ABC transporters have been discovered so far in humans, 14 of which are related to the efflux of xenobiotics from various cell types [27,28]. ABCs are expressed in a variety of tissues, often in normal epithelial cells such as the brush border membrane of intestinal enterocytes, the canalicular surface of hepatocytes, and the apical membrane of proximal renal tubular cells [27,29]. ABCs, in these tissues, are believed to play protective roles; intestinal ABCs limit the absorption of xenobiotics, whereas liver and kidney ABCs facilitate elimination of xenobiotics through biliary and urinary excretion, respectively [30]. Among the most studied ABC transporters is ABCB1, also known as *P-glycoprotein* (*P-gp*) or multidrug resistance protein 1 (MDR1). Highly expressed in the intestine, liver, and brain, ABCB1 functions as an efflux pump exhibiting broad substrate specificity, including affinity for many PSMs [27–30]. It is now well recognized that the phase III safeguard provided by ABCs works in concert with phase I and II enzymes to restrict systemic PSM exposure.

Two solute carrier families (SLC21 and SLC22) function in humans as uptake transporters for a variety of endogenous and exogenous anions, cations, and zwitterions [27]. Specific members of these families may be found on the apical and/or basolateral surfaces of cell membranes in a host of tissues [27,30]. It is as substrates for these polyspecific SLCs that many PSMs gain access to intestinal enterocytes and hepatocytes, at which point they can be acted on by XMEs and ABC transporters.

As a general rule, human XMEs and transporters exhibit broad substrate specificity, a characteristic that may be an adaptation to the selective pressures of an evolutionarily long and varied exposure to dietary PSMs [31]. In contrast, plant CYPs display much narrower substrate specificities [17,32]. Such differences underscore the discrete roles these proteins play in plants, where they function in PSM synthesis and accumulation, versus humans, where their purpose is to metabolize and excrete PSMs. At first glance, this impressive collection of plant and animal proteins appears to be working at cross-purposes, yet they actually share a common goal of defense of the organism.

16.4.1 PSM-Mediated Modulation of Human Drug Disposition

When consumed as part of the normal diet or as constituents in dietary supplements, many PSMs have the ability to modulate (inhibit or induce) the activity of human XMEs and transporters [33,34]. For most transporters, transferases, and CYPs, PSM-mediated inhibition at the protein level can be broadly classified as competitive or noncompetitive. Competitive inhibition occurs when PSMs simply compete with another substrate (e.g., drug) for either a transporter's binding domain or an enzyme's catalytic pocket, whereas noncompetitive inhibition involves binding at a position other than the active site (i.e., an allosteric site). In both competitive and noncompetitive inhibition, the effects are reversible. PSMs may, however, also produce irreversible inhibitory effects, to which CYPs are particularly susceptible [33]. This process, known as *mechanism-based inhibition*, involves catalytic activation of the PSM to produce a reactive metabolite (i.e., metabolic intermediate or MI) that irreversibly binds to and inactivates the CYP. Compared with reversible inhibition, mechanism-based inhibition of CYPs by PSMs more frequently cause clinically important herb–drug interactions, as the inactivated enzyme has to be replaced by newly synthesized CYP protein [33].

Induction of XMEs and transporters by PSMs generally occurs via ligand-activated transcription factors [35]. Distributed in the liver, intestine, and other tissues, these cytosolic proteins, or nuclear receptors, can sense a wide variety of xenobiotics and act as master regulators of XME and transporter expression [36]. Ligand-bound nuclear receptors form heterodimers with related coactivator proteins and translocate to the cell nucleus. There, these protein complexes bind several distinct DNA elements of XME and transporter genes to activate their transcription. Among the human nuclear receptors that recognize PSMs are the aryl hydrocarbon receptor (AhR), a regulator of CYP1A2 and ABCB1; the pregnane xenobiotic receptor (PXR), a promiscuous xenosensing regulator of CYP2A, CYP2B, CYP2C, CYP3A, UGT1A, SULT2A, GSTs, various ABC transporters; and the constitutive androstane receptor (CAR), which directs the expression of CYP2B6, CYP2C9, CYP3A4, UGT1A, SULT2A, GSTs, and certain ABC isoforms. Considerable cross talk also occurs among the nuclear receptors, as they can share ligands, DNA-binding elements, and coactivators [37]. Simultaneously with CYP and ABC upregulation, ligand-bound nuclear receptors may decrease SLC expression, a response in line with their function as xenosensors [27,37]. In short, these regulatory processes ensure optimized XME and transporter expression in the milieu of PSM exposure. Of the many XMEs and transporters under the control of nuclear receptors, CYP2E1 is an exception. Induction of CYP2E1 occurs posttranscriptionally, presumably by stabilization of enzyme degradation [38].

The heterogeneous nature of PSMs and their effects on human XME and transporter activity is exemplified by the sulfur-containing glucosinolates found in cruciferous vegetables. When ingested in sufficient quantities, vegetable glucosinolates are converted to isothiocyanates, which can inhibit certain CYP isoforms (e.g., CYP1A2 and CYP2E1) while inducing the activity of various GSTs and UGTs [39]. Other noteworthy examples include hyperforin, a prenylated phloroglucinol found in St. John's wort (SJW) (*Hypericum perforatum*), and isoquinoline alkaloids present in goldenseal (*Hydrastis canadensis*) [33]. Hyperforin, a potent PXR ligand, induces several CYPs, UGTs, and ABC transporters; while the isoquinoline alkaloids berberine and hydrastine are mechanism-based inhibitors of CYP3A4 and CYP2D6. From an evolutionary viewpoint, it is understandable why XME and ABC transporter induction can be beneficial to mammals, as this mechanism would facilitate elimination of potentially harmful dietary PSMs. From the plant's perspective, an ability to metabolically activate PSMs into more toxic species by stimulating XME expression in animals may be just one course of action in a multiple defense strategy. Many dietary PSMs, for example, are procarcinogens that require either CYP- (e.g., sinigrin, an allyl isothiocyanate found in brown mustard) or SULT-mediated activation (e.g., safrole and estragole) [40]. Granted, PSM-induced carcinogenesis is an unlikely mechanism for deterring herbivory, as cancer usually affects animals after the reproductive stage. These activated chemicals also would not have a detrimental effect on a population nor would they play a role in evolutionary selection over time [41]. Nevertheless, a means for generating toxic PSMs through bioactivation could be a beneficial adaptation for the plant.

Inhibiting animal XMEs may be another favorable defense tactic for plants, especially if the absorption of one group of PSMs is facilitated through competitive or noncompetitive inhibition mechanisms produced by a second group. A multitude of unique PSMs can be present in a given plant species and, when consumed, these selected chemical entities may work in concert through XME or ABC transporter inhibition—and possibly induction—to promote the exposure of one PSM at the

expense of another. An example familiar to most pharmacologists is the mechanism-based inhibition of human intestinal CYP3A4 by furanocoumarins present in Rutacea spp. (e.g., grapefruit, and Seville orange) [42]. When concomitantly ingested, the citrus furanocoumarins bergamottin and 6',7'-dihydroxybergamottin can significantly increase the oral bioavailability of various CYP3A4 drug substrates [43]. However, it remains to be determined if the absorption of other grapefruit PSMs are similarly affected by the inhibitory effects of furanocoumarins. In itself, grapefruit juice is not harmful to humans, but its ability to inhibit CYP3A may have evolved as a successful defense ploy against other species.

Of course, PSMs generated by plants in response to stresses imposed by one species may actually prove beneficial when ingested by another species. Fruits and vegetables as part of the human diet are a case in point. Owing to the variety of fruits and vegetables consumed by humans, PSM-mediated inhibition of XMEs and transporters may have salutary effects. For example, CYP2E1 catalyzes the activation of certain nitrosamines and other environmental carcinogens [44,45]. Inhibition of CYP2E1 activity and expression by organosulfur compounds present in garlic was correlated with a reduced tumor incidence in rat models of carcinogenesis involving nitrosamines [46,47]. Moreover, allyl sulfides in garlic oil and isothiocyanates from watercress have been shown to selectively inhibit human CYP2E1 *in vivo* [48,49]. While not conclusive, such effects likely represent one of many mechanisms to explain why diets rich in sulfur-containing PSMs appear to be chemopreventative [39,50]. Similar effects are likely related to interactions between PSMs and other XMEs and transporters.

The aforementioned examples illustrate that defensive strategies devised by both plants and animals, as a result of their continued "warfare," have garnered biological defeats and victories for both sides. From the human perspective, the savage nature of certain battles in this "war" is still visible within the genetic code.

16.4.2 Dietary PSMs and Human CYP Polymorphisms

For thousands of years, plants have been an integral component of the human diet and, as a result, man has developed an efficient means for detoxifying plant-derived xenobiotics. Several lines of evidence, however, point to man's continued exposure to unique phytochemicals, or lack thereof, as a possible mechanism for establishing genetic polymorphisms in XMEs [31,41,51]. Allelic variants in CYPs found among various ethnic groups are believed attributable, at least in part, to diets enriched by plants indigenous to specific geographic regions of the world [51]. Support for this hypothesis is coupled to the historical use of nitrogenous plant alkaloids among peoples of North Africa and the Middle East and the prevalence of the CYP2D6 ultrarapid metabolizer (UM) phenotype in these populations [52].

CYP2D6 is an important human CYP isoform with regard to xenobiotic metabolism in that it catalyzes the metabolism of many nitrogen-containing drugs and has a high affinity for plant alkaloids [52,53]. Two other characteristics are also notable for CYP2D6: the enzyme is polymorphic, and it is not inducible [52]. Allelic variants of the *CYP2D6* gene yield a range of xenobiotic metabolizing phenotypes: *poor metabolizer* (i.e., homozygous for inactive alleles), *intermediate metabolizer* (heterozygous for an active and inactive allele), *extensive metabolizer* (homozygous for active alleles), and *UM* (multiple copies of active alleles). Generally, most polymorphisms in CYP genes are of low frequency, but several are found with relatively high frequency in certain

populations. For example, the incidence of the CYP2D6 UM phenotype is ~10%, 20%, and 30% for people originating from Turkey, Saudi Arabia, and Ethiopia, respectively, whereas UMs are uncommon in Northern Europe (1–2%) and virtually absent in Asia [52,54]. This polymorphism occurs at rates far higher than can be accounted for by random mutation events and is believed to reflect selection pressures resulting from early exposure to local edible and medicinal plants [51,54,55]. Perhaps not coincidentally, North Africa and the Middle East are host to many plants whose nitrogenous alkaloids are CYP2D6 substrates. The opium poppy, a source of nitrogen-containing opioids, is native to the Turkish region, and khat (a plant containing amphetaminelike compounds) is native to Northeast Africa and is ubiquitously chewed in Ethiopia and the Arabian Peninsula [55].

That CYP2D6 is not inducible may have rendered it more susceptible to dietary pressures that select for expression of multiple copies of functional alleles. This adaptation would have allowed subjects to more efficiently detoxify plant toxins. It has been proposed that this selection pressure occurred in Northeast Africa ~5000–10,000 years ago during periods of starvation, which favored survival of those subjects exhibiting the UM phenotype [54,56]. Such a selection would have increased the number of plants available to provide useful food. Subsequent migrations to the Mediterranean area may also explain the high frequency of UMs in Southern Europe [52,56].

The reasons for mutant CYPs, which gave rise to slow-metabolizer phenotypes, are not known, but it is possible that during the course of human evolution the requirement for detoxification of nitrogenous plant alkaloids became unnecessary because of dietary changes. Thus, allelic variants, which would have spontaneously arisen, may have become established in the human genome due to lack of coevolutionary pressures to suppress them [31]. In other words, elimination of dietary pressures that had selected for functional XMEs now favored selection of polymorphs with reduced function. Similar arguments have been made to explain the frequency of CYP2A6 reduced function alleles among Japanese [55], the slow-acetylator phenotype for *N*-acetyltransferase among Caucasians [51], and the ethnic distribution of glucose-6-phosphate dehydrogenase mutations [51]. One prominent researcher in the field provided a succinct summation of the theory: “It is very likely . . . that 6000 years of a diet principally of goat meat and milk products—compared with that principally of tropical fruits and plants—might lead to XME polymorphisms” [51].

Over the last 10,000 years, the advent of agricultural practices and plant domestication has, in many instances, reduced the number and type of toxic PSMs commonly encountered in the human diet, and increased the content of beneficial phytochemicals [57]. This circumstance has become even more pronounced in the last 100 years with the global distribution of domesticated grains, fruits, and other plant foodstuffs, in effect leavening man’s exposure to more common PSMs. Today, many PSMs present in fruits and vegetables (e.g., flavonoids and polyphenolics) are thought to possess beneficial health effects, especially with regard to the prevention of chronic diseases (e.g., cancer, cardiovascular disease, and diabetes) [58–63]. As mentioned previously, modulation of XMEs and transporters are among the various pharmacologic mechanisms underlying the salutary effects of fruit and vegetable consumption. Yet, when taken concurrently with conventional medications, these mechanisms may give rise to clinically important drug interactions. Although this chapter focuses on botanical dietary supplements and their potential to effect clinically

important herb–drug interactions, two examples that involve foods—cruciferous vegetables and citrus juices—merit discussion.

16.5 FOOD–DRUG INTERACTIONS: CRUCIFEROUS VEGETABLES AND CITRUS JUICES

That food can affect drug disposition has been recognized in the pharmacy profession for decades [64–67]. The effect of meals and their composition (i.e., protein, carbohydrate, lipid) on the rate and extent of drug absorption remains an area of active research [68–71], with several new emerging theories on the role of dietary lipids in enhancing bioavailability [72,73]. Only recently, however, has an appreciation developed for the ability of dietary phytochemicals to alter drug absorption and disposition. Of the myriad foods present in the modern diet, cruciferous vegetables and citrus juices provide two classic illustrations of how common dietary phytochemicals may underlie clinically important food–drug interactions.

16.5.1 Cruciferous Vegetables

Cruciferous crops belong to the family Brassicaceae (also known as *Cruciferae*) and include such vegetables from the genus *Brassica* as broccoli (*Brassica oleracea* var. *italica*), brussels sprouts (*B. oleracea* var. *gemmifera*), cabbage (*B. oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *botrytis*), mustards (*Brassica juncea*), turnips (*Brassica rapa* var. *rapa*), as well as watercress (*Nasturtium officinale*), radish (*Raphanus sativus*), and others. Unique to dietary crucifers are the glucosinolates, a group of sulfur-containing PSMs whose hydrolyzed metabolites appear to have cancer preventative properties. Synthesized by various plant CYPs, the glucosinolate molecule consists of a β -thioglucose unit, a sulphonated oxime unit, and a variable side chain derived from an amino acid. (Fig. 16.1) [74]. When cut or chewed, cruciferous vegetables release myrosinase, an enzyme that catalyzes the hydrolysis of glucosinolates to form isothiocyanates, epithionitriles, or thiocyanates (Fig. 16.1). Cooking (mild or prolonged) may inactivate myrosinase affecting the type and content of hydrolytic metabolites formed. As a result of cooking, those glucosinolates that escape myrosinase hydrolysis may still be converted to isothiocyanates in the gut via microbial activity, although the bioavailable content is much reduced [39,74].

The chemopreventative effects of cruciferous vegetables and their associated PSMs have been widely studied in a variety of experimental models [75]. To date, the preponderance of these studies have focused on isothiocyanates, which are believed to protect against the carcinogenicity of various environmental chemicals (e.g., nitrosamines, heterocyclic amines, and polyaromatic hydrocarbons). In animal models, isothiocyanates appear to inhibit CYP1A2, CYP2E1, and CYP3A4, while the activities of various GSTs, UGTs, and SULTs are induced [50,75]. This combination of CYP inhibition and transferase induction is believed to account, in part, for the chemoprotection offered by cruciferous vegetables [39].

Clinical studies employing cruciferous vegetable diets, while providing less-compelling proof of chemoprevention, have also demonstrated differential modulatory effects on human XMEs and transferases. More than 30 years ago, diets supplemented with brussels sprouts (150 g, twice daily) and cabbage (100 g, twice daily) were found

to increase the clearance and reduce plasma concentrations of the nonspecific phase I probe, antipyrine [76], as well as the drug acetaminophen [77]. For acetaminophen, the diets significantly enhanced glucuronide metabolite formation [77]. Soon after, watercress (50 g, once daily) was shown to decrease plasma concentrations of CYP2E1-generated acetaminophen metabolites [78], while the AUC of chlorzoxazone, a recognized CYP2E1 substrate [49], was markedly increased. Watercress also increased glucuronidation of a nicotine metabolite [79], but seemed to have little effect on nicotine oxidation, a marker of CYP2A6 activity. Surprisingly, broccoli (500 g/day for 12 days) had no effect on chlorzoxazone metabolism, but appeared to increase the caffeine/paraxanthine metabolic ratio, an indicator of CYP1A2 activity [80]. Such observations appeared to correlate with *in vitro* CYP-inhibitory effects of individual isothiocyanates prevalent in each crucifer species [75]. Phenylethyl isothiocyanate (PEITC, Fig. 16.1), the hydrolytic metabolite of gluconasturitiin (watercress's principal glucosinolate) and an inhibitor of murine CYP2E activity, was believed responsible for inhibition of human CYP2E1 [78]. For broccoli, whose principal isothiocyanate, sulforaphane, possesses a methylsulfinylbutyl side chain (Fig. 16.1), the induction of CYP1A2 seemed linked to sulforaphane exposure [39]. Moreover, both PETIC and sulforaphane appeared to stimulate UGT activity. From these early studies, it became clear that cruciferous vegetables exert differential effects on XMEs—inhibiting some isoforms while inducing others—and these outcomes depend on the type and quantity of glucosinolates present in each crucifer form.

The last decade has seen a modest increase in the number of clinical trials assessing the impact of cruciferous vegetables on human drug metabolism. In a series of healthy volunteer studies evaluating diets containing broccoli (~300 g/meal), brussels sprouts

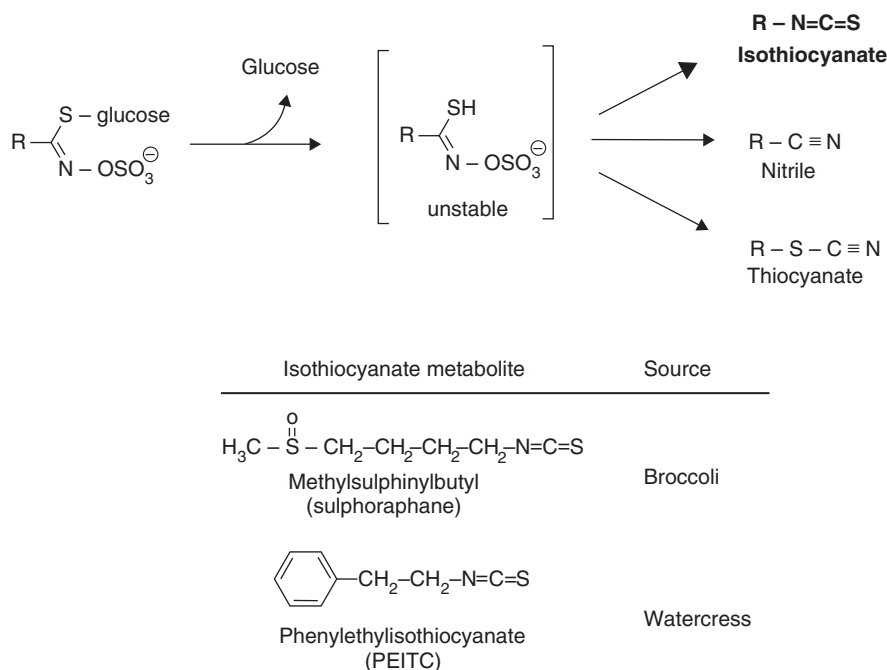


Figure 16.1 Representative phytochemicals (isothiocyanates) present in cruciferous vegetables.

(~300 g/meal), cauliflower (~300 g/meal), cabbage (~150 g/meal), and radish sprouts (~32 g/meal), significant increases in phenotypic measures of CYP1A2 [81,82] and UGT1A1 [83] activity, as well as elevated concentrations of GSTA1 and GSTM1 [84–87], were consistently observed. An investigation utilizing isolated human jejunal segments (Loc-I-Gut procedure) found that sulforaphane produced rapid (~2 h) and significant (approximately twofold) increases in GSTA1 and UGT1A1 mRNA from exfoliated enterocytes [88]. Collectively, these findings bolster numerous *in vitro* and animal studies that specific isothiocyanates can function as ligands for nuclear receptors that upregulate CYP1A2 (i.e., AhR) [89] and various transferases (i.e., nuclear factor erythroid-2 related factor 2, Nrf2) [39,90].

Interestingly, sulforaphane also downregulates CYP3A4 expression in human primary hepatocytes [91,92], purportedly by acting as a selective antagonist for PXR [93]. As the only natural PXR antagonist discovered to date, sulforaphane effectively impedes PXR-mediated CYP3A4 expression *in vitro* [93]. While yet to be confirmed *in vivo*, this last activity may not only represent another mechanism for sulforaphane chemoprevention, but may reduce adverse drug responses that arise through induction of *CYP3A4* and other PXR target genes.

Although not as intensively studied as their effects on XMEs, *in vitro* methods identify isothiocyanates as both substrates and inhibitors of ABC efflux transporters [94–96]. As inhibitors, however, they are not especially potent. Since isothiocyanate concentrations in excess of 50 μ M are necessary for *in vitro* inhibition (levels rarely achieved in the normal diet), the clinical risk for crucifer-ABC transporter interactions appears minimal.

In summary, when consumed in sufficient quantities, cruciferous vegetables can modulate XME activity; however, the amounts administered in the studies described above far exceed those commonly encountered in normal diets. Thus, with customary usage, the risk for clinically important crucifer–drug interactions seems limited.

Interaction risk: Low.

16.5.2 Citrus Juices

Unlike cruciferous vegetables, whose phytochemicals may affect human drug metabolism when consumed in excess, certain citrus fruit juices, especially grapefruit juice, pose a greater risk for food–drug interactions. Grapefruit juice (GFJ, *Citrus paradisi*) increases the oral bioavailability (a pharmacokinetic parameter designated by the letter *F*) of many drugs that are CYP3A4 substrates [97–99]. This effect was first observed two decades ago when concurrent administration of GFJ with either felodipine or nifedipine increased the area under the plasma concentration–time curve (AUC) for these two calcium channel blockers by more than 250% and 135%, respectively [100,101] (Fig. 16.2). The clinical relevance of this interaction was heightened by the occurrence of adverse hemodynamic side effects. Since that time, considerable research into the effects of citrus juices on drug disposition has been conducted making it the most widely studied food–drug interaction to date, with more than 350 scientific articles on GFJ-related interactions alone. From these efforts, a clearer understanding of the mechanisms underlying these interactions and their clinical relevance has come into view [42,99,102–104].

It is now recognized that fruits of various citrus plants (Rutaceae) produce a group of phytochemicals known as *linear furanocoumarins* (psoralens) (Fig. 16.3), which

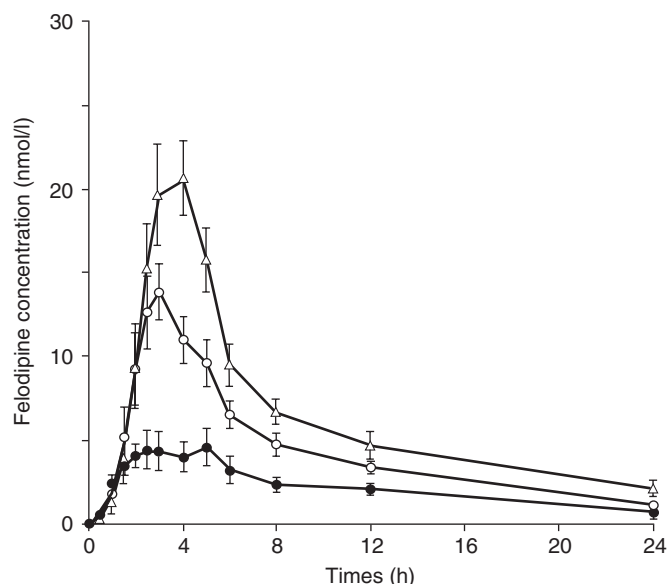


Figure 16.2 Effect of grapefruit juice on mean felodipine plasma concentrations. Felodipine plasma concentrations after the oral administration of 10-mg felodipine with either water (●), a single 8-oz. glass of grapefruit juice (○), or after five days of thrice daily administration of grapefruit juice (Δ). Bars indicate standard errors. Reprinted with permission. Ref. 100.

are mechanism-based inhibitors of CYP3A4 [43,105,106]. Those furanocoumarins most important for CYP3A inhibition are (in order of decreasing potency) the paradisins (a group of dihydroxybergamottin dimers), 6',7'-dihydroxybergamottin, 6',7'-epoxybergamottin, and bergamottin [107,108] (Fig. 16.3). Because furanocoumarins cause rapid degradation of CYP3A4, de novo enzyme synthesis—a process often taking more than 24 h—is necessary for recovery [109]. It appears that intestinal CYP3A4 isoforms are most readily affected. As little as 10 fluid ounces of regular strength GFJ is capable of producing significant inhibition [42,99,110]; however, excessive quantities or prolonged use may influence hepatic CYP3A activity [42,111]. The magnitude of the effect is dependent on furanocoumarin exposure, which is a function of type and quantity of juice and duration of juice ingestion [42,99,105,109]. Of the citrus juices most widely consumed, GFJ is of greatest concern as it contains the highest concentrations of furanocoumarins. Even unprocessed grapefruit segments, a common breakfast item, contain enough furanocoumarins to increase absorption of several drugs [112]. Seville orange (*Citrus aurantium*, bitter orange) juice, a widely studied variety, also contains high concentrations of furanocoumarins and can increase the bioavailability of many CYP3A substrates [113,114], but its bitter taste relegates it primarily to research purposes. Aqueous extracts of *C. aurantium* containing significant quantities of the sympathomimetic agent, synephrine, are common components of many weight-loss dietary supplements, yet they pose little risk for CYP3A-mediated interactions, since the highly lipophilic furanocoumarins are not extracted due to poor water solubility [115]. Pomelo (*Citrus grandis*) juice also contains furanocoumarins, and its use has been linked to interactions involving the immunosuppressant drugs, tacrolimus [116] and cyclosporine [117]. Other citrus juices such as

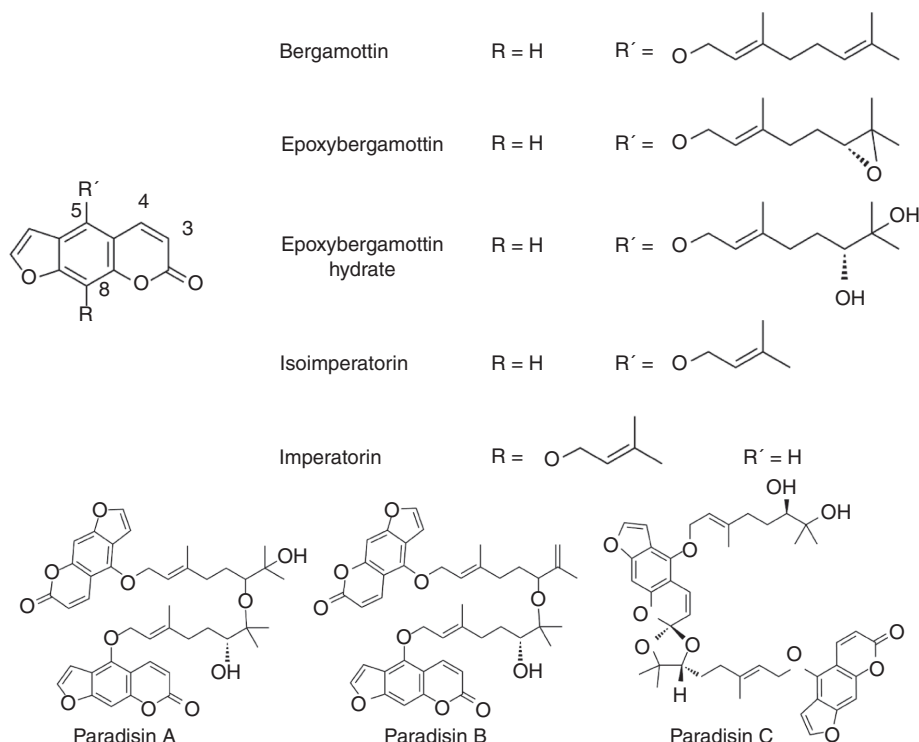


Figure 16.3 Representative phytochemicals (furanocoumarins) present in grapefruit juice.

orange (*Citrus sinensis*), lemon (*Citrus limon*), lime (*Citrus aurantifolia*), and tangerine (*Citrus reticulata*) are generally lower in their furanocoumarin content, or they differ in their furanocoumarin profile and do not interact with CYP3A substrates [42,102,118]. *C. sinensis* is almost devoid of furanocoumarins while the prevalent species in *C. limon* and *C. aurantifolia* are imperatorin and isoimperatorin [118] (Fig. 16.3).

In the small intestine, furanocoumarin-mediated inhibition of CYP3A4 increases circulating levels of various drugs that may lead to an enhanced pharmacodynamic effect and possible toxicity. Drugs most affected by GFJ are CYP3A4 substrates with narrow therapeutic indices that undergo extensive presystemic intestinal metabolism [42,99,119]. Accordingly, the already low oral bioavailability (i.e., $F < 25\%$) of such drugs can be dramatically increased, oftentimes to values several fold greater than normal. Of the many compounds whose pharmacokinetic profiles are influenced by GFJ, only a few fulfill these criteria. Those medications posing the greatest risk for clinically significant GFJ-related interactions are listed in Table 16.1. These include selected calcium channel blockers, certain HMG-CoA reductase inhibitors, calcineurin-inhibitor immunosuppressants, and a variety of others [99,103]. In lieu of potentially harmful drug toxicity, the labeling for some medications includes warnings or precautionary statements regarding GFJ interaction potential [120].

To what extent citrus juices affect xenobiotic transporter function *in vivo* remains to be fully determined. Clinical evidence suggests that GFJ and other citrus juices are not potent modulators of human ABC efflux transporter activity. Support for this view stems from GFJ's inability to negatively affect the clinical pharmacokinetic profile of

TABLE 16.1 Drugs Posing Significant Risks for Clinically Important Interaction When Taken with Grapefruit Juice and Other Citrus Juices

<i>Drugs exhibiting increased oral bioavailability (grapefruit juice)</i>
Calcium channel blockers: felodipine, nifedipine, nisoldipine, nitrendipine, manidipine
HMG-CoA reductase inhibitors: atorvastatin, lovastatin, simvastatin
Calcineurin-inhibitor immunosuppressants: cyclosporine, tacrolimus, sirolimus
Others: amiodarone, buspirone, carbamazepine, cisapride, diazepam, halofantrine
<i>Drugs exhibiting reduced oral bioavailability (grapefruit juice, orange juice, others)</i>
Fexofenadine, cefiprolol

digoxin, a putative ABCB1 substrate exhibiting good bioavailability [121,122]. Like CYP3A4 substrates, if low bioavailability is a necessary precondition for the development of clinically relevant GFJ-ABCB1 transporter interactions, digoxin may not be an ideal candidate from which to draw conclusions. Because many drugs affected by GFJ are dual substrates for CYP3A4 and ABCB1 (e.g., cyclosporine, simvastatin, and lovastatin), it appears that the brunt of GFJ's effect is borne by CYP3A4. Citrus juices can, however, inhibit the function but not expression of human intestinal uptake transporters like the solute carrier organic anion transporter family member 1A2 (SLCO1A2), which appears to be particularly affected [123,124]. The oral bioavailability of fexofenadine and cefiprolol, two substrates of human SLCO1A2, are substantially reduced in the presence of GFJ and orange juice [125–128]. It appears that citrus flavonoids (e.g., naringin and hesperidin), and not furanocoumarins, propagate the SLCO1A2 inhibitory effects of these fruits [129]. Even noncitrus juices (e.g., apple) have been recognized for their ability to reduce SLCO1A2 substrate bioavailability [126].

In summary, unique PSMs found in citrus fruits used by millions of people daily can, in normal servings, markedly affect human drug disposition. Given their phytochemical complexity, it is likely that an array of PSMs (e.g., furanocoumarins, flavonoids, and others) working in concert—perhaps synergistically—contributes to the varied effects fruit juices have on drug absorption [130].

Interaction risk: High.

16.6 DSHEA, DIETARY SUPPLEMENTS, AND THE RISK FOR HERB–DRUG INTERACTIONS

Botanicals have been the archetype for medicines since ancient times, and still today they serve as sources for modern drug discovery. While their use in contemporary American medicine has all but disappeared, they still underpin the philosophies of TCM, Ayurvedic medicine (India), and Kampo medicine (Japan). Up until the early twentieth century, monographs for botanicals and botanical extracts composed a significant part of the United States Dispensatory and the US Pharmacopeia, but the coming of the modern drug industry supplanted their use, relegating them to relative obscurity. That is until 1994, when the United States Congress passed the Dietary Supplement Health and Education Act (DSHEA), legislation that characterized botanical extracts as dietary supplements [131]. A dietary supplement, as defined by the act, is “a product (other than tobacco) added to the total diet that contains at least one of the following ingredients: a vitamin, mineral, herb or botanical, amino acid, metabolite, extract, or combination of any ingredient described previously.”

Often formulated as tablets and capsules, they are sold alongside conventional over-the-counter (OTC) medications in retail outlets; and while not intended to treat, cure, mitigate, or prevent any disease, many consumers view dietary supplements as substitutes for conventional medications [132]. Since their introduction in 1994, dietary supplements have gained a significant foothold in the American health care system. As testament to their popularity, 18% of US adults regularly consume botanical supplements, spending more than \$14 billion annually [133,134]. Current surveys indicate that 20–30% of prescription drug users take medications concomitantly with herbal supplements, oftentimes without notifying their physician or other health care professional [135,136]. Moreover, 70% of regular users of botanical supplements also take prescription medications [135]. Prospects for herb–drug interactions, therefore, are considerable.

Most vulnerable to these types of interactions are the elderly. Adults aged 65 years or older are the greatest consumers of prescription medications, and concurrent use of multiple medications (polypharmacy) is common in this population [137]. When coupled with age-related changes in drug disposition—a normal consequence of growing older—polypharmacy predisposes the aged to adverse drug reactions as well as drug–drug interactions. Over the last five years in the United States, a noticeable increase in botanical supplement usage has occurred among seniors [136,138], with as many as 40–70% of survey respondents reporting regular use of herbal products [136,139,140]. Surveys also indicate that <50% of Americans over age 65 disclose their herbal supplement use to physicians [139,141,142]. This combination of polypharmacy, botanical supplementation, and reticence places the elderly at an increased risk of herb–drug interactions.

16.7 UNCERTAINTY IN PREDICTING AND INTERPRETING HERB–DRUG INTERACTIONS

The molecular mechanism(s) underlying most herb–drug interactions and the clinical magnitude of their effects are oftentimes difficult to discern. The vagaries of these processes are rooted in both plant and human physiology. Factors influencing whether herb–drug interactions are of clinical concern include variable enteric CYP3A/ABC content and activity, human pharmacogenetics, phytochemical synergy, irregularities in phytochemical content, and incongruities between *in vitro* predictions and *in vivo* realities. Other confounders unrelated to physiological issues involve adulteration or contamination of botanicals with prescription medications or other toxins. As seen throughout the remainder of this chapter, inconsistent findings among clinical herb–drug interaction studies are quite common.

16.7.1 Variability in Enteric CYP3A/ABC Content/Activity

CYP3A4 catalyzes the biotransformation of more drugs than any other CYP isoform. Highly expressed in the liver and small intestine, it is a major determinant of drug bioavailability. ABC efflux transporters, localized on the apical surface of intestinal enterocytes, work in concert with CYP3A to regulate drug absorption. Intestinal CYP3A and ABCB1 expression can be extremely variable, with baseline enteric content/activity differing among individuals by as much as 8- and 10-fold, respectively

[143,144]. Intersubject variability in protein expression is also evident for an assortment of SLC transporters as well [145]. Such large interindividual variabilities can have profound effects on drug pharmacokinetic profiles and must be taken into consideration when interpreting clinical herb–drug interaction studies, especially those involving CYP3A4/ABCB1 substrates.

16.7.2 Human Pharmacogenetics

Pharmacogenetics—the study of genetic variation on human drug response—is gaining attention for its impact on the outcome of herb–drug interaction studies [146]. As discussed earlier, certain genetic polymorphisms of human XMEs and transporters may have evolved in response to selection pressures of diets rich in certain plant PSMs [51,52,54]. Individuals expressing mutant XME or transporter alleles, therefore, may respond to the modulatory effects of PSMs differently than subjects homozygous for the wild-type allele. Ginkgo (*Ginkgo biloba*), Baikal skullcap (*Scutellaria baicalensis*), garlic (*Allium sativum*), and milk thistle (*Silybum marianum*) are examples of botanicals whose effects on drug disposition appear to correlate with allelic variants. In a cohort of Chinese volunteers, *G. biloba* supplementation significantly enhanced omeprazole hydroxylation in a CYP2C19 genotype-dependent manner [147]. For those subjects exhibiting a poor metabolizer CYP2C19 phenotype, ginkgo-mediated induction was more pronounced. Similarly, baicalin, a flavone glucuronide isolated from Baikal skullcap, reduced plasma concentrations of rosuvastatin in an SLCO1B1 haplotype-dependent manner [148]. Allicin, an active ingredient in garlic, increased omeprazole AUC in a genotype-dependent manner for homozygous *CYP2C19*1* and heterozygous *CYP2C19*1/*2* carriers, but subjects homozygous for *CYP2C19*2* were not affected [149]. An increase in losartan AUC was also noted for volunteers with the *CYP2C9*1/*1* genotype, but not in *CYP2C9*1/*3* carriers when milk thistle (*S. marianum*) flavanolignans (e.g., silymarin, 140 mg) were administered thrice daily for 14 days [150]. From the preceding examples, it is clear that certain herb–drug interactions may be genotype dependent.

16.7.3 Phytochemical Synergy

Pharmacologic synergy is believed central to PSM defense mechanisms [151,152]. In combating enemies, particularly those having evolved resistance to a specific PSM, plants often utilize multiple phytochemicals whose total effect is greater than the sum of their individual actions. It is believed that phytochemical synergy also underpins the health benefits of fruits, vegetables, and medicinal plants [151,152], and likely orchestrates the interplay among XMEs and transporters when plant-based foods and drugs interact. This purported synergy may involve protection of an active substance from degradation by enzymes; it may facilitate transport across membrane barriers by overcoming multidrug resistance mechanisms; it may improve the aqueous solubility of lipophilic compounds, or involve a combination of measures. Numerous *in vitro* studies reveal an abundance of botanical extracts and purified phytochemicals capable of modulating XME and transporter activity [153–189]. Considering the wide array of PSMs to which intestinal enterocytes are subjected, synergy among components, or at least additivity, may explain the enhanced effects botanicals often exhibit relative to purified phytochemicals [172]. As an illustration of phytochemical pharmacodynamic

synergy, investigators recently demonstrated that the antidepressant properties of SJW extracts hinged on the joint activities of several structurally unrelated PSMs (e.g., hypericins, flavonol glycosides, and hyperforins) [190]. This pharmacodynamic synergy appeared linked to an assemblage of “coeffector” phytochemicals (e.g., procyanidin dimmers, procyanidin trimers, and polyphenols) whose combined effects at improving the solubility and transport of hypericin, a highly lipophilic naphodianthrone, produced a pharmacokinetic synergy [190].

Simply put, when plant foods or extracts are ingested along with highly purified, single-ingredient drug products, cellular defense mechanisms may be overwhelmed by a plethora of chemically diverse PSMs working in concert to affect drug uptake and disposition. Oftentimes, the winner of these isolated “battles” in the “war” between plants and animals will dictate the clinical magnitude of an herb–drug interaction.

16.7.4 Phytochemical Content Variability

When comparing results of clinical herb–drug interaction studies, variability in the phytochemical content of products evaluated is a common confounder. It is not unusual for large variations in phytochemical composition to exist among different brands of the same botanical species and amid batches of the same brand [191–193]. This drawback, often overlooked by clinical scientists, is dependent on the plant species utilized (e.g., *Echinacea purpurea* vs *Echinacea pallida*), country of origin, production practices (e.g., cultivation vs collection), climatic conditions, time of harvest, postharvest storage conditions, plant part utilized (e.g., fruit, leaves, stem, and root), extraction procedure (e.g., aqueous vs nonaqueous solvent extraction), and extract dosage form (e.g., powder, tablet, capsule, and liquid) in creating the final product. Variations in phytochemical type and content are perhaps more important to the safety and efficacy of botanical dietary supplements than for plant-based foods [194]. Unlike fruits and vegetables, where phytochemical exposure is dependent on the quantity ingested, exposure to PSMs in herbal products is dependent on the dose and type of preparation ingested. Botanical products come in a number of different dosage forms including teas (for aqueous extracts), tinctures (alcohol extracts), and capsules and tablets of extracts (aqueous, ethanolic, CO₂, or other extraction solvents). Among extracts, the type of solvent used will determine the PSM profile of a finished product. Many products contain the same relative amounts of PSMs found in the unprocessed herb while others are formulated to contain added levels of “active” compounds. Such concentrated or otherwise modified extracts are more likely to influence XME and/or transporter activity and to produce interactions with concomitantly administered drugs.

Plant extracts are not created equally. Extracts “standardized” to contain known quantities of specific phytochemicals may differ considerably in the content of “non-standardized” components [195,196]. Standardized extracts may also yield dissimilar quantities of PSMs when formulated into various dosage forms by multiple manufacturers [191–193,197]. Dissimilar PSM profiles, in turn, produce disparate effects on XMEs [198]. Variability in manufacturer recommended dose and duration of use are also factors for consideration, not to mention the ingestion of multiple supplements. Moreover, inconsistencies in dosage form performance (e.g., disintegration and dissolution) can also affect the rate and extent at which PSM concentrations reach the intestinal lumen [199]. This is exemplified by an examination of nine separate milk thistle formulations whose 1-h percent release rates of silibinin into an aqueous buffered solution

(pH 7.5, 37°C) ranged from 0% to 85% [200]. Brand-related disparities in phytochemical release rates have been observed for other botanical supplements [201,202]. At present, pharmacopeial recommendations for dissolution tests as a quality control measure for dietary supplements are limited to only a few botanicals [203]. Only recently have good manufacturing practices (GMPs) for dietary supplements been promulgated in the United States [204]; nevertheless, discrepancies in actual phytochemical content versus the stated label claim continue to plague many products [191,205]. Such discrepancies may profoundly affect the interpretations of clinical herb–drug interaction studies, especially, if a botanical supplement’s phytochemical content is not independently verified.

16.8 DISCONNECT BETWEEN *IN VITRO* PREDICTIONS AND *IN VIVO* REALITIES

Most of the published research on herb–drug interactions has focused on *in vitro* evaluations of botanical constituents in microsomal systems, supersomes, expressed enzymes or cell culture systems such as transfected cell lines, primary cultures of human hepatocytes, and tumor-derived cells. As a result, a substantial body of literature—of which only a modest portion is referenced here—indicates that many botanicals and individual phytochemicals appear capable of modulating XME and transporter activity [153–189]. In addition to *in vitro* studies, numerous *in vivo* investigations have been carried out in both animals and humans. Quite often, significant discrepancies exist between *in vitro* predictions and *in vivo* realities. Moreover, the results of animal experiments may not always coincide with clinical findings. Because the study of herb–drug interactions involves a host of complexities, each approach has its advantages and limitations [194,206,207].

16.8.1 *In Vitro* Methods (Advantages and Limitations)

In vitro studies are valuable for evaluating both purified phytochemicals and multicomponent extracts. They can provide mechanistic information about potential interactions, and are relatively simple and inexpensive to perform. Several limitations, however, hamper their utility as predictors of *in vivo* responses [195,206,207]. First, most methods employ solubilizing agents [e.g., dimethylsulfoxide (DMSO), ethanol, and acetonitrile] to facilitate exposure and uptake of poorly water-soluble phytochemicals into cells or microsomes. This practice, however, can exaggerate experimental findings because solubility issues that otherwise limit phytochemical bioaccessibility *in vivo* are circumvented. Second, phytochemical concentrations necessary to modulate enzyme or transporter activity often exceed those achieved *in vivo*. Third, studies examining purified compounds may not reflect the phytochemical complexity typical of extracts that may contribute to the net inhibitory or inductive effects observed. For example, many lipophilic aglycones examined *in vitro* exist as hydrophilic glycosides in extracts, a condition that may limit cellular uptake *in vivo*. Fourth, many PSMs undergo extensive intestinal and/or hepatic metabolism, a condition that may affect their net modulatory effects *in vivo*. Despite these limitations, *in vitro* screening methods can identify botanicals/phytochemicals with inhibitory or inductive potencies that may warrant further examination *in vivo*, even though few significant drug interactions

have actually been discovered or accurately predicted with *in vitro* methods to date [206,207].

16.8.2 *In Vivo* Animal Methods (Advantages and Limitations)

Herb–drug interaction studies conducted in small animals (e.g., mice, rats, and dogs) have advantages of being less expensive than human clinical studies and can often provide an initial assessment of bioavailability either for phytochemicals in pure form or in the context of a complex extract. Limitations of animal studies are that large, nonphysiological doses are often administered and, because of species variation in metabolism and transport, results are rarely generalizable to humans [194,206].

16.8.3 *In Vivo* Human Studies (Advantages and Limitations)

The importance of *in vivo* human studies for investigating herb–drug interactions came to the forefront in 2000 when a succession of clinical case reports revealed that SJW drastically reduced plasma concentrations of the immunosuppressant cyclosporine, precipitating graft rejection in organ transplant recipients [208–210]. Because cyclosporine is a substrate of CYP3A4 and ABCB1, SJW was suspected of inducing the activity of these two important drug disposition proteins. Coincident with the case study series, conflicting *in vitro* studies indicated that SJW either inhibited CYP3A4 [153] or was a potent ligand of the orphan nuclear receptor PXR [211,212]. Only after a series of prospective clinical studies incorporating other CYP3A4 and ABCB1 substrates did it become clear that SJW was indeed a potent inducer of these regulatory proteins [213–217].

Since 2000, substantial numbers of prospective clinical studies incorporating a variety of experimental designs have been conducted (for recent reviews, see Refs 218–228). Typically, subjects receive a single dose of a test drug or a cocktail of drugs that are markers for various enzymes. Individual drug phenotypes (e.g., pharmacokinetic profiles and drug/metabolite ratios) are then determined [68,206]. This is followed by daily multiple dosing of the botanical supplement, usually for 14–30 days, whereupon phenotypic profiling of the test drugs is repeated. A comparison of pre- and post-treatment pharmacokinetic parameters or phenotypic measures is then used to gauge how XME or transporter activities were affected. The principal advantage of this approach is that it offers a definitive means of assessing the magnitude and implications of herb–drug interactions.

Several common oversights, however, can influence the outcome and interpretation of human studies. First, because botanical supplements often exhibit significant brand-to-brand and lot-to-lot variability, commercially available products must be analyzed independently for phytochemical characterization and content. Simply relying on stated label claims is insufficient. Second, disintegration and dissolution characteristics for specific dosage forms should be conducted before study implementation. Dosage forms failing to properly disintegrate or release their purported “marker” compounds within a reasonable time period will preclude any meaningful results. Failure to address these issues makes it difficult to extrapolate from one formulation to another. Third, an assessment of phytochemical bioavailability or detection of marker compounds in circulating plasma or urine provides evidence of exposure as well as subject compliance.

Although desirable, this prerequisite is often limited by the paucity of validated analytical methods available for measuring unique phytochemicals and/or their metabolites in biologic fluids. Furthermore, because of the complexity of botanical extracts, detection of specific marker compounds in biologic fluids may not necessarily establish an individual phytochemical as a causative agent in an observed phenotype change. Fourth, very few studies incorporate positive controls for inhibition (e.g., clarithromycin as CYP3A4/ABCB1 inhibitor) or induction (e.g., rifampin as CYP3A4/ABCB1 inducer) as a means of gauging clinical relevance [229,230]. This last aspect provides a practical framework from which to compare and interpret the clinical relevance of human herb–drug interaction studies.

Recently, the US Food and Drug Administration (FDA) released a set of guidelines for interpreting the clinical significance of drug interactions in the United States [231,232]. These guidelines are based on clinical experience with several well-defined drug interactions, mainly involving the inhibition of CYP3A4. For this interaction mechanism, the benzodiazepine midazolam (MDZ) was shown to be a reproducible probe allowing quantitative determination of the interaction potential of an enzyme inhibitor. For MDZ, the extent of interaction can be measured in the form of an increase in the AUC. The guidelines propose that inhibitors producing changes in MDZ AUC amounting to less than a twofold increase should be classified as “weak.” Increases ranging from two- to fivefold were classified as “moderate,” whereas a strong interaction is marked by increases in MDZ AUC exceeding fivefold. For other CYPs and ABCB1, the guidelines suggest increases in the AUC of specific probes (e.g., warfarin, CYP2C9; omeprazole, CYP2C19; dextromethorphan, CYP2D6; and digoxin, ABCB1) of twofold or higher may constitute clinically significant interactions. Rifampin was identified as an acceptable probe for examining the induction of CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and ABCB1. A reduction in the AUC values of substrates by 30% or greater would constitute a significant induction interaction. For drugs with narrow therapeutic indices, demonstration of a potentially “weak” interaction by a botanical could result in significant adverse side effects. Therefore, for purposes of this review, botanical formulations producing increases in probe drug AUCs of twofold will be classified as “clinically significant” inhibitors, and those reducing probe drug AUCs by 25% or more will be considered “clinically significant” inducers [231,232].

One final limitation of clinical studies is that they can be costly, and it is often difficult to obtain mechanistic information. Nevertheless, clinical studies represent the only reliable means for realistically assessing a botanical supplement’s herb–drug interaction potential [206,207].

16.9 ADULTERANTS AND CONTAMINATION

Dietary supplements have also faced challenges with regard to contamination and adulteration. Contamination with heavy metals, microbial pathogens, pesticide residues, and improperly identified plant material as well as adulteration with toxic plant extracts, pharmaceutical agents, and other inappropriate additives have generated concern among the medical community and lay public [233]. While contamination or adulteration with less noxious substances or botanicals may not be harmful, other additives have been the cause of serious toxicity [233]. Contamination with heavy metals and various toxic herbs is generally more problematic for imported supplements than those produced

domestically [234]. More disconcerting than unintended product contamination has been the seemingly purposeful adulteration of dietary supplements with conventional drugs [235,236]. The FDA has removed a variety of dietary supplements from the market containing undeclared prescription medications and anabolic steroids, several of which have produced toxic manifestations in some consumers [237]. Most supplement manufacturers have avoided this problem through rigorous, proactive implementation of GMPs. Others, however, have been less sedulous in their approach to product quality. Beginning in 2008, the FDA implemented GMPs for supplements, addressing issues with species identification, contamination, and adulteration. Under the GMPs, adulterants continue to be illegal, and testing for certain contaminants is now required. Implementation of these regulations should significantly reduce incidences of adulteration and contamination. Nevertheless, from an herb–drug interaction perspective, effects attributable to botanicals must be differentiated from those produced by adulterants.

16.10 POPULAR DIETARY SUPPLEMENTS AND THEIR DRUG INTERACTION POTENTIAL

What follows are assessments of the available clinical evidence regarding herb–drug interaction potentials for several popular botanical supplements sold in the United States. Following each summary, a probability assessment (low, moderate, or high) will be provided.

16.10.1 Black Cohosh

Actaea racemosa L. (syn. *Cimicifuga racemosa* [L.] Nutt.) or black cohosh, is a perennial herb native to North America used traditionally by Native Americans for female reproductive system ailments and now popular for the relief of menopausal symptoms such as hot flashes [238,239]. The purported ability of black cohosh to help alleviate climacteric symptoms, premenstrual syndrome, and osteoporosis has secured its ranking among the 10 top-selling supplements in the United States [240]. Spiroketal triterpene glycosides (Fig. 16.4) are believed responsible for black cohosh's pharmacological activity even though they are not phytoestrogens [241,242]. As such, most commercial black cohosh products are chemically standardized to triterpene glycosides, with 23-*epi*-26-deoxyacteoin (also known as 27-*deoxyacteoin*) being the most abundant congener [243].

At present, black cohosh does not appear to be a potent modulator of human drug metabolism. *In vitro* studies found individual triterpene glycosides to be relatively weak inhibitors ($IC_{50} > 100 \mu M$) of human CYP3A4 [244,245], while whole extracts elicited greater inhibition, a finding suggestive of synergy [244]. The inhibitory effects of whole black cohosh extracts may stem not from triterpene glycosides but rather fukinolic and cimicifugic acids. These compounds are potent ($IC_{50} < 13 \mu M$) inhibitors of CYP1A2, 2D6, 2C9, and 3A4 *in vitro* [245]; however, their quantities vary considerably among commercially available black cohosh products [246], a factor that can profoundly affect their inhibitory activity *in vivo*. In human liver microsomes, IC_{50} values for CYP2B6, 2C19, and 2E1 were ~ 50 , 30, and $10 \mu g/mL$, respectively for methanolic extracts of black cohosh [189]. However, when compared to standard regimens of clarithromycin (500 mg daily for 7 days) or rifampin (600 mg daily for 7 days)

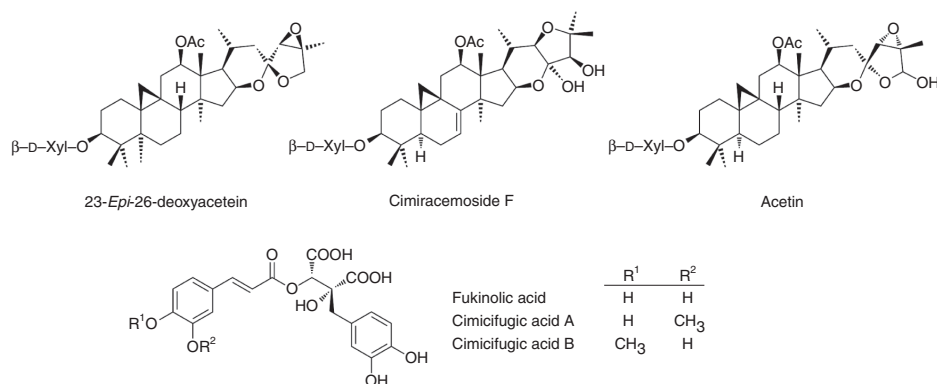


Figure 16.4 Representative phytochemicals (triterpene glycosides, phenylpropanoids) present in black cohosh.

black cohosh supplementation (40–80 mg extract daily delivering 3–6 mg triterpene glycosides for 14 days) produced no demonstrable effects on MDZ and digoxin pharmacokinetics [229,247]. These findings suggest that black cohosh is not a potent modulator of human CYP3A4 or ABCB1 activity *in vivo*. Black cohosh supplementation also had no clinically significant effects on phenotypic measures of human CYP1A2, 2E1, or 2D6 activity [248]. When administered orally, black cohosh's principal triterpene glycoside, 23-*epi*-26-deoxyacteoin, reaches the systemic circulation intact, albeit in very low concentrations (<10 ng/mL) [243]. This apparent lack of biotransformation bolsters the idea that black cohosh triterpene glycosides are unlikely to be inhibitors of human XMEs *in vivo*. Black cohosh extracts incorporating DMSO as a cosolvent moderately inhibited (~47%) uptake of estrone-3-sulfate, a SLCO2B1 substrate, into human embryonic kidney cells stably expressing the transporter [171]. Whether this effect translates to the *in vivo* condition remains to be determined.

As with most commercially available botanical supplements, black cohosh products exhibit considerable variability in phytochemical profiles, and label claims for “standardized” marker compounds can deviate considerably from actual content [246]. Such variations can have considerable influence on how the results of clinical studies evaluating black cohosh efficacy or its herb–drug interaction potential are interpreted. Nevertheless, based on the currently available data, standardized black cohosh supplements, when taken at recommended doses, pose little risk for herb–drug interactions.

Interaction risk: Low.

16.10.2 Echinacea spp

Echinacea spp. (e.g., *E. purpurea* [L.] Moench, *Echinacea angustifolia* DC., *E. pallida* [Nutt.] Nutt.) of the family Asteraceae are North American perennials, whose roots and aerial parts have been used traditionally for a variety of medicinal purposes [249,250]. *Echinacea* formulations containing either root or whole plant extracts are marketed for their “immune stimulatory” properties and for the prevention of colds [249,250]. *Echinacea*'s popularity as an immune stimulator has placed it among the 10 top-selling botanicals in the United States for many years. While evidence from

in vitro and animal studies lend credence to *Echinacea* preparations as immunomodulators, clinical findings remain equivocal (for reviews of clinical efficacy, see Refs 249–253). The three species most commonly encountered are chemically dissimilar. Both *E. purpurea* and *E. angustifolia* contain alkamides as their major lipophilic constituents, although of differing structural types (Fig. 16.5). By contrast, the lipophilic fraction of *E. pallida* is characterized by polyacetylenes and is practically devoid of alkamides. These phytochemical dissimilarities also extend to their respective plant parts (i.e., roots vs aerial parts). As PSMs, polyacetylenes and alkamides are natural pesticides that, when ingested in relatively high amounts, can be toxic. In low concentrations, however, alkamides appear to have beneficial effects [118]. Other PSMs such as caffeic acid esters (e.g., cichoric acid and echinacoside), polysaccharides, and alkenes are also thought to contribute to echinacea's activity. Because commercially available *Echinacea* supplements often consist of extracts from various species and plant parts, considerable variation in phytochemical profile and content is common among products [250,254].

Permeability [255,256] and pharmacokinetic [257–259] studies indicate that several alkamides, but not caffeic acid conjugates, cross the intestinal mucosa reaching the systemic circulation intact. Following single doses of *Echinacea* alkamides (~11 mg) administered as tablets manufactured from ethanolic liquid extracts, plasma concentrations varied considerably with maximum plasma levels not exceeding 350 ng/mL [259]. Individual *Echinacea* alkamides are metabolized to varying degrees by several human CYPs, but when combined (as in an extract) their metabolism is markedly reduced [260–262]. This appears to arise from

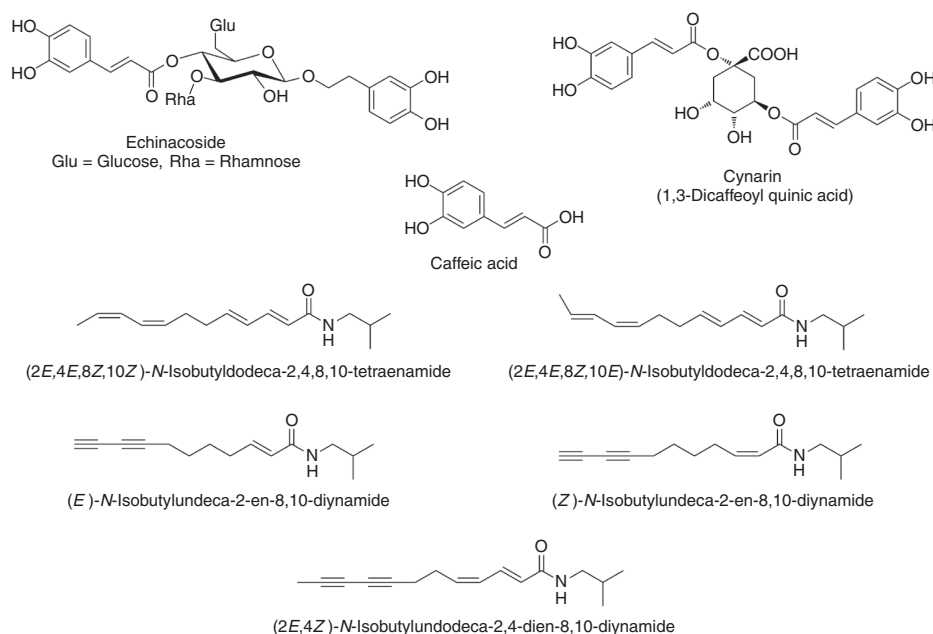


Figure 16.5 Representative phytochemicals (alkamides and phenylpropanoids) present in *Echinacea* spp.

(2*E*)-*N*-isobutylundeca-2-ene-8,10-diynamide (Fig. 16.5), which contains a terminal alkyne and may act as a mechanism-based inhibitor [260]. A considerable number of *in vitro* studies have examined the XME and ABCB1 modulatory effects of echinacea extracts and individual PSMs [154,157,164,169,175,177–180,263–268]. A recent review of the bulk of these studies concluded that alkamides exhibit at least mild to moderate inhibition of CYP3A4 in most of the model systems tested, with the magnitude dependent on alkamide content [262]. This conclusion is strengthened by a recent animal study, in which standardized *E. purpurea* extracts reduced rat CYP3A mRNA levels by 40% [269]. Expression of CYP1A mRNA, however, was increased by 80%. Mild inhibitory effects on ABCB1- [267,268] and SLCO2B1-mediated [171] transport have also been demonstrated.

Very few prospective clinical studies examining the interaction potential of *Echinacea* supplements have been conducted in humans. Using the CYP3A probe MDZ, Gorski *et al.* [270] concluded that eight days of *E. purpurea* supplementation selectively modulated CYP3A activity in the intestine (inhibition) and liver (induction) of healthy volunteers. The authors concluded that because of *Echinacea*'s seemingly opposing effects on intestinal and hepatic CYP3A4, any interaction would depend on the substrate's hepatic and intestinal extraction ratio. Mild inhibitory effects were observed for CYP1A2 and CYP2C9, while CYP2D6 was unaffected. In this instance, however, *Echinacea*'s effect pales in comparison to those reported by these same investigators for known CYP3A4 inhibitors (e.g., clarithromycin) and inducers (rifampin) of MDZ metabolism [271,272]. It would appear that *Echinacea*'s effect on CYP3A expression is modest at best, as neither standardized extracts or purified alkamides upregulated CYP3A4 mRNA levels when exposed to HepG2 cells for 96 h [265]. In contrast, rifampin exposure increased CYP3A4 mRNA expression by 3.8-fold. Using various phenotypic probe drug ratios to assess CYP1A2, CYP2D6, CYP2E1, and CYP3A4 activity, Gurley *et al.* [273] found that healthy adults supplemented with *E. purpurea* for either 14 (800 mg extract daily) or 30 days (1500 mg extract daily) [115] produced no clinically significant changes in CYP phenotypes. More recently, Penzak *et al.* [274] reported that 28 days of *E. purpurea* supplementation in healthy volunteers (500 mg, thrice daily) produced modest reductions in MDZ AUC (27% decrease), but had no significant effects on the pharmacokinetics of lopinavir and ritonavir (CYP3A4 substrates) or fexofenadine (a purported ABCB1 and SLCO substrate). This study, like that of Gorski *et al.*, suggests that *E. purpurea* may have mild inductive effects on human CYP3A4 *in vivo*. However, when compared to rifampin's effects on the pharmacokinetics of CYP3A4 substrates [229,272], any clinically important drug interactions with *Echinacea* seem remote.

To date, only two clinical studies have evaluated *Echinacea*'s impact on drug transporters. Fourteen days of supplementation with a standardized, well-characterized *E. purpurea* product (800 mg extract daily) had no effect on digoxin (an ABCB1 substrate) pharmacokinetics in healthy volunteers [230]. By contrast, seven days of rifampin (600 mg daily) or clarithromycin (500 mg daily) produced marked reductions and increases, respectively in digoxin AUC, a finding that underscores *Echinacea*'s clinically insignificant effect on these transporters. In addition, the pharmacokinetics of fexofenadine, a nonspecific ABCB1 and SLCO substrate, were not impacted by 28 days of *E. purpurea* supplementation [274]. Taken together, these data render it unlikely that *E. purpurea* will produce clinically relevant interactions with coadministered drugs through ABC or SLCO modulation.

On the basis of the collected evidence to date, it appears that dietary supplements formulated with standardized *Echinacea* extracts—when ingested per label recommendations—are not likely to yield alkamide concentrations sufficient enough to dramatically modulate human CYP, ABC, and SLCO isoforms *in vivo*. Therefore, echinacea supplements pose minimal risks for interacting with most conventional medications, an opinion concordant with that of other recent reviews [262,275].

Interaction risk: Low.

16.10.3 Garlic

Members of the family Alliaceae have been an important part of the human diet for thousands of years. *Allium* spp., such as garlic (*Allium sativum* L.) and onions (*Allium cepa* L.) are a rich source of sulfur-containing compounds, many of which are volatile and give rise to the characteristic flavor and aroma of these species. Fresh garlic has little smell but tissue damage by cutting, crushing, or biting results in alliin (2-propenyl-L-cysteine sulfoxide) (Fig. 16.6) being cleaved by the enzyme alliinase resulting in the formation of allicin (diallyl thiosulfinate), which gives crushed garlic its characteristic smell. Because allicin is very unstable to heat, cooking results in its degradation to a number of organosulfur compounds including diallylsulfides and ajoenes [130] (Fig. 16.6). Bad breath, which follows the ingestion of garlic products, is due to a range of sulfide compounds that appear in the systemic circulation and expired air.

Garlic is reputed to have benefits for protection against cardiovascular diseases, cancer, microbial infections, and vampires! Although there is some evidence for the first two effects, the rest are largely the result of speculation and folklore [130]. The putative antihypercholesterolemic effect of garlic supplements makes them one of the most widely used botanical supplements in the United States. Their efficacy, however, remains in doubt because of conflicting results from numerous published clinical trials [276–279]. This is probably a function of the type of product used, its quality, and poor characterization of the phytochemical agent(s) responsible for garlic's lipid lowering effect.

Three general categories of garlic supplements are available commercially: garlic oil, dehydrated garlic powder, and aged garlic extract (AGE), each with their own unique composition of purported bioactive components [280–282]. Within these products a plethora of organosulfur compounds, steroid saponins, and other phytochemicals have been identified [74,280–283]. Of these, the oil-soluble organosulfur compounds including allyl thiosulfonates (e.g., allicin), alkyl sulfides (e.g., diallyl sulfide), vinylthiins, and ajoene have received the most attention (Fig. 16.6). Allicin has long been touted as the agent responsible for garlic's lipid lowering effects, yet the compound is unstable in the gastrointestinal tract, is not bioavailable, and is rarely found in commercial products [280–282]. Allicin's degradation products such as diallyl sulfide, diallyl disulfide, diallyl trisulfide, dithiin, and ajoene may contribute to the lowering of serum cholesterol levels; however, many products, particularly those containing garlic oil, have relatively poor efficacy [284,285]. In addition, many *in vivo* studies indicate that garlic oil and individual alkyl sulfides, most notably diallyl sulfide and allylmethylsulfide, inhibit murine and human CYP2E1 [46,75,286,287]. This is likely a result of the CYP2E1-catalyzed biotransformation of diallyl sulfide to diallyl sulfoxide and diallyl sulfone, in which the latter is a mechanism-based inhibitor of the enzyme [46,288]. Despite

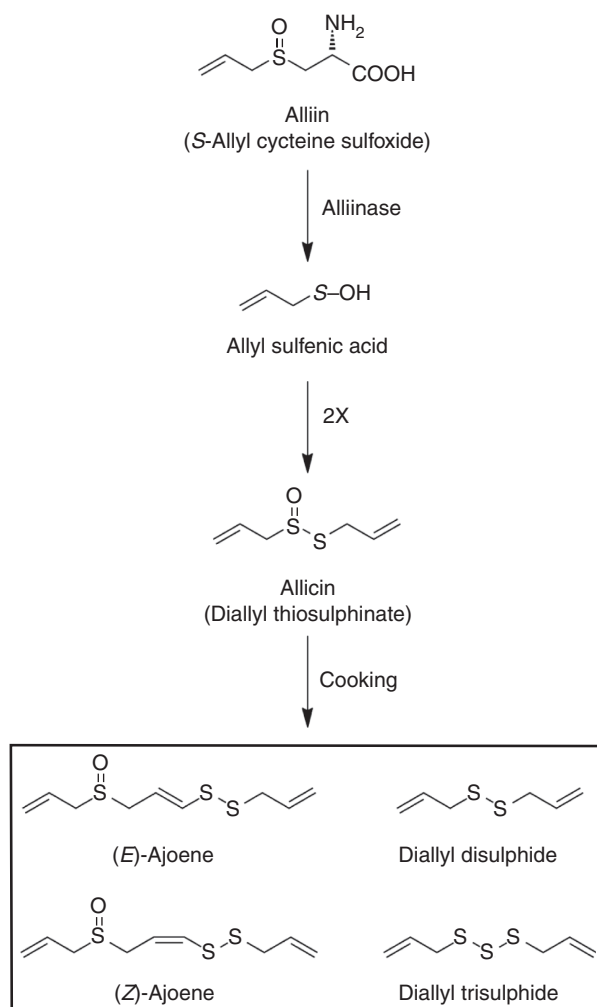


Figure 16.6 Representative phytochemicals (sulfur-containing compounds) present in garlic.

inhibition of human CYP2E1 *in vitro*, few interactions involving garlic products and CYP2E1 substrates have been reported, a consequence that probably reflects the paucity of drugs metabolized by this enzyme. Conversely, prolonged administration of diallyl sulfide and diallyl disulfide induced other hepatic and intestinal murine CYP subfamilies (e.g., CYP1A, CYP2B, and CYP3A), in addition to various transferases (e.g., GSTs and UGTs) [75,289–295] through activation of CAR and Nrf2 nuclear receptors [296].

The ability of garlic to inhibit other human CYP isoforms also appears dependent on product type and composition. *In vitro* assessments of allicin, fresh garlic, or commercially prepared supplements (e.g., garlic oil and garlic powder) either found no effect [164,297] or modest inhibition (<50%) of human CYP2C9, CYP2C19, and CYP3A4 isoforms [155,298]. A freeze-dried garlic supplement, however, produced significant inhibition of CYP3A4 *in vitro* (>95%) [298]. Because the chemistry of garlic

is complex and because different types of processing can significantly alter its phytochemical composition, lyophilization may stabilize those organosulfur compounds capable of inhibiting CYP3A4. AGEs, on the other hand, do not appear to inhibit any of the major CYP isoforms present in human liver microsomes [299]. This may stem from a dearth of oil-soluble organosulfur compounds in AGE and a preponderance of water-soluble components (e.g., *S*-allyl-L-cysteine, saponins) [280]. The shift from oil- to water-soluble ingredients arises from the aging process and subsequent aqueous extraction, which makes AGE distinct from the other types of garlic products.

On the basis of *in vitro* and murine *in vivo* findings, garlic's effects on transporter activity also appear dependent on the hydrophilicity of specific organosulfur compounds. The preponderance of *in vitro* data regarding ABC isoforms implies that garlic's oil-soluble organosulfur compounds are not potent inhibitors of the efflux transporter ABCB1 [95,181,298,300,301], but may, however, induce ABCC2 expression [301]. Interestingly, lipophilic compounds (e.g., diallyl sulfide and diallyl disulfide) increased ABCB1 efflux through rat ileum but not through Caco-2 cell monolayers [302]. When exposed to both Caco-2 cell monolayers and rat intestine at high concentrations ($\sim 12 \mu\text{g/mL}$), water-soluble compounds in AGE (e.g., *S*-allyl-L-cysteine) induced the activity of ABCB1, ABCC2, and SLCO transporters [302,303]. In a HepG2 cell line, however, ABCC2 activity was reduced, suggesting that AGE-mediated regulation of ABCC2 proteins in the liver is different from that in intestine [304]. Tissue-specific regulation of ABCC2 activity has also been observed for other xenobiotics [305], and this may only add to the difficulties of predicting garlic–drug interactions.

A modest number of prospective human studies have investigated the drug interaction potential of garlic supplements or purified garlic organosulfur compounds [48,150,306–316]. Owing to the variety of products evaluated and diverse dosages of garlic phytochemicals administered, results have been mixed. A number of drugs, whose biotransformation pathways run the gamut of human XMEs, have been assessed (e.g., acetaminophen, alprazolam, caffeine, chlorzoxazone, cyclosporine, debrisoquine, docetaxel, dextromethorphan, MDZ, omeprazole, ritonavir, saquinavir, and warfarin), yet the evidence implies that only substrates of CYP2E1 are significantly affected [48,307,313]. The first prospective study by Gwilt *et al.* [306] found that daily administration of AGE ($\sim 19 \text{ mg}$ *S*-allyl-L-cysteine) produced no significant effects on the pharmacokinetics of acetaminophen, a putative CYP2E1 substrate. Later, Loizou and Crocker observed that administration of diallyl sulfide ($\sim 0.2 \text{ mg/kg}$) to healthy volunteers reduced plasma 6-hydroxychlorzoxazone/chlorzoxazone ratios (a phenotypic measure of CYP2E1 activity) by an average of 31% [307]. This finding confirmed in humans what had been previously observed in murine models that lipophilic, and not hydrophilic, organosulfur compounds were CYP2E1 inhibitors. Subsequent studies confirmed that prolonged garlic oil supplementation (500 mg, thrice daily for 28 days) inhibited human CYP2E1 activity in both young [48] and elderly adults [313] by almost 40% and 25%, respectively; however, no modulatory effects were noted for CYP1A2, CYP2D6, or CYP3A4. In contrast, 21 days of twice daily supplementation with garlic powder ($\sim 9 \text{ mg}$ allicin and 23 mg alliin) reduced by 50% the mean AUC, 8-h trough concentrations, and mean maximum plasma concentrations (C_{max}) of the protease inhibitor, saquinavir [308]. The authors concluded that the garlic powder supplement might have induced intestinal CYP3A4 and/or P-gp, since saquinavir is a substrate for both proteins. A similar, although less dramatic, effect on ritonavir AUC, was observed after a four-day course of

garlic extract (5 mg, twice daily) [309]. Subsequent studies examining the prolonged effects of garlic supplementation on other CYP3A4 substrates, however, failed to note any significant changes [310–312]. Also, any clinically important garlic-mediated interactions have not been reported for warfarin, a recognized substrate for CYP2C9 and 3A4 [315,316]. However, large doses of the organosulfur compound allicin (~150 mg daily) did reduce the metabolism of omeprazole by inhibiting CYP2C19 activity in individuals both homozygous and heterozygous for the *CYP2C19*1* allele, but not for those homozygous for *CYP2C19*2* [150]. Interestingly, the CYP3A4-mediated omeprazole sulfone pathway was not affected. The extent to which garlic supplementation affects human ABC and SLCO substrates *in vivo* remains to be determined. Perhaps reductions in saquinavir and ritonavir concentrations noted in early studies could have been attributable to garlic-mediated upregulation of intestinal ABCB1 or ABCC2 activity as was demonstrated in several recent *in vitro* investigations [300–305].

Taken together the accumulated findings imply that most commercially available garlic supplements pose only a limited risk for producing clinically important herb–drug interactions. Presently, only human CYP2E1 appears to be inhibited by garlic oil products, but because only a few drugs are substrates of CYP2E1 and most of those have fairly broad therapeutic indices, this interaction is not a cause for great concern.

Interaction risk: Low.

16.10.4 Ginkgo biloba

Termed a *living fossil*, Ginkgo trees have existed since the early Jurassic period of 150 million years ago. The lone species that escaped extinction (*G. biloba* L.) is now cultivated in Asia, Europe, North America, New Zealand, and Argentina. Ginkgo is a popular ornamental tree recognizable by its unusual fan-shaped leaves that turn bright yellow in autumn. In Asia, the tree has long been held sacred for its therapeutic value. Today, dosage forms incorporating *G. biloba* leaf extracts are used throughout the world for the treatment of insufficient blood flow, memory deficits, cognitive disorders, Alzheimer's disease, depression, vertigo, tinnitus, and intermittent claudication [317]. Ginkgo's popularity has made it one of the most intensely studied botanicals in the world. Currently, more than 2500 articles related to *G. biloba* have been published in the medical literature.

Clinical trials on the efficacy of *G. biloba* extracts are numerous and controversial [318–322]. Much of the research has centered on products formulated with EGb 761, an extract produced by the German company, Schwabe. EGb 761 is a standardized, concentrated extract containing 24% flavonoid glycosides (e.g., quercetin, kaempferol, and isorhamnetin), 6% terpene lactones (3.1% ginkgolides A, B, C, and J and 2.9% bilobalide), 5–10% organic acids, and other constituents [317]. Several other companies produce *Ginkgo* extracts with similar chemical profiles. Terpene lactones are unique to *G. biloba* and include the ginkgolides, a group of diterpene trilactones (e.g., ginkgolide A, B, C, J, and M), and bilobalide, a sesquiterpene lactone (Fig. 16.7). *G. biloba* flavonoids occur principally as glycoside derivatives, with quercetin, kaempferol, and isorhamnetin being the most prevalent. Other PSMs present in *G. biloba* that may have allergenic, immunotoxic, and other undesirable properties (e.g., ginkgetin, amentoflavone, ginkgolic acids, ginkgotoxin, and others) are typically removed during processing [317].

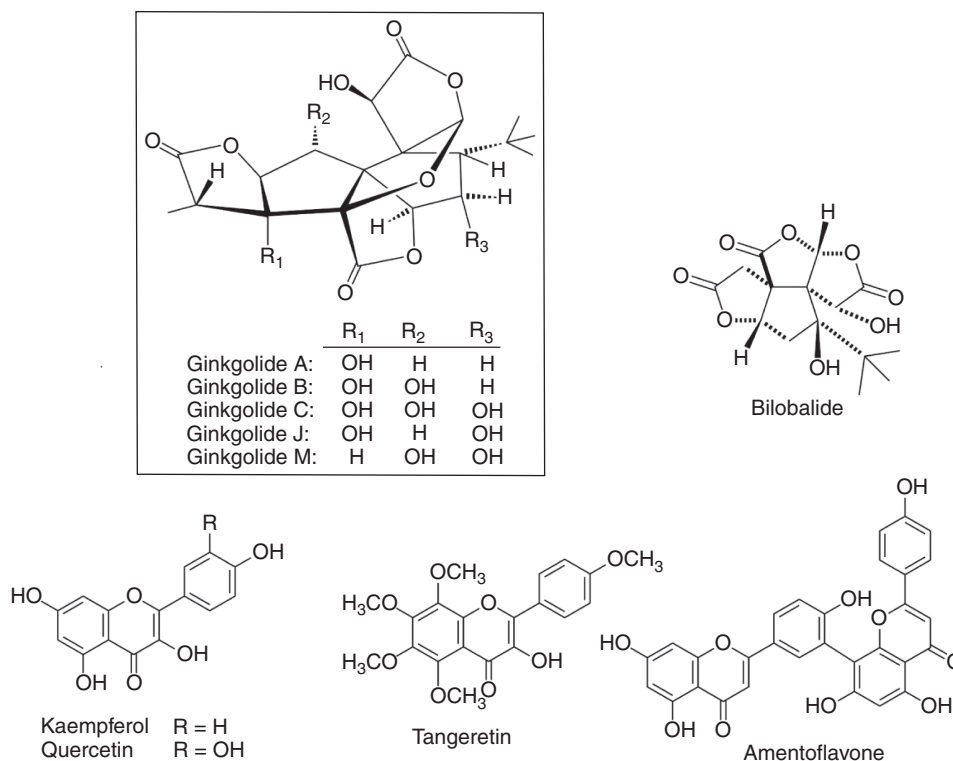


Figure 16.7 Representative phytochemicals (terpene lactones and flavonoid glycosides) present in *Ginkgo biloba*.

Discrepancies among clinical studies regarding efficacy may be traced to significant interproduct variability in phytochemical content and biopharmaceutical characteristics of ginkgo extract dosage forms (e.g., disintegration, dissolution, and bioavailability) [323,324]. These same discrepancies may also underly the confusion surrounding the herb–drug interaction potential of *G. biloba*. Depending on which experimental model is utilized, different interpretations of ginkgo’s drug interaction capabilities emerge. In many *in vitro* experimental systems, ginkgo extracts [152,169,171,174,176,177,180,184,325–329] as well as individual terpene lactones [162,323–329] and flavonoid glycosides [162,171,185,188,325–328,330] have been shown to inhibit various XMEs and transporters, although in most instances IC₅₀ values were well in excess of 20 μM. Aglycones of quercetin, kaempferol, and isorhamnetin seem to have the greatest inhibitory capacity [162,166,185,188,326,329–332], while ginkgolides and bilobalide exhibit the least and, in many cases, none at all [155,166,326–330,333]. In contrast, ginkgo extracts and individual terpene lactones appear capable of inducing the expression of several CYP, UGT, and ABC isoforms in rat [334–336] and human primary hepatocytes [176,184,336,337] as well as human mammary epithelial cells [338]. Cell-based reporter assays in HepG2 [335,337], LS180 [182], and other cell lines [338,339] revealed that ginkgolides A and B are activators of PXR, whereas quercetin and kaempferol activated PXR, CAR, and AhR. Bilobalide exerted no effects on nuclear receptors in these assays. The discovery that specific

ginkgolides and flavonoids are ligands for several xenobiotic receptors provides an explanation for a host of recent *in vivo* studies in which prolonged administration of *G. biloba* extracts to rats, often in high doses, not only induced a multitude of XMEs [340–345] but reduced efficacy for several drug substrates: nicardipine [346], tolbutamide [347], phenobarbital [348], propranolol [349], cyclosporine [350], and theophylline [351]. Concomitant administration of *G. biloba* also enhanced the hepatotoxicity of acetaminophen via CYP3A induction [352]. However, several of these *in vivo* studies contradicted cell-based reporter assay findings when bilobalide was implicated as an XME activator [353–355].

Evidence from *in vitro* and animal investigations clearly points to *G. biloba* extracts and their constituents as inducers of XME and transporter activity. These findings have led investigators to warn of significant drug interactions with *G. biloba*. Clinical evidence substantiating these claims, however, is not as compelling. To date, 29 prospective clinical trials assessing the effect of *G. biloba* supplementation on the pharmacokinetics of a variety of drugs, including several specific CYP probes, have been published [48,147,313,344,356–380]. Twenty-nine different drugs whose metabolism or transport is mediated by various CYP isoforms (e.g., CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) or transporters (e.g., ABCB1, SLCO1B1, and SLCO2B1) were evaluated. The drugs included aspirin [371], alprazolam [364], antipyrine [356], bupropion [378], caffeine [48,313,358], chlorzoxazone [48,313,358], cilostazol [366], clopidogrel [366], dapsone [358], debrisoquine [48,313,358], dextromethorphan [361], diazepam [379], diclofenac [367], digoxin [360], donepezil [363], endogenous steroids [344,365], fexofenadine [374], flurbiprofen [368], lopinavir [374], mephenytoin [358], metformin [373], MDZ [48,313,370,374], nifedipine [357,362], omeprazole [147], talinolol [376,377], ticlopidine [369,380], tolbutamide [367,370], voriconazole [375], and warfarin [359,364,372]. The majority of these studies followed a *G. biloba* supplementation regimen of 240 mg leaf extract twice daily from as few as 3 to as many as 90 days, while others utilized smaller doses. In addition, most utilized products containing the standardized EGb 761 extract. Of the 29 studies (many of which evaluated more than one drug), 23 observed no significant effects of *G. biloba* on drug disposition, 5 observed a modest inhibitory effect [357,358,370,376,377], and 3 demonstrated evidence of CYP3A4 [372], CYP2C9 [368], and CYP2C19 [147] induction. Moreover, when compared to FDA guidelines on drug interaction criteria [231,232], those few studies demonstrating a modulatory effect of *G. biloba* on drug disposition do not appear to be clinically important.

Discrepancies in *G. biloba*'s effect on rat and human XMEs *in vivo* do not appear to reflect species differences in PXR activation [335], but rather seem to be dose related [341,344]. Most rat studies administered *G. biloba* extracts at 100 mg/kg/day, a dose comparable to 6000 mg/day or greater in humans; however, most human studies incorporated doses of 240 mg/day or less.

Additionally, several studies have assessed the pharmacokinetics of ginkgolides and bilobalide in human volunteers. Following 240 mg doses of standardized *G. biloba* extracts, $C_{\max}$ values for ginkgolides and bilobalide rarely exceeded 40 ng/mL [381–383], concentrations well below the 4000 ng/mL at which ginkgolides A and B activated PXR *in vitro* [182]. It is possible that concentrations exceeding 40 ng/mL may be achieved within intestinal enterocytes, but this depends on disintegration and dissolution profiles of individual dosage forms (characteristics that can vary considerably between brands [384,385]), as well as the permeability of individual

terpene lactones or flavonol glycosides. In Caco-2 and MDR1-MDCK cell monolayers, ginkgolides and bilobalide exhibited low absorptive permeability and high efflux [386]; two conditions that would further preclude their exposure to enteric XMEs.

In summary, dosage forms containing standardized *G. biloba* extracts, when administered at doses of 240 mg/day or less, do not pose a risk for clinically relevant herb–drug interactions. However, daily doses exceeding 240 mg/day may increase prospects for interactions.

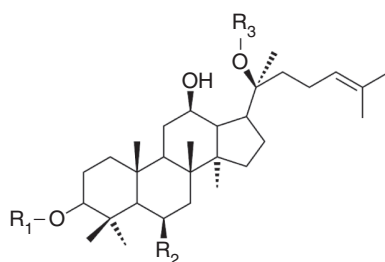
Risk: Low, at doses 240 mg/day or lower.

16.10.5 Ginseng

Of the five major *Panax* spp. worldwide, Asian ginseng (*Panax ginseng* C. A. Meyer) and American ginseng (*Panax quinquefolius* L.) are the most widely used and extensively studied. *P. ginseng* root has an almost 2000-year history of use in TCM as an adaptogen (a plant that increases resistance to stress and fatigue) and a restorative tonic. Today, ginseng root, either as a TCM or dietary supplement, is one of the most popular herbs in the world; used to improve libido and sexual performance, prevent cancer, regulate blood sugar, lower blood pressure, improve cognition, fight fatigue, and boost immunity [387]. With the possible exception of its mild hypoglycemic effect, ginseng's efficacy remains questionable, as many clinical trials have produced confounding results [388–390]. Ambiguity in clinical findings may be linked to differences in the ginsenoside content of products evaluated.

Ginsenosides, a group of triterpene glycosides (steroidal saponins), are unique to *Panax* spp. More than 40 ginsenosides have been identified in the roots of *P. ginseng* and *P. quinquefolius*. Ginsenoside nomenclature employs the designation Rx, where x represents the retention factor (Rf) value from the sequence of spots (from bottom to top) on thin-layer chromatography plates. Ginsenosides exhibit considerable structural variation. They differ from one another by the type, number, and site of attachment of sugar moieties. The two major subtypes of ginsenosides—protopanaxadiol and protopanaxatriol—are classified according to the arrangement and number of sugar (glucose, rhamnose, xylose, and arabinose) residues on the steroidal skeleton [391]. Rb1, Rb2, Rc, and Rd are examples of protopanaxadiol ginsenosides, while Re, Rf, Rg1, and Rg2 are examples of protopanaxatriols (Fig. 16.8). *P. ginseng* and *P. quinquefolius* differ significantly in type and proportion of ginsenosides, with *P. ginseng* having a high Rg1:Rb1 ratio and *P. quinquefolius* a low Rg1:Rb1 ratio [392]. Such distinctions may account for differences in purported efficacy between the two species. Ginseng root extracts are often standardized to contain a particular percentage (~4%) and ratio of ginsenosides. When ingested, ginsenosides undergo partial hydrolytic deglycosylation in the stomach and are further deglycosylated in the large intestine through enzymatic glucosidase activity of gut microflora [391,393–395]. It is these metabolites (e.g., Compound K, Fig. 16.8) that ultimately reach the systemic circulation and are alleged to have pharmacologic activity [393,395–397].

From a drug interaction perspective, clinical and nonclinical evidence regarding ginseng extracts and their effects on XMEs and transporters is particularly confusing. This confusion stems from an assortment of variables affecting ginsenoside disposition. These include, but are not limited to, variability in ginsenoside type and content [391,392], degree of preabsorptive deglycosylation [393–396], poor membrane permeability [398], interindividual differences in gut microflora [393–395], and enteric



Compound	R ₁	R ₂	R ₃
Protopanaxadiol derivatives			
Rb ₁	–Glc ² – ¹ Glc	–H	–Glc ⁶ – ¹ Glc
Rb ₂	–Glc ² – ¹ Glc	–H	–Glc ⁶ – ¹ Araf
Rc	–Glc ² – ¹ Glc	–H	–Glc ⁶ – ¹ Araf
Rd	–Glc ² – ¹ Glc	–H	–Glc
Rg ₃	–Glc ² – ¹ Glc	–H	–H
Rh ₂	–Glc	–H	–H
Compd K (C–K)	–H	–H	–Glc
20(S)-protopanaxadiol (Ppd)	–H	–H	–H
Protopanaxatriol derivatives			
Re	–H	–O–Glc ² – ¹ Rha	–Glc
Rg ₁	–H	–O–Glc	–Glc
Rg ₂	–H	–Glc ² – ¹ Rha	–H
Rh ₁	–H	–OGlc	–H
F ₁	–H	–OH	–Glc
20(S)-protopanaxatriol (Ppt)	–H	–OH	–H

Figure 16.8 Representative phytochemicals (ginsenosides) present in *Panax* spp. Arap = α -L-arabinopyranosyl; Araf = α -L-arabinofuranosyl; Glc = β -D-glucopyranosyl; Rha = α -L-rhamnopyranosyl.

efflux by ABC transporters [399,400]. It is now clear that highly polar, extensively glycosylated ginsenosides (e.g., Rb₁, Rb₃, Rg₃, Re, Rg₁, and Rg₂) are relatively weak modulators of human XMEs and transporters [162,179,329,401–408]. Ginsenoside concentrations necessary to inhibit most CYPs and transporters *in vitro* are not only high ($>50\mu\text{M}$), but are not likely to be realized *in vivo*, especially if normal dosing recommendations of ginseng products are followed [162,179,333,401–408]. This also holds true for the even higher ginsenoside concentrations ($>100\mu\text{M}$) required for inducing CYP1A1 [409], CYP2C9, and CYP3A4 [401] activity in human liver cells and microsomes. However, the products of ginsenoside hydrolysis (e.g., deglycosylated metabolites) have been shown to competitively inhibit a variety of CYPs and transporters, oftentimes at concentrations well below $20\mu\text{M}$ [404–409].

Only a limited number of animal studies have examined the effects of orally administered ginseng extracts ($>30\text{ mg/kg}$) on drug disposition. From these, it appears that *P. ginseng* or *P. quinquefolius* either mildly induce [410–412] or have no significant effects [413–415] on rat XMEs and ABCB1 activity *in vivo*. Substantially more prospective human trials have investigated the effects of *P. ginseng* and *P. quinquefolius* supplementation on human drug disposition, with the CYP2C9 substrate,

warfarin, being examined the most. Like the murine studies, clinical results fall into two categories: no effect [48,313,416–420] or mild induction [372,418,421,422].

Concerns regarding possible ginseng–drug interactions first surfaced when two case reports speculated that ginseng reduced warfarin anticoagulation [423,424]. As individual case reports cannot establish causation, a prospective clinical trial by Yuan *et al.* [422] demonstrated that international normalized ratios (INR, a measure of anticoagulation), peak plasma warfarin levels, and warfarin AUCs were reduced by *P. quinquefolius* in healthy volunteers, and these effects reached statistical significance. Subsequent clinical trials with *P. ginseng*, however, have failed to observe any influence on warfarin pharmacokinetics [372,417] or pharmacodynamics [372,417,419]. Such discrepancies may reflect the disparity in ginsenoside profiles between *P. ginseng* and *P. quinquefolius*; however, several methodological concerns have been raised about the Yuan *et al.* study including it being underpowered, that inadequate sampling was used to determine AUC, and a nonstereoselective assay was used to measure warfarin enantiomers [372]. Therefore, it appears that whatever effect(s) *P. quinquefolius* extracts might have on human CYP2C9 activity, their magnitude does not reach clinical significance. As a result, the available evidence that links ginseng supplementation to potentially harmful drug interactions remains unconvincing.

Interaction risk: Low.

16.10.6 Methylenedioxyphenyl-Containing Phytochemicals

The plant kingdom is replete with species that harbor phytochemicals whose structures contain methylenedioxyphenyl (MDP) moieties (Fig. 16.9). Popular botanical supplements known to contain substantial quantities of MDP-containing PSMs (MDP-PSMs) include goldenseal (*Hydrastis canadensis*), kava kava (*Piper methysticum*), black pepper (*Piper nigrum*), and *Schizandra* spp. In these species, MDP-PSMs often function as insecticides [40], but when consumed by humans they can act as mechanism-based inhibitors of CYPs [425,426]. This type of inhibition is thought to arise from CYP-dependent oxidation of the methylenedioxy carbon to a carbene that subsequently interacts with CYP heme iron to produce a stable heme-adduct, termed a *metabolic-intermediate* (MI) *complex* [426]. It is through the formation of MI complexes that CYP isoforms are inactivated by MDP-PSMs [426,427]. Because MDP-PSMs can function as mechanism-based CYP inhibitors, they too pose significant risks for herb-drug interactions.

Prolonged administration of MDP-containing compounds has also been shown to induce CYP1A and CYP2B expression in several animal species [426–428]. Induction of CYPs by MDP-PSMs may be mediated by AhR or through mechanisms that promote protein stabilization [427,428]. Whether CYP inhibition or induction predominates *in vivo* may depend on the length and bulk of MDP side chains as well as the individual CYP isoform examined [429]. MDPs with long bulky side chains appear to be more potent inhibitors of CYP1A2, CYP2C9, CYP2D6, and CYP3A4, whereas CYP2B6 and CYP2C19 are not inactivated [429].

16.10.6.1 Goldenseal. Extracts of goldenseal root (*H. canadensis* L.), a perennial herb indigenous to eastern North America, are often taken as an antimicrobial to prevent common colds and upper respiratory tract infections. Often formulated with *Echinacea* spp., goldenseal ranks among the top-selling botanicals in the United States.

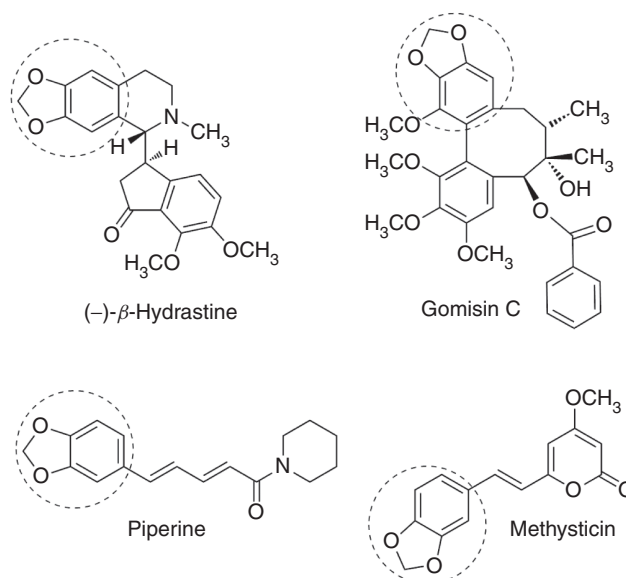


Figure 16.9 Representative methylenedioxyphenyl-containing phytochemicals. (Broken circle = methylenedioxyphenyl moiety).

Goldenseal's medicinal properties are attributed to several isoquinoline alkaloids, of which berberine and hydrastine (Fig. 16.10) are the most prevalent. Both berberine and hydrastine are MDP-PSMs that inhibit various CYP isoforms *in vitro* [154,157,159,175,178,179,189]. Using fluorometric microtiter plate assays, Budzinski *et al.* [154] first noted that commercial extracts of *H. canadensis* were potent *in vitro* inhibitors of CYP3A4. Follow-up investigations by these authors found that goldenseal reduced CYP3A-mediated conversion of testosterone to its 6β-hydroxy metabolite by 88% [157]. Chatterjee and Franklin later confirmed the inhibition of testosterone 6β-hydroxylation by goldenseal extracts and extended their findings to include individual isoquinoline alkaloids [159]. They demonstrated that hydrastine was a more potent inhibitor of CYP3A4 ($IC_{50} = 25 \mu M$) than berberine ($IC_{50} = 400 \mu M$).

It is seldom that prospective clinical studies corroborate *in vitro*-based predictions of CYP-mediated herb–drug interactions; however, goldenseal appears to be an exception. Using phenotypic measures of CYP activity, Gurley *et al.* [248] first observed that goldenseal supplementation significantly inhibited CYP2D6 and CYP3A4 in healthy volunteers. In subsequent investigations with the CYP3A4 probe MDZ, Gurley *et al.* further demonstrated that 14 days of goldenseal supplementation (~209 mg isoquinoline alkaloids daily) significantly increased MDZ AUC, C_{max} , and elimination half-life, and that the effects were proportional to those produced by the well-recognized, mechanism-based inhibitor clarithromycin (1000 mg daily) [430]. In addition, goldenseal's inhibition of human CYP2D6 *in vivo* was confirmed with other commercially available supplement brands [273]. Interestingly, another prospective study evaluating the influence of goldenseal supplementation on the pharmacokinetics of indinavir (a protease inhibitor and CYP3A4 substrate) failed to register any significant effects [431]. This discrepancy may stem from the relatively

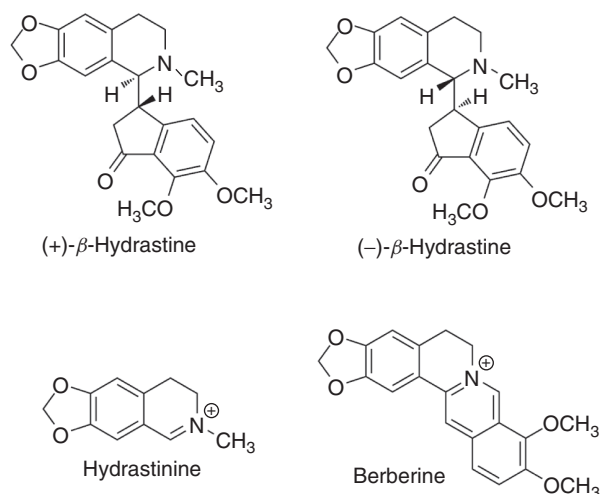


Figure 16.10 Representative phytochemicals (isoquinoline alkaloids) present in goldenseal.

high oral bioavailability of indinavir, which renders it a less effective probe for assessing herb-mediated changes in CYP3A activity. Thus, like GFJ furanocoumarins, goldenseal may have its greatest impact on those CYP3A substrates exhibiting high intestinal metabolism and low oral bioavailability.

In China, berberine (an OTC remedy for diarrhea of bacterial origin) has also been shown to significantly increase the AUC, $C_{\max}$, and trough concentrations of the immunosuppressive drug, cyclosporine [432,433]. Since cyclosporine is both a CYP3A4 and ABCB1 substrate, the activity of both of these proteins could conceivably be affected by hydrastine and berberine. There is convincing *in vitro* and animal evidence that berberine is a substrate for certain ABC isoforms [434–438], and thus, it may compete with other ABC substrates for efflux across intestinal and canalicular membranes. Recent clinical evidence, however, indicates that goldenseal's effect on digoxin (an ABCB1 substrate with a narrow therapeutic index) disposition in humans is not significant [439]. Therefore, goldenseal's likelihood for eliciting herb–drug interactions appears limited to those mediated by CYPs.

Dissolution profiles of commercially available goldenseal supplements reveal that berberine and hydrastine are rapidly and completely released from extract formulations (Gurley, 2010, Personal communication), producing intestinal luminal concentrations comparable to those required for CYP inhibition *in vitro* [430]. In humans, both berberine and hydrastine are readily absorbed and extensively metabolized, with berberine phase I metabolites being preferentially sulfated, while those of hydrastine are primarily glucuronidated [440]. Extensive metabolism is conducive to the formation of MI complexes and may explain goldenseal's penchant for CYP inhibition. Given that goldenseal isoquinoline alkaloids significantly inhibit both CYP3A4 and CYP2D6 activity (the two most important drug-metabolizing enzymes in humans), its herb–drug interaction potential is deemed considerable.

Interaction risk: High.

16.10.6.2 Kava Kava. Kava kava (*Piper methysticum* G. Forst.) has long been a traditional beverage consumed among South Pacific islanders to imbue psychotropic, hypnotic, and anxiolytic effects [441]. Since the 1990s, commercial kava extracts formulated as tablets and/or capsules have been marketed as dietary supplements for the alleviation of stress, anxiety, or insomnia [441,442]. Reports linking kava use to liver toxicity has led to the removal of these products from Australia, Canada, and several European countries, and prompted the FDA to issue warnings of possible hepatotoxic side effects associated with kava supplementation [441]. For those case reports documenting possible kava-related hepatotoxicity, prolonged usage (>60 days) and comedication with prescription drugs or other botanical supplements were frequent and confounding variables [441]. Modulation of human drug metabolism and/or transport has been postulated as an underlying mechanism for kava-induced liver toxicity. Accordingly, a significant body of literature exists exploring kava's ability to alter XME and transporter activity. The preponderance of *in vitro* data points to kava as an inhibitor of various CYPs and ABCB1 [155,156,161,442–446], whereas other evidence suggests that kava may activate PXR to induce CYP and ABCB1 activity [158,447,448]. In accordance to kava phytochemicals acting as nuclear receptor ligands, most studies that administered high doses of kava extracts to murine species for prolonged periods observed an induction in CYP1A and 3A activity and expression [445,449–452].

The kavalactones (e.g., kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin) are a collection of phytochemicals unique to kava (Fig. 16.11). Of these, methysticin and dihydromethysticin are both MDP-PSMs and potent ($\leq 10\mu\text{M}$) mechanism-based inhibitors of CYPs *in vitro* [156,444]. Dihydromethysticin, like some other MDP-containing compounds, also appears to induce CYP3A isoforms *in vitro* through activation of human PXR [447]. In addition, both methysticin and dihydromethysticin have good dissolution profiles in simulated intestinal fluid at pH values >4 , a property favorable for producing gut lumen concentrations in excess of $10\mu\text{M}$ (Gurley, 2010, Personal communication). As a result, one might anticipate that kava supplementation would significantly modulate of human XMEs and transporters *in vivo*.

To date, only five prospective clinical studies have attempted to evaluate the drug interaction potential of kava and the results have been mixed [248,273,430,439,453]. Russman *et al.* found that in chronic users of traditional kava-containing beverages (e.g., 7–27 g kavalactones per week) CYP1A2 activity was significantly reduced, yet this practice had no effect on phenotypic markers of CYP2C19, 2D6, 2E1 or 3A4 function [453]. In contrast, Gurley *et al.* observed that 30 days of kava supplementation in healthy volunteers had no effect on phenotypic markers of CYP1A2, CYP2D6, or CYP3A4 activity, however, CYP2E1 activity was significantly reduced [248].

Discrepancies between these studies with regard to kava's effect on CYP1A2 and 2E1 may be traced to variations in extract composition. Traditional kava beverages are made from aqueous extractions of *P. methysticum* root, whereas formulations of many commercially available kava supplements are products of nonaqueous solvent extractions. Kavalactone profiles can vary considerably among the two extraction processes [442]. Subsequent studies conducted by Gurley's group found that, when compared to known CYP inducers and inhibitors, 14 days of supplementation with well-characterized kava products had no clinically relevant effects on human CYP2D6, CYP3A4, or ABCB1 activity [273,430,439].

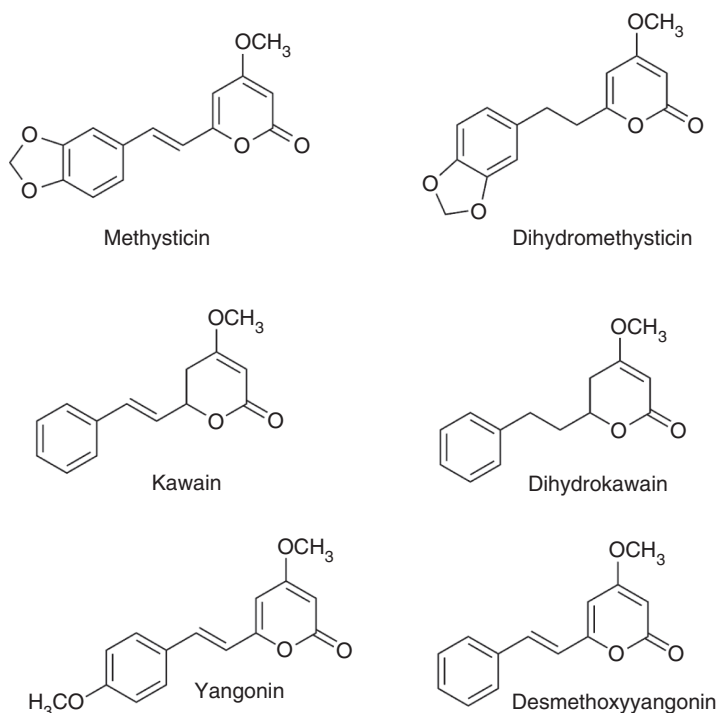


Figure 16.11 Representative phytochemicals (kava lactones) present in kava kava.

The apparent low potency of methysticin and dihydromethysticin as mechanism-based inhibitors *in vivo* may stem from the structure of their MDP side chains. In examining a series of MDP-containing compounds, Nakajima *et al.* noted that those with short, nonbulky side chains were less effective inhibitors of CYP activity [429]. This, coupled with the fact that kava extracts often have lower amounts of methysticin and dihydromethysticin compared to the isoquinoline alkaloid content of goldenseal, may explain the difference in herb–drug interaction potentials for these two MDP-containing species. In short, consumption of commercially available kava supplements per product label recommendations is not likely to affect the efficacy or toxicity of conventional medications.

Interaction risk: Low.

16.10.6.3 Piperine/Black Pepper. Dried ground black pepper (*Piper nigrum* L.) has been used since antiquity as both a flavoring agent and medicine [454]. In fact, for almost two millennia *Piper* spp. (e.g., *P. nigrum* and *P. longum*) have been essential components of several ayurvedic medicine preparations [455]. Black pepper is one of the most commonly used spices and may be found on nearly every dinner table in the industrialized world. Black pepper is produced from green unripe berries of the pepper plant; the fruits are dried following a heat treatment that releases browning enzymes from the cell walls [456]. The spiciness of black pepper is because of the MDP-containing phytochemical, piperine, and related pungent alkaloids known as

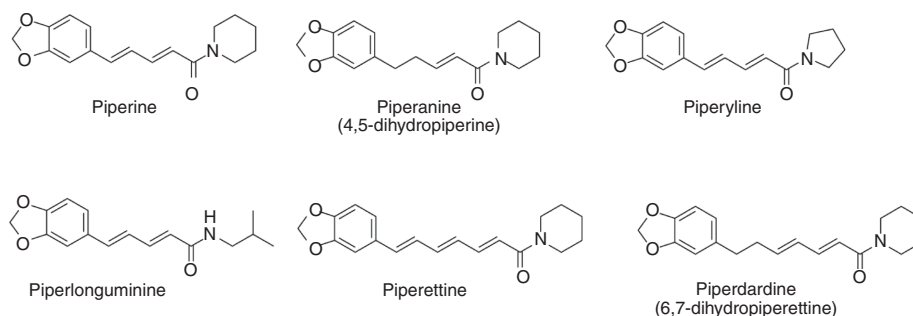


Figure 16.12 Representative phytochemicals (piperamides) present in black pepper.

piperamides [454] (Fig. 16.12). These compounds function as insecticides in the pepper plant [457–459], but in mammalian systems they are inhibitors of various XMEs [460–471] and transporters [186,187,472–475]. While inhibition is clearly the most prevalent finding, especially on acute exposure, several animal studies report upregulation of GSTs [476], certain CYPs [477,478], and ABCs [186,472,474] with long-term exposure to piperine, a finding in line with other studies examining chronic feeding of MDP-PSMs.

Piperine's ability to inhibit drug metabolism was first recognized by Atal *et al.* [460] more than 30 years ago when its administration to rats increased the oral bioavailability of the alkaloids sparteine and vasicine by factors of two and three, respectively. In addition, piperine increased the pharmacodynamic effects of hexobarbital and zoxazolamine in a dose-dependent manner [460]. Follow-up studies by Atal's group observed that piperine administration was a noncompetitive inhibitor of various murine hepatic monooxygenases as well as UGTs [461]. Subsequent studies confirmed piperine's effect on murine XMEs [462–467,470] and also found that it and other piperamides were selective inhibitors of human CYP3A4, CYP2D6, and ABCB1 *in vitro* [187,468,469,471]. Piperine's inhibitory effect on UGTs is more pronounced in intestinal epithelial cells than hepatocytes [464]. Moreover, structure–activity relationship studies involving more than 35 separate analogs revealed that piperine is especially suited for CYP inhibition [467]. Modifications to either the MDP moiety or piperidine side chain significantly reduced its potency [467]. (Millions of years of plant–animal warfare have clearly optimized piperine pharmacology.) In addition to its inhibitory effects on XMEs and ABCB1, piperine may also promote drug absorption by modulating the permeability characteristics of intestinal membranes as well as through stimulating increases in microvilli length [479].

One of the most compelling aspects of piperine is its ability to dramatically enhance the oral absorption of concomitantly administered medications [460,473,475,480–485] (Fig. 16.13). To date, every prospective human trial investigating black pepper's and/or piperine's effect on drug pharmacokinetics has demonstrated a profound improvement in oral bioavailability. Drugs affected and the observed percent increase in mean AUC include phenytoin (16–133%) [481,483,484], rifampicin (69%) [480], propranolol (103%) [482], theophylline (96%) [482], and nevirapine (170%) [485]. In practically every case, piperine's effect can be considered clinically relevant. This is especially so for drugs with narrow therapeutic indices.

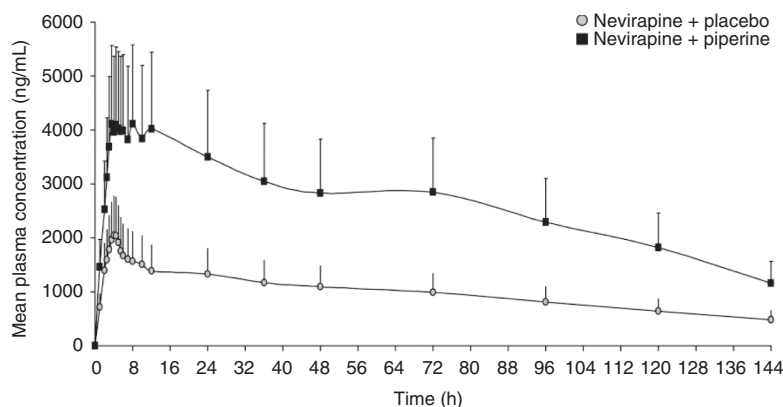


Figure 16.13 Plasma concentration–time curve for nevirapine in healthy subjects when coadministered with piperine (■) or placebo (○). (Mean $\pm$ standard error).

Recognizing piperine's utility as a bioavailability enhancer, many dietary supplement manufacturers incorporate *P. nigrum* or *P. longum* extracts into botanical formulations as a means of improving phytochemical efficacy. This positive herb–herb interaction is best exemplified by curcumin, a dietary phytochemical in tumeric with promising chemopreventative properties but exceedingly poor oral bioavailability due to extensive CYP- and UGT-mediated metabolism [486,487]. When administered with 5 mg of piperine, a twofold increase in curcumin AUC was observed in healthy volunteers [486]. In another study, 20 mg of piperine produced an almost 20-fold increase in curcumin AUC [488]. Similar bioavailability enhancing effects on green tea polyphenols have also been reported [489]. As an added benefit to enhancing phytochemical bioavailability, piperine also has a broad safety profile [454].

When used in quantities typical for flavoring food, black pepper is not likely to affect the disposition of most medications. However, excessive use of black pepper or intake of dietary supplements formulated with *P. nigrum* or *P. longum* extracts may produce clinically significant interactions with drugs. This may be of particular concern when CYP3A and/or ABCB1 substrates are ingested concomitantly with piperine or piperamides in excess of 10 mg.

Interaction risk: High.

16.10.6.4 *Schisandra* spp. Preparations of fruits from woody vines in the family Schisandraceae are a staple in traditional Chinese, Japanese, and Russian medicine [490]. Among their many uses, berry extracts of *Schisandra* spp. (e.g., *S. chinensis* (Turcz.) Baill. and *Schisandra sphenanthera* Rehder & E. H. Wilson) are often prescribed for their adaptogenic and hepatoprotective properties [490,491]. In the United States, extracts of *S. chinensis* and *S. sphenanthera* are often incorporated into multi-component dietary supplement formulations.

A host of unique MDP-PSMs including gomisins A-C, schisandrin, schisandrol B, and schisantherin D are found in *Schisandra* spp. (Fig. 16.14). Like many MDP-PSMs, those present in *Schisandra* spp. are modulators of mammalian XMEs and transporters. Recognition of these properties is important as *Schisandra* products are often taken in conjunction with conventional medications in China, Japan, Russia, and

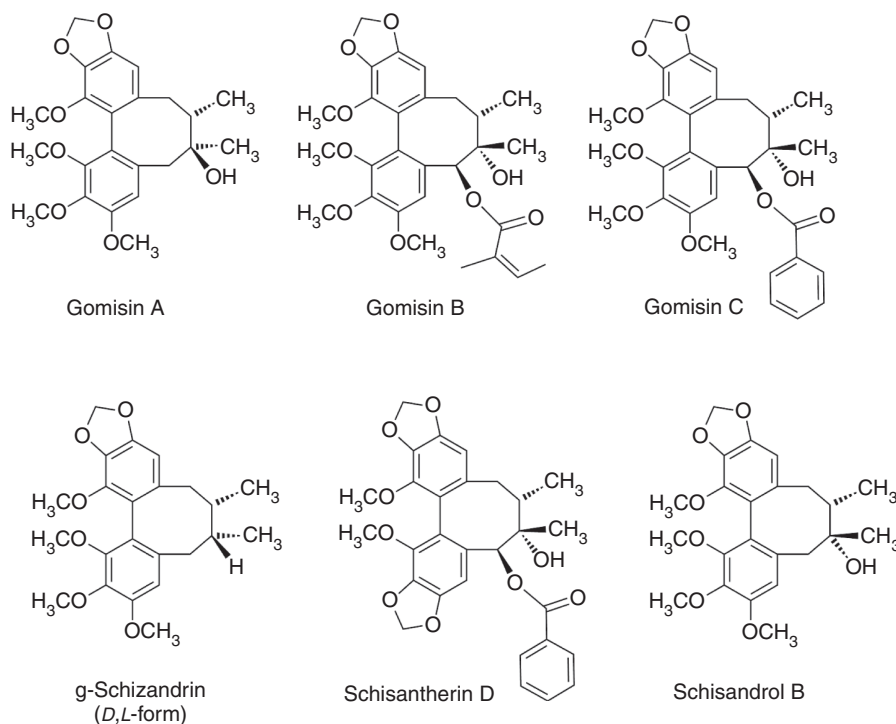


Figure 16.14 Representative methylenedioxyphenyl-containing phytochemicals present in *Schisandra* sp.

other Asian countries. In addition, dietary supplements containing *Schisandra* extracts are becoming more popular in many Western cultures. This too may increase the chance of *Schisandra*-related herb–drug interactions.

Ample evidence points to *Schisandra* MDP-PSMs as both substrates and inhibitors of ABC efflux transporters *in vitro* [492–498]. A number of *in vitro* studies also point to *Schisandra* MDP-PSMs as both competitive and noncompetitive inhibitors of human CYP isoforms [160,167,499–502]. In particular, gomisins B, C, and G were shown to be particularly effective inhibitors of human CYP3A4 with IC_{50} values $<1.5\ \mu\text{M}$ [167]. In the presence of NADPH, gomisin C's inactivation of CYP3A4 was time- and concentration-dependent, as well as irreversible, characteristics indicative of mechanism-based inhibition. Moreover, the inhibitory effect of gomisin C was stronger than that of ketoconazole, a known potent CYP3A4 inhibitor [167]. In contrast, reporter gene assays demonstrated that *S. chinensis* extracts and the individual constituents schisandrin and schisandrol activated rat and human PXR [500], whereas certain natural gomisin H analogs (e.g., tigloylgomisin H and angeloylgomisin H) significantly activated phase II detoxification gene expression via the Nrf2 nuclear receptor pathway [503].

Of the available animal studies investigating *Schisandra*'s effect on xenobiotic metabolism, two outcomes emerged (inhibition or induction), each dependent on the duration of administration. When single doses of *Schisandra* extracts ($\leq 250\ \text{mg/kg}$) were administered to rats concomitantly with CYP3A and/or ABCB1 substrates (e.g., MDZ [501], nifedipine [499], paclitaxel [504], and tacrolimus [502]), drug AUCs more

than doubled suggestive of inhibition. However, when administration period exceeded six days, rat XME and transporter function was consistently induced [500,501,505,506].

Unlike the murine study results, prospective clinical trials assessing *Schisandra*'s effect on CYP3A and/or ABCB1 substrate pharmacokinetics were not biphasic. Whether *Schisandra* was administered once or for up to 14 consecutive days, AUCs of talinolol (ABCB1 substrate) [376], tacrolimus (CYP3A4/ABCB1 substrate) [507,508], and MDZ (CYP3A4 substrate) [509] were increased 1.5-, 2.1-, and 2.0-fold, respectively. On the basis of these data, it appears that *Schisandra* is a potent inhibitor, not an inducer, of human XMEs and transporters. Such species differences may stem from the fact that humans not only received lower milligram per kilogram doses of *Schisandra*, but that gomisins C concentrations necessary for PXR activation in humans were twice that required for murine species [501]. Accordingly, the currently available clinical data strongly suggests that *Schisandra* extracts pose a significant risk for elevating blood levels of drugs that are CYP3A and/or ABCB1 substrates.

Interaction risk: High.

16.10.7 Milk Thistle

Silybum marianum L. Gaertn., commonly known as *milk thistle*, is an herbaceous plant native to the Mediterranean region, although it has been naturalized throughout the world [510]. Extracts of milk thistle fruits (achenes) yield a collection of flavanolignans and flavonoids collectively known as *silymarin* [510,511]. The principal phytochemical components in silymarin are silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin [511] (Fig. 16.15). Milk thistle extracts are touted for their antioxidant and hepatoprotectant properties, and their utility as therapy for treating various liver diseases (e.g., cirrhosis, hepatitis, and hepatotoxicity) has been examined in numerous clinical trials [512–516]. While many smaller trials have revealed improvements in various clinical indices, several larger studies have yielded equivocal results. However, an unequivocal conclusion gleaned from the clinical studies is that milk thistle has an excellent safety profile [512–516].

Milk thistle is one of the few popular botanicals in which the pharmacokinetic profile of its principal phytochemicals has been thoroughly examined in humans [517–521]. Components of standard milk thistle extracts, as a general rule, have very low oral bioavailability and short elimination half-lives. This stems from a combination of the poor water solubility of silymarin phytochemicals coupled with extensive presystemic metabolism (both phase I and II) and biliary secretion. As an example, $C_{\max}$ values for unconjugated silybin A and B (the two most prevalent flavanolignans in milk thistle products) rarely exceeded 20 ng/mL for single 600 mg doses of milk thistle extract in healthy volunteers [520]. Even when milk thistle extract (700 mg) was administered every 8 h for seven days, the maximum steady state concentrations of silybin A and B rarely exceeded 1.5 μ g/mL [521]. At doses exceeding 1500 mg daily, there was also evidence of nonlinear pharmacokinetics [521]. Liver disease, however, does increase milk thistle flavanolignan exposure. Systemic concentrations of silybin and isosilybin diastereomers may be three- to fivefold higher in patients with cirrhotic or nonalcoholic fatty liver disease, which may account for milk thistle's purported efficacy in these populations [519,521].

Given its popularity as one of the most utilized botanical supplements in the world, milk thistle's ability to modulate human XMEs and transporters has received

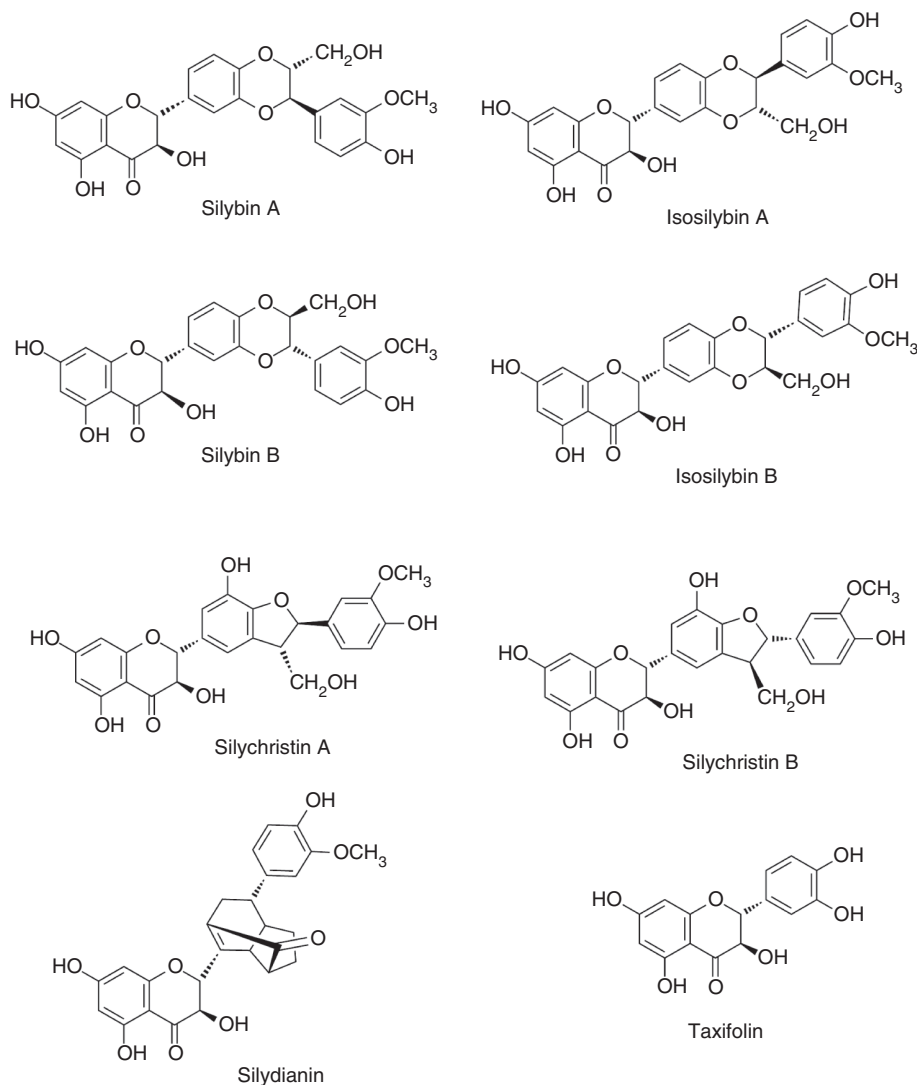


Figure 16.15 Representative phytochemicals (flavanolignans) present in milk thistle.

considerable attention. A variety of *in vitro* methodologies have been utilized to assess the effect of milk thistle extracts or individual flavanolignans on XME [174,178,179,189,522–530], ABC efflux transporter [174,178,179,527,531–536], and SLC1B1 uptake transporter [537] activity. The majority of studies are in general agreement that flavanolignan concentrations in excess of 10 μM are required for inhibition of most CYP isoforms and even higher concentrations are needed for ABCB1 and ABCG2 inhibition. Of all the CYP isoforms tested, CYP2C9 appears to be the most sensitive with IC_{50} values $\sim 8 \mu\text{M}$ for human liver microsomes. At concentrations exceeding 30 μM , silybin diastereomers have been reported to function as mechanism-based inhibitors of CYP2C9 and 3A4 [526]. UGT1A1, however, may

be the XME most easily inhibited by milk thistle with an IC_{50} value of $1.4 \mu M$ [526]. SLC1B1 also exhibited an IC_{50} value $<4 \mu M$ for silymarin [537].

Because milk thistle flavanolignans exhibit poor oral bioavailability, several technologies have been employed to enhance this property [538]. One of the more successful has been complexation of silymarin components with phosphatidylcholine. Marketed as Silipide[®] and Siliphos[®], these phosphatidylcholine complexes, or phytosomes, exhibit improvements in flavanolignan bioavailability three to five times that of conventional milk thistle extract formulations [538–542]. In addition, recent clinical studies in prostate cancer patients have shown that doses of Siliphos ranging from 2.5 to 20 g daily for up to four weeks produced C_{max} values between 10 and $100 \mu M$ for unconjugated silybin diastereomers [543,544]. Conceivably, these concentrations are sufficient for inhibition of various XMEs and efflux transporters, and episodes of hyperbilirubinemia reported in these studies may indeed reflect inhibition of UGT activity [543,544].

To date, a considerable number of prospective human studies have examined milk thistle's drug interaction potential. Supplementation regimens utilizing standard milk thistle extracts had no observable effects on the clinical pharmacokinetics of aminopyrine (nonspecific CYP probe) [545], caffeine (CYP1A2 probe) [115], chlorzoxazone (CYP2E1 probe) [115], debrisoquine (CYP2D6 probe) [115,273], digoxin (ABCB1 substrate) [247], indinavir (CYP3A4 substrate) [546–548], irinotecan (CYP3A4/UGT1A1 substrate) [549], MDZ (CYP3A4 probe) [115,229], nifedipine (CYP3A4 substrate) [550], phenylbutazone (nonspecific CYP probe) [545], ranitidine (CYP3A4/ABCB1 substrate) [551], and rosuvastatin (ABCB1/SLC1A1 substrate) [537]. Taken together, these findings imply that standard milk thistle products generate flavanolignan concentrations *in vivo* that are incapable of affecting most human XMEs and transporters. However, a few clinical studies challenge this assumption. For example, 14 days of silymarin supplementation (140 mg, thrice daily) increased the AUC of talinolol (ABCB1 substrate) in healthy volunteers by 36% (a finding suggestive of ABCB1 inhibition); however, this effect is not considered clinically relevant [552]. In contrast, 140 mg of silymarin administered to healthy adults for nine days reduced the mean AUC for metronidazole (CYP3A4/ABCB1 substrate) by 29% [553], and while this effect hints at possible induction, it too is considered clinically insignificant. More concerning is a recent finding that silymarin inhibits the metabolism of losartan to its active metabolite E-3174, and that the magnitude of the interaction is dependent on *CYP2C9* genotype [149]. In subjects with the *CYP2C9**1/*1 genotype, a 14-day course of silymarin produced a twofold increase in losartan AUC and C_{max} , but these parameters were not affected in subjects with the *CYP2C9**1/*3 genotype. This finding supports that of Brantley *et al.* [530] who noted that *CYP2C9* appeared most vulnerable to inhibition by clinically achievable concentrations of silybin B.

At present, no prospective studies have examined the effects of phytosomal milk thistle preparations on the pharmacokinetic profiles of conventional medications. As such, it remains to be seen whether milk thistle products with enhanced bioavailability pose a greater risk for herb–drug interactions. A recent clinical trial of Siliphos for the treatment of chemotherapy-related hepatotoxicity in childhood acute lymphoblastic leukemia suggests otherwise, as investigators observed no adverse interactions between milk thistle and methotrexate, 6-mercaptopurine, or vincristine during the 28-day course of supplementation [554]. Therefore, based on the collective clinical data currently available, the drug interaction risk for milk thistle products appears minimal.

Interaction risk: Low.

16.10.8 St. John's Wort

With more than 2000 peer-reviewed articles published on its safety and efficacy, SJW (*H. perforatum* L.) is the most studied botanical dietary supplement in the world. *H. perforatum* is a yellow-flowering, perennial herb indigenous to Europe that has been introduced to many temperate areas of the world and grows wild in many meadows. The common name comes from its traditional flowering and harvesting on 24 June, the birthday of John the Baptist (St. John's Day).

Extracts of *H. perforatum* have gained international recognition for their antidepressant activity although the efficacy of many SJW products remains questionable [555]. Nevertheless, many clinical trials have demonstrated efficacy superior to placebo and comparable to standard antidepressants, but with fewer side effects than conventional antidepressive agents [556]. When used as a single agent, a favorable risk/benefit ratio has made SJW one of the most readily consumed dietary supplements in the world. In turn, the popularity of SJW has also contributed to its distinction as being one of the most problematic dietary supplements with regard to herb–drug interactions.

Both the antidepressant effect and drug interaction potential of SJW hinge on the extract's content of hyperforin, a bicyclic polyprenylated acylphloroglucinol found exclusively in *Hypericum* spp. [557] (Fig. 16.16). As an antidepressant, hyperforin functions as broad-based neurotransmitter reuptake inhibitor, affecting the synaptosomal uptake of serotonin, dopamine, norepinephrine, glutamate, and γ -aminobutyric acid with similar efficiency. Hyperforin's mode of action appears unique in that the phytochemical does not interact directly with uptake transporters but elevates intracellular sodium concentration, thereby inhibiting gradient-driven neurotransmitter reuptake [557]. As previously mentioned in Section 16.4.1, recent investigations reveal that hyperforin and other SJW-related phytochemicals (e.g., adhyperforin, hypericins, and flavanol glycosides) act synergistically through both pharmacodynamic and pharmacokinetic mechanisms to alleviate depression [190].

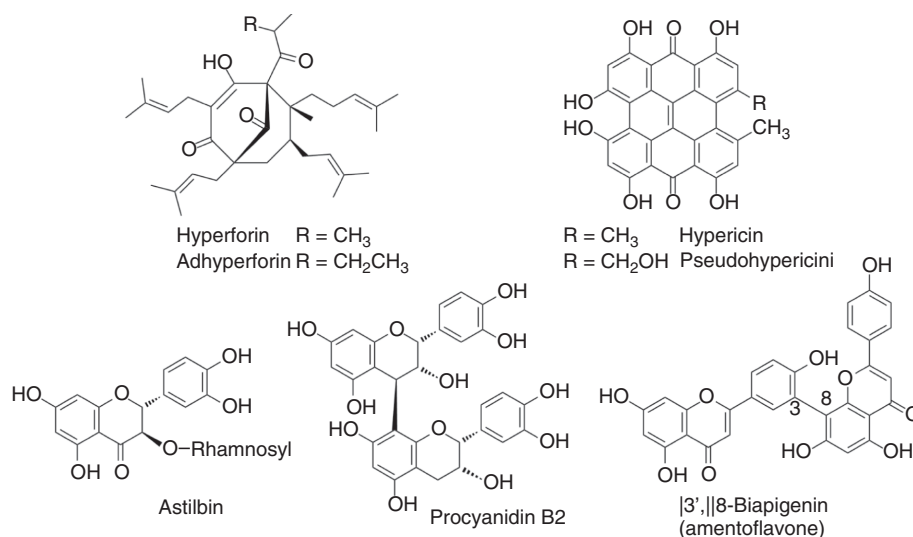


Figure 16.16 Representative phytochemicals (phloroglucinols, naphthodianthrone, and flavanol glycosides) present in St. John's wort.

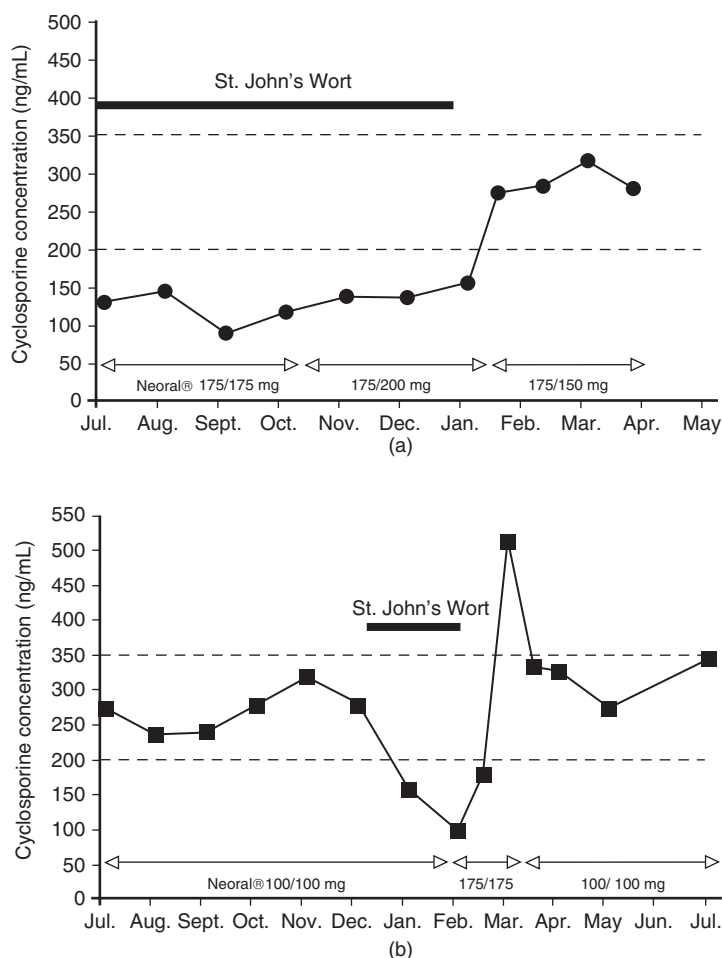


Figure 16.17 Effect of St. John's wort on cyclosporine trough concentrations in renal transplant recipients who did not (a) and who did (b) develop graft rejection. Broken lines represent cyclosporine therapeutic range. Reprinted with permission [558].

While recognized as a natural antidepressant, SJW is equally well known for its ability to induce the activity of several XMEs and transporters, thereby reducing the efficacy of a multitude of prescription medications. As discussed earlier in Section 16.7.3, the clinical severity of SJW-mediated interactions was first recognized in 1999–2000. At that time, several clinics around the world reported that concomitant use of SJW and cyclosporine produced dramatic reductions in blood levels of the immunosuppressant among organ transplant recipients resulting in graft rejection (Fig. 16.17) [208–210,558]. Since that time a plethora of clinical studies have borne out SJW's effect on the pharmacokinetics of various medications [226,559–562].

Hyperforin, a principal mediator of SJW's antidepressive action, lies at the heart of the herb's drug interaction potential. The prenylated phloroglucinol is a high affinity ligand for human PXR, an orphan nuclear receptor selectively expressed in the liver and intestine that mediates the induction of XME and efflux transporter gene transcription [211,212,563]. According to one estimate, hyperforin is the most potent

TABLE 16.2 Drug Interactions with St. John's Wort Confirmed in Human Studies

Drug Category	Effect of SJW on Drug Concentration	References
<i>Antianginals</i>		
Ivabradine	Decreases plasma level of drug	571
<i>Antiarrhythmics</i>		
Digoxin	Decreases plasma level of drug	213,230,573,574
<i>Anticoagulants</i>		
Warfarin	Decreases plasma level of drug	372,417
<i>Anticonvulsants</i>		
Mephenytoin	Decreases plasma level of drug	575
<i>Antidepressants</i>		
Amitriptyline	Decreases plasma level of drug	576
<i>Antifungals</i>		
Voriconazole	Decreases plasma level of drug	577
<i>Antihistamines</i>		
Fexofenadine	Mixed, generally decreases plasma level of drug	578–580
<i>Antihyperlipidemics</i>		
Simvastatin	Decreases plasma level of drug	216
Atorvastatin	Decreases plasma level of drug	581
<i>Anxiolytics</i>		
Midazolam	Decreases plasma level of drug	48,215,313,570,578,580,582
Alprazolam	Decreases plasma level of drug	583,584
Quazepam	Decreases plasma level of drug	585
<i>Antivirals</i>		
Indinavir	Decreases plasma level of drug	586
Nevirapine	Decreases plasma level of drug	587
<i>β-Adrenergic Blockers</i>		
Talinolol	Decreases plasma level of drug	588
<i>Calcium Channel Blockers</i>		
Verapamil	Decreases plasma level of drug	589
Nifedipine	Decreases plasma level of drug	357,572
<i>Cancer Chemotherapeutics</i>		
Irinotecan	Decreases plasma level of drug	590
Imatinib	Decreases plasma level of drug	591,592
<i>Hormonal Contraceptives</i>		
Ethinylestradiol	Decreases plasma level of drug	593–595
Norethindrone	Decreases plasma level of drug	593,594
Ketodesogestrel	Decreases plasma level of drug	595
<i>Hypoglycemic Drugs</i>		
Gliclazide	Decreases plasma level of drug	596

(continued overleaf)

TABLE 16.2 (continued)

Drug Category	Effect of SJW on Drug Concentration	References
<i>Immunosuppressants</i>		
Cyclosporine	Decreases plasma level of drug	210,578,597
Tacrolimus	Decreases plasma level of drug	598–600
<i>Opioids</i>		
Methadone	Decreases plasma level of drug	601
<i>Proton-Pump Inhibitors</i>		
Omeprazole	Decreases plasma level of drug	602
<i>Skeletal Muscle Relaxants</i>		
Chlorzoxazone	Decreases plasma level of drug	48,313
<i>5α-Reductase Inhibitors</i>		
Finasteride	Decreases plasma level of drug	603

PXR activator discovered to date [211], with a half-maximal effective concentration (EC₅₀) of 23 nM—a value well below plasma concentrations often achieved in humans (~100–300 nM) [564,565]. (Unlike many of the unique phytochemicals described previously, hyperforin is bioavailable and exhibits an elimination half-life of 8–12 h, thus allowing for significant accumulation with repeated dosing [564,565].) Of the XMEs and transporters regulated by PXR, those most affected by SJW are CYP subfamilies 2C and 3A as well as several ABC efflux transporters [559–562]. As a result, more than 70% of all prescription medications are susceptible to SJW-mediated interactions, the consequences of which are decreased oral bioavailability, enhanced systemic clearance, and reduced drug efficacy.

As with all botanicals, phytochemical content can vary considerably (Section 16.7.4) and such is the case with hyperforin [566,567]. Most SJW extracts are currently standardized to contain 3% hyperforin, yet many brands may possess amounts well below this value. Several clinical studies have demonstrated that SJW extracts containing <1% hyperforin are less likely to produce clinically relevant herb–drug interactions [567–570]. Unfortunately for consumers, few SJW products are specifically labeled as having low hyperforin content. Accordingly, to avoid significant drug interactions, consumers should avoid concomitant use of SJW and prescription medications.

In addition to content variability, another factor affecting the magnitude of hyperforin's effect on CYP3A expression and drug efficacy is PXR haplotype. Like many CYPs, PXR is polymorphic, and certain mutations produce significant functional defects in terms of CYP3A transcription. Recent studies indicate that individuals with the H1/H1 haplotype pair appear more susceptible to SJW-mediated CYP3A induction than subjects with H1/H2 or H2/H2 pairings [571]. Furthermore, once SJW has been discontinued, as much as a week may be required before CYP3A activity returns to basal levels [572].

SJW's penchant for producing herb–drug interactions has been scrutinized in several recent reviews [226,559–562]. Table 16.2 summarizes those drugs in which SJW produces clinically significant interactions.

Interaction risk: High.

16.11 CONCLUSIONS AND FUTURE PERSPECTIVES

Millions of years of “plant–animal warfare” have led to a complex and proficient system of human XMEs and transporters that work in concert to preclude absorption and facilitate elimination of numerous structurally diverse dietary phytochemicals. As a result, most plant-based foods and dietary supplements pose only minimal risks for modulating human drug metabolism. However, several distinct PSMs can either inactivate or hijack the controls of this innate gastrointestinal defense network. PSMs such as GFJ furanocoumarins, functioning as mechanism-based inhibitors of CYPs, may potentiate the toxicity of allopathic medications, whereas potent nuclear receptor ligands such as hyperforin may dramatically reduce drug efficacy. What differentiates these PSMs as significant modulators of human drug disposition from the multitude of others is their combination of favorable physicochemical properties and unique pharmacophores. As discussed in previous sections, many PSMs modulate human XMEs and transporters *in vitro*, but because of various factors, this activity is rarely realized *in vivo*.

Recognizing that poor dissolution characteristics represent major obstacles to phytochemical bioavailability and efficacy, several dietary supplement manufacturers have recently incorporated novel methods of formulating botanical extracts as a means of improving oral absorption. Such innovative formulation technologies include liposomes, self-emulsifying microemulsions, microspheres, phosphatidylcholine complexation (phytosomes), and nanoparticles [604], as well as incorporation of piperine as a CYP3A4 and ABCB1 inhibitor [468,469]. In each instance, when compared to conventional extract formulations, the oral bioavailability of various phytochemicals can be increased several fold [604]. To date, no clinical assessments of the herb–drug interaction potential of botanical extracts incorporating these novel formulations have been reported. Accordingly, many botanicals whose drug interaction potential is minimized when administered as conventional dry extracts may increase significantly on ingestion of formulations utilizing novel delivery systems. Phytochemicals that heretofore had been casualties of man’s xenobiotic defense system may emerge as inducers and/or inhibitors of human XMEs and transporters. Improved phytochemical delivery, therefore, may open up a new theater of operations in the “drug war” between humans and plants.

Currently, thousands of botanical supplements are available in the United States and abroad, and hundreds of new products are introduced onto the market each year. Not surprisingly, the vast majority of these botanicals have yet to be evaluated in a clinical setting. From a practical perspective, most may not warrant clinical study; logistics alone clearly preclude such an endeavor. Nevertheless, *in vitro* studies and compelling case reports suggest that many more botanical supplements may indeed be potent modulators of human drug disposition.

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17 Idiosyncratic Drug Reactions

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17.1 Summary	1
17.2 Introduction	2
17.3 Characteristics and mechanisms of idiosyncratic drug reactions	3
17.4 Role of reactive metabolites in idiosyncratic drug reactions	6
17.5 Animal models of idiosyncratic drug reactions	7
17.6 Predicting idiosyncratic drug reactions	18
17.7 Conclusions	19
References	20

17.1 SUMMARY

Idiosyncratic drug reactions (IDRs) are adverse drug reactions (ADRs) that are not related to the known pharmacological properties of the drug, occur in only a small percentage of the population (accounting for 6–10% of all ADRs), and do not show any apparent dose–response relationship. The unpredictable nature of IDRs together with the often serious adverse effects encountered, pose a major health concern in the development and clinical usage of pharmaceuticals. As opposed to direct toxicity, which generally occurs rapidly following drug exposure, IDRs typically have a delayed onset of reaction following drug administration and may involve various organs, including liver, skin, bone marrow, or may be a generalized systemic response. The mechanism of IDRs is not clearly understood and is the subject of considerable debate. Several theories have been proposed, but IDRs can be generally categorized mechanistically as either immune-mediated or nonimmune-mediated. Currently, the major hypotheses for the mechanism behind the development of IDRs include the hapten hypothesis, the reactive metabolite hypothesis, the metabolic idiosyncrasy hypothesis, the pharmacological interaction (p-i) concept, the danger hypothesis, the mitochondrial injury hypothesis, and the inflammagen hypothesis. For both immune-mediated and nonimmune-mediated mechanisms, there is circumstantial evidence that reactive metabolites play a role in IDRs. In general, most drugs that cause idiosyncratic reactions are not sufficiently reactive in themselves, but most are metabolized to chemically reactive metabolites

that may covalently bind to cellular proteins. Recent investigations continue to support the association of reactive metabolites and immune-mediated responses with IDRs. In drug development, IDRs can lead to drug failure, which is further complicated by the difficulty in predicting the clinical risk of IDRs and by the ineffectiveness of current nonclinical toxicity testing strategies to identify drugs that may potentially cause an IDR. Investigations in animal models have had limited success owing to the unpredictability of IDRs in animals, which is similar to the situation in humans. However, IDRs in animals do share some characteristics and mechanisms observed in humans, supporting the need for further study of animal models. Examples of animal models that most closely reflect human IDRs include sulfonamide hypersensitivity in dogs, nevirapine rash in rats, propylthiouracil (PTU)-induced autoimmunity in cats, and D-penicillamine autoimmunity in Brown Norway (BN) rats. Although attempts with other animal models, such as procainamide-induced lupus and felbamate-induced liver toxicity and aplastic anemia, have been unsuccessful to date, they are examples of compounds where reactive metabolites are thought to play a role in ADRs and may provide some insight into possible mechanisms that may be relevant to IDRs. As evidenced from the many hypotheses that have been proposed, the mechanisms involved in the occurrence of IDRs are complex and the development of appropriate animal models will be key to developing a better understanding of the basic underlying mechanisms involved in IDRs.

17.2 INTRODUCTION

ADRs are among the top 10 leading causes of death [1,2]. It is estimated that 100,000 deaths per year are attributed to ADRs [3]. They are a major contributor to hospitalization throughout the world and have resulted in health care costs in excess of \$3.6 billion annually [1,4,5]. Drugs most frequently associated with ADRs are diuretics, opioid analgesics, antipsychotics, anticoagulants, and antirheumatism drugs [4–7]. A secondary outcome to ADRs is often the immediate removal of the drug from the market with the consequent loss of a potentially promising new medicine and the associated financial costs to the pharmaceutical industry [8,9]. Troglitazone and felbamate are two examples of drugs that either have been withdrawn or have restricted usage because of ADRs [10–12].

ADRs can be variably classified as predictable or unpredictable [13]. Type A ADRs are predictable, attributed to an augmented primary or secondary pharmacological effect, and are usually dose dependent; whereas, type B ADRs are considered unpredictable or idiosyncratic [13–15]. IDRs are also referred to as *drug-induced hypersensitivity reactions* or *allergic reactions*. IDRs are ADRs that are not related to the known pharmacological properties of the drug, occur in only a small percentage of the population, and do not show any apparent dose–response relationship [13,16]. Type A ADRs are more common than type B ADRs, accounting for >80% of ADRs, whereas IDRs (i.e., type B ADRs) are rare, occurring with an incidence ranging from 1 in 100 to 1 in 100,000 [17,18]. Although the incidence of IDRs is not as high as type A ADRs (IDRs accounting for 6–10% of all ADRs), the occurrence of IDRs is still of considerable concern because of the number of drugs involved, the number of people taking these drugs, and the unpredictable and serious nature of the ADRs that may account for many drug-related deaths [13,16,19,20]. IDRs can be very serious

medical conditions when manifested as hepatotoxicity, hemolytic anemia, agranulocytosis, erythema multiforme, toxic epidermal necrolysis (TEN), or Stevens–Johnson Syndrome (SJS) [13,21–24]. IDRs are generally drug and individual specific [16,25]. Genetic variants that cause susceptibility to a drug reaction are becoming increasingly more important in understanding the potential causes of ADRs [26].

In drug development, IDRs can lead to drug failure, which is further complicated by the difficulty in predicting the clinical risk of IDRs and by the ineffectiveness of current nonclinical toxicity testing strategies to identify drugs that may potentially cause an IDR [16]. Investigations in animal models have had limited success owing to the unpredictability of IDRs in animals, which is similar to the situation in humans. However, IDRs in animals do share some characteristics and mechanisms observed in humans, supporting the need for further study of animal models [27,28].

17.3 CHARACTERISTICS AND MECHANISMS OF IDIOSYNCRATIC DRUG REACTIONS

As opposed to direct toxicity, which generally occurs rapidly following drug exposure, IDRs typically have a delayed onset of reaction following drug administration and may involve various organs, including liver, skin, bone marrow, or may be a generalized systemic response. In most cases, this delay is in the order of a week or more following first exposure, but on occasion, may occur within days of administering the first dose of drug [16]. For example, the delay in onset of idiosyncratic drug-induced hepatitis may be more than a year for troglitazone [29]. However, on rechallenge, the time of onset for many drugs may be much shorter and may even be in the order of minutes for an anaphylactic reaction [16]. IDRs can occur when there is a convergence of risk factors, which may involve both drug-related risk factors (e.g., metabolism, bioactivation, covalent binding, and inhibition of key cell functions) and patient-related risk factors (e.g., age, sex, concurrent disease, comedications, nutritional status, immune function, physical condition, and genetic predisposition)[30,31].

The mechanisms of IDRs are not clearly understood and are the subject of considerable debate. Several theories have been proposed, but IDRs can be generally categorized mechanistically as either immune-mediated or nonimmune-mediated [16,32]. Currently, the major hypotheses for the mechanism behind the development of IDRs include the hapten hypothesis, the reactive metabolite hypothesis, the metabolic idiosyncrasy hypothesis, the pharmacological interaction (p-i) concept, the danger hypothesis, the mitochondrial injury hypothesis, and the inflammagen hypothesis [33,34]. In the hapten hypothesis, small molecules (e.g., a drug or a chemically reactive metabolite) themselves are not immunogenic, but when a small molecule (i.e., hapten) is bound irreversibly to protein, the modified protein acts as an immunogen to elicit an immune response [35]. The reactive metabolite hypothesis proposes that while most reactive metabolites are detoxified, when detoxification systems (e.g., glutathione (GSH)) become depleted, reactive metabolites can act as haptens, which fits with the hapten hypothesis [34]. In some cases of IDRs, particularly those involving idiosyncratic liver toxicity, characteristics of an immune-mediated reaction (i.e., fever, rash, eosinophilia, antidrug antibodies) are not present. In these cases, the metabolic idiosyncrasy hypothesis postulates that the drug-metabolizing polymorphism may be a risk factor, although clear evidence of the polymorphism being responsible for the idiosyncratic nature of

an IDR is lacking [34]. In the p-i concept, it is proposed that some drugs are able to initiate an immune response by reversible interaction with the major histocompatibility complex (MHC) or the T-cell receptor and that this interaction more closely resembles a pharmacological response [36]. The danger hypothesis postulates that the immune system responds with tolerance to most foreign antigens, and that an immune response is triggered by an innate “danger signal” rather than by the foreign antigen [18,37–41]. Although the exact nature of the stimuli that trigger a “danger signal” is not entirely certain, it is plausible that cell damage or cell stress (e.g., heat shock proteins) is a major stimulus with consequent activation of antigen-presenting cells (APCs) [34]. In the mitochondrial injury hypothesis, heterozygous mice deficient in mitochondrial superoxide dismutase (complete deficiency is lethal) treated with troglitazone or flutamide developed a delayed-onset hepatotoxicity [42,43]. This is a very attractive model because, unlike many models, the toxicity is delayed in onset, thereby mimicking clinical idiosyncratic hepatotoxicity [33]. The inflammagen hypothesis proposes that “inflammagens,” such as lipopolysaccharide (LPS), which we are commonly exposed to, in combination with a drug (e.g., ranitidine) can result in drug-induced idiosyncratic hepatotoxicity [44–46].

On the basis of the characteristic delay observed between initiation of drug administration and the onset of the adverse reaction and the decreased delay observed following rechallenge, many IDRs appear to be immune-mediated [27,47–50]. Clinically, immune-mediated events are sometimes manifested by blood and tissue eosinophilia associated with fever, which subside quickly following drug withdrawal, unless there is progression to drug-induced autoimmunity [25]. For both immune-mediated and nonimmune-mediated mechanisms, there is circumstantial evidence that reactive metabolites play a role in IDRs [14,27]. In general, most drugs that cause idiosyncratic reactions are not sufficiently reactive in themselves, but most are metabolized to chemically reactive metabolites that may covalently bind to cellular proteins [27]. However, covalent binding to a protein is not in itself enough to cause an IDR since many drugs or metabolites that bind to proteins do not result in a significant incidence of IDRs [51].

Although many consider IDRs to be dose independent, an increase in incidence of IDRs may be seen with increasing dose within the therapeutic range for many drugs consistent with a dose–response relationship [16,52,53]. Although most IDRs are associated with a clinical dose range, there is almost always a dose level that will not elicit an IDR. There is evidence that IDRs rarely occur with drugs administered at doses less than 10 mg/day, which suggests that there is a threshold for the most common types of IDR [16].

Another characteristic of IDRs is adaptation or tolerance, in which clinical symptoms ameliorate or completely resolve in the face of continued treatment, particularly in situations where a low dose is administered initially, followed by dose escalation [14,16]. The reduced risk of developing an IDR clinically may be attributed to the low dose being insufficient to trigger an idiosyncratic reaction but sufficient to induce immune tolerance [14]. In a murine model of 2,4-dinitrochlorobenzene (DNCB)-induced delayed-type hypersensitivity (DHT) reaction, hepatic Kupffer cells (KCs) were shown to play an important role in mediating tolerance against drug–protein adducts. KCs were found to suppress T-cell activation by the production of prostaglandins (PGE₂ and 15 d-PGJ₂). These findings suggest that KCs, in addition to regulating immune reactions in the liver, may play a role in liver-mediated systemic

immune tolerance [54]. Furthermore, any factors (e.g., genetic and environmental) that can impair hepatic KC function leading to decreased production of prostaglandin may result in an increased risk of developing IDRs [55].

IDRs share characteristics and pathology with other disease processes, which makes diagnosis difficult. For example, hepatic IDRs may present with a similar histopathologic appearance as viral hepatitis or idiopathic hepatitis, which precludes identification of the cause or initiating events [16]. The inflammatory response in the liver in these cases may appear similar but is not pathognomonic for the cause of the hepatitis. However, eosinophilia in cases of hepatitis is considered evidence of an immune-mediated response consistent with hepatic IDR. Similarly, the cause of most IDR-induced skin rashes cannot be readily differentiated from viral or other causes unless they present with specific diagnostic features as those that occur in fixed drug eruptions or TEN, which are pathognomonic for an idiosyncratic drug response [16].

Other risk factors identified for IDRs include sex, age, weight, and disease state. However, these factors appear to be associated with specific IDRs [16]. Although women appear to be at increased risk for IDRs caused by some drugs (e.g., halothane-induced hepatitis) and for idiopathic autoimmune reactions (e.g., lupus and autoimmune thyroid disease), other autoimmune diseases, such as type 1 diabetes, do not have such a female predominance [16,56]. Similarly, while the risk of drug-induced liver toxicity (e.g., isoniazid and acetaminophen) increases with age, the risk of valproic acid liver toxicity is greater in children than adults [16]. Halothane-induced hepatotoxicity occurs more commonly in obese patients, which is attributed to the distribution of halothane to fat, thereby requiring higher doses of drug to achieve effect [56]. Although it is generally believed that preexisting liver disease does not increase susceptibility to hepatic IDRs, there appears to be exceptions to this dogma [16,57]. Patients with hepatitis C appear to be at greater risk for developing veno-occlusive disease following myeloablative therapy [58]. Other types of diseases associated with increased risk of IDRs include mononucleosis [59], HIV infection [60], and human herpesvirus 6 infection [61].

Although the acute hepatotoxic effects of some drugs, for example, acetaminophen, are well understood and clearly dose dependent, there are many other drugs that are potentially hepatotoxic at therapeutic doses, for which the key susceptibility factors are not known [62]. An underlying disease state can further complicate and influence the toxic response to a drug through modulation of toxicokinetics (e.g., metabolism, disposition, and clearance) or altered expression of key genes by a disease that may render an individual more susceptible to a drug's adverse effects [62]. Mice subjected to environmental stress through immobilization in restraint cages demonstrated enhanced liver injury to CCl₄ or allyl alcohol at doses that did not cause liver injury in unrestrained animals [63]. This suggests that "psychogenic stress" may enhance susceptibility to liver damage by hepatotoxicants and may be a predisposing factor to liver injury after drug administration [63]. The underlying mechanism is considered to be the result of increased hepatic KC activation, with subsequent release of soluble mediators that sensitize liver cells to hepatotoxicants [63]. Proinflammatory conditions can significantly alter the hepatic response to drugs as a result of mediators and cytokines released from inflammatory cells. [62]. Endotoxemia is an example of a proinflammatory condition in which bacterial LPS is released as a result of liver disease, gastrointestinal disease, or drug effects on the intestinal mucosa [62]. Release of massive amounts of LPS can lead to circulatory shock, multiorgan failure, and death, whereas less amounts of LPS

release can cause a clinically silent inflammatory condition associated with increased release of eicosanoids, tumor necrosis factor alpha (TNF- α), reactive oxygen species (ROS), or reactive nitrogen species (RNS) [62,64,65]. In rats, administration of nontoxic amounts of LPS induces a low level endotoxemia resulting in a mild noninjurious inflammatory state that can significantly increase susceptibility to hepatotoxicants [62]. Pretreatment of rats with the H₂ receptor antagonist ranitidine before administration of a nontoxic dose of LPS produces hepatotoxicity [44,62]. Idiosyncratic liver toxicity occurs in <0.1% of people taking ranitidine, and while most hepatic reactions are mild and reversible, extensive liver damage and death may occur [64]. Ranitidine hepatotoxicity is typical of an IDR, where the time of onset varies greatly relative to the initiation of treatment. However, unlike other drugs where rechallenge results in a shorter time of onset of adverse effects, rechallenge with ranitidine does not inevitably result in the recurrence of hepatotoxicity [64]. This suggests that a mechanism other than immune-mediated is involved. However, this hypothesis predicts a random time to onset rather than a typical time to onset of one to three months, and IDR very rarely occurs immediately on first exposure to a drug (see *Idiosyncratic Drug Reactions and the Potential Role of Metabolism* in Volume VI).

In terms of patient susceptibility to IDRs, there is currently little evidence of a genetic predisposition to most IDRs. Certain IDRs such as abacavir-induced hypersensitivity are linked to a specific gene (*HLA-B*5701*) and flucloxacillin-induced hepatotoxicity is linked to the same gene [34,66]. However, there does not appear to be one gene that is linked to all IDRs. The *HLA-B*1502* gene is thought to contribute to the pathogenesis of carbamazepine-induced SJS/TEN, and the genetic susceptibility to carbamazepine-induced cutaneous ADRs is phenotype specific [67]. Although a few associations with genetic polymorphism for metabolizing enzymes have been identified, most investigations into this association have been negative [16,68]. This suggests that differences in drug metabolism do not represent major risk factors in the pathobiology of IDRs [16]. Furthermore, population studies do not currently fully support a genetic predisposition to IDRs [34]. Whole genome single nucleotide polymorphism (SNP) profiling may provide additional insight in determining genetic predisposing factors for ADRs [15].

17.4 ROLE OF REACTIVE METABOLITES IN IDIOSYNCRATIC DRUG REACTIONS

Although the mechanism of most idiosyncratic reactions is unknown, several characteristics of idiosyncratic reactions are suggestive of involvement of the immune system [69]. These include a lag period of more than a week between starting the drug therapy and the development of toxicity, immediate recurrence of the idiosyncratic response on reexposure to the drug, lack of correlation between dose and risk of toxicity, and the presence of eosinophilia [70]. In some cases, antibodies have been found to support an “allergic” nature of certain idiosyncratic reactions [70]. However, the molecular weight of molecules that are antigenic is generally greater than 1000 Da, whereas most drugs are not this large [70,71]. This suggests that molecules must bind to proteins in order to be antigenic (i.e., hapten) [69]. It also appears that covalent binding is required for immunogenicity since other types of chemical interactions such as ionic and hydrophobic binding are insufficient [71,72]. Furthermore, it appears that most drugs do not directly react with proteins to form covalent bonds [70]. This

implies that metabolism of a drug is critical to forming a hapten. Most drugs are metabolized to reactive metabolites that can covalently bind to proteins, and there appears to be a good correlation between the quantity of reactive metabolite formed and the risk of developing an idiosyncratic reaction [69]. Since the structure of the metabolite–protein adduct is likely very different from the parent molecule, the antibodies induced by the adduct may not bind to the drug itself [70]. There are several examples of metabolism of drugs producing metabolites involved in idiosyncratic reactions, such as halothane, isoniazid, and sulfonamide [69,70,73]. The reactivity of the metabolic intermediates together with their occurrence in certain tissues and their localization within a specific subcellular fraction of those tissues may be significant in the manifestation of toxicity in target organs [74]. Many drugs are target-organ specific; for example, halothane is metabolized in the liver and the toxicity of halothane is manifested selectively in the liver. Other end organs such as the lung and skin may also be sites of metabolism. In addition, many drugs (e.g., procainamide, sulfonamides) may also be metabolized by leukocytes to reactive intermediates, which may then lead to other types of hypersensitivity reactions such as agranulocytosis, drug-induced lupus, or generalized idiosyncratic reactions [70,75,76]. Many drugs that possess arylamine, sulfhydryl, or other readily oxidizable moieties are particularly susceptible to oxidation by activated leukocytes to reactive intermediates [70]. However, since activation of leukocytes is a necessary condition for the development of a leukocyte-associated idiosyncratic reaction, this implies that concurrent conditions such as infection or unspecified inflammation may be contributing risk factors [70]. Consistent with this hypothesis, a higher incidence of idiosyncratic reactions has been observed with some drugs in association with certain viral infections [70].

17.5 ANIMAL MODELS OF IDIOSYNCRATIC DRUG REACTIONS

On the basis of the uncertainty of the various proposed mechanistic hypotheses for IDRs and the potential for overlap of mechanisms involved, identification of a single *in vitro* or *in vivo* model to enable prediction of IDR has been difficult at best. In fact, current hypotheses preclude the possibility of reproducing such reactions *in vitro* [16,27]. Therefore, biomedical research with animal models represents the most probable direction for exploring models of IDRs. Idiosyncratic reactions have been reported in pet animals (dogs and cats) by veterinarians and with low incidence as in humans [77–79]. Unfortunately, IDRs in animals are also idiosyncratic, rarely occurring in preclinical species used in drug development and therefore unpredictable in animals, thus further complicating this approach to understanding the mechanisms involved in IDRs [16,80]. Furthermore, for an animal model to truly represent the human condition in IDR, it must possess a similar mechanism as that in humans. Although all of these factors need to be taken into account, animal models still represent the best approach to studying IDRs and may provide some mechanistic clues for understanding the nature of IDRs in humans. Not knowing the precise mechanism for human IDRs poses a quandary to establishing a relative animal model. Even in cases where the pathophysiology of a disease is well characterized, the toxic response to a drug may be significantly altered under these circumstances, a situation that is not often investigated in preclinical toxicity testing in drug development [62]. Nevertheless, to date, several animal models for IDRs have been investigated, which hold some promise and can be categorized according to putative mechanism (Table 17.1).

∞ **TABLE 17.1 Animal Models of Idiosyncratic Drug Reactions**

	Toxic Reactive Metabolite	Mechanism of IDR	Animal Model
<i>Animal Models of Drug-Induced Hypersensitivity</i>			
Sulfonamide (antibacterial)	Hydroxylamine and nitroso derivatives	Immune mediated (genetic predisposition? immune status? and antidrug antibodies)	Dog
<i>Animal Models of Idiosyncratic Liver Toxicity</i>			
Halothane (anesthetic)	Trifluoroacetyl chloride	Immune mediated (TFA-protein-adduct antibodies)	Rat, guinea pig, mouse
Isoniazid (antitubercular drug)	Acyl carbocation and free radical	Immune mediated? (protein acylation)	Rat, rabbit
Ticrynafen (tienilic acid) (antihypertensive)	Thiophene epoxide	Immune mediated (Cyp protein adduct and anti-CYP450 antibodies)	Rat
Acetaminophen	<i>N</i> -acetyl- <i>p</i> -benzoquinone imine	Direct cytotoxicity with involvement of innate immune system	Rat, mouse
Mitochondrial superoxide heterozygote mouse model	Troglitazone: quinone; flutamide: quinone imine	Mitochondrial dysfunction with oxidant stress	Sod2 ^{+/-} knockout mouse
<i>Animal Models of Drug-Induced Autoimmunity</i>			
D-Penicillamine (antirheumatic)	(IDR attributed to chemically reactive parent drug)	Immune mediated (genetic predisposition? and antinuclear antibodies)	Rat, guinea pig
Propylthiouracil (antithyroid drug)	Sulfonic acid	Immune mediated (antinuclear and antimyeloperoxidase antibodies)	Cat
Procainamide (cardiovascular agent)	Hydroxylamine and nitroso metabolites	Immune mediated? (antinuclear antibodies to histone)	Rat, mouse
<i>Animal Models of Drug-Induced Skin Rash</i>			
Nevirapine (antiretroviral agent)	Benzyl sulfate	Immune mediated (genetic predisposition? T-cell involvement?)	Rat
<i>Animal Models of Hematotoxicity, Bone Marrow and/or Liver Toxicity</i>			
Amodiaquine (antimalarial)	Quinone imine	Immune mediated (protein adduct and antidrug antibodies)	Rat
Felbamate (anticonvulsant)	Atropaldehyde	Immune mediated? (protein adduct)	Rat

? indicate mechanisms of IDR that are postulated but unproven.

17.5.1 Sulfonamide-Induced Hypersensitivity

Sulfonamide hypersensitivity is well recognized in humans and is manifested as fever, dermatopathy (i.e., rash), lymphadenopathy, and, occasionally, liver, kidney, or bone marrow dysfunction (e.g., agranulocytosis, eosinophilia, thrombocytopenia, or aplastic anemia) [81]. Clinically, the incidence of sulfonamide hypersensitivity may vary from <3% to 65% depending on an individual's immune status, occurring with higher incidence in immunosuppressed patients (e.g., HIV-positive individuals) [82,83]. Sulfonamide hypersensitivity has been commonly described in various dog breeds and has been categorized as polyarthritis/fever, cutaneous drug eruptions, hepatotoxicity, and blood dyscrasias [84,85]. The onset of clinical signs in dogs following initiation of treatment may range from 5 to 36 days [85]. Giger *et al.* [86] were able to demonstrate that the polyarthritis, glomerulonephropathy, and focal retinitis observed in Doberman pinschers following administration of trimethoprim sulfadiazine was due to the sulfadiazine since the recurrence of the polyarthritis occurred with rechallenge of sulfadiazine alone but not with trimethoprim alone. The more rapid onset with rechallenge with sulfadiazine was consistent with an immune-mediated reaction. Additionally, hypersensitivity in dogs has been shown to be associated with antidrug, antimyeloperoxidase, and anti-cathepsin G antibodies [87,88]. It is thought that the immune reaction to sulfonamide is triggered by a reactive metabolite since sulfonamides are metabolized to hydroxylamines that may be further oxidized to nitroso derivatives, which in turn may form protein adducts, culminating in antibody- and/or cell-mediated immune responses [88]. A dog may be a very good mechanistic model for sulfonamide hypersensitivity in humans since this syndrome in dogs shares many similarities with that in humans. The metabolism of sulfonamides is similar in dogs and humans, and antidrug antibodies and drug-serum adducts have been identified in dogs and humans with sulfonamide hypersensitivity [87]. However, the low incidence of sulfonamide hypersensitivity in dogs and the ethical and practical difficulties of working with dogs preclude the utility of this animal model [27]. Unfortunately, a rodent model is not available for sulfonamide hypersensitivity.

17.5.2 Halothane-Induced Liver Toxicity

Halothane may cause severe idiosyncratic liver toxicity in humans, albeit at low incidence [89]. Halothane-induced hepatotoxicity in guinea pigs is attributed to oxidative biotransformation [90]. In rats, halothane was demonstrated to cause liver toxicity but only if the rats were first pretreated with phenobarbital and then exposed to halothane under hypoxic conditions [91]. Halothane-induced hepatotoxicity in rats was attributed to hypoxia in combination with enzyme induction, leading to free radical formation by a reductive or non-oxygen-dependent metabolic pathway [92]. Halothane-induced hepatotoxicity in humans has been linked to the formation of antibodies to a reactive metabolite, trifluoroacetyl (TFA) chloride, covalently bound to a hepatic protein [93,94]. Multiple exposures to halothane is recognized as a major risk factor and halothane-induced hepatotoxicity is often associated with autoantibodies (e.g., autoantibodies to CYP2E1) and sometimes eosinophilia [95,96]. Taken together, these observations are consistent with an immune-mediated mechanism in humans [27]. Drug-specific T cells are also believed to play a role in the mechanism of halothane-induced liver injury ("immunoallergic hepatitis"), and it is considered that the balance

between different subsets of T cells (i.e., T-helper (Th) cells and T suppressor cells) influences individual susceptibility and the extent of hepatic injury [97]. Thus, it is likely that the mechanism involved in halothane-induced hepatotoxicity in humans differs from that in rats because different metabolites are involved and the immune-mediated mechanism of the human idiosyncratic reaction is not apparent in the rat model [27].

In guinea pigs, halothane-induced hepatotoxicity is observed when coadministered with buthionine sulfoxide (to deplete GSH), resulting in death within 48–72 h after a single exposure to halothane, which is more consistent with a direct toxicity rather than an immune-mediated mechanism [98]. However, antibodies to TFA-albumin have been observed after a single exposure to halothane in guinea pigs, and subsequent exposure to halothane also results in anti-TFA antibodies, although hepatotoxicity was not significantly altered and anti-TFA antibody titers were similar to those of the initial response [99,100]. These findings may be indicative of immune tolerance, similar to that observed in patients [27]. A cellular immune response has also been demonstrated in guinea pigs following halothane exposure, although the relationship to liver toxicity is not certain [101–103]. Thus, the guinea pig model may be useful in providing insight into the humoral and cellular immune responses involved in the human halothane-induced idiosyncratic reaction [98].

Halothane also causes acute hepatotoxicity in mice. It apparently involves the innate immune system because it is associated with an increase in TNF- α , interleukin (IL)-1 β , IL-6, and IL-8 levels, as well as recruitment of neutrophils [104]. Furthermore, neutrophil depletion was protective. It was also found that IL-17 level was elevated in this model and depletion of IL-17 was protective [105]. Although IL-17 is the cytokine after which Th-17 cells were named, it is also produced by other cells and can even be elevated in acetaminophen-induced hepatotoxicity, which is unlikely to be mediated by Th-17 cells. The presence of CYP2E1 IgG₄ autoantibodies in patients with anesthetic drug-induced liver injury (DILI) suggests that IL-4 may be involved in immune-mediated halothane hepatotoxicity [106]. The involvement of IL-4 in many autoimmune disease models (e.g., autoimmune diabetes, experimental autoimmune encephalitis, collagen-induced arthritis) is well known, but its immunomodulatory role is complex and multifaceted and may follow pro- or anti-inflammatory pathways [107]. In comparison to IL-4^{-/-} knockout (KO) mice, wild-type (WT) Balb/c developed more hepatitis, TFA and S100 antibodies, and T-cell proliferation following TFA-S100 immunization (oxidative metabolism of halothane by CYP2E1 produces TFA hapten, which is covalently bound to S100 hepatic protein) [106]. In this mouse model of anesthetic DILI, suppression of regulatory responses (IL-10, IFN- γ , and possibly IL-6) to CYP2E1 autoantigen and promotion of proinflammatory responses (TNF- α , IL-1 β , or IL-6) to TFA hapten suggest that IL-4 may play a dual role in immune-mediated halothane hepatotoxicity [106,107]. The mouse model appears to be significantly different than classic halothane-induced hepatotoxicity in humans; however, it is likely that the mechanism of IDRs can be different in different individuals.

17.5.3 Isoniazid-Induced Liver Toxicity

Isoniazid is an antitubercular drug that has been associated with liver injury following prolonged usage [108]. It is proposed that the hepatotoxicity is caused by acetylation

of isoniazid followed by the hydrolysis of *N*-acetylisoniazid [3,109]. Acetylhydrazine has been identified as a metabolite of isoniazid in humans, and *in vitro* studies have shown that acetylhydrazine may be further oxidatively metabolized by microsomal enzymes to a reactive acylating species that covalently binds to tissue macromolecules [108]. Further support for the involvement of a metabolite of acetylhydrazine in hepatotoxicity is provided, in that only a small protective effect is observed with the rapid acetylator phenotype contrary to what would be expected if hepatotoxicity only involved direct oxidation of isoniazid to a reactive metabolite [70]. Studies in rats have demonstrated that a similar pathway is followed for isoniazid-induced hepatotoxicity; however, the toxicity in the animal model occurs acutely, whereas hepatotoxicity in humans is usually delayed for a month or more [70]. This suggests that this IDR may be immune-mediated, which is further supported by the effect being largely dose independent. In humans, isoniazid hepatotoxicity may be due to an immune response to a metabolite bound to a protein that is then recognized as foreign [70]. Antibodies to isoniazid have been demonstrated in humans exposed directly or indirectly to isoniazid [110–112]. Rabbits may provide an alternate model for isoniazid-induced hepatotoxicity, exhibiting many features similar to isoniazid toxicity in humans, including the need for repeated dosing, a slight delay in the onset of release of hepatic enzymes, variability in the severity of toxicity, and histopathologic findings of liver necrosis and steatosis [113]. Additionally, the acetylation rate in rabbits is a genetically determined polymorphic trait, and so, similar to humans, rabbits may be classified as “fast” or “slow” acetylators [113].

17.5.4 Ticrynafen-Induced Liver Toxicity

Ticrynafen (tienilic acid) is a diuretic with uricosuric activity and is indicated for the treatment of hypertension. Shortly after it was introduced to the market in the United States in 1979, it was quickly withdrawn after it was determined to be associated with severe hepatotoxicity [114]. Similar hepatotoxicity was not observed in nonclinical studies in animals [70]. The delay in onset of hepatotoxicity following initiation of treatment was suggestive of an immune-mediated idiosyncratic reaction [70]. Antibodies to a hepatic cytochrome P450 isoenzyme were detected in patients with ticrynafen-associated hepatotoxicity [115]. The CYP450 involved (CYP2C9 in humans, CYP2C11 in rats) appears to be responsible for oxidation of the thiophene ring of ticrynafen to a reactive metabolite (arene oxide), which is highly electrophilic and immediately reacts with the CYP450 molecule that formed it [70,116,117]. A reaction occurs leading to alkylation of a thiol group in the enzyme's active site. The reactive intermediate that inactivates the CYP2C9 is the thiophene epoxide as determined by HPLC/electrospray ionization mass spectrometric analysis [118]. The reactive metabolite of ticrynafen covalently binds to the enzyme, thus inhibiting the enzyme irreversibly. It is postulated that this altered protein is immunogenic, leading to antibody formation against this cytochrome [70]. Since the CYP450 that ticrynafen reacts with is one of the common genetically variable CYP450s, individuals who lack this isozymes are likely to be protected from ticrynafen toxicity. Much of our understanding of the metabolic pathways of ticrynafen is based on *in vitro* investigations. Until recently, the putative metabolites of ticrynafen involved in idiosyncratic hepatotoxicity had not been detected *in vivo* [114]. However, a GSH–tienilic acid adduct has been identified in the bile of rats administered ticrynafen.

Coadministration of tienilic acid and the GSH biosynthesis inhibitor L-buthionine-(*S*, *R*)-sulfoximine (BSO) resulted in increased serum alanine transaminase (ALT) and hepatic necrosis which could be prevented by the CYP-inhibitor, 1-aminobenzotriazole. Thus, electrophilic metabolites of ticrynafen from CYP-mediated bioactivation may contribute to ticrynafen-induced hepatotoxicity, particularly where GSH biosynthesis may be impaired by a mechanism that causes electrophilic or oxidative stresses on hepatocytes [114]. However, the association of anti-CYP2C9 antibodies with clinical toxicity suggests that the hepatotoxicity in humans is mediated by the adaptive immune system, although the toxicity may be cell mediated rather than being antibody mediated.

17.5.5 Acetaminophen-Induced Liver Toxicity

Hepatotoxicity attributed to acetaminophen is well recognized in man and animals [97,119,120]. Acetaminophen-induced hepatotoxicity is attributed to an electrophilic CYP450 reactive metabolite, *N*-acetyl-*p*-benzoquinone imine, which covalently binds to cellular macromolecules and depletes GSH, leading to hepatocellular damage [3,119,121]. Although the mechanism of acetaminophen-induced hepatotoxicity has been attributed to direct liver cytotoxicity, more recent investigations have shown that the immune system may play a role [121,122]. In rats, pretreatment with inhibitors of macrophage function, such as dextran sulfate or gadolinium chloride, reduced serum transaminase levels and prevented centrilobular liver necrosis compared to rats administered only acetaminophen [27,122]. This suggests that activated macrophages play a role in acetaminophen-induced hepatotoxicity through the release of proinflammatory mediators (e.g., nitric oxide, IL-1 β , and TNF- α) [122]. Similar results have been observed in mice pretreated with gadolinium chloride [123].

Treatment of mice with liposome-entrapped clodronate to deplete KCs (the major resident macrophage in the liver) before administration of acetaminophen led to more severe hepatic necrosis and significantly elevated transaminase levels compared to mice treated with acetaminophen alone, in contrast to the effects observed with gadolinium chloride pretreatment [123]. The basis for the different effects of gadolinium chloride and liposome-entrapped clodronate is unclear. However, this suggests that KCs have a protective function in acetaminophen-induced hepatotoxicity possibly through the elaboration of cytokines and other mediators that are hepatoprotective [27,123]. IL-10 expression was demonstrated to be decreased in KC-depleted mice, suggesting that KCs are an important source of IL-10 and that this cytokine may be involved in cytoprotection [123]. In addition, IL-10 KO mice (IL-10^{-/-}) depleted of KCs have been shown to be more susceptible to acetaminophen-induced hepatotoxicity than intact mice, suggesting that other factors may be involved [27,123]. Other types of KO mice, including IL-6^{-/-}, tumor necrosis factor receptor 1 (TNFR1^{-/-}), cyclooxygenase-2 (COX-2^{-/-}), are also being used to investigate other cytokines or mediators that may be involved in acetaminophen-induced liver toxicity [27,124,125]. Thus, the occurrence and severity of acetaminophen-induced liver injury are influenced by factors that affect upstream events (i.e., drug metabolism) and downstream events (i.e., immune response) [120]. Although acetaminophen-induced liver injury is not considered an “idiosyncratic drug reaction” per se, the study of KO mice may provide insights into immune-mediated mechanisms of IDRs involving other drugs [27].

17.5.6 Mitochondrial Superoxide Heterozygote Mouse Model

One hypothesis for the mechanism of hepatic IDRs is that they involve damage to mitochondria [126]. Boelsterli developed an animal model in which Sod2^{+/-} mice heterozygous deficient in mitochondrial superoxide dismutase (a clinically silent genetic deficiency in mitochondrial function; complete deficiency is lethal) were treated with troglitazone (a thiazolidinedione antidiabetic drug) or flutamide (a nonsteroidal antiandrogen drug) and developed a delayed-onset hepatotoxicity [42,43, 127–129]. This is a very attractive model because, unlike many models, the toxicity is delayed in onset, thereby mimicking clinical idiosyncratic hepatotoxicity [33]. However, in the case of the troglitazone experiments, the toxicity was relatively mild (a twofold increase in ALT levels) and other groups have been unable to reproduce the results [130]. One possible source of the difference in results is that in the Boelsterli study, troglitazone was dissolved in Solutol HS 15/water solution and administered by intraperitoneal injection, while in the Fujimoto study, it was administered by oral gavage. Solutol HS 15 has the potential to cause mitochondrial damage because it contains polyglycol esters of 12-hydroxystearic acid.

It is postulated that the underlying impairment of mitochondrial function in this model sensitizes hepatic mitochondria to an additional oxidant stress by drugs that target mitochondria, such as troglitazone and flutamide [42,43]. The mechanisms by which these drugs potentiate oxidant stress are as yet poorly understood. One possibility is that ROS are generated metabolically and the increased superoxide levels overwhelm the antioxidant defenses over time [127]. Metabolism of both troglitazone and flutamide produces reactive intermediates. The major CYP3A4-dependent metabolite of troglitazone in mice, rats, and humans is a quinone, and quinones may undergo redox cycling thereby causing oxidative stress [131–133]. Troglitazone is also an inducer of CYP3A4, which may further contribute to its toxicity by autoinduction and generation of reactive intermediates [132,133]. Metabolism of troglitazone at the thiazolidinedione ring (mediated largely by CYP3A) may result in the formation of thiol-reactive reactive intermediates and, subsequently, the formation of GSH conjugates, which may also add to oxidant stress [132,134,135]. It is also possible that formation of troglitazone phenoxy radicals by peroxidase/H₂O₂ demonstrated *in vitro* may deplete GSH and contribute to its prooxidant activity and hepatotoxicity [136]. Flutamide is also known to produce reactive metabolites from metabolism by CYP1A2, CYP3A4, and CYP2C19 and the carboxylesterase pathway [137]. Hepatic protein adducts (e.g., GSH conjugates, mercapturic acid conjugates) are formed from N-hydroxylation of flutamide metabolites, which is a proposed mechanism of flutamide-induced liver injury [137–143]. Aromatic hydroxylation of flutamide metabolites followed by oxidation results in the formation of a reactive quinone imine [140]. Additionally, flutamide contains a nitroaromatic group that can undergo redox cycling of the intermediates, which may enhance hepatocellular cytotoxicity by its detrimental effect on mitochondria [140,144,145]. Reduction of the nitro group to an amine is followed by oxidation to the diimine intermediate, which would be subject to subsequent nucleophilic attack by GSH at three unsubstituted positions of the aromatic ring to form GSH adducts [140,145]. Flutamide has also been shown to be an inducer of CYP1A2, which could also contribute to hepatotoxicity by further increasing oxidant stress [146]. More studies will be required to determine if this model represents the mechanism of idiosyncratic liver toxicity that occurs in humans.

17.5.7 D-Penicillamine-Induced Autoimmunity

D-Penicillamine (dimethyl cysteine) as a chelating agent has been shown to be efficacious in the treatment of Wilson's disease and cystinuria and also in the treatment of rheumatoid arthritis because of its immunologic properties [147]. However, its clinical use has been somewhat limited because of the occurrence of ADRs manifested by a broad range of autoimmune syndromes including lupuslike syndrome, pemphigus, myasthenia gravis, agranulocytosis, and rash [148–150]. In guinea pigs, chronic administration of D-penicillamine elicited electrophysiologic, serological, and histopathologic changes resembling experimental allergic myasthenia gravis and allergic myositis [147]. D-penicillamine causes similar autoimmune reactions in BN rats but not Lewis or Sprague-Dawley (SD) rats [151,152]. This suggests that genetic factors are important in D-penicillamine-induced ADRs in rats and that it is idiosyncratic in that it is strain specific [28]. The autoimmune syndrome in BN rats shares many features with that in humans, including antinuclear antibodies (ANAs) and immune complex deposition in the kidneys. Other manifestations include rash, weight loss, and, in cases of prolonged treatment, death [28,151]. Similar to humans, the onset of these effects in BN rats occurs after several weeks of treatment with D-penicillamine [16]. The mechanism of this idiosyncratic reaction in BN rats is clearly immune-mediated [153]. Although the incidence of immune disease does not increase at doses above 20 mg/day, tolerance is induced at lower doses. This tolerance can be transferred to naive rats by spleen cells or T cells from tolerized rats and is mediated by CD4⁺ regulatory T cells [154–156]. If animals are rechallenged with D-penicillamine after recovery, the onset of autoimmunity is similar to that observed following initial exposure [16]. Additionally, the onset, incidence, and severity of D-penicillamine-induced autoimmunity can be increased by administration of the Th1 cytokine inducer, poly I:C (a synthetic polymer of cytosine and inosine), which further supports an immune-mediated mechanism [157]. High throughput screening of hepatic mRNA using reverse transcriptase polymerase chain reaction (RT-PCR) has revealed that *Kid-1*, *sgk*, and *cinc* genes may be involved in determining sensitive and resistant animals [158]. Although reactive metabolites are postulated to play a role in IDRs, the mechanism of D-penicillamine-induced IDRs involves a chemically reactive drug rather than a reactive metabolite. It is still uncertain how D-penicillamine triggers the immune reaction, but it is postulated that D-penicillamine reacts with aldehydes to form an irreversible thiazolidine ring on APCs (i.e., macrophages), which become activated and may interact with T cells through formation of a reversible imine bond [159–161]. Transcriptome profiling of splenic macrophages obtained from BN rats following D-penicillamine treatment revealed upregulation of macrophage-activating cytokines (i.e., IFN- γ and GM-CSF [granulocyte-macrophage colony-stimulating factor]) and downregulation of IL-13 indicative of NK (natural killer) cell activation, as well as increased levels of TNF- α , IL-6, and IL-23 in a D-penicillamine-treated murine macrophage cell line (RAW264.7), further supporting the role of macrophages in the initiation of immune responses that, in some cases, may lead to generalized autoimmunity [162].

17.5.8 Propylthiouracil-Induced Autoimmunity

PTU therapy in humans for treatment of goiter has been associated with immune-mediated disease similar to lupus characterized by agranulocytosis, thrombocytopenia,

polyarthritis, vasculitis, hepatotoxicity, skin rashes, fever, and the development of ANAs but not hemolytic anemia [163–165]. Although efforts to induce immune-mediated diseases in animals have been largely unsuccessful, an animal model with clinical and serological characteristics of PTU-induced disease has been developed in cats [164]. Normal healthy cats administered 150 mg of PTU orally developed a disease syndrome characterized by lethargy, fever, weight loss, lymphadenopathy, Coombs' positive hemolytic anemia, a positive direct antiglobulin test (DAT), antimyeloperoxidase antibodies, and ANAs [28,164]. Onset of clinical signs varied from four to eight weeks of PTU administration, whereas the development of a positive DAT and/or ANAs occurred after three to eight weeks of treatment [164]. Clinical signs and serological changes resolved within two to four weeks of cessation of PTU administration [164]. On rechallenge, time to onset of PTU-induced autoimmunity was similar to the initial PTU-treatment period [27]. In humans and animals, drugs containing a sulfhydryl group have been associated with various immune-mediated diseases [151,164]. This hypothesis is supported by findings in the cat model, where resolution of both clinical and serological signs of disease occurred following the substitution of PTU administration with 150 mg of 6-propyluracil (PU), an analog of PTU that does not contain a sulfur moiety but has similar bioavailability [164]. Although antithyroid drugs do not bind to lymphocytes, the interaction of mononuclear phagocytes (i.e., macrophages) with lymphocytes is postulated to play a role in the development of PTU-induced immune-mediated disease [164]. Macrophages contain free sulfhydryl residues on their cell membranes that are essential to their function and the elaboration of various mediators [164]. PTU is metabolized by the myeloperoxidase (MPO)/H₂O₂/Cl⁻-system to reactive sulfonic acid metabolites [70]. Interaction of the reactive intermediates of sulfur-containing drugs, such as PTU, with sulfhydryl residues on macrophages may modulate the immune response through stimulation or suppression of lymphocyte proliferation, as well as promotion or suppression of immunoglobulin production, such as autoantibodies [164]. Consistent with this hypothesis, PTU is oxidized to reactive metabolites by the MPO system of macrophages, and PTU-induced autoimmunity in cats is associated with antimyeloperoxidase antibodies [166,167].

17.5.9 Procainamide-Induced Lupus

Procainamide is widely used as an antiarrhythmic drug. However, its chronic use has been associated with the development of an autoimmune disorder similar to lupus, agranulocytosis, and hepatotoxicity [32,70,168]. Lupus is characterized by the formation of antigen–antibody complexes and complement activation [70]. DNA is the principal antigen in antigen–antibody complexes in idiopathic lupus, whereas in procainamide-induced lupus, ANAs bind primarily to histone proteins rather than the DNA itself [70]. It is uncertain how procainamide antibodies form and bind to histone. Procainamide undergoes metabolic oxidation by liver microsomes to form reactive hydroxylamine, and nonenzymatically, it forms nitroso metabolites [169,170]. Nitroso-procainamide covalently binds histone and other proteins, whereas *N*-acetylprocainamide is not metabolized to a reactive metabolite and does not induce lupus [170,171]. The procainamide–histone protein if recognized as foreign could induce an immune reaction resulting in the production of ANAs [170]. Since procainamide metabolites are further metabolized in the liver, it is difficult to understand how systemic manifestations of procainamide-induced lupus occur. It has

been shown that activated mononuclear leukocytes and neutrophils generate H_2O_2 and MPO, which can metabolize procainamide to hydroxylamine [171,172]. Investigations of administration of procainamide to human neutrophils *in vitro* and rat tissues *in vitro* and *in vivo* confirmed the bioactivation of procainamide to reactive metabolites by activated neutrophils and subsequent covalent binding of reactive metabolites to the cell membrane of neutrophils where oxidation via the MPO system occurs [173]. Similarly, procainamide reactive metabolites may be formed and may bind to the cell surface of activated monocytes, which may influence antigen processing, a key function of monocytes [172]. Thus, the binding of reactive metabolites of procainamide to neutrophils and monocytes may be an initial step in triggering an immune response [171]. Antimyeloid cell antibodies have been identified in cases of procainamide-induced agranulocytosis [174]. Since most reactive metabolites have a short half-life, the formation of the reactive metabolites of procainamide in proximity to neutrophils in bone marrow and circulating monocytes provides a plausible mechanism for an idiosyncratic adverse reaction resulting in agranulocytosis and lupus through an immunologic mechanism [175].

Although an immune response manifested as a DHT reaction and a low titer of antibody to the drug was demonstrated in guinea pigs administered with procainamide in combination with complete Freund's adjuvant (CFA), attempts to develop an animal model of procainamide-induced lupus have generally been unsuccessful [176]. Administration of procainamide hydroxylamine intrathymically to C57BL/6 $\times$ DBA/2 F1 mice resulted in an initial and transient production of IgM antidenatured DNA antibodies, which was followed by a delayed and protracted IgG antichromatin antibody response [177]. Although the loss of IgG tolerance to chromatin in mice is consistent with findings in procainamide treatment in humans, the animals do not develop any clinical signs of autoimmunity, indicating a limited utility for this animal model [27,177]. Procainamide has also been shown to inhibit DNA methylation and induce reactivity to $CD4^+$ T cells, and therefore, this epigenetic effect appears to play an important role in the induction of lupus [178,179].

17.5.10 Nevirapine-Induced Skin Rash

Nevirapine is a nonnucleoside reverse transcriptase inhibitor (NNRTI) intended for the treatment of HIV-1 infections [180]. However, its use has been associated with a high incidence of idiosyncratic skin rash and liver toxicity, which may be life threatening [27,181]. At 400 mg/day, the incidence of skin rash can range from 17% to 48% depending on dosing regimen, with 0.3% of rashes considered severe, that is, SJS, SJS/TEN transition syndrome, or a syndrome of drug reaction with eosinophilia and systemic symptoms (DRESS) [180,182–185]. Sixteen percent of patients treated with nevirapine developed a skin rash, and of them, 65% developed the rash in less than six weeks following nevirapine treatment [182]. In clinical trials, 1.0% of nevirapine-treated patients developed clinical hepatitis, usually occurring within the first five weeks of treatment but delayed as late as 18 weeks in some cases [182,183].

Administration of nevirapine to BN rats and SD rats is also associated with skin rash but not liver toxicity [16,28]. However, Lewis rats are resistant to nevirapine-induced rash, which is consistent with the idiosyncrasy of the skin reactions and suggests that genetic factors may play a role in the development of skin lesions [27,28]. The similarity of the characteristics of nevirapine-induced skin rashes in rats and humans suggests

a common mechanistic pathway [27,180]. In rats, skin lesions occur two to three weeks after nevirapine administration; however, if administration is stopped followed by rechallenge after the rashes have resolved, then the reappearance of red ears occurs in <24 h and the rash is associated with more severe systemic manifestations than the original rash [186]. Histopathologically, the skin lesions are characterized by an inflammatory infiltrate of predominantly lymphocytic cells consisting largely of CD4⁺ and CD8⁺ T cells, as well as macrophages [187]. These findings provide strong evidence for an immune-mediated reaction. Depletion of CD4⁺ T cells, but not CD8⁺ T cells, was partially protective, and sensitivity can be transferred by isolated CD4⁺ T cells, but not CD8⁺ T cells, from sensitized to naive rats [180,188]. Furthermore, tolerance can be induced in BN rats by administration of low doses of nevirapine (50–75 mg/kg/day) followed by a higher dose (150 mg/kg/day), a dose typically associated with 100% incidence of rash [28,180]. The incidence and severity of nevirapine-induced skin rash appears to be related to the rat strain and gender [16,187]. The incidence of rash is higher in female BN rats than in male BN rats given the same dose, which appears to be related to kinetic factors; male BN rats metabolize nevirapine much more rapidly than female BN rats [28,187]. However, male BN rats develop skin rashes following administration of a cytochrome P450 inhibitor, aminobenzotriazole, which inhibits 2- and 3-hydroxylation but not 12-hydroxylation of nevirapine [28,189]. Mechanistically, the skin rash is related to 12-hydroxylation of nevirapine, rather than the parent drug, and it is postulated that the 12-hydroxy metabolite is converted by sulfation to a reactive quinone methide metabolite in the skin [16,180,189]. Since sulfotransferases are known to occur in skin, this provides a plausible explanation for the development of nevirapine-induced skin rashes [16,180]. Studies on the specificity of lymphocytes in nevirapine-treated BN rats have demonstrated that reactive metabolites may be responsible for initiation of an immune response but in addition, the immune response may be extended in that lymphocytes may also become reactive to the parent drug [190]. The BN rat appears to be a good model for the relatively mild maculopapular rashes that occur in some patients, which are likely mediated by CD4⁺ T cells, but the more severe rashes such as TEN are more likely mediated by CD8⁺ T cells [191].

17.5.11 Amodiaquine-Induced Liver Toxicity and Agranulocytosis

Amodiaquine, a 4-aminoquinoline, is an antimalarial drug [27,192]. The use of this drug has been restricted because of its association with hepatotoxicity and agranulocytosis [192]. Anti-amodiaquine IgG antibodies have been found in patients exhibiting adverse reactions to amodiaquine [193]. Amodiaquine is oxidized by liver microsomes and peroxidases to a reactive quinone imine, which can covalently bind to proteins [193,194]. In Wistar rats, administration of amodiaquine induces the production of specific IgG anti-amodiaquine antibodies after four days of treatment [195]. Antibody titers in rats were dose dependent and correlated with immunogenicity [195]. Activated polymorphonuclear cells have been shown to convert amodiaquine to reactive intermediates *in vitro* that can react with proteins, and therefore, it is plausible that a similar mechanism may occur *in vivo*, leading to an immunogenic response [195,196]. The specificity of the antibody to conjugates of amodiaquine quinone imine (via *p*-hydroxyanilino group) and cysteine residues in cellular proteins was determined by hapten inhibition [195]. At high doses, a significant decrease in peripheral white blood cell counts was observed in rats, which is suggestive of agranulocytosis, but it was not

reported if neutrophil counts were decreased, as would be expected in agranulocytosis [195]. Although ALT levels were increased in rats at high doses of amodiaquine, there was no evidence of hepatotoxicity histopathologically [195]. Peripheral white cell counts and ALT levels returned to normal within two weeks following discontinuation of treatment [195]. At lower doses of amodiaquine, there was an antibody response, but no alterations in peripheral white blood cell counts or ALT levels were observed [195]. Thus, amodiaquine is immunogenic at doses below which leukopenia or hepatotoxicity occurs [195]. Resolution of the adverse effects in rats may be indicative of immune tolerance [27]. The findings in amodiaquine-treated rats suggest that this may represent a suitable animal model of an immune-mediated idiosyncratic reaction in humans, involving a specific antibody response [28,195].

17.5.12 Felbamate-Induced Liver Toxicity and Aplastic Anemia

Felbamate (2-phenyl-1,3-propanediol dicarbamate) is an antiepileptic drug (AED) whose use has been restricted because of hepatotoxicity and aplastic anemia [11,197]. In contrast to humans, felbamate is essentially nontoxic in animals (i.e., rat, dog, and monkey) even at high doses [80]. Felbamate is metabolized by esterases to a monocarbamate, which is then oxidized by alcohol dehydrogenase to an aldehyde carbamate [11]. The aldehyde carbamate spontaneously gets converted to 2-phenylpropenal, an α,β -unsaturated aldehyde, also known as *atropaldehyde*, which is considered to be the reactive metabolite that may covalently bind to proteins and is responsible for the idiosyncratic toxicity of felbamate [11,198]. Although atropaldehyde, is found in rats, it has not been possible to develop an animal model for felbamate-related idiosyncratic toxicity [27]. The reason for the difference in toxicity between rats and humans may in part be related to the levels of atropaldehyde generated, which are lower in rats (~1%) than in humans (~6%) [199]. This may be attributed to the lower esterase activity in rats, which could reduce the amount of monocarbamate formed, in combination with enhanced CYP450 metabolism, increased detoxification by aldehyde dehydrogenase (which is present at higher levels in rats than in humans), and/or GSH conjugation of atropaldehyde [199]. Attempts to produce felbamate toxicity in rats through inhibition of protective metabolic pathways (i.e., aldehyde dehydrogenase, CYP450, GSH *S*-transferase, and glucuronosyltransferase) have failed possibly because of the inherent toxicity of the inhibitors [27]. In BN rats, coadministration of felbamate with poly(I:C) and ketoprofen, compounds known to increase the incidence, onset, and/or severity of the immune response in the penicillamine-treated rat model, also failed to produce liver or bone marrow toxicity, which is possibly related to immune tolerance [27,153,157]. Although felbamate has minimal effects on covalent-binding parameters, its effect on oxidative stress/reactive metabolite (OS/RM) gene expression in rat liver is quite pronounced, and this combination of assays may provide a useful alternative approach to screening compounds for the potential to form reactive metabolites that may lead to idiosyncratic toxicity [80].

17.6 PREDICTING IDIOSYNCRATIC DRUG REACTIONS

The unpredictable nature of IDRs makes them virtually impossible to prevent and difficult to study [16,70]. Identifying which patients may develop an idiosyncratic ADR

on the basis of the drug's chemical structure is also currently not possible [200]. Furthermore, despite years of extensive research, it is still impossible to predict the toxicologic potential of compounds that are metabolically activated [51]. Although the exact mechanism of IDRs is not known, reactive metabolites are considered to play a key mechanistic role [70]. Reactive metabolites can covalently bind to cellular proteins or oxidize thiol or amine groups that can result in tissue damage [200]. This can lead to cell death through apoptosis or necrosis, alteration of the immune system, or depletion of defense mechanisms such as GSH [200]. Advances in analytical methodology (e.g., mass spectrometry and NMR spectroscopy) have improved the detection of chemically reactive moieties, termed *toxicophores*, contained within the molecule, thereby assisting in the identification of potential targets of metabolism, which may form reactive metabolites [51,200]. Similarly, small-molecule trapping agents and covalent-binding studies using radiolabeled compounds have facilitated identification of reactive intermediates [51]. Although some animal models have been developed to investigate IDRs, these models suffer the same unpredictability for IDRs, as in humans [28]. Nevertheless, research with animal models has provided both insight into potential mechanisms that may be involved in IDRs and some control over certain variables (e.g., genetic, dietary, stressors) that can occur in human studies [201]. *In vitro* investigations have provided information on specific factors or pathways that may be involved in IDRs but lack the complexity of the numerous interactions and factors that occur *in vivo* [202]. To date, there are no methods sensitive enough to detect weakly sensitizing chemicals or robust enough for the purpose of routine screening [203].

Genetic linkage analyses have shown that multiple genes contribute to hypersensitivity [204]. However, prediction of hypersensitivity reactions is still hampered by both the complexity of the genetic mechanisms involved and our limited understanding of those mechanisms [204]. As cytokines are key regulators of immune responses, transgenic mouse models with overexpression or deletion of cytokine genes hold promise as animal models for investigation of the pathophysiological mechanisms of drug hypersensitivity [204]. Mice overexpressing Th2 cytokines (e.g., IL-4, IL-5, and IL-13) are of particular interest as models of hypersensitivity reactions [204].

17.7 CONCLUSIONS

There is circumstantial evidence that suggests that most idiosyncratic reactions are hypersensitivity reactions initiated by the formation of chemically reactive metabolites that bind to proteins and induce an immune-mediated response [70]. Toxicity may be limited to a target organ where the metabolism occurs and where consequently the largest concentrations of reactive metabolites may be present, such as liver and skin [160]. As a result of their reactivity, the response to reactive metabolites will typically occur in proximity to their formation [70]. For example, halothane is largely metabolized in the liver and halothane toxicity is selective for the liver. The role of activated leukocytes in the generation of reactive metabolites could explain IDRs in tissues other than the liver (e.g., agranulocytosis). Reactive metabolites binding to cellular proteins and thus making them appear "foreign" may also induce an immune reaction to "native" proteins, resulting in a more generalized drug-induced autoimmunity characterized by generation of autoantibodies [160]. Recent investigations continue to support the association of reactive metabolites and immune-mediated responses with IDRs, although

there may be exceptions (e.g., ximelagatran) [34]. Although many hypotheses have been proposed, the mechanisms involved in the occurrence of IDRs are complex and the development of appropriate animal models will be key to a better understanding of the basic underlying mechanisms involved in IDRs.

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18 **Animal Models of Idiosyncratic, Drug-Induced Liver Injury: Emphasis on the Inflammatory Stress Hypothesis**

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18.1	Introduction	1
18.2	Overview of idiosyncratic, drug-induced liver injury (IDILI)	1
18.3	Proposed explanations for IDILI	3
18.4	Overview of the inflammatory response	7
18.5	Models of drug–inflammation interaction	11
18.6	Conclusions	18
	References	18

18.1 INTRODUCTION

In this chapter, several hypotheses that have been proposed to explain the basis of idiosyncratic, drug-induced liver injury (IDILI) reactions are reviewed briefly, with an emphasis on the inflammatory stress hypothesis. A short description of the inflammatory response is presented, and how it relates to recently developed animal models of idiosyncratic hepatotoxicity is described. The latter emphasizes sulindac (SLD), a nonsteroidal anti-inflammatory drug (NSAID) that has caused IDILI in human patients.

18.2 OVERVIEW OF IDIOSYNCRATIC, DRUG-INDUCED LIVER INJURY (IDILI)

Adverse drug reactions (ADRs) remain a major issue in affected patients and a huge challenge to health providers. The safety of a new compound is sometimes not well

understood until it has been on the market as a drug for several years. As a result, serious ADRs often emerge only after approval of a drug by the US Food and Drug Administration (US FDA). More than 10% of newly approved drugs from 1975 to 2000 in the United States either had to be withdrawn from the market or received a warning because of adverse reactions [1]. The liver, which plays an important role in the metabolism of drugs, is a frequent target of ADRs.

Hepatotoxic drug reactions are often categorized as one of two types: dose-related reactions (type A reactions) and idiosyncratic reactions (type B reactions). Type A reactions are dose-dependent and most likely occur in overdosed individuals. A typical example is an adverse reaction due to acetaminophen overdose [2,3]. Idiosyncratic ADRs differ from type A reactions in that they are unpredictable and occur in a minority (often a quite small percentage) of patients at therapeutic doses. The dose dependency in a population is obscure, although it is likely that within an individual the response is dose-related. IDILI typically does not occur by the same mechanisms that underlie the drug's pharmacological actions [4,5]. Many drugs have been associated with IDILI reactions (Table 18.1).

IDILI, which can result in permanent disability or death, has obvious importance to human health. In addition, these reactions are a major issue for the pharmaceutical

TABLE 18.1 Some Drugs Associated with IDILI in Humans

Acitretin	Lumiracoxib
Allopurinol	Minocycline
Amoxicillin/clavulanic acid	Naltrexone
Amiodarone	Nefazodone
Amitriptyline	Nevirapine
Azathioprine	Nimesulide
Benoxaprofen	Nitrofurantoin
Bosentan	Pemoline
Bromfenac	Penicillamine
Carbamazepine	Phenytoin
Chlorpromazine	Procainamide
Cyclosporine	Propylthiouracil
Dacarbazine	Quinidine
Dantrolene	Ranitidine
Diclofenac	Rifampicin
Dihydralazine	Stavudine
Disulfiram	Sulindac
Erythromycin	Tacrine
Estrogen	Tamoxifen
Felbamate	Terbinafine
Flutamide	Tienilic acid
Gemtuzumab	Tolcapone
Halothane	Troglitazone
Iproniazid	Trovaflaxacin
Isoniazid	Valproic acid
Ketoconazole	Ximelagatran
Labetalol	Zafirlukast
Leflunomide	Zileuton

industry because they lead to withdrawals and restrictions in the use of efficacious drugs. An example of a drug that caused idiosyncratic hepatotoxicity is troglitazone (TGZ), which was withdrawn from the market by the US FDA in 2000 [6]. TGZ is a peroxisome proliferator-activated receptor (PPAR)- γ agonist that was used to treat type 2 diabetes. The main reason for its withdrawal was that TGZ was associated with the development of acute liver failure [7], and it accounted for 10% of all idiosyncratic drug reactions between 1998 and 2001 [6]. Thus, although TGZ was beneficial in reducing insulin resistance, among 1.92 million patients who took the drug, 94 cases of liver failure were reported (89 acute, 5 chronic) [8]. A majority of these patients died, whereas others required liver transplants.

The risk for IDILI reactions is difficult to predict, and most idiosyncratic reactions are not discovered until a drug is on the market. That is in part because a mechanistic understanding of IDILI remains absent, and IDILI is generally not reproducible in traditional animal models used in preclinical safety evaluation. For example, oral administration of TGZ at large doses (60- to 120-fold larger than the therapeutic dose) to monkeys for 52 weeks did not increase levels of serum liver enzymes and had little gastrointestinal, hematologic, or hepatic effects [9]. The lack of preclinical animal models causes mechanisms of IDILI to remain poorly understood, so that appropriate action cannot be taken to prevent or treat these reactions.

18.3 PROPOSED EXPLANATIONS FOR IDILI

Although the mechanisms are still not fully understood, extensive studies on IDILI have been performed. Several hypotheses have been put forth to explain the modes of action and mechanisms of these responses. These hypotheses are described briefly, and the inflammatory stress hypothesis is described in more detail below.

18.3.1 Metabolic Polymorphism Hypothesis

The metabolic polymorphism hypothesis suggests that drug metabolites are responsible for IDILI and that susceptible patients metabolize the offending drug differently from the majority of the population. Some drugs are metabolized into electrophiles or free radicals, which can bind covalently to proteins and unsaturated fatty acids and/or can induce lipid peroxidation [10,11]. As a result, cell functions can be impaired and cytotoxicity initiated through cell death signaling pathways. Cytochrome P450s (CYPs) comprise a superfamily of enzymes that can transform drugs into reactive metabolites. Overexpression of CYPs in a fraction of patients can lead to excessive reactive metabolite formation and accumulation in the liver that can result in hepatotoxicity. Accordingly, polymorphisms that result in increased expression or activity of CYPs might be responsible for drug-induced hepatotoxicity in a fraction of patients. Alternatively, polymorphisms that result in diminished CYP activity could lead to hepatic accumulation of a drug that is toxic in its parent form.

TGZ is metabolized into its reactive metabolites by CYP3A4 [12]. CYP3A4-mediated oxidative cleavage of the thiazolidinedione ring generates a highly electrophilic metabolite, TGZ quinone, which covalently binds to cellular macromolecules [13]. This is supported by evidence that administration of TGZ to HepG2 cells transfected with CYP3A4 led to increased cytotoxicity [14]. On the other hand,

it has been suggested that TGZ quinone is less toxic to hepatocytes and HepG2 cells than TGZ itself [15]. Despite these results *in vitro*, the role of CYP3A4 in TGZ-induced toxicity *in vivo* is unclear.

Polymorphisms in enzymes responsible for conjugation reactions or in drug transport proteins also hold the potential to influence susceptibility to drug toxicity. Indeed, a variety of such polymorphisms have been associated with a number of drugs that cause IDILI [16]. For example, polymorphism of the *N*-acetyltransferase 2 gene has been identified as a susceptibility factor for IDILI from treatment with antituberculosis drugs such as isoniazid (slow acetylators are more susceptible) [17]. Polymorphisms of glutathione *S*-transferase M1 and T1 have also been associated with IDILI reactions from these drugs [18]. Variants of the genes for UDP-glucuronosyltransferase and for the multidrug resistance transport protein ABCC2 have been associated with diclofenac-induced IDILI [19]. It should be noted, however, that a causal link has not been established between these genetic polymorphisms and hepatotoxicity. It might be that for some drugs, metabolic polymorphisms play a permissive role but additional factors are required for the development of IDILI.

18.3.2 Hapten Hypothesis

The hapten hypothesis suggests that drugs, or more likely their reactive metabolites, form covalently bound adducts to cellular proteins [5,20]. The drug–protein adducts are recognized as nonself antigens by the immune system and are processed by antigen-presenting cells. The processed adducted peptides are presented to T cells, and this leads to the expansion of effector T and B cells that recognize the adducted peptides. On reexposure or continued exposure to the drug (i.e., drug challenge), an active immune response is elicited, which is associated with the production of specific antibodies to adducts and activation of cytotoxic T cells. This can result in a cascade of events that damage host tissue.

This hypothesis is supported by case reports in some patients with IDILI. Clinical characteristics accompanying some cases, such as skin rash and eosinophilia, are consistent with an adaptive immune response. In addition, antibodies have been detected in plasma after exposure to halothane, diclofenac, or TGZ, all of which cause IDILI in people [21–23]. However, evidence against this hypothesis has also accumulated for some drugs. Autoantibodies were present in only a fraction of the patients treated with diclofenac or halothane who developed liver damage and were detected in some patients, who did not experience hepatotoxicity [24,25]. Moreover, IDILI is induced in some patients after the first exposure to a drug [26,27], whereas a second exposure is needed to precipitate an adaptive immune response.

Several animal models of drug-induced adaptive immune response have been developed, including penicillamine-induced autoimmune syndrome and nevirapine-induced skin rash in rats [28,29]. Although antibodies against penicillin-modified proteins were present in rats treated with penicillin, liver injury was not induced in the animal model [30]. Similarly, liver damage was not observed in the nevirapine-induced skin rash model. Therefore, the results in animal models and human patients suggest that the autoantibodies are not necessarily pathogenic, and additional evidence supporting this hypothesis is needed.

18.3.3 Danger Hypothesis

The immune response induced by a foreign antigen can be weak in the absence of an adjuvant, which is a possible explanation for why an adaptive immune response induced by a drug itself is typically insufficient to cause liver injury [5]. The danger hypothesis was proposed as a modification of the adaptive immune hypothesis [31,32]. It suggests that necrosis or cell stress imposed by reactive drug metabolites provides a “danger signal” that activates macrophages, antigen-presenting cells, or other cells. The danger signals from stressed cells lead to upregulation of costimulatory molecules or production of cytokines that cause an enhanced antibody- or T-cell-mediated specific immune response. It is not clear what these danger signals are as they relate to drug-induced liver injury. Activation of the innate immune system, that is production of inflammatory mediators, was proposed to be a potential danger signal [4].

18.3.4 Mitochondrial Dysfunction Hypothesis

This hypothesis proposes that the mitochondria are targeted by drugs that cause idiosyncratic reactions, and the resulting mitochondrial damage is the underlying cause of some IDILI reactions. Mitochondria are critical players in mediating cell death and also common targets of xenobiotics [33]. Numerous drugs associated with IDILI, such as TGZ, tolcapone, nimesulide, and valproic acid, can cause mitochondrial dysfunction *in vitro* [34–38]. There is also clinical evidence associating IDILI reactions with mitochondrial dysfunction. For example, in one case report, mitochondrial swelling, loss of cristae, and reduced matrix density were observed in hepatocytes of a patient with tolcapone-induced liver injury [39].

An inherited mitochondrial dysfunction or mutation in mitochondrial DNA might make people more susceptible to drug-induced toxicity. In one case of valproate-induced liver injury, an inherited dysfunction of mitochondrial electron transport chain complexes was observed in the patient [40]. For many unrelated drugs, age is one of the risk factors for IDILI. This observation is of interest because mitochondrial DNA mutations increase with age [41].

Superoxide dismutase (SOD) eliminates the reactive oxygen species (ROS), superoxide anion, in mitochondria, and heterozygous SOD2 (SOD2^{+/-}) mice have a decreased ability to manage oxidative stress in the liver. These mice have been used to develop animal models of IDILI to test the mitochondrial dysfunction hypothesis. Nimesulide caused permeability transition of isolated mitochondria [42], and chronic treatment with this drug led to hepatocellular apoptosis in SOD2^{+/-} mice but not in wild-type mice [43]. TGZ, which injures isolated mitochondria and causes hepatocellular death *in vitro* [44], caused mild hepatocellular necrosis in SOD2^{+/-} mice, whereas no injury was observed in wild-type mice [45]. Similar results were obtained after administration of flutamide to these mice [46]. Accordingly, it is likely that mitochondrial toxicity occurs during IDILI, but whether it is a direct effect of drug/metabolite exposure or a distal event in a cascade that leads to cell death *in vivo* remains to be explored.

18.3.5 Failure to Adapt Hypothesis

Recent evidence suggests that it is not uncommon for drug treatment to lead to increases in plasma alanine aminotransferase (ALT) activity, a marker of hepatocellular damage

[47]. Despite continued exposure to the drug, ALT activity returns to normal in most patients [47]. This observation has given rise to the hypothesis that a fraction of patients who fail to adapt to this initial perturbation are those who progress to clinically significant liver injury. Mechanisms for this failure to adapt are unknown, and no animal models specifically designed to address this hypothesis have been established. Nonetheless, it is possible that factors involved in other hypotheses, such as metabolic polymorphisms and inflammatory stress, could underlie an inability to recover from the initial insult.

18.3.6 Multiple Determinant Hypothesis

This hypothesis states that IDILI is a result of the occurrence of multiple, critical, and discrete events or factors and that the probability of the occurrence of IDILI is the product of the probabilities of each event or factor [48]. This product would be small, thereby explaining the rare occurrence of these reactions. The critical events could include chemical properties of the drug, exposure (dosing regimen), environmental factors, and genetic factors. This hypothesis is consistent with other hypotheses described here, inasmuch as genetic polymorphisms, adaptive immune responses, mitochondrial toxicity, danger signals, and inflammation might each represent one event or factor that contributes to the overall probability of an IDILI response.

18.3.7 Inflammatory Stress Hypothesis

Inflammatory episodes are commonplace in humans and can be precipitated by infections, disease, or exposure to microbial products such as bacterial endotoxin. The principal biologically active component of endotoxin is lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. LPS is a potent inflammagen that at great enough doses can cause damage to several organs, including the liver [49]. Various conditions, including alcohol consumption, gastrointestinal distress, changes in diet, antibiotic treatment, and surgery, can increase LPS concentration in plasma of humans [50]. Such exposure may not be sufficient by itself to cause overt illness, but it might potentiate the toxicity of xenobiotics by activating inflammatory cells and cytokines. Previous studies have shown that inflammatory stress enhances the hepatotoxicity of numerous xenobiotic agents, for example, allyl alcohol, monocrotaline, and aflatoxin B1 [51–53].

The characteristics and effects of inflammation described above led to the inflammatory stress hypothesis. This hypothesis proposes that an inflammatory episode can render an individual susceptible to IDILI by decreasing the dose of the drug required for drug toxicity (Fig. 18.1) [54]. As modest inflammation is episodic in nature, IDILI arising from drug–inflammation interaction would be expected to have an inconsistent temporal relationship to drug exposure. Furthermore, because mild inflammatory responses can go unnoticed in humans, IDILI responses involving inflammation might not appear to be dependent on the dose of the drug. Accordingly, this hypothesis can provide an explanation for the characteristics of IDILI reactions.

There are many challenges to demonstrating that inflammatory stress contributes to IDILI. One is that by the time such reactions are suspected, a patient could have signs and symptoms of inflammation that have arisen as a consequence of initial

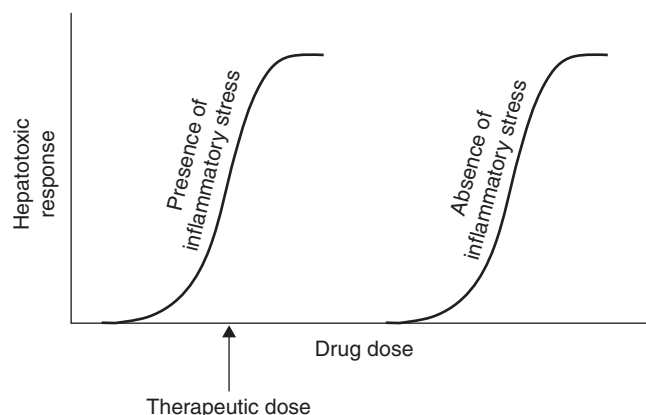


Figure 18.1 Illustration of the inflammatory stress hypothesis of IDILI. The normal, therapeutic dose lies to the left of the dose–response curve for toxicity in the absence of inflammatory stress. Inflammatory stress shifts the dose–response relationship for hepatotoxicity such that it intersects the normal, therapeutic dose, precipitating an idiosyncratic adverse reaction.

damage. Nonetheless, it is interesting that prodromal signs indicative of inflammation are common in people experiencing IDILI from some drugs [55,56]. Furthermore, many NSAIDs, which are often prescribed for inflammatory conditions, cause IDILI in humans.

Several rodent models of IDILI have been developed that support the inflammatory stress hypothesis. Specifically, diclofenac, SLD, halothane, chlorpromazine, ranitidine, and trovafloxacin, when administered at nonhepatotoxic doses, induced significant liver injury in rodents in the presence of a concurrent inflammatory stress [55–61]. The remainder of this chapter focuses on models of IDILI developed on the basis of this hypothesis. This begins with a brief description of inflammatory mediators that have been implicated in liver injury in drug–inflammation interaction models.

18.4 OVERVIEW OF THE INFLAMMATORY RESPONSE

Inflammation is a first-line defense mechanism by the innate immune system. It occurs in response to infection by pathogens, noninfectious disease, or tissue injury [62]. Tissue injury leads to activation of the innate immune response after trauma, which when severe can present as a systemic inflammatory response syndrome associated with multiple organ failure [63]. Inflammation is also associated with conditions such as anemia [64], Parkinson’s disease [65], diabetes [66], obesity [67,68], heart disease and other vasculopathies [69], and cancers [70,71]. Furthermore, inflammagens released into the circulation from periodontal disease are thought to be a risk factor for cardiovascular disease [72,73].

Inflammation is a complex response coordinated by various inflammatory cells and involves numerous soluble mediators (Fig. 18.2). Activation of toll-like receptors (TLRs) and the resultant intracellular signaling are involved in the initiation of the inflammatory response [74]. TLRs are expressed by inflammatory cells and recognize

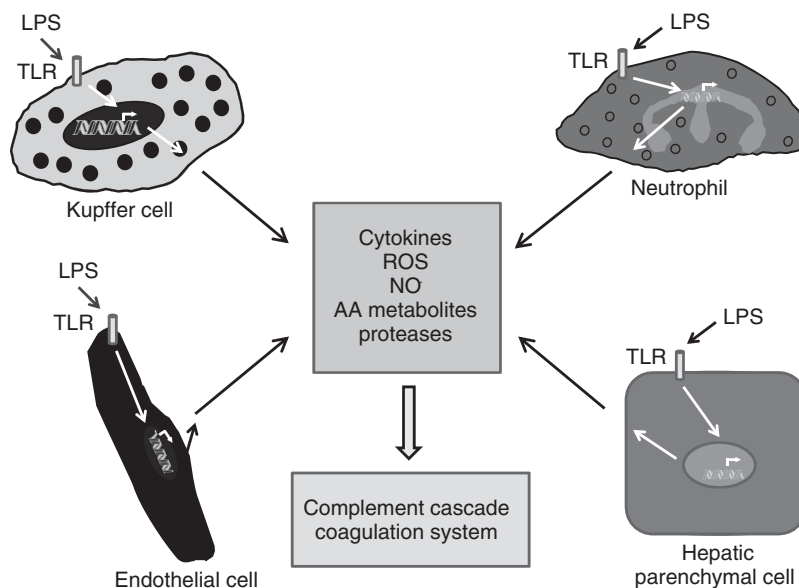


Figure 18.2 Mediators of inflammation. In the liver, Kupffer cells, neutrophils, endothelial cells, hepatic parenchymal cells, and other cells such as NK/NKT (natural killer T) cells (not pictured) are involved in inflammatory responses. Inflammagens such as LPS bind to Toll-like receptors (TLRs) to activate cells, resulting in release of soluble mediators and activation of the complement cascade and coagulation system. Some of these changes are mediated by increased gene transcription. AA, arachidonic acid; NO, nitric oxide. (See color insert.)

specific structures conserved among microorganisms [75]. LPS binds to TLR4, resulting in intracellular signal transduction that leads to a cascade of inflammatory events [69]. Macrophages are the primary immune cells responding to LPS, although TLR4 is also expressed on the plasma membranes of hepatocytes, endothelial cells, and mast cells [76].

Neutrophils, macrophages, endothelial cells, epithelial cells, and platelets are all activated by inflammatory stimuli [54]. With the exception of platelets, activation results in increased expression of a variety of genes. Proteases and ROS are released by neutrophils and macrophages and can kill parenchymal cells directly. Proinflammatory cytokines, such as tumor necrosis factor- α (TNF), are produced and released by each of the cell types listed previously. These cytokines can either activate cell death signaling pathways or initiate other inflammatory events. The coagulation system as well as the complement system can also be activated as a result of inflammation. Some of these mediators are described in greater detail in subsequent sections.

18.4.1 Tumor Necrosis Factor- α (TNF)

TNF is a proinflammatory cytokine that is produced by many cell types, such as macrophages, mast cells, endothelial cells, and stellate cells, in response to LPS [77,78]. Kupffer cells are resident macrophages that exist in liver in close apposition to the luminal surface of endothelial cells in liver sinusoids [79], and they are the major macrophage population exposed to blood-borne inflammagens. After LPS binds to

TLR4, pro-TNF is produced by Kupffer cells as a result of activation of the NF- κ B (nuclear factor kappa B) signaling pathway. Pro-TNF is a 26-kDa membrane-bound precursor form that is not biologically active [80]. Tumor necrosis factor converting enzyme (TACE), a membrane-bound metalloprotease, recognizes a cleavable signal sequence on pro-TNF and mediates shedding of the active 17-kDa form of TNF from the cell membrane [81,82].

TNF exerts its biological effects through two plasma membrane receptors: tumor necrosis factor receptor 1 (TNF-R1) and tumor necrosis factor receptor 2 (TNF-R2). In a few cell types, for example, T cells, the binding of TNF to TNF-R2 leads to proliferation, NF- κ B activation, and cytokine production [83,84]. However, in most cells, TNF-R2 has no direct effect on signal transduction and plays an indirect role in TNF-R1 responses by delivering TNF to the lower affinity TNF-R1. The ligand-passing activity of TNF-R2 allows fine tuning of TNF-R1-mediated signal transduction.

TNF plays an important role in regulating liver homeostasis [82]. TNF-R1 activation leads to hepatocyte proliferation via the activation of NF- κ B, the initiation of mitogen-activated protein kinase (MAPK) cascades, or cell death. Whether TNF leads to cell survival or death is determined by NF- κ B [82]. NF- κ B plays a protective role against cell death by inducing the expression of several antiapoptotic genes, including caspase-8 inhibitor c-FLIPL, the Bcl-2 family members Bcl-xL and A1/Bfl-1, and X-linked inhibitor of apoptosis (XIAP) [85–87]. Moreover, prolonged activation of c-Jun N-terminal kinase (JNK), resulting from ROS production or TNF signaling, causes cell death [88], and NF- κ B indirectly downregulates TNF-induced JNK activation [89].

In addition to its role in cell death and survival, TNF regulates production of other inflammatory mediators. It positively controls production of a variety of cytokines and chemokines including interferon gamma (IFN- γ), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein (MIP)-1 α [90]. TNF also promotes a procoagulant state by upregulating tissue factor, plasminogen activator inhibitor 1 (PAI-1), and platelet-activating factor and by downregulating tissue plasminogen activator [91]. Thus, TNF has multiple roles in inflammation and inflammation-mediated injury.

18.4.2 The Hemostatic System and Hypoxia

Vascular hemostasis is regulated by coagulation and fibrinolysis. The coagulation system is activated in many inflammatory diseases [92]. It can be activated through the extrinsic or the intrinsic pathway. In the extrinsic pathway, tissue factor is exposed to blood and forms a complex with factor VII, which activates factor X through the activation cascade. Factor X cleaves prothrombin into thrombin, which converts fibrinogen into fibrin monomers. Fibrin can cross-link to form fibrin clots in blood vessels [93]. This process is balanced by fibrinolysis, in which fibrin clots are degraded by plasmin [94]. Plasminogen activators (PAs) cleave plasminogen to active plasmin. PAs are inhibited by active PAI-1, which is synthesized by hepatocytes, endothelial cells, and platelets in response to TNF or inflammagens such as LPS [95,96]. Therefore, an increase in active PAI-1 can dampen the fibrinolytic system and thereby enhance fibrin deposition.

Fibrin deposition controls the magnitude and area of infection and by doing so plays a protective role against inflammagen exposure. However, detrimental effects can also occur when fibrin deposited in liver sinusoids impairs blood flow and causes

tissue hypoxia. Hypoxia activates numerous intracellular signaling pathways related to the transcription of hypoxia-responsive genes, mostly mediated by hypoxia-inducible factors. ROS that are produced after exposure to hypoxia lead to the mitochondrial permeability transition [97,98]. Energy deficit (loss of ATP) and inhibition of aerobic metabolism also result from the decrease of available oxygen. Physiological function is impaired, and liver damage can occur [99]. Hypoxia enhances LPS-induced liver damage *in vivo* [100] and potentiates the toxic effects of polymorphonuclear leukocyte (PMN)-derived proteases on hepatocytes *in vitro* [101].

Thrombin and PAI-1 are the two critical factors in regulating the hemostatic system and fibrin deposition, but they also play a role in activating inflammatory cells. Thrombin cleaves and activates G-protein-coupled receptors, including protease-activated receptor 1 (PAR-1), which significantly increases production of proinflammatory cytokines (TNF, IL-1, and IL-6) from target cells [102]. *In vitro*, PAI-1 potentiates LPS-induced neutrophil activation through a JNK-mediated pathway and by enhancing nuclear translocation of NF- κ B. The latter leads to the production of IL-1, TNF, and MIP-2 [103]. Studies *in vivo* also suggest that PAI-1 contributes to the production of TNF, IL-10, keratinocytes chemoattractant (KC), and MCP-1 [104].

18.4.3 Neutrophils (PMNs)

PMNs are abundant blood leukocytes that play an important role in the innate immune response. They protect tissues from pathogens but can also contribute to tissue injury in conditions such as ischemia-reperfusion and alcoholic hepatitis [105]. PMNs are involved in several models of drug-induced liver injury and in drug–LPS interaction models of idiosyncratic liver injury [57,105–108].

PMNs migrate from vessels into tissues and are primed and activated by systemic or local exposure to proinflammatory mediators. TNF, IL-1, or CXC chemokines (cytokine-induced neutrophil chemoattractant-1 (CINC-1), MIP-2) increase the expression of integrins on the surface of PMNs as well as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the surface of endothelial cells [109,110]. The binding of an adhesion molecule to its integrin receptor results in tight adherence of PMNs to endothelial cells, which is necessary for migration into tissue. Selectins are another type of adhesion molecule that is responsible for accumulation of PMNs in the vasculature of some tissues; however, in the liver, PMN accumulation in sinusoids can occur independently of selectins [111]. PMN migration from the vasculature and infiltration into the parenchyma is a prerequisite for neutrophil-induced cytotoxicity [109] and is mediated by several mechanisms. CXC chemokines as well as products released by dying cells, such as high mobility group box 1 (HMGB1), can instigate PMN migration [112,113]. Anti-HMGB1 antibody significantly reduced the production of proinflammatory cytokines and PMN infiltration in an ischemia-reperfusion model of liver injury [113]. Apoptotic cells might also trigger PMN migration [114]. It has been proposed that gaps in the sinusoidal endothelial cells may facilitate the direct contact of PMNs with altered membranes of apoptotic cells [114].

After PMNs cross the endothelial cell barrier, they localize close to hepatocytes and degranulate, leading to the release of proteases. They also produce ROS, including hydrogen peroxide and hypochlorous acid [115]. Both proteases and ROS contribute to liver injury in animal models [114]. Cathepsin G and elastase are two cytotoxic proteases released by activated PMNs. They are important mediators of

hepatic parenchymal cell killing *in vitro* [116], and inhibition of elastase protected against liver injury in drug–inflammation interaction models [106,108,136]. Besides a direct effect on hepatocytes, proteases released by PMNs can also contribute to fibrin deposition by activating PAI-1 [106].

18.4.4 Reactive Oxygen Species (ROS)

ROS comprise free radical and nonradical, reactive oxygen molecules. They include hydrogen peroxide, superoxide, and hydroxyl radical, and their reaction with cellular constituents can lead to the generation of other reactive species such as lipid free radicals and unsaturated aldehydes. ROS are produced by several sources within the cell. Mitochondria, in which the electron transport chain transfers electrons to oxygen, are a major source of ROS. It has been estimated that during normal cellular respiration about 2% of electrons escapes, and this leads to the production of superoxide anion [117]. As described in previous sections, TNF and hypoxia can increase ROS generation in a mitochondria-dependent manner. Another major source of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. This complex assembles on phagosomal, plasma, and granule membranes of cells, including activated PMNs, endothelial cells, and macrophages [115]. NADPH oxidase reduces oxygen to superoxide as it oxidizes NADPH [118]. In addition, many other enzymes, including xanthine oxidase, cyclooxygenases (COX), lipoxygenases, myeloperoxidases, hemoxygenases, monoamine oxidases, aldehyde oxidase, and CYPs, can produce ROS. However, the capacity of these enzymes to generate ROS is typically less robust than the mitochondrial electron chain complex or NADPH oxidase [119].

Excessive ROS can tilt the prooxidant–antioxidant balance within cells, causing oxidative stress. ROS can directly oxidize proteins and lipids, as well as nucleic acids, leading to cellular damage and dysfunction [119]. In addition, ROS can regulate the activities of numerous enzymes. For example, they inactivate JNK phosphatase, which leads to prolonged activation of JNK [88]. Activated JNK phosphorylates antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL) and inactivates them [120,121], thereby predisposing cells to apoptosis.

Oxidative stress plays a role in several models of liver injury. For example, it contributes to ischemia-reperfusion injury in mice [122], alcoholic liver disease [123], and the pathogenesis of inflammatory liver diseases [124]. Oxidative stress has also been proposed as a potential mechanism of NSAID-induced hepatotoxicity [125].

18.5 MODELS OF DRUG–INFLAMMATION INTERACTION

There are few animal models to study the mechanisms of drug-induced idiosyncratic hepatotoxicity. One reason for this is that idiosyncratic hepatotoxicity induced by drugs only occurs in a small fraction of animals, as it often does in humans. However, imposing a stress on animals can reveal the hepatotoxic effects of some drugs. According to the hypothesis introduced above, that inflammatory stress precipitates idiosyncratic drug toxicity, several animal models of IDILI based on drug–inflammation interaction have been developed. The focus of the next two sections of this chapter is on a model developed for SLD-induced hepatotoxicity.

18.5.1 IDILI from Sulindac (SLD)

SLD is a prodrug in the therapeutic class of NSAIDs. It was introduced into the market in 1978 to relieve pain, tenderness, swelling, and stiffness caused by osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis. SLD has a half-life of 8 h, and the typical dose for human patients is 150–200 mg, twice per day. The bioavailability of SLD is more than 90%. It is absorbed rapidly on oral administration and reaches a peak concentration in human plasma in 2–4 h [126]. SLD and its metabolites are secreted into bile, undergo enterohepatic circulation, and are excreted in urine and feces [127].

Bioactivation of SLD to its active metabolite, SLD sulfide, is a reversible reaction. Methionine sulfoxide reductase in both liver and gut flora is the enzyme primarily responsible for reduction of SLD to SLD sulfide, and a flavin-containing monooxygenase catalyzes the reverse reaction [128]. This same flavin-containing monooxygenase converts SLD to SLD sulfone irreversibly (Fig. 18.3). SLD sulfide is responsible for the pharmacological activity to inhibit COX 1 and 2 [129], with subsequent reduction in synthesis of prostaglandins.

The use of SLD in the United States was associated with several hundred cases of hepatic injury in the period 1978–1986 [130]. The US FDA Arthritis Advisory Committee wrote “the potential for producing liver injury is a class characteristic of NSAIDs” [130,131]. The incidence of SLD-induced hepatotoxicity was about 1 in

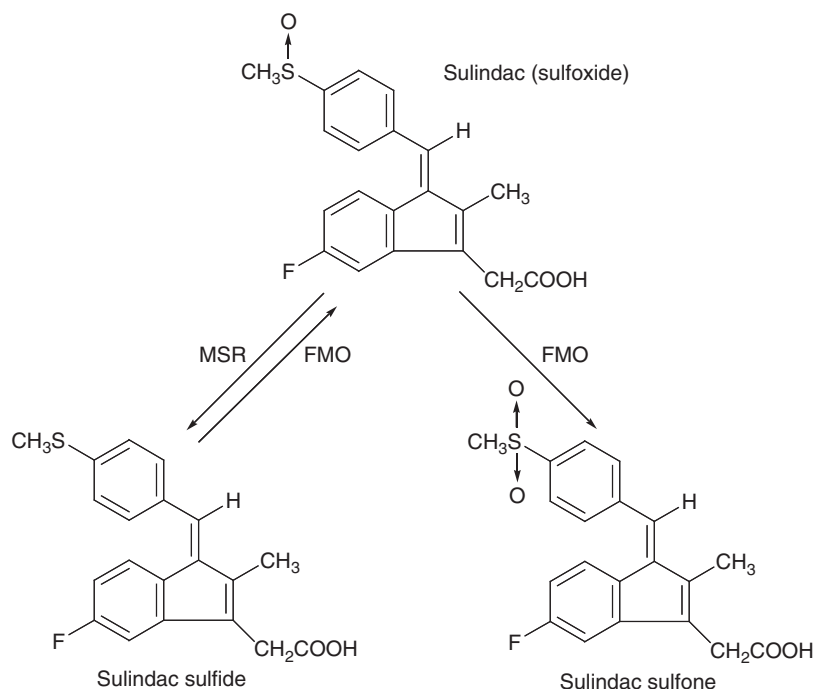


Figure 18.3 Metabolism of sulindac. Sulindac (i.e., the parent drug, sulindac sulfoxide) is metabolized by methionine sulfoxide reductase (MSR) to its active metabolite, sulindac sulfide. The reverse reaction is catalyzed by flavin-containing monooxygenase (FMO). FMO also catalyzes conversion of sulindac to sulindac sulfone.

100,000 patient-years of NSAID use [132]. According to the analysis of cases reported to the US FDA, SLD-induced liver injury most often occurred within eight weeks of maintenance therapy, whereas about 20% of the reported reactions occurred later [130]. Females were more susceptible to SLD toxicity than males, and two-thirds of the patients were over 50 years of age. Clinical chemistry parameters identified hepatocellular, cholestatic, and mixed patterns of injury, and histopathologically, the pattern of SLD-associated liver injury was cholestatic or hepatocellular. In the cases of hepatocellular injury, the lesions were panacinar in most cases (eight out of nine). Portal inflammation and eosinophil infiltration were observed in a portion of patients.

Mechanisms of SLD-induced idiosyncratic liver injury are not well understood. Because clinical characteristics consistent with hypersensitivity were observed in some patients, it was proposed that hypersensitivity accounts for a significant proportion of SLD-induced liver injury. However, no direct evidence for this has been found. Mitochondrial injury has also been proposed as a mode of action. A variety of NSAIDs (e.g., nimesulide, diclofenac, and SLD) or their metabolites injure mitochondria of hepatocytes *in vitro* [34–38]. There is little evidence *in vivo* supporting this hypothesis, and an animal model of idiosyncratic liver injury from SLD based on this hypothesis has not emerged.

18.5.2 An Animal Model of SLD IDILI Based on the Inflammatory Stress Hypothesis

To develop a liver injury model of SLD–inflammation interaction, rats were given two administrations of SLD (p.o.) with a 16-h interval [58]. Half an hour before the second administration of SLD, a nonhepatotoxic dose of LPS was administered (i.v.) to rats. Rats treated only once with SLD and LPS did not develop liver injury consistently. However, as assessed from increases in the activity of ALT in serum, liver injury was evident by 8 h and was statistically significant by 12 h in rats given LPS and two administrations of SLD. Markers of biliary injury were also increased. Midzonal hepatocellular necrosis was observed histopathologically only in livers of rats cotreated with SLD (two administrations) and LPS (Fig. 18.4). These results suggested that SLD interacts with inflammatory stress induced by LPS to produce liver injury.

Mediators of inflammation, for example, some cytokines, can affect the expression of enzymes involved in drug metabolism. Inflammation is most often associated with reduced expression of drug-metabolizing enzymes, and this could contribute to IDILI responses by decreasing clearance of drugs or their toxic metabolites [133]. In rats treated with SLD, the concentrations of SLD and its metabolites, that is, SLD sulfone and SLD sulfide, in plasma and livers were decreased by LPS cotreatment [134]. In contrast, their concentrations in the gastrointestinal tract and feces were increased by LPS cotreatment, suggesting decreased absorption of the drug. Thus, hepatotoxicity seen in SLD/LPS-cotreated rats was not due to the enhanced uptake of the drug, increased bioactivation of SLD to its toxic metabolite, SLD sulfide, or increased accumulation of SLD sulfide in the liver.

Several mediators potentially involved in the pathogenesis were investigated. Hemostatic factors, including PAI-1 and thrombin, were elevated in the plasma by LPS, and this effect was magnified by SLD cotreatment [58]. As a result, significant fibrin deposition was observed in liver sinusoids of rats cotreated with SLD/LPS compared to those treated with SLD or LPS alone. Hypoxia occurred in the livers of rats treated

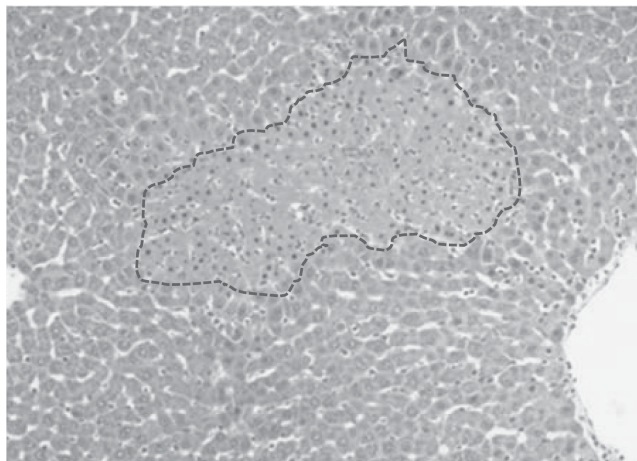


Figure 18.4 Histopathologic changes in liver of rats treated with SLD/LPS. Vehicle (Veh) or SLD was administered at -16 and 0 h, and Veh or LPS was given at -0.5 h. The liver was taken at 12 h, fixed, processed, and stained with hematoxylin and eosin. No necrosis was observed in liver sections from rats treated with Veh, LPS, or SLD only. In this liver section from a rat treated with SLD/LPS, a large area of hepatocellular necrosis can be seen. The lesion (demarcated by a dashed line) is characterized by hepatocytes with pyknotic nuclei and eosinophilic cytoplasm.

with SLD/LPS, presumably as a result of impaired blood flow due to microvascular occlusion by fibrin clots. The anticoagulant heparin attenuated fibrin deposition and hypoxia in the liver and protected against hepatocellular necrosis induced by SLD/LPS. Moreover, hypoxia increased SLD-mediated cytotoxicity in isolated hepatocytes [58]. These results suggested that the hemostatic system and tissue hypoxia resulting from fibrin deposition contributed to the pathogenesis.

As noted earlier, TNF is a central mediator of inflammation. In rats treated with SLD/LPS, the LPS-induced increase in plasma TNF concentration was enhanced by SLD [134]. TNF neutralization using etanercept decreased serum ALT activity in SLD/LPS-cotreated rats, demonstrating an important role for TNF in the development of liver injury.

SLD sulfide was more cytotoxic to HepG2 cells and primary rat hepatocytes than SLD itself [134]. However, *in vivo*, the amount of SLD sulfide produced was insufficient to cause liver injury by itself, and another factor arising from administration of LPS was required. A direct cytotoxic interaction between SLD sulfide and TNF was suggested by the observation that cell killing by SLD sulfide *in vitro* was synergistically enhanced by TNF [135]. However, additional events contribute to hepatotoxicity *in vivo*. LPS treatment led to PMN accumulation in the liver, and SLD enhanced this effect [136]. Interestingly, activation of the accumulated PMNs was only evident in the livers of rats cotreated with SLD/LPS. Thus, the drug–inflammation interaction was required for activation of these cells. Either antibody-induced depletion of PMNs or administration of a PMN protease inhibitor, eglin C, attenuated liver injury, indicating that toxic proteases released from activated PMNs participate in the pathogenesis [136]. *In vitro*, PMN elastase acted synergistically with hypoxia to kill hepatocytes [101], raising the possibility that this same interaction might contribute to SLD/LPS-induced liver injury in the rat model.

Additional evidence suggested complex interactions among inflammatory mediators involved in SLD/LPS-induced liver injury. For example, neutralization of TNF or PAI-1, both of which are important mediators in liver injury induced by SLD/LPS, decreased PMN activation but did not affect PMN numbers in the liver. On the other hand, neutralization of TNF had no effect on PAI-1 production, suggesting a lack of dependence on TNF for PAI-1 involvement in toxicity [136].

SLD/LPS cotreatment produced a unique gene expression profile in the liver compared to treatments with SLD alone or LPS alone [135]. Analysis of the genes specifically changed by SLD/LPS at a time before the onset of injury suggested that a pathway associated with oxidative stress was perturbed. The concentration of protein carbonyls in mitochondria, a marker of oxidative stress, was increased only in rats cotreated with SLD and LPS [135]. Antioxidants protected against liver injury induced by SLD/LPS in rats and reduced cytotoxicity caused by SLD sulfide and TNF interaction *in vitro* [135]. Furthermore, TNF enhanced the cytotoxicity of hydrogen peroxide in HepG2 cells, suggesting the possibility of TNF-ROS interaction *in vitro* and *in vivo*.

Results of these studies suggest a complex, liver-damaging cascade of inflammatory events that is summarized in Fig. 18.5. LPS stimulates accumulation of PMNs in the liver, release of TNF, and initiation of coagulation. SLD enhances each of these initial responses. Increased TNF concentrations lead to PMN activation and subsequent release of proteases that are necessary for hepatocellular killing. Enhanced activation of coagulation and inhibition of fibrinolysis result in fibrin deposition in the liver and hepatic hypoxia. Hypoxia increases SLD sulfide-mediated cytotoxicity and has the potential to augment cell killing by proteases. In addition, cotreatment with LPS and SLD causes oxidative stress, and evidence from *in vitro* studies suggests that TNF increases ROS-mediated cell killing. Accordingly, TNF, the hemostatic system,

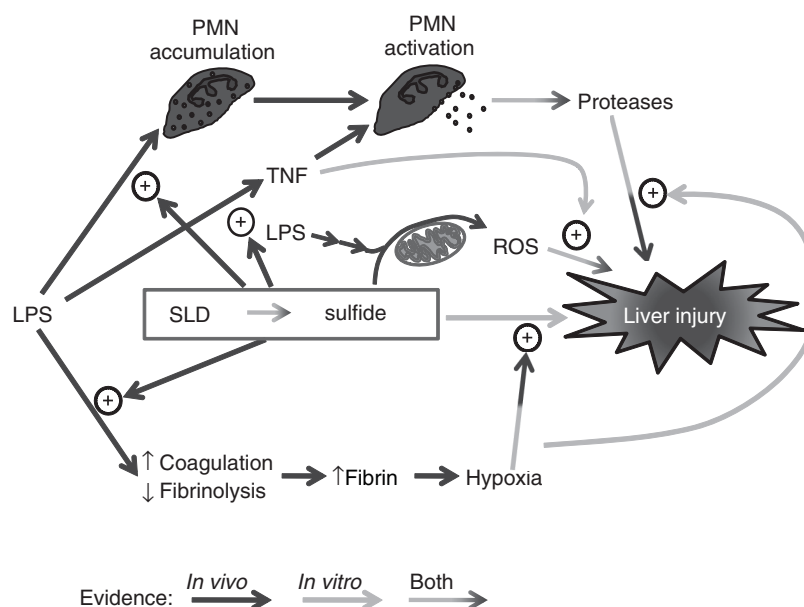


Figure 18.5 Proposed mechanism of LPS/SLD interaction that leads to liver injury.

hypoxia, and PMNs, as well as ROS, play important roles in the pathogenesis of SLD/LPS-induced liver injury.

18.5.3 Commonalities and Differences Among Liver Injury Models of Drug–Inflammation Interaction

Evidence in animal models supporting the inflammatory stress hypothesis has been accumulating. In rodents, inflammation precipitates liver injury induced by several drugs associated with IDILI in humans. These drugs include SLD, diclofenac, trovafloxacin, halothane, ranitidine, and chlorpromazine [55,56,58–61,137]. The characteristics and mechanisms involved in the pathogenesis have been studied to various extents, and the results are summarized in Table 18.2 and recent reviews [138,139]. Some commonalities and differences are mentioned here.

Most models have been developed using LPS, a product of gram-negative bacteria, to induce inflammation. Other inflammatory stimuli have been used with similar results, however, suggesting that the effect is related generally to the inflammatory response rather than to LPS specifically. For example, a gram-positive stimulus (a peptidoglycan–lipoteichoic acid mixture) or the viral RNA mimetic (polyI:C) increased liver damage from trovafloxacin or halothane, respectively [108,140]. In the trovafloxacin/LPS model in mice, replacing LPS with TNF also resulted in liver injury, supporting the idea that TNF is an early and central mediator in these models [141]. The routes of drug administration are different among the models, suggesting that liver injury is not dependent on a specific route of drug administration. The SLD/LPS model is the only one explored thus far, in which the drug was administered twice; one dose of SLD failed to induce reproducible liver injury in rats. The durations between drug and inflammation administration needed to elicit a toxic response also vary among different models.

SLD, trovafloxacin, and ranitidine models have been relatively well investigated using LPS as an inflammatory stimulus. Enhanced TNF release, activation of the hemostatic system, and PMN accumulation in liver have been observed in all of these models. Neutralization or inhibition studies showed that each of these mediators is critical to the pathogenesis of liver injury induced by the interaction with LPS [58,61,90,101,104,106,134,136,142,143]. PMNs are also critical to liver injury in rats cotreated with LPS and diclofenac; [57] however, the role of PMNs in chlorpromazine-induced liver injury has not been tested. Taken together, these results suggest that TNF, hypoxia, and PMNs are important players shared by several models of liver injury from drug–inflammation interaction. The results also indicated that these mediators are not independent but interact with each other; however, their interactions differ somewhat among models.

Some mediators have been found to be critical in the pathogenesis in a single model but have not been tested in other models; for example, IFN- γ and IL-18 are both involved in trovafloxacin/LPS-induced liver injury [144]. Oxidative stress proved to play a role in SLD/LPS-induced liver injury [135]. The roles of these mediators in other models have not been investigated.

In summary, evidence is accumulating to support the hypothesis that inflammation precipitates or enhances drug-induced liver injury for drugs that cause IDILI in humans. TNF, the hemostatic system, and PMNs are involved in the pathogenesis of all the liver injury models of drug–inflammation interaction in which their roles have been tested.

TABLE 18.2 Characteristics and Underlying Mechanism of IDILI in Models Based on the Inflammatory Stress Hypothesis

Drug	SLD	DCLF	TVX	RAN	CPZ	HAL
Inflammagen	LPS	LPS	LPS ^a or PGN + LTA	LPS	LPS	PolyI:C ^a or LPS
Route of drug administration	p.o.	p.o.	i.p.	i.v.	i.p.	i.p.
Number of drug administrations	2	1	1	1	1	1
Time (h) drug was given relative to LPS	–15.5 and 0.5	2	–3	2	2	–6
Hours after the drug administration when liver injury was observed	12 after second SLD	6	12	6	24	15
Drug enhanced TNF production	Y		Y	Y		Y
TNF involved in pathogenesis	Y		Y	Y		Y
Hemostatic system activated	Y		Y	Y		
Fibrin deposition and hypoxia involved in pathogenesis	Y		Y	Y		
PMN accumulation	Y	Y	Y	Y	Y	
PMN activation	Y			Y		
PMNs involved in pathogenesis	Y	Y	Y	Y		
Injury is IFN/IL-18-dependent			Y			
ROS involved in pathogenesis	Y					
Hemostasis affected by TNF	N		Y	Y		
PMN accumulation increased by TNF	N		N	N		
PMN accumulation affected by hemostasis				N		
PAI-1 increased by TNF	N		Y	Y		
PMNs activated by PAI-1 and TNF	Y			Y		
Kupffer cells and NK cells involved						Y

Abbreviations: Y, the phenomenon or mechanism is observed in the model; N, the phenomenon or mechanism does not occur in the model. SLD, sulindac; DCLF, diclofenac; TVX, trovafloxacin; RAN, ranitidine; CPZ, chlorpromazine; HAL, halothane; PGN, peptidoglycan; LTA, lipoteichoic acid.

A blank entry means the feature has not been tested or reported (see text for references).

^aIndicates the inflammagen used in the mechanism studies.

The roles of oxidative stress or proinflammatory cytokines other than TNF as well as other potential mediators remain to be determined for models involving drugs besides trovafloxacin and SLD. Studies of drug–inflammation models have generally clarified several important mediators involved in the liver injury observed in these models using drugs that cause IDILI in humans. However, the mechanisms of drug–LPS interaction remain incompletely understood. For example, the cellular sources of inflammatory mediators important in these responses have not been identified, and exploration into the signaling pathways leading to liver injury has only just begun. Importantly, the

molecular target to which drug binds to initiate interaction with inflammatory pathways is unknown.

18.6 CONCLUSIONS

Idiosyncratic drug reactions resulting in liver injury remain a health concern and a major reason for loss of therapeutically useful drugs from the market. Despite their importance and years of study, the mechanisms by which IDILI arises are still not understood, and animal models that could aid in understanding and predicting these reactions have only recently begun to appear. Numerous hypotheses have emerged suggesting that the reactions might occur from polymorphisms in drug-metabolizing enzymes or transporters; from adaptive immune reactions to haptens formed by reactive metabolites; from mitochondrial dysfunction rendered injurious by drug exposure; from failure to adapt to modest, drug-induced injury; from the intersection of multiple genetic, environmental, and drug-related factors; or from an injury-precipitating interaction of the drug with an episode of inflammation. Of these theories, the inflammatory stress hypothesis has thus far led to the most robust animal models. Drug–LPS interaction models have revealed that several drugs can potentiate the activation of inflammatory cells and the generation of inflammatory mediators that act synergistically with the drug to cause hepatocellular necrosis and/or that precipitate a complex cascade of inflammatory events that results in liver injury. These events include but are probably not limited to cytokine release, inflammatory cell stimulation, and coagulation system activation. Despite progress in developing these as well as models based on other hypotheses, the ability to predict which drug candidates are likely to cause IDILI remains poor. Further development and refinement of animal models is likely to enhance understanding of IDILI pathogenesis, and this in turn should lead to *in vitro* and *in silico* models that will improve preclinical prediction and lead to the discovery of better biomarkers for IDILI and strategies to prevent it.

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19 Regulation of Drug Metabolizing Enzymes and Transporters in Infection, Inflammation, and Cancer

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19.1	Summary	1
19.2	Introduction	2
19.3	The innate immune system and its activation in inflammation and infection	4
19.4	Considerations for predicting the effects of inflammation on drug disposition and drug-disease-drug interactions	8
19.5	Regulation of the CYP1 family	11
19.6	Regulation of the CYP2 family	12
19.7	Regulation of the CYP3 family	16
19.8	Regulation of the CYP4 family	17
19.9	Flavin monooxygenases	17
19.10	Conjugation enzymes	18
19.11	Extrahepatic metabolism	19
19.12	Drug transporters	21
19.13	Roles of cytokines	25
19.14	Mechanisms of regulation	29
19.15	Conclusions and future perspectives	31
	References	31

19.1 SUMMARY

Under conditions of innate immune system activation (i.e., inflammation), the functions of specific cytochrome P450 enzymes, other drug metabolizing enzymes (DMEs), and drug transporters (DTs) are altered in the liver, small intestine, lung, kidney, and central

nervous system (CNS). Many of these effects are primarily manifest at the transcriptional/RNA level, leading to corresponding changes in protein levels and function. This not only leads to altered drug and xenobiotic toxicity and action in diseased humans, but also has importance for disease therapy with biologic drugs that target inflammatory mediators or their receptors. Major roles for proinflammatory cytokines such as interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor- α (TNF α) are inferred from the abilities of these agents to affect DMEs and DTs in cultured cells and *in vivo*, but the *in vivo* contributions of cytokines to regulation of these proteins in different inflammatory disease states is still poorly understood.

19.2 INTRODUCTION

As is elaborated in detail in the ensuing pages, inflammation and infection in humans or experimental animals are associated with changes in the expression and function of many DMEs and DTs. Especially for the DMEs, in the majority of cases these effects result in an impairment of their function (enzyme or transporter activity), although some proteins can be induced.

It will become evident that the great majority of information in this area comes from studies on the cytochrome P450 (P450) enzymes. However, research on the regulation of other DMEs and DTs is progressing, and will be summarized hereunder. The majority of studies in animals have been performed on acute models of infection or inflammation [e.g., the bacterial lipopolysaccharide (LPS) model]. In most cases where the time course has been studied, the effects are transitory and drug metabolism or disposition normalizes with the resolution of the inflammation. However, there is also the potential for long-lasting effects of disease, as observed for flavin monooxygenase (FMO)-3 that remains downregulated in the livers of mice treated with *Citrobacter rodentium* long after the infection and concomitant colonic inflammation have resolved [1].

19.2.1 Effect of Disease on Clinical Drug Therapy

Downregulation of hepatic DMEs consequent to activation of host defense (the innate immune system) can be expected to reduce the hepatic clearance of drugs that are eliminated by the specific enzymes affected, resulting in elevated plasma levels of drug and possible toxicity if doses are not adjusted. For example, an outbreak of influenza virus was associated with increased incidence of theophylline toxicity in asthmatic children, concomitant with a 67% increase in theophylline half-life (corresponding to approximately a 40% decrease in clearance) [2]. Even relatively modest effects like this clearly pose a risk of drug toxicity and indicate the need for dose adjustment, when the drug affected has a low therapeutic index (such as theophylline, warfarin, and cyclosporine). More recently, a 90% decline in hepatic activity of CYP2D6 in human immunodeficiency virus (HIV)-infected patients was reported [3], indicating the potential for much greater effects on drug clearance in humans.

Conversely, induction of DMEs due to inflammation or infection would be expected to cause elevated clearance and reduced plasma levels of affected drugs, possibly resulting in therapeutic inefficacy. For example, in a review of the literature on pharmacokinetics in HIV/AIDS (acquired immunodeficiency syndrome), Dickinson *et al.* [4] concluded that there was a general trend toward reduced plasma levels

of protease inhibitors (CYP3A4 substrates), especially azatanavir, in people with HIV/AIDS, although no single study showed any significant differences. This would imply the induction of CYP3A4 and/or P-glycoprotein (PGP), although this has not been demonstrated.

The consequences of DT regulation by inflammatory factors may be anticipated. Downregulation of efflux transporters would be expected to result in increased bioavailability and tissue accumulation of drugs, whereas the same effect on uptake transporters would be expected to result in reduced bioavailability and tissue concentrations of drug. The reverse would be true for induction of the transporters.

Overall, the information on the impact of disease on drug metabolism and disposition in humans is scanty, and much more research is needed. However, such studies are very difficult because rarely does the researcher have access to patients with untreated disease, and the ethical issues associated with withholding or delaying treatment are manifest.

19.2.2 Drug–Drug Interactions (DDIs)

Many, if not all, of the effects of inflammatory diseases on drug metabolism are thought to be due to the action of proinflammatory cytokines on hepatocytes and other cells. To date, TNF α , IL-1 β , IL-6, and interferons (IFNs) have been implicated as the most important. While TNF α , IL-1, or IL-6 are not clinically used drugs, IFN α , β , and γ are all used to treat various cancers and are capable of downregulating P450 expression in cell culture and in animals *in vivo*. Therefore, they have the potential to interact with other medications. However, effects reported on drug metabolism in humans are inconsistent [5].

19.2.3 Drug-Disease-Drug Interactions (DDDI)

The recent advent of the so-called biologic drugs targeting proinflammatory cytokines or their receptors (Table 19.1) has resulted in a new type of drug–drug interaction (DDI) that is disease dependent, which we will call drug-disease-drug interaction (DDDI). Originally, it was thought that biologic drugs did not pose a risk for interactions with small molecule drugs, because the biologics are metabolized by completely different enzymes, and do not inhibit or induce DMEs. However, the results from two animal studies in which the downregulations of hepatic CYP3A enzymes were reversed by a polyclonal antibody to IL-6 [6] or by infliximab, a monoclonal antibody to TNF α marketed for the treatment of arthritis and other autoimmune diseases [7], demonstrated the possibility for disease-dependent biologic-small molecule interactions in humans. Recently, the Food and Drug Administration (FDA) approved a humanized monoclonal antibody to the IL6 receptor, tocilizumab, for the treatment of rheumatoid arthritis. The report of the FDA's Arthritis Committee notes that plasma levels of simvastatin (a CYP3A4 substrate) and omeprazole (a CYP2C19 substrate) were reduced in patients treated with tocilizumab, consistent with the ability of IL-6 to downregulate these P450s in primary human hepatocyte cultures [8]. Thus, biologics can reduce the therapeutic efficacy of small molecule drugs, by relieving the inflammatory downregulation of the enzymes involved in their clearance.

The extent of DDDIs is only beginning to be understood. When a drug neutralizes a cytokine that can directly regulate DMEs in the hepatocyte, a DDDI of the type described might reasonably be expected. However, other cytokines might act

TABLE 19.1 Biologic Drugs Targeted to Inflammatory Diseases

Drug	Target	Diseases/Indications
Adalimumab	TNF α	Rheumatoid arthritis
Certolizumab-pegol	TNF α	Crohn's disease
Etanercept	TNF α	Rheumatoid arthritis
Infliximab	TNF α	Rheumatoid arthritis, Crohn's disease
Golimumab	TNF α	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis
Canakinumab	IL-1 β	Cryopyrin-associated periodic syndromes
Rilonacept	IL-1 β	Cryopyrin-associated periodic syndromes
Anakinra	IL-1 receptor	Rheumatoid arthritis
Daclizumab	IL-2 receptor	Acute organ rejection, asthma, multiple sclerosis, autoimmune
Basiliximab	IL-2 receptor	Acute organ rejection
Tocilizumab	IL-6 receptor	Rheumatoid arthritis
Ustekinumab	IL-12 and IL-23	Psoriasis
Abatacept	B7, T-cell comodulator	Rheumatoid arthritis

The above drugs are monoclonal antibodies or Fab fragments except anakinra, which is a receptor antagonist; etanercept, which is a cytokine receptor fragment; and abatacept and rilonacept, which are fusion proteins.

redundantly with the one targeted by the biologic, such that no effect would have been seen. On the other hand, it is possible that any drug that resolves inflammation, regardless of its mechanism, has the potential to cause a DDDI by reducing production of the cytokines that act directly on the liver. It remains to be seen whether well-characterized nonbiologic drugs such as nonsteroidal anti-inflammatories might be involved in DDDIs.

19.3 THE INNATE IMMUNE SYSTEM AND ITS ACTIVATION IN INFLAMMATION AND INFECTION

19.3.1 Cellular and Molecular Aspects of the Innate Immune System

Mammals and other host organisms deal with invading organisms via the two branches of the immune system, that is, the innate and the adaptive immune system [9]. The innate immune system has developed to survey the plasma and tissues for signs of foreign organism invasion, and to respond rapidly to eliminate the danger. The adaptive immune system mounts a slower but more specific response, designed to remember the molecular characteristics of a pathogen or toxin and thus enable the host to respond rapidly to a subsequent infection weeks to years later. Cells and molecules associated primarily with innate immunity have been implicated in the modulation of drug metabolism and transport, and therefore we will concentrate on the innate arm of the immune system.

Inflammation is caused by the response of the innate immune system to invading organisms or to tissue damage. It can be thought of as an adaptive homeostatic mechanism that becomes detrimental if inappropriately stimulated or dysregulated [10]. The cellular response of the innate immune system to infection or the presence of damaging toxins is primarily mediated by granulocytic leukocytes of the myeloid

lineage: monocytes, macrophages, neutrophils, mast cells, basophils, and eosinophils [9]. Natural killer (NK) cells, a type of T cell, also participate. The innate immune system is activated by invading pathogens via several families of proteins, called *pattern-recognition receptors*, which recognize structural motifs common to many invading pathogens or the molecules they secrete [11]. These pathogen-associated molecular patterns (PAMPs), described in detail in the ensuing section, are characteristics of molecules such as LPS, double-stranded viral RNA, and bacterial muramyl dipeptide.

The pattern-recognition receptors are expressed primarily in macrophages, but also in monocytes and neutrophils as well as in fixed tissue cells such as epithelial cells and keratinocytes [9]. Engagement of PAMPs with their receptors initiates specific signaling pathways in the cells, which result in the secretion of cytokines such as $\text{TNF}\alpha$, IL-1, IL-6, and $\text{IFN}\gamma$ to initiate the inflammatory response. $\text{TNF}\alpha$ and IL-1 are so-called proinflammatory cytokines, whereas IL-6 can be either pro- or anti-inflammatory. These initiator cytokines then activate other immune and nonimmune cells to produce additional cytokines and prostanoids, as well as chemokines (chemotactic cytokines) such as macrophage inflammatory protein-1 α (MIP-1 α , chemotactic for neutrophils and NK cells) monocyte chemoattractant protein-1 (MCP-1, chemotactic for monocytes and basophils), and IL-8 (chemotactic for neutrophils). Chemokines induce the expression of adhesion molecules on host cells so that effector cells such as neutrophils and NK cells, recruited to the site of injury by the chemokines, attach to the host cells. The identities of the chemokines induced, and thus the immune cells recruited, depends on the initial cytokine pattern which in turn depends on the initiating signal and the tissue(s) in which they occur [12].

Of special interest for drug disposition are the specialized macrophages of the liver, Kupffer cells (KCs), which represent an estimated 80–90% of the fixed macrophages in the body [13]. KCs are found in the sinusoidal lumen, where they are well positioned to detect bacteria, their toxins, and breakdown products absorbed from the gastrointestinal tract. When activated by bacterial endotoxin or by complement, KCs release $\text{TNF}\alpha$, IL-1, and IL-6 as well as nitric oxide, superoxide (from NADPH oxidase), prostaglandins, and thromboxanes. Therefore, it should be apparent that the local concentrations of these agents in the vicinity of the hepatocytes is likely to be much greater during inflammation caused by, for example, the injection of LPS than during inflammation elicited at a distant anatomical site. In the latter case, regulation of the hepatocytes will occur via circulating cytokines.

19.3.2 Pattern-Recognition Receptors

The best-understood family of pattern-recognition receptors is the family of integral membrane glycoproteins called *toll-like receptors* (TLRs) [11]. TLRs 1–9 are common to humans and mice, whereas the additional species-specific TLRs (TLR10 in humans and TLRs 11–13 in mice) are of unknown function. The TLRs recognize PAMPs associated with bacterial, fungal, and viral organisms (Table 19.2) [11]. TLR4, the best-characterized and prototypic TLR, was discovered as the gene whose mutation rendered mice (HeJ strain) insensitive to LPS. However, binding of LPS to TLR4 absolutely requires the presence of a soluble accessory protein MD-2 [14]. CD14 is another TLR4 accessory protein that sensitizes cells to LPS by facilitating ligand transfer to the receptor complexes and/or directing these complexes to signaling centers such as lipid rafts [14]. There is recent evidence that CD14 may also fulfill similar functions with

TABLE 19.2 Characteristics of the Toll-Like Receptors

TLR	Ligands	Signaling Pathways	Cellular Context	Hepatic Expression
1	Triacyl lipoprotein	MyD88/TIRAP	PM	SE KC SC
2	Lipoproteins	MyD88/TIRAP	PM	H SE KC SC
3	ds RNA	TRIF/TRAM	Endosomes	H SE KC SC
4	LPS	MyD88/TIRAP TRIF/TRAM	PM	H SE KC SC
5	Flagellin	MyD88/TIRAP	PM	SE KC SC
6	Diacyl lipoprotein Zymosan	MyD88/TIRAP	PM	SE KC SC
7	ss RNA, siRNA	MyD88/TIRAP	Endosomes	H
8	ss RNA	MyD88/TIRAP	Endosomes	SE KC SC
9	Unmethylated CpG-DNA	MyD88/TIRAP	Endosomes	SE KC SC

Abbreviations: PM, plasma membrane; H, hepatocytes; SE, sinusoidal endothelial cells; KC, Kupffer cells; SC, stellate cells.

TLR2 and other TLRs. For TLR4, CD14 also directs whether the responses will be via the MyD88/Mal or TRIF-TRAM (TIR-domain-containing adapter-inducing interferon- β /TRIF-related adaptor molecule) pathways (see below), this being dependent on the ligand [14].

While most TLRs homodimerize in response to ligand binding to initiate signaling, TLR2 heterodimerizes with either TLR1 or TLR6 to recognize bacterial, mycobacterial, and fungal products. Thus, TLR1/2 recognizes triacyl lipopeptides produced by gram-positive bacteria and mycobacteria, and TLR1/6 recognizes diacyl lipopeptides and fungal zymosan. TLR5 recognizes flagellin from flagellated bacteria.

TLRs 1, 2, 4, 5, and 6 are expressed on cell membranes, but TLRs 3, 7, 8, and 9 are found in intracellular membranes where they recognize PAMPs associated with intracellular pathogens. TLR3 recognizes double-stranded viral RNA, and is the receptor for the commonly used synthetic agonist polyI:rC. TLRs 7 and 8 recognize single-stranded viral RNA, and TLR9 distinguishes unmethylated CpG motifs associated with DNA from viral and bacterial pathogens.

The TLRs (mainly 2 and 4) also recognize damage-associated molecular patterns (DAMPs) on host molecules such as heat shock proteins and hemin, so that inflammation consequent to different types of tissue or cellular damage is also dependent on TLR2 or TLR4. There is some debate as to whether DAMPs themselves activate TLRs, or if they act by sensitizing cells to low levels of PAMPs [15].

Upon ligand binding, the TLRs dimerize to initiate signal transduction through two pairs of adaptor molecules: MyD88 and Mal/TIRAP (toll-interleukin 1 receptor (TIR) domain containing adaptor protein); or TRIF and TRAM (Fig. 19.1). However, only TLRs 2 and 4 need Mal/TIRAP or TRAM (Fig. 19.1). Both pathways result in the activation of NF- κ B, a transcription factor that is a master regulator of inflammatory gene expression, as well as activation of MAP (Mitogen Activated Protein) kinases that can regulate other transcription factors such as AP-1. Among the many targets of NF- κ B are proinflammatory cytokine genes such as IL-1, TNF, and IL-6, and also several hepatic acute-phase genes. Signal transduction via TRIF/TRAM results in the activation of transcription factors IRF3 and IRF7, causing the induction of IFNs α and β , which have antiviral activities. Activation of NF- κ B and MAP kinase (MAPK)

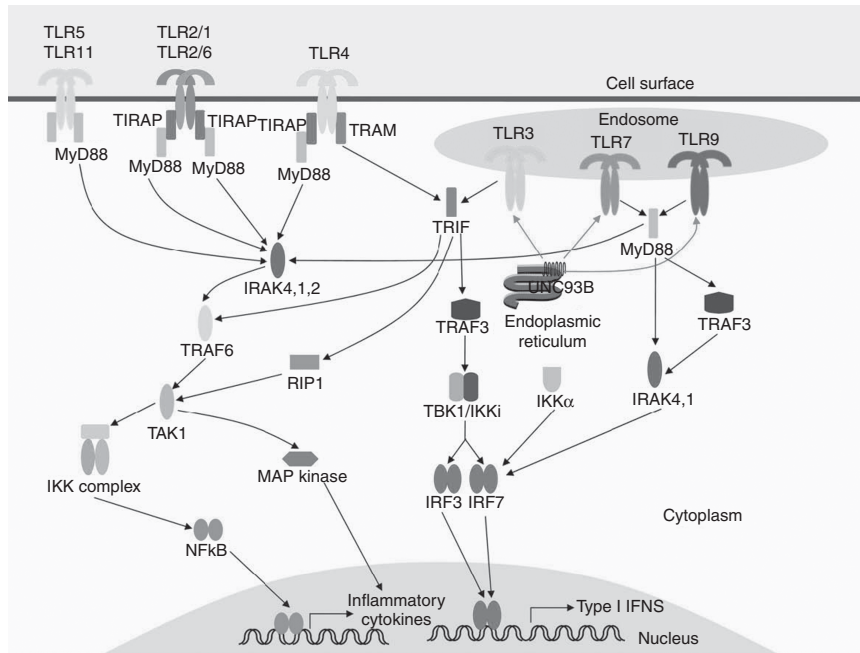


Figure 19.1 TLRs and their cellular signaling mechanisms. *Source:* Reprinted from Kumar H, Kawai T, Akira S. *Biochem Biophys Res Commun* 2008;388 with permission from Elsevier. (See color insert.)

activation through the two adaptor molecules may be kinetically different and also cell-type dependent. Thus, the result of activation of a given TLR in two different cell types can be different [16].

While TLRs 1, 5, 6, 8, and 9 have not been reported on hepatocytes, they are all expressed in liver nonparenchymal cells (Table 19.2). Moreover, agonists of all TLRs evoked $\text{TNF}\alpha$ and IL-6 release by KCs, except for TLR5 that only evoked IL-6 release [16]. TLR3 and TLR4 agonists stimulated $\text{IFN}\beta$ release from KC as well. Liver sinusoidal epithelial cells released $\text{TNF}\alpha$ in response to TLR2, 3, 4, and 8 activation; and IL-6 in response to TLR3 or 4 activation [16]. Similarly, stellate cells express mRNAs for all nine TLRs, especially when activated [17]. Thus, any TLR agonist has the potential to affect hepatocyte DME and DT expression either directly or indirectly.

The roles of TLRs in regulation of DMEs and DTs have been explored to a limited extent. Perhaps not surprisingly, the downregulation of P450 enzymes [18–20] as well as of UDP-glucuronosyltransferase 1a1 (UGT1A1) [19] and the uptake transporter Oatp4 [21] in the LPS model of inflammation requires a functioning TLR4. On the other hand, the regulation of hepatic P450 enzymes in the liver during intestinal infection with *C. rodentium* is independent of TLR4 [20]. This demonstrates that gram-negative bacterial infection can influence hepatic gene expression via different mechanisms.

A second family of pattern-recognition receptors is the NLR (nucleotide-binding, leucine-rich repeat-containing) family, of which there are more than 20 in humans [22]. These intracellular sensors detect PAMPs such as muramyl dipeptide, LPS, flagellin, and bacterial or viral RNA, as well as DAMPs such as uric acid crystals, exhibiting significant overlap with TLR ligands. Ligand binding of NLRs results in

nucleotide-dependent activation of a large protein complex called the *inflammasome*, that in turn results in cleavage of procaspase-1 [22]. Active caspase-1 then cleaves pro-IL-1 and pro-IL-18 to their active forms, initiating an inflammatory response. NLR ligand binding can also mediate the activation of NF- κ B and MAP kinase pathways, which further stimulate the production of inflammatory cytokines, antimicrobial peptides, and reactive oxygen species [23]. In addition, NLR activation can be associated with both caspase-1-dependent and -independent cell death (specialized mechanisms of necrosis). This denies intracellular pathogens a cell in which to replicate, and also results in release of cellular components that can stimulate inflammation via TLRs [22].

The potential roles of NLRs and the inflammasome in regulation of DMEs during infection and inflammation are unknown. Mouse hepatocytes express the NLRs NOD1 and NOD2 and respond to NOD1 ligands with the activation of NF- κ B and MAP kinases [24]. Hepatic sinusoidal endothelial cells are capable of mounting an Nalp3 inflammasome response in response to DNA from apoptotic hepatocytes [25]. The potential for NLR involvement in DME regulation will be an important area of future research.

19.3.3 The Acute-Phase Response and the Liver

The acute reaction of the body to infection or tissue injury is termed the *acute-phase response* (APR), which encompasses all of the cellular and molecular events described above as well as the inflammatory symptoms of fever, redness, swelling, and pain that accompany them. The hepatic component of the APR includes the increased synthesis and secretion of a battery of proteins called *acute-phase proteins* (APPs) or *acute-phase reactants* [26,27] within a few hours of the initiating stimulus, and the reduced synthesis of other hepatic proteins called *negative APPs* (such as albumin). Thus, the many DMEs and DTs that are downregulated in inflammation can be considered to be negative APPs. APP synthesis is regulated largely by IL-1, TNF α , and IL-6, with IL-6 playing the dominant role [26]. In addition to acting directly on hepatocytes, however, IL-1 and TNF α can also stimulate KCs to release IL-6, affecting APP synthesis indirectly [27].

During chronic inflammation, serum levels of APPs are often elevated and are used as markers of inflammatory burden in humans. Because their expression and secretion integrate multiple upstream inflammatory signals including proinflammatory cytokines, APP levels tend not to fluctuate as much as those of the cytokines which are relatively short-lived [28]. Thus, serum APPs are being used to assess human health and predict the outcomes of some diseases including cancer [28].

19.4 CONSIDERATIONS FOR PREDICTING THE EFFECTS OF INFLAMMATION ON DRUG DISPOSITION AND DRUG-DISEASE-DRUG INTERACTIONS

The ultimate goal of research in this area is to develop biomarkers and decision-making tools that will allow prediction of changes in drug metabolism and disposition in an individual or a population due to a specific disease, and the prediction or exclusion of DDIs in people treated with anti-inflammatory biologic drugs. This would allow physicians to prescribe safer and more efficacious drug regimens for their patients, and also facilitate the drug development process. To achieve this goal, we must fully understand the multiple factors that can alter drug metabolism during inflammation and infection, and also the mechanisms by which these effects are achieved.

19.4.1 Roles of Cytokines and Other Mediators

Proinflammatory cytokines such as $\text{TNF}\alpha$ and IL-6 are capable of potent regulation of DMEs and DTs in hepatocytes and some other cell types, with a degree of specificity. Studies in cytokine or cytokine receptor knockout mice, or studies using cytokine antibodies, provide evidence for *in vivo* roles of these cytokines in certain inflammatory disease states. However, the effect of removing a cytokine either genetically or immunologically is dependent on the disease model and is not generalizable to all DMEs or transporters. This is most likely because of redundancy and/or cross-talk of cytokine pathways, as well as qualitative, quantitative, spacial, and temporal differences in cytokine secretion in different disease states.

As mentioned above, studies on cytokine regulation of DMEs and DTs to date have focused mainly on the ones known to be involved in hepatic APP regulation. However, there are receptors for many other cytokines and chemokines on hepatocytes and nonparenchymal cells in the liver (Table 19.3), and there is a need to discover which of these could be involved in DME and transporter regulation.

In certain diseases, DME and transporters could be regulated by factors other than cytokines. Activation of TLRs or other pattern-recognition receptors on hepatocytes could trigger changes in DME expression. Nonproteinaceous inflammatory mediators such as prostaglandins, or proteins or small molecules secreted by invading bacteria or parasites could also play a role. However, good evidence for or against such regulation is lacking at the present time.

19.4.2 Inflammation and Chronic Diseases

Research on the impact of inflammation on DMEs and DTs has tended to focus on acute inflammation and diseases or models of diseases that have inflammation as a

TABLE 19.3 Hepatic Target Cells of Cytokines and Chemokines

Cytokine/chemokine	Target Cells in Liver	References
IL-1	H SE KSC	29–32
IL-2	H K SC	33–35
IL-6	H SE KSC	36–38
IL-8	H	39
IL-12	Not described	—
IL-13	H SC	40,41
IL-17	H L	42,43
IL-21	Not described	—
$\text{TNF}\alpha$	K	44
$\text{IFN}\alpha/\beta$	H K SC	45,46
$\text{IFN}\gamma$	H SE K SC	31,47,48
MCP-1	H K SC	49–51
MIP-1 α	K	52
RANTES	K SC	52

In this table, a cell type is designated to be a target cell if receptors or a specific functional response have been detected. *Abbreviations:* L, liver-cell type unspecified; H, hepatocytes; SE, sinusoidal endothelial cells; KC, Kupffer cells; SC, stellate cells.

TABLE 19.4 Disease States with Inflammation as a Primary or Underlying Component

Primary	Underlying
Infection, septic shock	Cancer
Alcoholic and nonalcoholic fatty liver disease	Congestive heart failure
Rheumatoid arthritis	Hypertension
Gouty and osteoarthritis	Atherosclerosis
Tendinitis	Sickle cell disease
Fibromyalgia	Multiple sclerosis
Lupus (SLE)	Parkinson's disease
Asthma, chronic obstructive pulmonary disease (COPD)	Alzheimer's disease
Inflammatory bowel diseases	Chronic kidney disease/hemodialysis
Psoriasis	Obesity, metabolic syndrome, diabetes
Injury	

cause or primary component (Table 19.4). However, inflammation is now recognized as a condition associated with, or underlying, many chronic diseases and therefore has the potential to impact drug metabolism and disposition (Table 19.4). For example, inflammation is intimately involved in the development and progression of cancer, with inflammatory cytokines being produced both by immune cells and by the cancer cells themselves [53]. Cancer in humans is associated with reduced activity of CYP3A4, as detected by reduced clearance of its substrates erythromycin and docetaxel [53]. Clearance was negatively correlated with serum concentrations of APP and IL-6 levels [53,54], suggesting that the effect is dependent on the degree of inflammation associated with the disease. Examples of other diseases that have inflammation as an underlying component will be presented, but much more work is needed in this area.

19.4.3 Biomarkers

It will be apparent from the above discussion that cytokines themselves could be prognostic biomarkers for drug metabolism and disposition. This will be especially true for diseases in which a given cytokine is demonstrated to be mechanistically involved. Likewise, serum APP levels are used to measure inflammatory loads and are associated with disease outcomes such as in cancer [53]. C-reactive protein (CRP) is an APP that is readily detectable at low concentrations in human plasma, and which is measured in many routine health screening tests. As mentioned above, its plasma levels are negatively correlated with CYP3A4-catalyzed drug clearance in cancer patients, and it deserves further scrutiny as a predictive biomarker in cancer and other inflammatory diseases. If CRP levels are elevated, then one can infer that IL-6 and/or TNF α signaling pathways have been activated in hepatocytes suggesting that some DMEs and/or DTs will be affected. However, if other mechanisms of DME regulation predominate in a particular disease state, changes in drug metabolism or disposition may well occur before or without changes in APP secretion. This illustrates the need to understand the mediators and mechanisms of DME and DT regulation.

19.5 REGULATION OF THE CYP1 FAMILY

Sections 19.5–19.8 focus on hepatic expression of the major drug metabolizing P450 enzymes.

19.5.1 Bacterial Infection

Bacterial infections have been reported to downregulate protein and (or) CYP1A-associated activity in humans infected with *Helicobacter pylori* [55] or bacterial pneumonia [56]; pigs infected with *Actinobacillus pleuropneumoniae* [57]; and both mice and *Cyprinus carpio* (carp) infected with *Listeria monocytogenes* [58–60]. Following intracerebroventricular (i.c.v.) injection of *L. monocytogenes* in rats, CYP1A activity was significantly elevated at 48 h but returned to normal levels by 72 h [61]. In mice that developed hepatitis due to *Helicobacter hepaticus* infection, Cyp1a protein levels were induced [62], but the infection of mice with *C. rodentium*, a mouse intestinal pathogen, had no effect on Cyp1a2 mRNA levels measured seven days post infection [63,64].

19.5.2 Viral Infection

It has been known for several decades that humans infected with the influenza virus have depressed CYP1A activity as shown by their depressed theophylline clearance [2,65]. Hepatitis caused by infection with either the B or C virus also depressed CYP1A-associated activities and/or proteins in humans and mice [66–68]. Infection with HIV, on the other hand, had no significant effect on CYP1A2 activity as determined by urinary caffeine metabolite ratios [3].

19.5.3 Parasitic Infection

Both the hookworm (*Ancylostoma ceylanicum*) and liver fluke (*Fasciola hepatica*) downregulate hepatic mammalian CYP1A activity [69–71]. The caffeine and theophylline clearance of both children and adults infected with *Plasmodium falciparum* malaria is reduced [72,73]. The infection of rodents with *Plasmodium berghei* (ANKA strain), *P. falciparum*, or *Plasmodium chabaudi chabaudi* depresses their CYP1A activity levels [74–77]. Mice with schistosomiasis (infection of *Schistosoma mansoni*) have reduced hepatic 7-methoxy- and 7-ethoxyresorufin-*O*-deethylase (EROD), both Cyp1A-associated activities, during the early phases of infection and upregulation in chronic infection [78–80]. The hepatic microsomes of rats infected with *Taenia taeniformis* had lower EROD activity than those of rats that were uninfected [81].

19.5.4 Sterile Inflammation

Injection of LPS is a model of bacterial sepsis, and evokes an acute systemic inflammatory response. Owing to its ease of use and the large responses observed, it has been used in many laboratories to study how inflammation can regulate DME and DT expression. It is important to keep in mind that this model of end-stage disease is unlikely to predict how these systems will be affected in diseases where inflammation

is more localized and/or less fulminant. Most reports indicate that i.p. injection of LPS, derived from either *Escherichia coli* or *Salmonella abortus equi*, results in the down-regulation of CYP1A mRNA, protein levels, or activity in of rats or mice [64,82–84] as well as human beings [85,86]. However, LPS upregulates EROD activity in juvenile carp [87].

The administration of dextran sulfate sodium (DSS) is a well-characterized model of chemically induced inflammatory bowel disease (IBD) that has been used to study the regulation of hepatic P450 function in animals. In (DSS)-induced colitis as well as in collagen-induced arthritis or local inflammation induced by subcutaneous injection of casein or silver nitrate, downregulations of CYP1A-associated activities were observed [82,88,89]. The induction of cirrhosis by either carbon tetrachloride, thioacetamide or by bile duct ligation has been reported to cause the downregulation of CYP1A [90,91], which is likely to have inflammatory components. Conversely, Nakajima *et al.* [91] reported that D-galactosamine-induced hepatitis does not affect rat CYP1A protein levels. While both the Bacillus Calmette Guérin (tuberculosis, BCG) and influenza vaccines have been reported to downregulate theophylline clearance in humans [92,93], in another study the BCG vaccine given to rats did not affect phenacetin O-de-ethylation [94].

19.5.5 Cancer

Rodents with different sarcomas have been reported to have decreased CYP1A protein and (or) activity when compared to animals free of this condition [54,95]. In humans, the presence of hepatocellular carcinoma was reported to both to have no effect [68] and to downregulate phenacetin-deethylation [67], an activity which is associated with CYP1A.

19.6 REGULATION OF THE CYP2 FAMILY

19.6.1 CYP2A

Unlike most other CYPs, the CYP2A enzymes are often upregulated in inflammation or infection. This could be due to the fact that these enzymes are regulated by the redox-sensitive transcription factor Nrf2 [96,97].

Murine hepatitis caused by bacterial infection with *H. hepaticus* has been reported to elevate levels of Cyp2a5 [62], while infection with *C. rodentium* did not affect mRNA levels of the same enzyme [63,64]. In mice, hepatitis B virus infection has been shown to upregulate Cyp2a5 protein levels as well as activity [66,98,99]. In human beings on the other hand, when compared to uninfected controls, those infected by the hepatitis A virus had decreased coumarin-7-hydroxylation (a marker of CYP2A6 activity) [100], while infection with the hepatitis B and C viruses increased CYP2A6 protein levels [101].

The effect of parasite infestation on CYP2A-dependent coumarin 7-hydroxylation has been investigated in both rats and mice. Female C57BL/6 mice were not affected by infection with either *P. berghei* or *P. chabaudi chabaudi*, but age-matched animals with a DBA/2 background had elevated Cyp2a5 activity [76]. In rodents infected with *T. taeniformis* [81,102] and hamsters infected with the liver fluke, *Opisthorchis*

viverrini [103] the activity and (or) protein levels of CYP2As were elevated. Using mice suffering from schistosomiasis, Conte *et al.* [78] reported that there was no change in coumarin 7-hydroxylase activity but Manhaes-Rocha *et al.* [79] noted that while infestation did not change this activity in females, there was significant downregulation in male mice.

The administration of *E. coli* LPS downregulates Cyp2a5 mRNA levels but not the 7 α -hydroxylation of testosterone in several strains of mice [20,64,83,84,104,105]. Using a cDNA expression array, Fang *et al.* [106] reported the downregulation of CYP2A1 in rats given 0.5 mg/kg *S. abortus equi* LPS. Administration of lipotechoic acid, a component of gram-positive bacteria and a TLR2 agonist, transiently downregulated hepatic Cyp2a4 [107]. Colitis induced by 3% DSS in drinking water over seven days did not affect Cyp2a5 mRNA levels in the mouse [20].

19.6.2 CYP2B

Bacterial infections tend to downregulate CYP2B isoforms. The infection of either mice or rats with *L. monocytogenes* resulted in lower rates of CYP2B-dependent 7-pentoxoresorufin-O-dealkylation (PROD) and 7-benzoyloxyresorufin-O-dealkylation (BROD), respectively [59,61]. Pigs infected with *A. pleuropneumoniae*, mice infected with *C. rodentium*, and tuberculous guinea pigs all exhibited downregulated levels of CYP2B isoforms [20,57,64,108].

Humans infected with hepatitis B or C have been reported to have higher levels of hepatic CYP2B6 than uninfected controls, as assessed via immunohistochemical detection [101]. On the other hand, transgenic hepatitis V virus (HBV) mice had CYP2B protein levels and pentoxoresorufin O-dealkylase (PROD) activity comparable to ordinary mice [66,98]. Parasitic infection with liver flukes (*F. hepatica*) downregulated the N-demethylation of benzphetamine in hepatic microsomes of rats [70] and sheep [109]. In mice, infection with malaria downregulates BROD activity in a strain- and time-dependent pattern [75,110]. When using DBA/2 and Swiss Webster mice, Manhaes-Rocha *et al.* [79] reported that schistosomiasis generally did not affect Cyp2b-associated activity, while Conte *et al.* [78] reported a consistent downregulation of the same. In a different model of parasitic infection, microsomes from rats infected with tapeworms (*T. taeniformis*) had a lower activity of PROD than their uninfected counterparts [81].

The administration of *E. coli* LPS downregulates CYP2B in human beings [85,86], rodents [20,64,111], and in cell culture [112]. The TLR2 agonist lipotechoic acid greatly downregulates Cyp2b10 in mouse liver via a KC-dependent mechanism [107]. The downregulation of CYP2B isoforms has been reported for several chemically induced inflammatory conditions including collagen-induced arthritis [88] and thioacetamide-induced cirrhosis in the rat and DSS-induced colitis in the mouse [20] but not D-galactosamine-induced hepatitis of the rat [91]. Male Fisher 344 rats with a pituitary mammotropic tumor had decreased CYP2B activity, measured by hexobarbital sleeping time, as compared to cancer-free animals [113].

19.6.3 CYP2C

Bacterial infection of mice with *C. rodentium* or *L. monocytogenes* as well as the guinea pig with tuberculosis downregulated various CYP2C mRNAs or activities

[60,63,108]. Viral infections also tend to downregulate CYP2C-related activity. Mice infected with encephalomyocarditis virus and humans carrying the HIV or diagnosed with viral hepatitis all had downregulated CYP2C activity [98,114–117]. Callahan *et al.* [118] reported that after a single intravenous dose of the recombinant adenovirus serotype 5 vector, ranging from 5.7×10^6 to 5.7×10^{12} virus particles per kilogram, the activity and mRNA levels of CYP2C11 were upregulated. In a subsequent publication the authors reported that the adenovirus, administered without a transgene, suppressed CYP2C11 activity [119]. The authors concluded that the immunogenic and biological nature of a transgene cassette can influence changes in the 2C11 isoform and can be used to study part of the host response to viral infection, via its action on TLR3. Seminal work by Renton and Mannering [120] found that the double-stranded RNA poly I:C, an “interferon inducer” and TLR3 agonist, reduced hepatic content and activities of unspecified P450 enzymes. Its administration to rats was subsequently shown to downregulate CYP2C11 and 2C12 mRNAs and proteins [121,122].

The demethylation of aminopyrine, used as a marker of CYP2C activity is mainly metabolized by CYP2C11 in male rats [123]. Parasitic infections have been reported to downregulate aminopyrine-N-demethylation activity in several mammals including hamsters infected with *Leishmania donovani* [124] or *A. ceylanicum* (hook worm) [69], rats and sheep infected with *F. hepatica* (liver fluke) [125,126], and mice suffering from schistosomiasis [80].

Regardless of the route of administration, whether i.p or by i.c.v. injection, the administration of LPS from *E. coli* or *S. abortus equi* downregulates the levels of CYP2C mRNAs, protein expression and activity [20,64,82,83,106,127]. Mice administered an i.p injection (40 mg/kg) of bacillus Calmette-Guerin vaccine had normal levels of Cyp2c29 after 24 h [128], but the influenza vaccine has been reported to decrease aminopyrine clearance in humans [129]. In rodents, barium sulfate, carrageenan, celite, D-galactosamine, and kaolin have been used as models of sterile inflammation and have resulted in the reduction of CYP2C11 activity and (or) protein in rats [82,91, 130–132] and Cyp2c29 mRNA in mice [20].

Several reports have been made of the downregulation of CYP2C isoforms by cancer including unspecified metastatic cancers in humans [133], Murphy-Sturm lymphosarcoma, pituitary mammotropic tumor, and Walker 256 carcinosarcoma in rats [95,113]. Interestingly, the Engelbreth-Holm-Swarm sarcoma in mice upregulated Cyp2c39 and did not affect Cyp2c37 mRNA [134].

19.6.4 CYP2D

Bacterial infection with *C. rodentium* infection in female mice has been reported to downregulate Cyp2d22 and upregulate Cyp2d9 mRNA levels up to 10 days post infection [63,64]. Using male Wistar rats, Glazier *et al.* [77] reported that there was no significant difference in CYP2D1 activity between control animals and those infected with *P. berghei*, as measured by the α -hydroxylation of metoprolol.

Perhaps in one of the most dramatic demonstrations of the effects of infection on human DMEs, Jones *et al.* [3] reported that compared with healthy volunteers,

HIV-infected subjects had 90% lower CYP2D6 activity determined by the O-demethylation ratio of dextromethorphan metabolites.

LPS downregulated Cyp2d22 mRNA levels in C57BL/6 mice, while levels of Cyp2d9 were induced at 24 h in Refs 20 and 64. Compared to healthy individuals, humans with systemic lupus erythematosus had a decreased ability to convert debrisoquine to 4-hydroxydebrisoquine [135]. While treatment of rats with 2,4,6-trinitrobenzene sulfonic acid resulted in the downregulation of lidocaine metabolism [136], propranolol-7-hydroxylation was neither affected by DSS nor LPS treatments [82]. Treatment of mice with DSS did not affect Cyp2d9 but upregulated Cyp2d22 [20]. The expression of CYP2D was downregulated in rats with carrageenan-induced granuloma [137].

When proteomic profiling of mouse hepatic tissue was performed by multiplexed tandem mass spectrometry after isobaric tag for relative and absolute quantitation (iTRAQ) labeling it was reported that Engelbreth-Holm-Swarm sarcoma downregulated Cyp2d9 but did not affect the expression of either Cyp2d10 or 2d26 [134].

19.6.5 CYP2E

Bacterial infections in the pig (*A. pleuropneumoniae*) and mouse (*C. rodentium*) have been reported to depress CYP2E1 levels [57,63,64]. In mice, the hepatitis B virus upregulated Cyp2e1-associated 4-nitrophenol hydroxylation [66], and a retroviral infection resulted in increased Cyp2e1 activity at week 8 that returned to normal levels by week 16 [138]. In a malarial parasitic infection model, mice infected with *P. berghei* had increased Cyp2e1-associated activity [75], while in rats CYP2E1 activities were either upregulated or not affected [74,139]. Swiss Webster rats with schistosomiasis had decreased 4-nitrophenol hydroxylase activity at 90 days post infection [78], but only infected male rats showed significant elevation of *N*-nitrosodimethylamine *N*-demethylation at 30 days post infection [79]. While infection of female rats with *T. taeniformis* did not have a significant effect on 4-nitrophenol hydroxylase activity [81], hamsters infected with leishmaniasis or hook worms (*A. ceylanicum*) as well as sheep and rats infested with liver flukes (*F. hepatica*) all had depressed CYP2E1-associated activity compared to control animals [69,109,124,125].

The administration of LPS to rats and mice has been demonstrated to downregulate both expression and activity of CYP2E1 [20,64,82–84,104,106,111,127], although in one study CYP2E1 mRNA was induced [140]. Neither inflammation induced by kaolin nor D-galactosamine had significant effects on CYP2E1 protein levels of rats [91,132]. DSS-induced colitis suppressed 4-nitrophenol hydroxylase activity in rats but had no significant effect on mRNA levels in mice [20,82]. Administration of the tuberculosis vaccine to rats downregulated chlorzoxazone 6-hydroxylation [94], while in humans CYP2E1 activity is not altered by influenza vaccination [141]. In contrast, Muntane *et al.* [137] reported that rats with carrageenan-induced granuloma showed a strong depression of CYP2E1 RNA during the acute phase and recovered during the chronic phase. The presence of Engelbreth-Holm-Swarm sarcoma in mice and of metastatic cancers in human beings have both been reported to have no effect on CYP2E1 expression [133,134].

19.7 REGULATION OF THE CYP3 FAMILY

19.7.1 Infections

Bacterial infections have been reported to downregulate mouse Cyp3a11, 3a25, and 3a41 as well as rat CYP3A2 [61,63,64]. Critically ill patients with septic shock have been reported to be unable to metabolize midazolam [142]. Both humans and rodents infected with different forms of malaria exhibit downregulation of CYP3A activity [74,139,143]. While infection with hepatitis B or C virus upregulates CYP3A proteins [66,101], HIV-infected subjects had 18% lower hepatic CYP3A4 activity compared with age and sex-matched healthy volunteers [3]. Following a single intravenous dose of the recombinant adenovirus serotype 5 vector, the activity and mRNA levels of rat CYP3A2 were downregulated [118].

19.7.2 Sterile Inflammation

Treatment with LPS causes downregulation of CYP3A1 and 3A2 in rats, and Cyp3a11, 3a25, and 3a41 but not Cyp3a13 in mice [20,64,82–84,104,127,128,144,145]. The TLR2 agonist lipotechoic acid causes a moderate and transient downregulation of Cyp3a11 in mouse liver [107]. Administration of the TLR3 ligand poly rI:C to rats downregulates CYP3A2 [146]. Humans with rheumatoid arthritis had the same amount of urinary 6 β -hydroxycortisol as individuals who did not [147], but those with systemic lupus erythematosus, another inflammatory condition, had higher estrogen 16 α -hydroxylase activity [148]. Downregulation of rat hepatic CYP3A mRNAs, proteins, and activities has been observed in two different models of arthritis [6,7].

Both rats and mice treated with the tuberculosis vaccine had reduced CYP3A-associated activity [94,128], while humans immunized against the influenza virus had similar CYP3A activity measured by erythromycin breath test as those not immunized [149]. Treatment of rats with the irritants barium sulfate, celite, D-galactosamine, or kaolin did not affect CYP3A2 protein levels [91,132], while induction of a local inflammatory response with turpentine in the rabbit resulted in the downregulation of CYP3A6 protein [150]. In rats, carrageenan-induced granuloma downregulated CYP3A1 mRNA and catalytic activity [137].

19.7.3 Cancer

There is good evidence that CYP3A-mediated clearance is inhibited in people with advanced cancer. Erythromycin clearance was reduced, and negatively correlated with plasma IL-6 and acute-phase protein markers, in individuals with various different associated cancers [53]. Another study found similar results with cancer patients having decreased midazolam clearance and increased exposure to docetaxel [151]. An earlier study on metastatic cancers found no effect on CYP3A protein levels in surgical liver samples [133]. In mice transgenic for a CYP3A4 promoter- β -galactosidase reporter gene, implantation of a murine sarcoma caused downregulation of the transgene as well as the native Cyp3a11 mRNA [54]. These were associated with elevated serum levels of IL-6.

19.8 REGULATION OF THE CYP4 FAMILY

19.8.1 CYP4A

Compared to uninfected animals, mice infected with *C. rodentium* had significantly lower hepatic levels of Cyp4a10 and 4a14 mRNA, and CYP4As were the most profoundly affected of all P450s [63,64]. Infection of mice with the parasite *S. mansoni* resulted in depressed Cyp4a protein levels in the liver [80].

In rats, we found that *E. coli* LPS treatment upregulated CYP4A2 and 4A3 mRNA levels [127]. While there was no difference between the ability of control and treated male Sprague-Dawley rats to carry out ω -lauric acid hydroxylation, the same treatment resulted in upregulation of the activity in male Fisher 344 rats. However, another study reported that treatment of male Wistar rats with *S. abortus equi* LPS caused downregulation of CYP4A1 and 4A3 [106]. In mice, hepatic lauric acid ω -hydroxylation and Cyp4a10 mRNA levels are depressed by treatment with *E. coli* endotoxin [20,64,83,152]. Modulation of Cyp4a14 appears to be strain-dependent in mice with LPS downregulating levels in C57BL/6J [64] but not in C3H strains [20].

Treatment of rats with the sterile irritants barium sulfate or celite upregulated CYP4A1 mRNA but did not affect either CYP4A2 or CYP4A3 [132]. Microsomes from rats with collagen-induced arthritis had higher lauric acid hydroxylation when compared to nonarthritis rats [88]. In contrast, DSS-induced colitis in mice downregulated Cyp4a10 and 4a14 mRNAs [20]. Muntane *et al.* [137] reported that the presence of carrageenan-induced granuloma in rat depressed CYP4A levels.

19.8.2 CYP4F

Unlike with CYP4As, relatively little is available on the modulation of CYP4Fs by either inflammation or infection. In mice infected with *C. rodentium*, Cyp4f15 mRNA was downregulated while Cyp4f18 was upregulated [63,64]. Treatment of rats with *E. coli* LPS suppressed CYP4F4 and 4F5 expression by 50% and 40%, respectively [153,154], whereas CYP4F5 mRNA was upregulated [153]. LPS from *S. abortus equi* also downregulated CYP4F4 in rat liver after 4 h [106]. In hepatocytes, LPS downregulated CYP2C11, 4F4, and 4F5 [154].

19.9 FLAVIN MONOOXYGENASES

Flavin-containing monooxygenases (FMOs, EC 1.14.13.8) oxygenate substrates with a soft nucleophile, usually nitrogen or sulfur. While in many respects FMOs are similar to P450s and have overlapping substrate specificities, they often yield distinct metabolites. In rats, LPS treatment evoked decreases in hepatic content of FMO1 mRNA and protein, as well as of FMO activities, and these effects were attenuated by inhibition of nitric oxide synthases, suggesting a role for NO in the effect [155]. LPS treatment of mice depressed hepatic FMO1, 3, and 5 mRNAs but did not affect FMO4 mRNA [1]. Mice infected with *C. rodentium* had downregulated levels of FMO1, 3, 4, and 5 [1], and the downregulation of FMO3 persisted for at least nine days after the infection was resolved. The oxidation of nicotine to nicotine-1'-N-oxide is catalyzed by FMOs [91], and this activity was not changed in rats with D-galactosamine-induced hepatitis, in

comparison with the controls, but was significantly decreased in thioacetamide-induced cirrhotic rats [91].

19.10 CONJUGATION ENZYMES

19.10.1 Uridine 5'-Diphospho-Glucuronosyltransferases

The UDP-glucuronosyl transferases (UGTs) are a superfamily of glycosyltransferases (EC 2.4.1.17) that catalyzes the glucuronidation reaction. Mammalian UGTs are divided into four families, UGT1, UGT2, UGT3, and UGT8.

In animals with bacterial or parasitic infections, UGTs were reported to be either downregulated or not affected. Carp infected with *L. monocytogenes*, mice infected with *Plasmodium berghei*, *P. chabaudi chabaudi*, or *S. mansoni* and pigs infected with *A. pleuropneumoniae* had UGT activity similar to uninfected animals [58,74,76,79]. On the contrary, *P. berghei* infected rats were reported to have depressed UGT activity [77,156,157]. Infection of mice with *C. rodentium* resulted in the downregulation of Ugt1a1, 1a9, and 2b5 mRNA but not Ugt1a2 or 1a6 in the liver [158].

The i.p. administration of LPS to rodents did not affect transcription of UGT1A2 and 1A6, but it downregulated Ugt1a1 and 1Aa9 in mice [158] and UGT1A1, 1A6, 2B1, and 2B6 in rats [159]. Turpentine administered intramuscularly to rats was reported to have no effect on 4-nitrophenol glucuronidation but downregulated hepatic UGT isoforms from the UGT1 and two families [160]. Analysis of mRNA extracted from liver tissue samples of patients with fibrosis revealed general downregulation for several UGT1A and UGT2B isoforms that reached statistical significance only for UGT1A4, 2B4, and 2B7 [161]. In the rat, carbon tetrachloride-induced cirrhosis upregulated UGT2B1 [162] but did not affect 4-nitrophenol conjugation by a perfused rat liver [163].

In rodents with induced cancer, the reported modulation of UGTs is varied. Rats with 2-acetylaminofluorane-induced liver preneoplastic nodules had upregulated mRNA [164], while mice injected with Engelbreth-Holm-Swarm sarcoma cells had normal levels of Ugt1a7 but reduced levels of Ugt2b5 [134].

19.10.2 Sulfotransferases (SULTs)

The sulfotransferases (SULTs) (EC 2.8.2) are a gene family of enzymes that catalyze the transfer of a sulfonate group (SO_3^-) from a donor molecule, 3'-phosphoadenosine 5'-phosphosulfate, to a variety of acceptor molecules in a process referred to as *sulfonation* or *sulfurylation*.

In the rat, parasitic infections have been reported both to upregulate [156] as well as downregulate [125,157] SULT activity, while the LP-BM5 retrovirus that causes murine leukemia did not cause significant differences in the recovered urinary acetaminophen sulfate between infected and uninfected mice [165]. Intraperitoneal injection, or the treatment of rodent perfused liver with LPS, has been reported to cause the downregulation of several isoforms in the SULT1 and SULT2 families. In comparison to healthy individuals, SULT1A1 activity increased almost 10-fold in patients with hepatocellular carcinoma secondary to chronic hepatitis B virus infection [68]. Transgenic mice injected with Engelbreth-Holm-Swarm sarcoma cells had higher levels of SULT1A1 but depressed SULT2A1 protein levels [134].

19.10.3 Glutathione S-Transferases (GSTs)

The glutathione S-transferases (GSTs) (EC 2.5.1.18) are a superfamily of enzymes that catalyze the nucleophilic attack of the sulfur atom of glutathione on an electrophilic group on a variety of lipophilic compounds. On the basis of amino acid sequence similarities, six classes of cytosolic GSTs are recognized in mammalian organisms, namely, alpha, mu, omega, pi, sigma, theta, and zeta designated A, M, O, P, S, T, and Z, while mitochondrial GSTs are designated kappa (K).

In humans, patients suffering from *Plasmodium vivax* malaria had about half the overall GST activity of patients without malaria [166]. De-Oliveira *et al.* [76] reported that when mice were infected with either *Plasmodium chabaudi chabaudi* or *P. berghei*, the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) in mouse liver did not change. All cytosolic GSTs, except class Theta are able to carry out this reaction. Infection with *Plasmodium yoelii nigeriensis*, on the other hand, reduced CDNB conjugation [167], while infection with *Angiostrongylus cantonensis* upregulated this activity [168].

Infection with hepatitis C virus (HCV) has been reported to upregulate levels of GSTA to over 150% when compared to HCV negative healthy subjects [169,170]. Viral infection of mice (hepatitis B or C) generally causes the upregulation of GST activity [66,169–171]. While the treatment of both F111 rat fibroblasts and human hepatoblastoma (HepG2) cells with SV40T antigen did not affect the GST levels as measured by Northern blotting, infection of HepG2 cells with the hepatitis B virus resulted in downregulation of GSTA [171].

The i.p. administration of up to 2 mg/kg *E. coli* endotoxin (LPS) has been reported to elicit no significant changes in mouse [145], juvenile carp (*C. carpio*) [87], or rat [111] GSTs. Choi and Kim [172] reported that i.v. LPS treatment downregulated GSTA and GSTM classes in rat liver. Endotoxin from *Salmonella typhimurium* and *S. abortus equi* have also been documented to reduce the expression or protein levels of GSTs in the A, M, and K classes [105,106,173] in rat liver.

19.10.4 Arylamine N-Acetyltransferases (NATs)

N-acetyltransferases (NATs) (EC 2.3.1.5) are enzymes that catalyze the transfer of an acetyl group from acetyl-CoA to an aromatic or heterocyclic amine, hydrazine, hydrazide, or N-hydroxylamine acceptor substrate. Humans have two isoforms, NAT1 and NAT2, while rodents have a third, Nat3 [174]. Compared with age and sex-matched healthy volunteers, HIV-infected subjects had 53% lower NAT2 activity [3].

19.11 EXTRAHEPATIC METABOLISM

19.11.1 Intestine

The intestinal P450 profile differs from that of the liver and shows large interindividual variation in the expression levels of individual P450s. CYP3A and CYP2C9 are the major intestinal P450s in humans, on average accounting for 80% and 15%, respectively [175]. The small intestine is the first site to metabolize orally ingested xenobiotics. Intestine-specific ablation of cytochrome P450 reductase in mice demonstrated that P450-catalyzed nifedipine metabolism in the small intestine plays an important role in the first-pass clearance of the nifedipine, and possibly other drugs [176].

CYP3A, P-glycoprotein (PGP) (mdr1), and MRP2 are important barriers to the absorption of many clinically important drugs in the intestine [177]. In the duodenum of pediatric patients with Crohn's disease, CYP3A mRNAs were higher compared with control groups [178]. Consistent with these observations, in a trinitrobenzenesulfonic acid model of colitis, rat colonic tissue samples showed increased activities of PGP-related efflux and CYP3A-catalyzed metabolism *ex vivo*. However, no change was observed in CYP3A metabolic activity and expression in the respective microsomal fractions [179]. The author suggested that increased metabolism could be due to infiltrating cells at the inflammation site [179]. Greer *et al.* studied the levels of nuclear receptors (NRs) and DME mRNAs in duodenum and colon biopsies of dogs with naturally occurring IBD or food responsive diarrhea (FRD) compared with healthy animals. In the duodenum, peroxisome proliferator-activated receptor- γ (PPAR γ), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and retinoid X receptor- α (RXR α) mRNAs were not different in any of the groups, while PPAR α was elevated in colon only. Duodenal MRP2, CYP3A12, and SULT1A1 mRNAs were elevated in both FRD and IBD, whereas MDR1 mRNA was only elevated in FRD [180].

MDR1, MRP2, and CYP3A mRNAs were decreased by 50%, and CYP3A-mediated metabolism of 7- amiodarone and 7-benzyloxyquinoline were also decreased by $\sim$ 50% in the jejunum of LPS-treated rats [177,181]. The activity of PGP and CYP3A was also downregulated [181].

19.11.2 Lung

In human lung, CYP1A1 (in smokers), CYP1B1, CYP2B6, CYP2E1, CYP2J2, and CYP3A5 proteins are all expressed, as well as FMO and phase II conjugating enzymes [182]. Somers *et al.* [183] compared the expression and metabolizing activities of phases I and II enzymes in freshly isolated human lung parenchymal cells and cryopreserved human hepatocytes, showing that P450 gene expression and activities were generally lower in lung than in liver, whereas expression of all sulfotransferase isoforms in lung was similar to or higher than that in liver.

Rat pulmonary CYP1A and CYP2B1 activities were reduced by 3- and 1.5-fold, respectively 24 h after administration of LPS, while LPS had no effect on basal GST activity in both lung and kidney [184]. Levels of Cyp4a12 mRNA, the only detectable Cyp4a in mouse lung, were enhanced by fourfold in animals injected with IL-1 β [185]. During the acute inflammatory phase of ovalbumin-induced allergic airway disease the pulmonary mRNA levels of Cyp2e1, Cyp2f2, Cyp2j6, Cyp4b1, and Cyp8a1 were decreased while the mRNA levels of Cyp4f18, Cyp5a1, and Cyp7b1 were elevated [186].

19.11.3 Kidney

The highest concentrations of P450 enzymes in the kidney are found in the S₃ segment of the proximal tubules in the renal cortex [187]. LPS treatment caused the induction of renal CYP4A mRNAs in both rats [132,188] and SV/129 mice [152]. In contrast, LPS injection decreased renal CYP 4A1 and CYP 4A activity 4 h after injection in rat [189].

Anwar-Mohamed *et al.* reported that LPS injection was associated with the down-regulation of CYP1A1, 1B1, 2C11, and 2J3 mRNAs in rat kidneys and hearts [188].

These authors also reported that CYP4F1, 4F4, and 4F5 were downregulated in the kidney, whereas Kalsotra *et al.* previously found no change of CYP4F1, 4F5, and 4F6 after LPS treatment [153]. The downregulation of the CYPs involved in arachidonic acid epoxidation (2C11 and 2J2) was accompanied by a concomitant decrease in epoxyeicosatrienoic acids produced by these enzymes [188].

CYP2E1-mediated chlorzoxazone-6-hydroxylase activity and CYP2E1 mRNA and protein levels, as well as mRNAs of CYP4A2 and 4A3 were increased in rat kidneys 48 h after treatment with the particulate irritants SiO₂, BaSO₄, and kaolin [132]. BaSO₄ also induced renal CYP4F1 and 4F6 expression [153].

Infection with *C. rodentium* did not affect renal expression of most P450 isoforms (Cyp1a2, 2a5, 2c29, 2e1, 3a11, and 3a13) in C3H/HeOuJ mice. However, renal Cyp4a10 and 4a14 mRNAs were significantly downregulated [190]. Renal UGT isoforms were also relatively unaffected, except for Ugt2b5 which was induced [158]. The oxidation of *trans*-diethylstilbestrol to diethylstilbestrol-4',4'-quinone by P450 in microsomes of estrogen-induced kidney tumors of male hamsters was only 10–20% of the rate compared to control kidney microsomes [191]. Significantly, total P450 content of the tissue surrounding the tumors was also reduced compared to control tissue. In contrast, traumatic brain injury in rats resulted in an elevation of renal CYP1A and CYP4F levels measured 24 h and two weeks after injury [153].

19.11.4 Central Nervous System

CYP1A1, CYP1A2, CYP2B1, CYP2B2, and CYP2E1 proteins are detected in the rat and/or human brain [192], and CYP2C29, CYP2D4, CYP2E1, CYP4F, and CYP3A9/11/13 are known to be expressed in brain [193]. Recently, higher levels of expression of CYP3A43 were discovered in brain as compared to liver across different ethnic populations [194].

The i.c.v. injection of LPS has been employed as a model of CNS inflammation, as it produces a robust inflammatory response in the brain characterized by the activation of microglia and astrocytes and the induction of inflammatory cytokine (TNF α , IL-6, and IL-1 β) production [18,195,196]. This causes downregulation of CYP1A proteins and associated ethoxyresorufin *O*-dealkylase (EROD) activity in the brain [196]; reduced EROD activity in the brain was also observed after systemic LPS injection [195].

In rats with inflicted traumatic brain injury, hippocampal expression of CYP4F4 and 4F5 is transiently elevated before being downregulated, and hippocampal CYP4F proteins are reduced to 20% of control within 24 h [154]. At this time point, CYP4F1, CYP4F4, CYP4F5, and CYP4F6 are all substantially downregulated in the frontal and occipital lobes of the brain as well [197].

19.12 DRUG TRANSPORTERS

DTs play important roles in drug metabolism and disposition, and they can be classified into efflux and uptake transporters. The largest family of efflux transporters is the ATP-binding-cassette (ABC) family, which are involved in the cellular efflux of many clinically important drugs. The family of uptake solute carriers (SLCs) also plays a central role in the cellular trafficking of numerous drugs [198].

Among the ABC drug efflux transporters, PGP/ABCB1, which is encoded by the multidrug resistance gene *MDR1* in humans and *MDR1a* and *MDR1b* in rodents, multidrug resistance-associated proteins (MRP1–3/ABCC1–3), and breast cancer resistance protein (BCRP/ABCG2) have particularly important roles in clinical drug disposition [198]. These transporters are highly expressed in epithelia of the intestine, liver, kidney, placenta, and blood–brain barrier, and they display a great deal of substrate overlap [198]. In addition, PGP, BCRP, and MDRs are known for their involvement in the extrusion of anticancer drugs from tumor cells and development of multidrug resistance. Members of the organic anion-transporting peptide (OATP, SLC21) family, especially OATP1 and OATP2, are important in the cellular uptake and oral bioavailability of a wide array of anionic drug substrates.

Inflammation also causes alterations in the expression and activity of DTs, contributing to changes in the absorption, distribution, and clearance of drugs and resulting in, for example, altered toxicity and compromised secretion of drugs into bile or urine [134,198]. Effects of inflammation on DT are described in a recent review by Teng and Piquette-Miller [199], and are summarized here in Table 19.5.

TABLE 19.5 Effect of LPS and Turpentine-Induced Inflammation on Various Drug Transporters

Transporter	Species	Tissue	LPS	Turpentine
MDR1a	Mouse	Liver	↓	↓
	Rat	Liver	↓	↓
	Rat	Intestine	—	↓
	Rat	Brain	↓	—
	Rat	Heart	↓	—
	Rat	Placenta	—	↓
MDR1b	Mouse	Liver	↓	↓
	Rat	Liver	↑	—
		Intestine	—	↔
	Rat	Placenta	—	↓
	Rat	Kidney	↔	—
ABCC1 (MRP1)	Rat	Brain	↓	—
		Placenta	↓	—
MDR2	Mouse	Liver	↓	—
	Rat	Liver	↓	—
MRP2	Mouse	Liver	↓	↓
	Rat	Liver	↓	
	Rat	Intestine	—	↓
MRP3	Mouse	Liver	↓	↓
	Rat	Intestine	—	↔
OATP2	Mouse	Liver	↓	↓
	Rat	Liver	↓	—
OATP4	Mouse	Liver	↓	—
BSEP	Mouse	Liver	↓	↓
NTCP	Mouse	Liver	↓	↓
	Rat	Liver	↓	—

Source: This table is adapted from the review article by Teng and Piquette-Miller [199].

19.12.1 LPS Model

As described above, LPS injection is a classic model of the inflammatory response, triggering the release of the proinflammatory cytokines IL-6, IL-1 β , and TNF α and eliciting numerous changes in hepatic DMEs and DTs. In rats, hepatic mRNAs of MRP2, MRP6, OATP1, OATP2, OATP4, sodium taurocholate cotransporting polypeptide (NTCP), bile salt export pump (BSEP), organic cation transporter (OCT)1, and OAT3 were dramatically and rapidly downregulated following injection of LPS. In contrast, the efflux transporter mRNAs MRP1, MRP3, and MDR1b were induced; and MRP5 and OAT2 were unaffected [200]. LPS injection in mice results in mRNA downregulation of many hepatic DTs including Mdr1a, Mdr1b, Mdr2, Mrp2, Mrp6, Oatp1, Oatp2, Oatp4, Bsep, and Ntcp [198,201]; whereas, Mrp1 and Mrp5 are induced [201]. Note that Mdr1b mRNA levels are decreased in mice and increased in rats. Nevertheless, in both species decreased hepatic PGP protein expression is seen [202–204]. The effects of LPS on transporter expression were similar in wild-type (WT) C57BL6/J mice and in mice null for TNF α -receptor 1, IL-1 receptor, IL-6 or inhibitor of κ B kinase β (IKK β) [201].

19.12.2 Sterile Inflammation

Injection of the TLR2 agonist lipotechoic acid caused a transient downregulation of Mrp2 mRNA in mouse liver, whereas Mdr1b was induced and Mrp3 was unaffected [107]. Initial studies detected decreases of 50–70% in the hepatic expression and activity of PGP within 24–48 h after administration of turpentine [205]. These reductions, which occurred at the level of mRNA, were shown to stem from suppression of MDR1a and MDR1b nuclear gene transcription in turpentine-treated rats [206]. The effects of turpentine on hepatic transporter expression in mice are well summarized by Teng and Piquette-Miller: Mdr1a, Mdr1b, Mrp2, Mrp3, Oatp2, Bsep, and Ntcp are all downregulated [207].

19.12.3 Viral Infection

Nakai and coworkers studied the hepatic expression of CYP enzymes and DTs in chronic hepatitis C patients. They found that relative mRNA levels of CYP3A4 and OATP-C all were substantially downregulated compared to non-HCV livers. This correlated with the stage of fibrosis but not with inflammation [208]. CYP 1A2, 2E1, NTCP, and OCT1 were apparently elevated in the F1 stage of fibrosis, and then declined back toward normal levels as fibrosis progressed [208].

Treatment of rats with the TLR3 ligand poly I:C resulted in elevated plasma concentrations of IFN γ , TNF α , and IL-6, and significant downregulation of hepatic MRP2, BCRP, SLCO1A4, and SLC10A1 mRNAs; whereas MDR1b and MRP3 mRNA levels were significantly induced. Hepatic PGP, MRP2, and BCRP were significantly downregulated at the protein level. Many placental transporter mRNAs were also downregulated [146].

19.12.4 Other Inflammatory Diseases

Cholestasis in mice caused by bile duct ligation caused a downregulation of hepatic Oatp1 and induction of Mrps 1, 3, and 5. In the same model Oatps 2, 4, 5, 9, 11,

12, and 14; Mrps 1, 2, 4, 6, 7, and 9; and Ntcp and Bsep were unaffected [201]. The changes in DT expression were similar in mice lacking the genes for TNF α -receptor 1, IL-1 receptor, IL-6, or IKK β , suggesting that compensatory or redundant mechanisms exist for this regulation [201]. In another model, mice inoculated with rotavirus (which causes intrahepatic cholestasis) had a downregulation of canalicular and basolateral hepatobiliary DTs and their regulatory NRs and a concomitant increase in inflammatory cytokines [209].

Several hepatic DTs, as well as CYP enzymes, are altered in animal models of end-stage renal disease (ESRD) [210–212]. For example, PGP is induced in the liver while OATP2 is downregulated. However, the possible contribution of inflammation to these effects is unknown, and uremic mediators may be responsible [211]. Diabetes is another disease with a strong inflammatory component. Streptozotocin-induced diabetic rats display decreased levels of MDR1a, MRP2, and BCRP and increased levels of MDR3 [213].

19.12.5 Regulation in Extrahepatic Tissues

Inflammation affects DT expression in the intestine, and can potentially affect drug absorption and oral bioavailability [198]. The mRNA and protein expression of PGP/MDR1a, MRP2, and CYP3A are downregulated by approximately 50% in the jejunum of LPS-treated rats [177]. The functional effects of these changes on absorption of PGP and MRP2 substrates were demonstrated in intestinal sections isolated from control and LPS-treated rats [177]. In the DSS model of colitis, mice have reduced expression of Mdr1a mRNA and PGP and PXR proteins in their large intestines [214]. In patients with active ulcerative colitis, mRNA and protein levels of BCRP and PGP were significantly reduced in the colons and rectums of individuals with active ulcerative colitis compared with controls or patients in remission, and were negatively correlated with the levels of IL-6 mRNA [215]. MRP2 mRNA, protein, or activity are decreased in the intestine of LPS-treated rats and in several other models of inflammation, including cholestasis and chronic renal failure [198].

PGP is expressed abundantly in several cell types within the CNS [198]. LPS-induced downregulation of PGP/MDR1a in brain tissues has been found to increase intracranial drug accumulation in rodents. Downregulation of PGP protein following i.v. LPS treatment was associated with increased accumulation of doxorubicin in mouse brains [216]. The i.c.v. LPS also downregulated CNS levels of PGP, Oatp2, and Mdr1a mRNAs, accompanied by elevated brain levels of the PGP substrate digoxin [204], demonstrating that localized CNS inflammation produces a loss of PGP function in the blood–brain barrier. The reduction of brain Mdr1a expression was blocked in mice with mutated TLR4, confirming that the activation of TLR4 receptor signaling is the major pathway involved in the reduction of brain Mdr1a expression [134].

Likewise, systemic and CNS inflammation produced by i.p. or intracranial administration of LPS triggered significant decreases in the expression of MDR1a mRNA in rat brain with analogous increases in the brain accumulation of PGP substrates such as ^{99m}Tc -sestamibi and digoxin [198]. Similar to liver, inflammation also resulted in a pronounced decrease of OATP2 in brain [204]. While hepatic mRNA levels of all Mdr isoforms are reduced in LPS-treated mice, Hartmann *et al.* reported that Mdr1b levels

are increased in kidney [217]. However, another study showed decreased renal expression of Mdr1a and Mdr1b mRNA [218]. Further studies are needed to understand the renal regulation of DTs respect to inflammation.

19.13 ROLES OF CYTOKINES

19.13.1 IL-1, TNF α , IL-6, and IFNs

Most investigations of the roles of cytokines in the regulation of DMEs and DTs have focused on IL-1, TNF α , and IL-6, as they are the major proinflammatory cytokines regulating hepatic acute-phase genes. IFNs have been studied because viral infections trigger the production of IFNs, and because IFN inducers can mimic the effects of viral infection on the P450 system [219]. Cytokines administered *in vivo* or incubated with cultured hepatocytes have enzyme-selective effects on CYP expression, and cultured hepatocytes have served as good models for the *in vivo* effects of cytokines [8,219–221].

IL-1, TNF α , and IL-6 as well as IFNs are capable of downregulating basal and drug-induced expression of many different CYP enzymes in cultured human and animal hepatocytes. For example, 72 h of IL-6, IL-1 β , or TNF α treatment resulted in downregulation of mRNA levels and enzyme activities of CYP1A2, CYP2C, CYP2E1, and CYP3A in human hepatocytes [222]. More recently, we showed that, whereas CYP3A4 and CYP2C8 mRNAs were downregulated by IL-6, TNF α , IFN γ , or IL-1 β in primary human hepatocytes within 24 h of treatment, other CYP2Cs and CYP2B6 showed cytokine-specific effects [8]. Of these cytokines, IL-6 downregulated the mRNAs of all CYPs tested except CYP2C18. Exposure of primary human hepatocytes to IFN γ resulted in downregulation of mRNA levels of influx transporters NTCP, OATP2B1, OATP1B1, and OATP1B3, as well as the efflux transporters MDR1, MRP2 protein, MRP3, BCRP, and BSEP [223].

In terms of inducible expression, IL-6, IL-1 α , or TNF α treatment caused suppression of β -naphthoflavone-induced CYP1A1/2 mRNAs (with TNF α as the most potent) and of rifampicin-induced CYP3A4 mRNA (with IL-6 as the most potent) [224].

IL-1 β , IL-6, TNF α , or IFN α all downregulated CYP2C11 mRNA in cultured rat hepatocytes. IL-6 was the most potent but least efficacious of these, and IFN γ had no effect [225]. However, IFN γ treatment did suppress both basal and induced CYP3A expression in rat hepatocytes [226]. Rat hepatocyte CYP4F5 is induced by IL-6, IL-1 β , or TNF α treatment [227]. IL-10, an anti-inflammatory cytokine, suppressed CYP4F1, 4F4, and 4F5 expression [227].

IL-6, IL-1 β , and TNF α suppress both P450- and UGT-dependent enzyme activities in primary pig hepatocytes, with IL-6 effects being slower than those of the other two cytokines [228]. IL-6 and TNF α produce stronger repression of CYP 1A1, 2C8, and 3A4 in porcine hepatocytes than in human hepatocytes [229].

IL- β , TNF α , or IL-6 can also downregulate DTs in hepatocytes [230]. In primary human hepatocytes TNF α or IL-6 each caused the mRNA downregulation of influx transporters NTCP, OATP1B1, OATP1B3, OATP2B1, OCT1, and OAT2, and induced MRP3; whereas, IL-6 but not TNF α suppressed MDR1, MRP2, and BCRP [231]. On the other hand, IFN γ treatment downregulated mRNAs for NTCP, OATP 2B1, OATP1B1, and OATP1B3 as well as the drug efflux pumps MRP1, MRP2, MRP3, BCRP, and BSEP [223].

Many of the effects of cytokines on hepatocytes described above have also been observed when the same cytokines are administered *in vivo*, and this has been reviewed extensively [219]. For example, IFN γ administration to male rats suppressed CYP3A2 mRNA expression and activity [232]. IL-1 and/or TNF α administration resulted in the suppression of total P450 content, activities and/or P450 gene expression in rat and mouse liver [219]. Injection of IL-1 β *in vivo* resulted in the suppression of rat female-specific CYP2C12 mRNA and protein [233], and also caused a decreased expression of CYP2C11 and CYP3A2, but not CYP2E1 in male rats [234]. TNF α administration causes the suppression of rat CYP3A2 and CYP2C11 mRNAs and proteins [235].

Administration of IL-6, IL-1 β , or TNF α to mice has been shown to alter the expression of several DTs, especially via downregulation. These effects are summarized in Table 19.6, where a comparison of the impacts of cytokines on DTs in mice *in vivo* with those in cultured human hepatocytes reveals much commonality between the two model systems [199,230]. IL-1 β , IL-6, and TNF α also caused differential effects on DT expression in human and murine hepatoma cell lines [199].

TABLE 19.6 Comparison of Cytokine Effects on Hepatic Transporter Expression in Mouse

Transporter	Liver and Human Hepatocytes		
	Cytokine	Mouse liver	Primary Human Hepatocytes
Mdr1a	IL-1 β	↓	↓
	IL-6	↔	↓
	TNF α	↔	↔
Mdr1b	IL-1 β	↔	—
	IL-6	↓	—
	TNF α	↑	—
Mdr2	IL-1 β	↓	—
	IL-6	↓	—
	TNF α	↔	—
MRP2	IL-1 β	↓	↓
	IL-6	↓	↓
	TNF α	↓	↔
MRP3	IL-1 β	↔	↓
	IL-6	↔	↔
	TNF α	↓	↔
OATP2	IL-1 β	↓	↓
	IL-6	↓	↓
	TNF α	↓	↓
BSEP	IL-1 β	↓	↓
	IL-6	↓	↔
	TNF α	↔	↓
NTCP	IL-1 β	↓	↓
	IL-6	↓, ↔	↓
	TNF α	↓	↓

Source: This table is adapted from the review articles of Teng and Piquette-Miller [199] and Fardel and Le Vée [230].

Most of the *in vivo* effects of cytokines on human drug metabolism have been observed with type I IFNs α or β , and these studies are well summarized in a recent review by Mahmood and Green [5]. The effects observed were highly variable and often contradictory. The response of DMEs to IFNs is obviously influenced by genetic, environmental, or other factors.

19.13.2 Other Cytokines

Data for cytokines other than the ones mentioned above is scant, and there is a need for more research in this area. The most information exists for IL-2, which is produced by T cells upon T-cell receptor activation, and functions in T-cell development and killer T-cell activation. Injection of IL-2 in rats modestly induced CYP2D mRNA and protein, as well as bufuralol demethylase activity. Several other microsomal drug metabolizing activities were also induced, whereas others were unaffected [236]. On the other hand, IL-2 treatment of cultured rat hepatocytes for 24 h was associated with downregulation of CYP3A2 and 2C11 mRNAs as well as proteins of the 1A, 2B, 2C, 2D, and 3A subfamilies [33], and these effects were attributed to increased expression of the transcription factor c-myc [237]. IL-2 receptor was identified on hepatocytes by immunofluorescence labeling. IL-2 also downregulated rifampicin induced, but not constitutive, CYP3A6 expression in cultured rabbit hepatocytes [238]. Patients with secondary hepatic cancer receiving treatment with IL-2 had reductions in the hepatic protein levels and activities of CYP1A2, CYP2C, CYP2E1, and CYP3A4 enzymes that were dependent on the IL-2 dose [239]. Cell culture studies revealed that IL-2 was able to downregulate CYP3A4 only when hepatocytes were cocultured with KCs [240]. Basiliximab and daclizumab are inhibitory anti-IL-2 receptor (α -chain) monoclonal antibodies approved by the FDA in 1998 to reduce organ transplant rejections. Daclizumab also tested successfully in a phase II trial for MS. Pediatric patients given basiliximab experienced high cyclosporine blood levels and toxicity [241]. This suggests that the antibody is countering an induction of CYP3A4 by IL-2, which is puzzling in view of the clinical observation of downregulation by IL-2 treatment [239].

IL-4 has important roles in T-cell development as well as in humoral and adaptive immunity. Its well-characterized targets are T cells and B cells. CYP2E1 mRNA was rapidly induced by IL-4 in human B16A2 hepatoma cells, which involved two independent signaling pathways: IRS1/2 via NFATc1 and a Stat6-dependent pathway [242]. In human hepatocytes, three days of IL-4 treatment induced the expression of CYP2E1 as well as GSTA1/2 mRNAs. [243], but the length of treatment may suggest an indirect effect.

IL-8 is a chemokine (chemotactic for neutrophils) and also has angiogenic activity. We found that IL-8 had no effect on the expression of CYP2C11 in rat hepatocytes, at concentrations up to 10 ng/mL [244]. However, IL-8 induces expression of the retinoic acid-catabolizing enzyme CYP26A1 in leukemia cells [245]. Since CYP26A1 contributes significantly to the hepatic clearance of the chemotherapeutic agent all-trans retinoic acid, its regulation in liver by IL-8 might be important to study [246].

IL-10 is a monocyte-derived cytokine that is considered to be anti-inflammatory, as it antagonizes many inflammatory pathways. However, when healthy humans were given 8 mg/kg IL-10 for six days, it evoked an APR characterized by reduced albumin and elevated ferritin in the serum [247]. CYP3A4-mediated midazolam clearance was

modestly inhibited by 12%, but there was no effect on CYP1A2-, 2D6-, or 2C9-catalyzed drug clearance [247]. Another study found no effect of IL-10 treatment on the pharmacokinetics of prednisolone, a CYP3A4 substrate [248,249]. We found that IL-10 suppressed CYP4F1, 4F4, and 4F5 expression in rat hepatocytes [227].

IL-11 is a member of the IL-6 family of cytokines whose receptors dimerize with the gp130 protein to initiate cellular signaling. It is an inducer of APPs, and we showed that it also downregulates CYP2C11 mRNA in rat hepatocytes with an EC₅₀ of ~100 pg/mL [244].

IL-17 is actually a family of highly related cytokines that are considered to be proinflammatory due to their ability to stimulate cytokine production from many cell types. It induces the expression of the cholesterol-catabolizing enzyme CYP7B1 in synovio-cytes, where it may be involved in maintenance of inflammation in rheumatoid arthritis [250]. The effects of IL-17 on liver or on drug metabolizing P450s are not known.

19.13.3 Knockout Mice

The aforementioned abilities of cytokines to downregulate P450s or DTs in hepatocyte model systems and in animals *in vivo* does not address their physiological importance. To address the question of what cytokines are important for the *in vivo* regulation of P450 isoforms and DTs caused by a given stimulus, cytokine null mice or cytokine receptor null mice have been used.

IL-6 is suggested to be the most important cytokine modulating the hepatic expression of APPs [84], and studies in IL-6-null mice also reveal it to be important in inflammatory regulation of P450 enzymes and DTs. Downregulation of Cyp1a2, Cyp2a5, and Cyp3a11 [84] and of Ntcp, Oatp1, Mrp3, Mrp2, and Bsep [251] mRNAs during turpentine-induced inflammation was abrogated in IL-6-deficient mice. IL-6-deficiency also blocked the downregulation of Cyp3a11 and 2c29 in mice treated with tuberculosis vaccine, while IL-1 β gene deletion had no effect on Cyp3a11 or 2c29 downregulation in tuberculosis vaccine-treated mice [128]. We recently reported similar evidence for the involvement of IL-6 in the regulation of hepatic Cyp2d9, 3a11, and 3a13, in mice infected with *C. rodentium* [64], although the majority of CYPs were regulated in an IL-6-independent manner. These findings were in sharp contrast to those in the LPS model of inflammation, in which IL-6-null mice exhibited regulation similarly to WT of all the P450 enzymes [84] and DTs [201] that were studied. Similarly in WT, IFN γ -null mice likewise had responses of hepatic P450s to LPS injection [64] similar to WT. Compared to WT, mice deficient in both TNF α receptors also had similar responses of P450 enzymes and activities to LPS [104]. Overall, the above results suggest a functional redundancy of cytokines during LPS-induced inflammation [84], such that the absence of any one will not greatly affect the regulation of DMEs or DTs.

19.13.4 Anti-Cytokine Antibodies and Biologic Drugs

One of the disadvantages of knockout mouse models is the possibility of compensatory mechanism developing over time. An alternative approach is to administer neutralizing antibodies to the cytokine in question. Ling and Jamali used this strategy to demonstrate the importance of TNF α to downregulation of CYP1A and CYP3A proteins. The therapeutic TNF α antibody infliximab partially inhibited the downregulation of these proteins in a rat model of adjuvant arthritis [7]. Similarly, in a different rat model

of rheumatoid arthritis, Ashino *et al.* demonstrated that polyclonal antibodies to IL-6 reversed the downregulation of CYP3A mRNA, protein and activity in the liver [6]. The fact that both TNF α and IL-6 seem to be involved in downregulation of CYP3As in arthritis probably reflects the fact that these cytokines can regulate each other's production. These observations in rats are proof of principle for the DDDIs discussed in Section 19.2.3. One such possible interaction for IL-2 is suggested by the study described in Section 19.13.2

19.14 MECHANISMS OF REGULATION

As described above, proinflammatory cytokines are thought to mediate most of the effects of inflammation on hepatic DME and DTs. Consequently, most mechanistic studies have focused on the effects of individual cytokines. Because cytokines exert enzyme-selective effects on P450 gene products, it is important to recognize that no single common pathway has been identified that is responsible for the downregulation of DMEs and DTs. Different mechanisms are likely to pertain depending on the specific gene and inflammatory stimulus and also on the time point in the response.

19.14.1 Transcriptional Regulation

In rat liver, the transcription of CYP2C11, 3A2, and 2E1 is suppressed to 20%, 30%, and 10% of control, respectively, within 1–2 h of LPS treatment in rats [252]. The swiftness and size of these effects suggest that transcriptional suppression is the primary mechanism for the decline of CYP mRNAs.

During an LPS-induced APR, mediated primarily by LPS and proinflammatory cytokines, the expression and activation states of many hepatic transcription factors change [253]. It must be recognized that the transcription factors that predominate in basal and drug-induced expression of DMEs and DTs are different, and that therefore the mechanisms of transcriptional downregulation will also be different between the two states [221].

The transcription factor NF- κ B, a major regulator of inflammation, can be activated by many stimuli including cytokines via their receptor-associated kinases [254], viruses, oxidative stress, and chemical agents [255,256]. NF- κ B has been implicated in the regulation of P450s via several different effects on their induction mechanisms [256,257]. The first mechanism is that NF- κ B can regulate NR-mediated, inducible DME gene expression indirectly by suppressing expression of NRs and/or modulating their binding partner (e.g., RXR). For example, hepatic CAR and RXR expression is repressed in LPS-treated mouse liver [258], and IL-6 stimulation causes a reduction of PXR expression in primary human hepatocytes [259]. IL-1 β decreases CAR expression and decreases phenobarbital-mediated induction of CYP2B6, CYP2C9, CYP3A4, UGT1A1, GSTA1, GSTA2, and SLC21A6 messenger RNA through chromatin remodeling by glucocorticoid receptor (GR)/NF- κ B interaction [260]. NF- κ B can interact with GR, PPAR, RXR, or farnesoid X receptor (FXR) [256].

The NF- κ B pathway can also interact directly with NRs governing DMEs and DT expression [256,257]. Thus, inflammatory stimuli can suppress the induction of CYP3A4 by binding of the NF- κ B p65 subunit to RXR, inhibiting PXR-RXR binding

to CYP3A4 promoter [261]. Inflammatory stimuli can regulate aryl hydrocarbon receptor (AhR) regulated genes (*CYP1A1*, *IA2*, and *1B1*). NF- κ B binds to AhR, and these two transcription factors can mutually repress each other's functions [262].

We found that NF- κ B plays a role in the downregulation of CYP2C11 in rat hepatocytes in response to LPS or IL-1 β , through direct binding to a negative response element spanning the transcription start site of the *CYP2C11* gene [263]. NF- κ B binding can also be identified to the promoter regions of the *CYP1A1*, *CYP2B1/2*, *CYP2C11*, and *CYP2D5* genes by electrophoretic mobility shift assay (EMSA) [256], although this binding has not been shown to be functional.

IL-6 causes a moderate induction of the mRNA of the transcription factor CCAAT-enhancer binding protein- β (C/EBP β , enriched in liver). However, a marked increase in the translation of a C/EBP β isoform lacking a transactivation domain resulted in downregulation of CYP3A4 mRNA in hepatoma cells [264]. C/EBP binding to the promoter regions of CYP2D5 and CYP2B1 measured by EMSA is increased by LPS-induced CNS inflammation, suggesting that C/EBP could negatively regulate the gene expression of CYP2B1 and 2D5 [265].

Hepatocyte nuclear factors (HNFs) regulate the basal transcription of many CYP genes, and many of these factors are downregulated in inflammation [253]. DNA binding activities of HNF1 α , HNF3 β , and HNF4 α are all rapidly suppressed in the rat liver following LPS treatment [252]. Therefore, it is likely that downregulation of HNF expression or activities during inflammation could contribute to the regulation of DME and DT expression [221]. Downregulation of HNF1 α by LPS administration appears to contribute to the downregulations of CYP27A [266], NTCP [267], and OATP4 [268].

The question of whether downregulation or antagonism of NRs responsible for the induction of DMEs and DTs is also a mechanism for the downregulation of basal DME and DT expression is still open to debate. On the one hand, the phenotypes of PXR and CAR-knockout mice suggest little importance of these receptors in basal DME expression. In agreement with this idea, we found that LPS treatment downregulates hepatic P450 expression to a similar extent in PPAR α or PXR-null mice compared to WT [83], indicating that downregulation of P450 during inflammation does not require PPAR α and PXR. On the other hand, siRNA-mediated knockdown of PXR attenuates downregulation of CYP3A4 in human hepatocytes [269]. This could reflect a species difference in the role of PXR in basal P450 expression.

19.14.2 Posttranscriptional Regulation

As detailed in a previous review [219], there is an abundance of kinetic evidence that posttranscriptional mechanisms such as regulation of RNA degradation, protein synthesis, and protein degradation also function in the inflammatory regulation of DMEs and DTs. As a recent example, hepatic CYP2E1 mRNA was rapidly induced in the livers of rats treated with LPS via the i.p. or i.c.v. routes, and this induction persisted for 24 h, during which period the CYP2E1 protein was downregulated [140]. The mechanism of this posttranscriptional regulation is not clear.

Nitric oxide (NO) is a short-lived free radical produced during inflammatory and infectious conditions. We have shown that in cultured hepatocytes, NO formed by inducible nitric oxide synthase in response to an inflammatory stimulus causes a post-transcriptional downregulation of rat CYP2B [270] and CYP3A [271], as well as human CYP2B6 proteins [272]. For the rat CYP2B and CYP3A enzymes this is due to

increased proteasomal degradation of the enzymes [271]. In addition, NO can inhibit the activities of P450 enzymes by coordination to the P450 heme [221]. The *in vivo* relevance of these NO-dependent mechanisms has yet to be established.

19.15 CONCLUSIONS AND FUTURE PERSPECTIVES

The data from animal studies and the limited experiments in humans demonstrate that drug metabolism and transport can be profoundly affected in either direction in inflammatory disease states, and crucially, that this regulation depends on both the nature of the disease/inflammatory stimulus as well as on the specific protein in question. Much more research is needed into the regulation of DMEs and DTs in humans in specific inflammatory disease states, including diseases such as diabetes that have a chronic inflammatory component, and also into identification of biomarkers that can predict these changes. This will require not only clinical research in humans, but also mechanistic research in animal and cell culture models to identify blood-borne mediators, receptors, and signaling mechanisms involved. The effects of many cytokines on drug metabolism and transport have yet to be studied, as have the roles of noncytokine mediators and NLRs.

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1 Bioanalytical Support for Both *In Vitro* and *In Vivo* Assays Across Drug Discovery and Drug Development

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1.1 Summary	1
1.2 Introduction	2
1.3 Bioanalytical strategies	3
1.4 <i>In vitro</i> assays	3
1.5 <i>In vivo</i> assays	8
1.6 Conclusions	11
References	11

1.1 SUMMARY

Bioanalysis (BA) in a drug metabolism environment can be defined as the analytical techniques that are utilized to assay various samples that are the result of *in vitro* or *in vivo* studies of one or more new chemical entities (NCEs). BA has continued to evolve in response to the needs of the drug discovery and drug development teams as well as the continuing improvement of the analytical technology that is used for the various assays. This chapter provides an overview of the various assays that are commonly used in various stages of drug discovery and drug development. The chapter is divided into three main sections: bioanalytical strategies, *in vitro* assays, and *in vivo* assays.

For most of the assays discussed in this chapter, the analytical tool is high performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS). HPLC-MS/MS is preferred because it provides excellent selectivity and is applicable to most small molecules. The strategy for how best to use HPLC-MS/MS for the different *in vitro* and *in vivo* assays is still a topic of debate within the bioanalytical community.

It is common to have different levels of analytical acceptance criteria that become stricter as one moves from the early lead optimization arena and moves into the development arena. Using this strategy, the higher throughput assays that are used in the

screening stage would have minimal standards needed as part of the assay. This makes sense because these assays usually are used for many compounds with only a small number of samples per compound. As one goes to the higher levels, relatively smaller numbers of compounds are being assayed and the number of samples per compound is higher. So, for the higher level assays, it makes sense to increase the number of standards that are required to perform the assay.

There are multiple *in vitro* assays that are used for drug discovery screening of NCEs. The basic strategy for utilizing these various *in vitro* assays is to use them to screen NCEs in order to select those that have druglike properties. These tests are commonly used as part of the absorption, distribution, metabolism, and excretion (ADME) optimization phase of new drug discovery. The most commonly used assays are designed to measure one of the following parameters: permeability, metabolic stability, enzyme inhibition, or metabolism. Each of these assay types is described in this chapter as a subtopic.

In a drug discovery setting, *in vivo* assays are similar to *in vitro* assays in terms of the need for providing the results in a timely manner. The main difference between *in vitro* and *in vivo* assays is that *in vivo* assays are often more challenging because the sample matrix is more complex. Most *in vivo assays* rely on some form of HPLC-MS/MS for the analytical step. The main topics covered in this chapter are: pharmacokinetic (PK) screening, PK studies, and metabolite identification.

In conclusion, mass spectrometry is used throughout new drug discovery and drug development for multiple assays. The type of mass spectrometer that is used depends on the type of assay that is being performed. As mass spectrometers evolve and are improved, it can be safely predicted that they will continue to be the premier analytical tool for many of the assays that are performed at various steps in this process.

1.2 INTRODUCTION

BA in a drug metabolism environment can be defined as the analytical techniques that are utilized to assay various samples that are the result of *in vitro* or *in vivo* studies of one or more NCEs. BA has continued to evolve in response to the needs of the drug discovery and drug development teams as well as the continuing improvement of the analytical technology that is used for the various assays. As shown in Fig. 1.1, BA can be considered the cornerstone for the whole drug discovery process. Multiple review articles and book chapters have been published on the topic of BA used in a drug metabolism environment, and the reader may want to consult these in order to gain additional perspectives on this topic [1–14]. For a comprehensive review of all of these assays, one could also consult the book by Kerns and Di [15].

As shown in Fig. 1.1, lead optimization is a critical step in the drug discovery process. One of the goals of the lead optimization step is to improve the drug metabolism and pharmacokinetic (DMPK) properties of the initial lead compounds in order to find new compounds that are active in the pharmacological model and have acceptable DMPK properties. In order to improve DMPK properties, hundreds of NCEs are tested in various *in vitro* assays. The goal of these studies is to find compounds that can be predicted to have human DMPK properties that are acceptable for the proposed use of the compound [14–21].

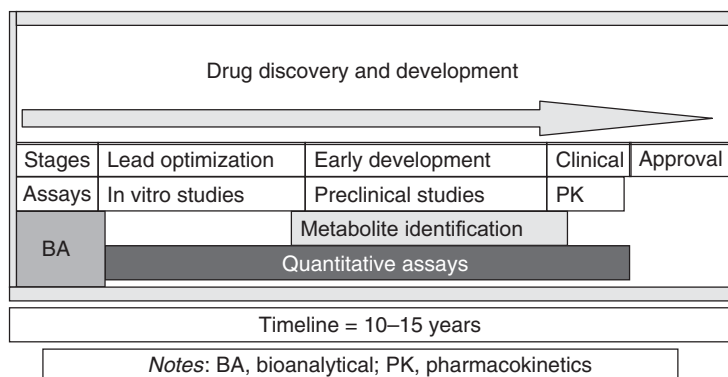


Figure 1.1 Schematic showing the various stages of new drug discovery and development as well as how bioanalysis (BA) is the cornerstone to the whole process.

This chapter provides an overview of the various assays that are commonly used in various stages of drug discovery and drug development. The chapter is divided into three main sections: bioanalytical strategies, *in vitro* assays, and *in vivo* assays. The chapter concludes with a summary that serves as a condensed version of the topic.

1.3 BIOANALYTICAL STRATEGIES

For most of the assays discussed in this chapter, the analytical tool is HPLC combined with MS/MS. HPLC-MS/MS is preferred because it provides excellent selectivity and is applicable to most small molecules [5,6,13,14,22–25]. The strategy for how best to use HPLC-MS/MS for the different *in vitro* and *in vivo* assays is still a topic of debate within the bioanalytical community.

It is common to have different levels of analytical acceptance criteria that become stricter as one moves from the early lead optimization arena and moves into the development arena. Table 1.1 shows how this type of strategy might be utilized.

Using this strategy, the higher throughput assays that are used in the screening stage would have minimal standards needed as part of the assay. This makes sense because these assays usually are used for many compounds with only a small number of samples per compound. As one goes to the higher levels, relatively smaller numbers of compounds are being assayed and the number of samples per compound is higher. So, for the higher level assays, it makes sense to increase the number of standards that are required to perform the assay.

1.4 IN VITRO ASSAYS

There are multiple *in vitro* assays that are used for drug discovery screening of NCEs [7,21,26–29]. The basic strategy for utilizing these various *in vitro* assays is to use them to screen NCEs in order to select those that have druglike properties [21,30–34]. These tests are commonly used as part of the ADME optimization phase of new drug discovery [14,35–37]. The most commonly used assays are designed to measure one

TABLE 1.1 Bioanalytical Strategies for Various Stages of Drug Discovery

Level	Drug Stage	Major Analytical Criteria	Example Assay	GLP ^{a?}
0	Early screening	No standard curve	Caco-2 cell	No
1	PK ^b screening	Two-point standard curve	CARRS	No
2	Lead optimization	Multipoint standard curve (no QCs ^c)	Rat PK study	No
3	Lead qualification	Multipoint standard curve plus QCs	Multidose rat PK study	No
4	Development	Follow GLP rules	TK ^d studies	Yes

^aGood laboratory practices.^bPharmacokinetic.^cQuality control.^dToxicokinetic.

of the following parameters: permeability, metabolic stability, enzyme inhibition, or metabolism. Each of these assay types is described in this section as a subtopic.

1.4.1 Permeability

The goal of the various permeability assays is to measure the ability of a compound to cross a cell membrane. This is an important characteristic of a druglike molecule that could be developed into an orally dosed drug. In order for an orally dosed drug to be effective, it must be absorbed across the intestinal gut membrane. The various permeability assays strive to measure the potential for a compound to be absorbed.

The first cell-based assay for permeability measurement is the Caco-2 assay [38]. The Caco-2 cell line is an immortal human colon carcinoma cell line that was commercialized for this assay. It is available in multiple formats, but each of these shares the same functional design. The experimental setup includes a chamber with two sides (apical and basolateral) that contain liquid and are separated by the cell membrane. The test compound can be added to either side. Once the experiment is completed, a sample of the liquid from each side is taken and assayed for the test compound.

The assay is typically performed using HPLC-MS/MS. Various HPLC-MS/MS procedures have been described in the literature [39–48]. Because the goal of the assay is to measure the ratio of the test compound concentrations of the two compartments, in some cases the HPLC-MS/MS analysis merely provides a response level for each compartment; the ratio of the response levels is equal to the ratio of the compound concentrations of the two compartments [49].

In addition to being used as a drug discovery screen, the Caco-2 assay has also been validated as an *in vitro* assay for development compounds to assess their potential to act as either substrates or inhibitors of P-glycoprotein (P-gp) [50]. When used in this manner, compounds are subjected to “bidirectional” Caco-2 assays, in which the test compound is added to the apical side and basolateral side in separate experiments. This test measures the efflux ratio for the compound to determine if it is a substrate for

P-gp. Additional measurements can then be made with known model compounds to determine if the test compound is an inhibitor of P-gp. These results are then suitable to be included in regulatory submission documents.

Another permeability assay is the parallel artificial membrane permeability assay (PAMPA). The advantage of PAMPA is that the artificial membrane is simple to maintain and the assay can be performed using either HPLC-ultraviolet (UV) detection or even a UV plate reader [15]. The main disadvantage of PAMPA is that it measures only passive diffusion (there is no P-gp component as there is with Caco-2). Some drug discovery practitioners have suggested using PAMPA as an initial screen for permeability and waiting until the end of the lead optimization stage to use the Caco-2 cell assay [51].

1.4.2 Metabolic Stability

The goal for the metabolic stability assay is to measure the metabolic liabilities of a test compound. The two most common metabolic stability assays are based on either liver microsomes or hepatocytes. The advantage of using liver microsomes is that they are relatively inexpensive. The disadvantage of liver microsomes is that they only demonstrate phase I (CYP-mediated) metabolites unless additional cofactors are added to allow for phase II (conjugated) metabolism. The disadvantage of hepatocytes is that they are relatively expensive. The advantage of hepatocytes is that they are able to produce both phases I and II metabolites. In some cases, liver microsomes are used as an early screen for metabolic stability and hepatocytes are reserved for those compounds that are potential candidates for development.

Regardless of the microsomal stability process that is chosen, the bioanalytical challenge is the same. For a higher throughput metabolic stability assay, one might need to develop HPLC-MS/MS assays for 100–200 NCEs per week. Typically, this requires automated MS/MS method development software tools in order to meet this analytical challenge. Currently, most vendors for triple quadrupole MS/MS systems will include this type of software in their system. Kieltyka *et al.* [52] have described the utility of QuickQuan™ (Thermo) for the automated MS/MS method development of 500 compounds per month in order to use HPLC-MS/MS to assay these NCEs for metabolic stability. In their application, the compounds were tested in human, rat, and mouse microsomes for metabolic stability. They reported a success rate of >95% when using the automated method development procedure on more than 25,000 compounds.

1.4.3 Enzyme Inhibition

The goal of enzyme inhibition assays is to estimate the potential for a compound to produce drug–drug interactions due to enzyme inhibition in a clinical setting. The assays used for this screen are based on testing the effect of the NCE on cytochrome P450 (CYP) activity by monitoring various well-known CYP substrates. There are a small number of CYP isozymes that account for most of the CYP activity. For example, 50% of all drugs are metabolized by CYP3A4 and 30% of all drugs are metabolized by CYP2D6 [15]. If an NCE was found to significantly inhibit CYP3A4 or CYP2D6 at concentrations in the 1–10 μ M range that could be a potential development issue for that NCE.

There are two common strategies for screening NCEs for their enzyme inhibition potential. The first strategy uses recombinant CYP isozymes and fluorogenic probe substrates. The assay readout is based on a fluorescence plate reader. The advantage of this assay is that it is high throughput and relatively low cost. The second strategy (considered the “gold standard” assay) is based on human liver microsomes (HLMs) and various probe substrates. The assay for this method is normally based on HPLC-MS/MS. Bell *et al.* [53] compared these two strategies and found that for NCEs, the fluorescence-based assay often produced results that did not correlate well with the HPLC-MS/MS-based assay.

Multiple authors have described procedures for assaying enzyme inhibition potential using HLMs with various probe substrates and an HPLC-MS/MS assay [54–62]. The main differences in these assays are: (i) how many probe substrates are measured and (ii) what methodology was used for the HPLC-MS/MS assay. Chu *et al.* [59] described a 0.5 min HPLC-MS/MS assay that was able to provide enzyme inhibition potential results for CYP3A4 and CYP2D6. Plumb *et al.* [60] made use of ultra-performance liquid chromatography (UPLC) combined with MS/MS to assay six probe substrates in 0.5 min.

1.4.4 Protein Binding

Protein binding is another *in vitro* assay that relies on HPLC-MS/MS for the analytical step. The goal of the assay is to measure the free (unbound) concentration of an NCE when it is in human or laboratory animal plasma. Various methods for performing the protein binding experiment have been described [15,63,64]. The typical current strategy for screening NCEs for protein binding is based on 96-well equilibrium dialysis [63,65]. Owing to the fact that many compounds are >99.5% bound, it is important to be able to measure the low concentration of the test compound in the free fraction in these studies. Owing to the low concentrations of some of these samples, it is important to use HPLC-MS/MS for the analytical step. Wan and Rehnberg [66] reported the use of a sample pooling approach for higher throughput screening of protein binding in a discovery setting: their assay was based on HPLC-MS/MS.

1.4.5 *In Vitro* Metabolite Identification

In vitro metabolism studies are a common part of the lead optimization strategy. In a drug discovery setting, the ability to provide metabolite information to the discovery teams is very helpful to the medicinal chemists. For example, knowing the site of oxidation of a compound that has a short *in vivo* half-life can lead to the synthesis of a new compound where that site is modified in a way that blocks the metabolic site; in some cases, this new compound may have a longer *in vivo* half-life. In another example, the metabolite may be active and have a better *in vivo* PK profile—in this example, the metabolite could become the new lead compound in the discovery program.

Typically, in order to perform an *in vitro* metabolism study, one will incubate the NCE in either liver microsomes or hepatocytes which will produce a sample that contains metabolites of the NCE. Microsomes provide a good picture of phase I metabolites (CYP-mediated reactions) while hepatocytes produce both phases I and II (conjugated) metabolites [25]. Multiple literature reviews and book chapters have been written on methods for performing metabolism studies in various matrices [9,25,27,67–75]. These

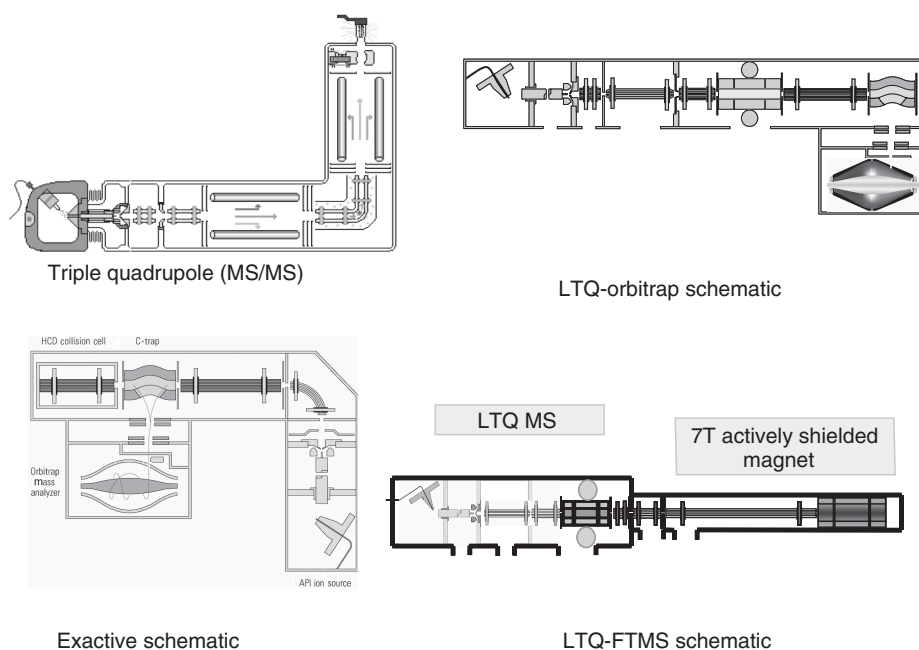


Figure 1.2 Schematic showing the multiple types of mass spectrometers that can be used for various BA assays in new drug discovery and development. *Source:* Figure provided by Jerry Pappas from Thermo-Fisher and used with permission. (See color insert.)

methods all rely on some form of HPLC-MS/MS analysis to find the metabolites and then to further characterize them in order to provide at least a partial structural identification.

There are multiple types of mass spectrometers that have been used for various BA assays. Figure 1.2 shows four example MS systems that have been used by various drug metabolism researchers. While various strategies have been used in the past to find the metabolites in the *in vitro* sample, currently, most researchers would use higher mass resolution mass spectrometers plus one or more software filtering tools to simplify the task of determining what metabolites are present in the sample. Most triple quadrupole mass spectrometers are unit mass resolution (not high mass resolution). Unit mass resolution is sufficient to separate a compound that has a mass of 520.4 from a second compound that has a mass of 521.4. A high mass resolution mass spectrometer can separate a compound that has a mass of 521.45 from a second compound that has a mass of 521.46. This capability (high mass resolution) is very helpful in finding metabolites in a complex matrix because most of the endogenous material will not have the same exact mass as the metabolites. Examples of mass spectrometers that have high mass resolution capabilities are quadrupole-time-of-flight (QTOF) MS systems as well as Orbitrap MS systems [76].

In addition, because many of the metabolites will have predictable masses (e.g., +16.0 for the addition of an oxygen atom to the NCE), one can use specialized software tools in conjunction with the high mass resolution mass spectrometers to look for metabolites. An example of this type of software is the “mass defect filter”

software described by Zhu *et al.* [75,77]. This filter takes advantage of the fact that most druglike chemicals have an exact mass that is different from most endogenous materials (even though the nominal mass may be the same).

1.5 *IN VIVO* ASSAYS

In a drug discovery setting, *in vivo* assays are similar to *in vitro* assays in terms of the need for providing the results in a timely manner. The main difference between *in vitro* and *in vivo* assays is that *in vivo* assays are often more challenging because the sample matrix is more complex. Most *in vivo* assays rely on some form of HPLC-MS/MS for the analytical step [5,6,22–24,78].

1.5.1 PK Screening

PK screening refers to higher throughput *in vivo* studies that are designed to differentiate multiple compounds in the lead optimization phase of new drug discovery. Typically, groups of compounds will be tested in a standardized manner in order to rank order them or screen out compounds quickly. Various researchers have proposed different types of PK screening assays; most of these assays are based on a rodent model.

One of the more popular assays for PK screening was based on the concept of using cassette dosing [79–84]. Cassette dosing makes use of the ability of HPLC-MS/MS to analyze multiple compounds in one assay. This ability allowed the researchers to dose multiple compounds into one laboratory animal and then obtain individual concentrations for each compound over a series of time points such that PK parameters such as area under the curve (AUC) or maximum concentration ($C_{\max}$) could be measured for each compound. Cassette dosing has the advantage that it requires fewer laboratory animals than would be needed for the standard individual dosing model. Several pharmaceutical companies found cassette dosing to be useful for screening NCEs. Cassette dosing also has disadvantages. For example, cassette dosing can produce incorrect PK parameters in many cases [85]. Another disadvantage is that the assay takes more effort to develop due to the mixture analysis and the planning has to take into account the possibility that compounds (or their metabolites) could interfere with each other if they are not separated chromatographically and are isobaric.

Korfmacher *et al.* [86] described a methodical PK screening system that was not based on cassette dosing. The method was called *cassette-accelerated rapid rat screen* (CARRS). The described system was based on cassette analysis where groups of six compounds are orally dosed individually into two rats and six time points (0.5, 1, 2, 3, 4, and 6 h) are taken from each rat. The samples are pooled across the two rats, yielding a total of six plasma samples per test compound. The assay used a simple two-point standard curve. The result was that all the samples for one cassette could be placed on one 96-well plate—this allowed for efficiencies in sample preparation and HPLC-MS/MS analysis. Mei *et al.* [87] reported on a retrospective study of the CARRS data and found that it correlated well with standard (“full”) PK studies. In addition, Mei *et al.* reported that CARRS provided a screening efficiency of about 50% (i.e., about one-half of the NCEs tested in CARRS were found to provide such low oral AUC levels that they were eliminated from further consideration as a potential-lead

compound). CARRS has been used successfully as a PK screening tool on over 20,000 compounds.

Liu *et al.* [88] described an oral PK screening model based on mice that they called *snapshot PK*. Their model used two mice per compound and provided an oral AUC value in a systematic manner similar to the CARRS model. The snapshot PK was reported to have a screening efficiency of >80%. In addition, because the snapshot PK model was based on mice, less compound was required for dosing the two animals than what would be needed for a similar screen in rats (e.g., CARRS).

1.5.2 PK Studies

PK studies whether in a discovery setting or in a “bridging to development” study are normally carried out in one of two ways [oral (PO) only or intravenous (IV)/PO]. If one is trying to get a full PK picture of a compound in a laboratory animal, then it would be common to dose one set of animals via PO administration and another set (same species) via intravenous IV administration at a similar dose. By comparing the AUC values of the IV and PO dose, one can obtain the important parameter, percentage bioavailability (F), using the following equation:

$$\%F = \frac{(AUC_{PO} \times Dose_{IV})}{(AUC_{IV} \times Dose_{PO})}$$

where

F = bioavailability

AUC_{PO} = area under the concentration versus time curve after PO dosing

$Dose_{IV}$ = dose for the IV administered compound

AUC_{IV} = area under the concentration versus time curve after IV dosing

$Dose_{PO}$ = dose for the PO administered compound.

In addition, multiple additional PK parameters can be derived from a single PO/IV PK study [7,15,89]. The second common PK study is a single rising dose (SRD) study. In a PO SRD study, several sets of laboratory animals are dosed with the test compound PO, but the dose is increased with each set. This study can be used to determine if the AUC increases with the increasing dose. A well-behaved (from a PK perspective) compound may show an almost linear relationship between dose and AUC, while many compound will show a nonlinear increase in the AUC versus the dose of the test compound.

There are multiple steps involved in the BA of a PK study. As shown in Fig. 1.3, these steps involve alignment and coordination across various specialties in order to obtain the test compound, dose the compound, collect the samples, and assay the samples. All of this needs to be done in a timely manner when working in a lead optimization environment.

Once a compound is in development, various safety studies are performed using laboratory animals (typically rat and dog or monkey). In these studies, it is common to take blood samples to measure the exposure of the test animals to the test compound—this type of study is referred to as *toxicokinetic (TK) study*. For TK studies, one must always use good laboratory practices (GLPs) when assaying the plasma samples from the study [90–94].

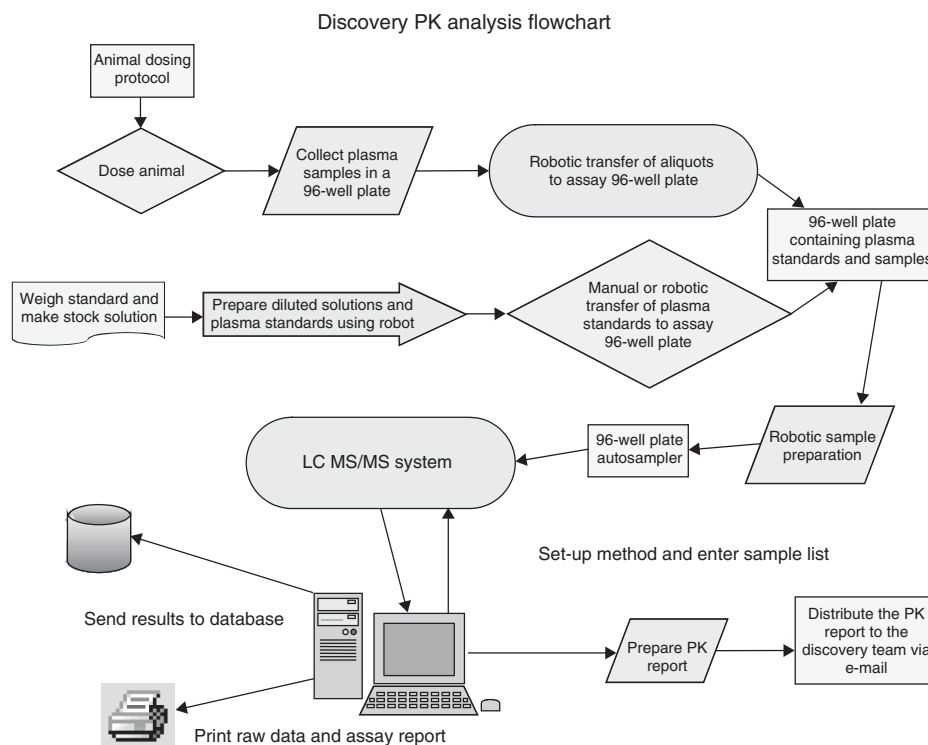


Figure 1.3 Schematic showing the various steps that are taken when assaying compounds in a discovery PK setting. *Source:* Figure was reprinted with permission from Korfmacher [5]. (See color insert.)

1.5.3 *In Vivo* Metabolite Identification

Metabolite identification is an important part of both new drug discovery and drug development [95]. During the new drug discovery stage, metabolite identification can be very helpful in guiding medicinal chemists in two ways: (i) by pointing toward the sites of metabolism on the NCEs, medicinal chemists can modify the lead compounds in ways that could block the metabolism; and (ii) in some cases, if the metabolite is active and has favorable PK properties, it may become the new lead compound in the series.

During the developmental stage, it is important to identify all major human metabolites of the test compound as well as its major metabolites in the rodent and large animal species used in the safety studies [96]. The Metabolites in Safety Testing (MIST) guidelines that were issued by the FDA (US Food and Drug Administration) have added a requirement that metabolite testing must be done using samples from studies where the test compound has reached steady state [97–100]. These requirements have mandated that pharmaceutical companies show that they have similar or higher exposure in animal safety studies than clinical studies for not only the test compound, but also for all major metabolites of the test compound [97–100].

Multiple authors have described various techniques for detecting or profiling metabolites during the new drug discovery stage as well as the development stage [1,3,27,68,69,72,74,101–109]. Most of these methods rely on using either a triple

stage quadrupole (TSQ) MS/MS system or a hybrid quadrupole-linear ion trap (QTrap) for detecting the metabolites and then getting product ion spectra that are needed to provide structural identification of the metabolites [72,110–114]. The QTrap MS/MS system has unique advantages that make it uniquely suitable for both quantitative assays as well as metabolite identification studies [111,115].

Higher mass resolution mass spectrometers have become an important tool for metabolite identification studies. The hybrid QTOF mass spectrometer has been used extensively for this purpose [101,116–118]. One major advantage of the QTOF mass spectrometer is that it can be used to obtain both high mass resolution mass spectra as well as high mass resolution product ion spectra of the metabolite.

If one obtains full scan data in a high mass resolution mode, then one can use special software tools to help differentiate the dosed compound and its metabolites from endogenous background materials. One of the software tools that can be used for this purpose is referred to as a *mass defect filter* [68,75,77,119–121]. The mass defect filter takes advantage of the fact that most pharmaceutical test compounds have an accurate mass that is shifted (less than) the exact nominal mass. This shift can be measured using a high mass resolution mass spectrometer and can be used to differentiate the compound and its metabolites from isobaric (same nominal mass) interferences. The mass defect filter is a software tool that makes this capability easy to use when looking for metabolites of a dosed compound. Zhu *et al.* [108,109] has recently described two additional high mass resolution software filters that can be used to help find metabolites when assaying complex biological matrices.

1.6 CONCLUSIONS

Mass spectrometry is used throughout new drug discovery and drug development for multiple assays. The type of mass spectrometer that is used depends on the type of assay that is being performed. As mass spectrometers evolve and are improved, it can be safely predicted that they will continue to be the premier analytical tool for many of the assays that are performed at various steps in this process.

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2 General Principles of Mass Spectrometry: GC-MS, LC-MS, and LC-MS/MS

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2.1	Summary	1
2.2	Introduction	2
2.3	Gas chromatography-mass spectrometry	3
2.4	Liquid chromatography-mass spectrometry	5
2.5	Single quadrupole mass spectrometry	5
2.6	Triple quadrupole/tandem mass spectrometry (MS/MS)	6
2.7	Ion-trap mass spectrometry (MS ⁿ)	9
2.8	Hybrid and high resolution mass spectrometry	11
2.9	Derivatization reactions to assist in metabolite identification using mass spectrometry	16
2.10	Metabolite identification strategies using LC-MS/MS	17
2.11	Metabolite identification strategies employing high resolution and accurate mass measurement	19
	References	25

2.1 SUMMARY

Gas chromatography (GC) and liquid chromatography (LC) based mass spectrometric approaches represent some of the most commonly employed and informative approaches available for bioanalytical studies. In targeted analyses, such as in metabolite identification or structural elucidation studies, signals from all other components are intentionally minimized by sample cleanup or by the judicious use of selected ion or reaction monitoring or simply ignored if present and separated from peaks of interest. Chemical derivatization and alternative modes of chromatographic separation, ionization, and detection (positive or negative ion) are also often used to separate and further distinguish molecules of interest from background noise and interferences. Information-dependent product ion scans, precursor ion scans, and neutral loss scans add both flexibility and selectivity to analyses, particularly when used in an iterative

manner, where information from previous runs is incorporated into data-acquisition and data-processing strategies. GC- and LC-coupled mass spectrometry (MS) can also be extremely useful for nonbiased or nontargeted analysis, where all chromatographic peaks, or all peaks above a certain intensity, can be characterized by their mass spectral patterns and GC retention indices, thereby enabling their reliable recognition in further samples [1] or their comparison to nontreated controls. The number of potentially reliable and comparable components can be increased by deconvolution of the spectral information using numerical methods incorporated into automated and optimizable software programs such as the automated mass spectrometry deconvolution and identification system (AMDIS, see Ref. 2 or ACD/IntelliXtract™, and for a review of a variety of spectral deconvolution and componentization software, see Ref. 3). Finally, the most robust techniques applied toward structural elucidation, metabolite identification, or the initial detection of putative biomarkers typically involve instruments with sufficient mass accuracy and resolution to provide possible molecular formula. With these most comprehensive approaches, further advances in software systems for spectral interrogation, information processing, and pattern recognition are still needed in order to more fully avail mass spectral investigations with relevant comparisons and scientifically sound conclusions. Thus, the continued development and application of such analytical approaches using time-of-flight (TOF) or ultrahigh mass resolution instruments appear poised to play a significant role in the future of bioanalytical chemistry, particularly as limitations on data processing and interpretation software are further refined.

2.2 INTRODUCTION

Metabolite identification and structural elucidation studies are often complex and time consuming, requiring chromatographic separation, concentration, and detection using numerous spectroscopic techniques such as MS and nuclear magnetic resonance (NMR) spectroscopy. However, such studies have been greatly facilitated by the availability of improved technologies, including (i) temperature and pressure programmable injection ports coupled to capillary GC columns and GC $\times$ GC separation; (ii) ultraperformance liquid chromatography (UPLC); (iii) in-line flow-through radiochemical detectors; (iv) high field (700 MHz or higher) NMR spectrometers capable of direct and indirect detection methods, pulsed field gradients, and liquid chromatography (LC)-coupled (flow-through) or microprobes to allow detection of most NMR active nuclei; and (v) mass spectrometers capable of providing MS^n , high resolution ($>50,000$), and accurate mass (<5 ppm). Advances in MS instrumentation and chromatographic methods have produced further increases in the sensitivity and selectivity of GC-MS and LC-MS techniques, which further facilitate metabolite identification and structural elucidation. Techniques that have increased or enhanced the signal available from the molecule (ion current) or the mass spectrometer include chemical ionization; the use of high energy dynode detectors; and the use of chemical derivatization to increase electronegativity (as in negative-ion chemical ionization), molecular weight, and/or chromatographic suitability. Similarly, advances have been made in methods to decrease the noise, or background, in a particular sample. Solid-phase extraction systems, negative-ion chemical ionization techniques, and MS/MS are examples of efforts to increase signal-to-noise ratios for given analytes by decreasing interferences. Advances in ionization

techniques are being made at a rapid pace, with improved electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and photoionization (PI) sources, for example, showing greater utility in a variety of applications. Finally, hybrid mass spectrometers, such as Q-trap and quadrupole time-of-flight (Q-TOF) systems, offer the additional sensitivity inherent in ion-trap technologies, and ion mobility/TOF mass spectrometers add shape resolution and accurate mass and high mass resolution to the separation potential of analytical methods. In the following sections, the general principles of MS are described, as they are typically applied to GC-MS, LC-MS, and GC-MS/MS or LC-MS/MS to qualitative drug metabolism and bioanalytical studies.

2.3 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

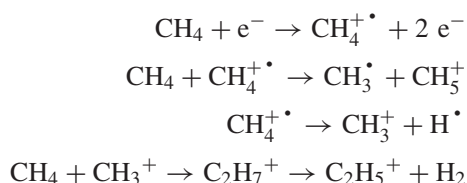
2.3.1 GC-MS Analysis Using Electron Ionization

Electron ionization (EI, also termed electron ejection ionization or electron impact ionization) MS combined with GC is a common method of quantitative and qualitative analysis. It offers the advantages of a relatively simple, reproducible ionization mode, a lower source pressure (less potential for source contamination), and a long history of use in the analysis of pharmaceuticals and drugs of abuse. In this mode of operation, electrons are produced by heating a wire filament that has electric current running through it. The electrons are accelerated and concentrated into a beam traveling in a perpendicular direction to the flow of analytes from the GC column. At around 70 eV, the de Broglie wavelength of the electrons matches the length of common bonds in organic molecules, and energy transfer to organic analyte molecules is maximal, leading to the strongest possible ionization and fragmentation. Under these conditions, about 1 in 1000 analyte molecules in the source is ionized. At lower energies, the interactions between the electrons and the analyte molecules transfer less energy and cause less ionization and fragmentation. At higher energies, the de Broglie wavelength of the electrons becomes smaller than the bond lengths in typical analytes, the molecules then become “transparent” to the electrons, and ionization efficiency decreases [4] and produces considerable fragmentation, resulting in the sample’s ion currents being distributed over a large number of m/z values. The distribution of the sample’s ion currents over a wide m/z range results in decreased sensitivity at any given m/z . If lower energies are used (<20 eV), fragmentation can be decreased, but sample ionization efficiency is also decreased.

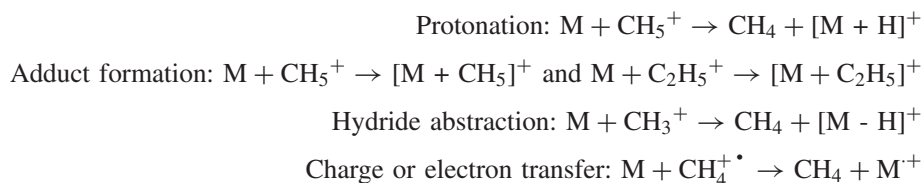
2.3.2 GC-MS Analysis Using Chemical Ionization

GC with chemical ionization MS offers some significant advantages in quantitative analyses. When using single-ion monitoring and an appropriate choice of a reagent gas, most drugs are softly ionized, generating ions at only a few m/z values. Thus, the sample’s ion currents are concentrated in fewer m/z . Furthermore, because the ions that are formed in chemical ionization mode are generally of higher mass than the base ion obtained for the same compound in electron ionization mode, there is a less likelihood of interferences from contaminants or matrix (higher masses are more unique in nature than lower masses). Although chemical ionization with some reagent gases, such as ammonia, is more difficult than electron ionization, the use and optimization of most chemical ionization gases have become fairly straightforward.

As previously noted, chemical ionization is typically a lower-energy mechanism than that occurring under standard electron ionization conditions, such that ions are more often formed that allow the identification of the molecular weight of the compound. In chemical ionization sources, these ions are produced by the collision of the analyte with ions of a reagent gas that are present in a large excess in the ion source. Some common reagent gases include methane, ammonia, and isobutane. Electrons entering the source preferentially ionize the more abundant reagent gas. The resulting collisions with other reagent gas molecules create an ionization plasma. With methane as the reagent gas, a variety of reactions occur, including the following:



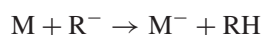
Under these conditions, there are several mechanisms of ionization that can occur with a molecule (M), including protonation, cationization or adduct formation, hydride abstraction, and electron capture or charge transfer:



Many compounds will accept a proton from a donor species to become ionized, and depending on the choice of the reagent gas, the transfer of the proton can occur readily and with excess energy, or will occur with little excess energy transfer. The greater the difference between the proton affinity of the sample and that of the reagent gas, the greater the energy transfer is to the sample during ionization. The excess energy that is transferred produces an analyte ion in a highly excited vibrational state, above a certain point that can lead the sample ion to fragment through the cleavage of covalent bonds. However, some compounds are not particularly stable to protonation (e.g., carbohydrates) or cannot accept a proton readily (e.g., hydrocarbons). In addition to simple protonation, adduct formation often occurs under GC-coupled chemical ionization conditions. For example, with methane as a reagent gas, positive ions at $M + 17$ $[\text{M} + \text{CH}_5]^+$, $M + 29$ $[\text{M} + \text{C}_2\text{H}_5]^+$, and $M + 41$ $[\text{M} + \text{C}_3\text{H}_5]^+$ are also often seen, which when paired with the $[\text{M} + \text{H}]^+$ ion, provide a relatively clear indication of the molecular weight of the analyte for structural elucidation or metabolite identification studies. It is commonplace to denote $[\text{M} + \text{H}]^+$ and $[\text{M} - \text{H}]^+$ ions as quasimolecular ions since these ions comprise the otherwise intact analyte molecule. In addition to protonation or adduct formation, hydride abstraction reactions also frequently occur under chemical ionization conditions, particularly with hydrocarbons and related compounds, such as 1-decanol, where a prominent $[\text{M} - \text{H}]^+$ ion at m/z 157 is observed, with isobutane as the reagent gas. Finally, in the presence of gases with high ionization potentials, electron-transfer reactions result in the production of a radical cation, as is

produced via electron ionization, but in this case, the radical cation is produced less energetically, resulting in diminished fragmentation.

Chemical ionization processes usually refer to reaction mechanisms that produce positive ions; however, negative-ion chemical ionization processes also exist, which can offer additional selectivity and sensitivity in ion formation and analyte detection. Indeed, negative-ion chemical ionization can dramatically increase the sensitivity of analyte detection in GC-MS applications compared with positive chemical ionization or electron ionization, particularly in compounds with electronegative moieties, such as halogen atoms in the xenobiotic itself; as in benzodiazepines or in chlorinated pesticides; or after corresponding derivatization, as for amphetamines, cannabinoids, or opioids [5]:



While several types of ion–molecule reactions can occur, the most common is proton abstraction as shown above, where the proton affinity of the reactant ions determines the propensity of the proton abstraction process.

2.4 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

LC-MS offers the advantage of soft-ionization modes (ESI, PI, laser-assisted photoionization (LAPI), and APCI), allowing the separation and ionization of a wide range of volatile and nonvolatile analytes, including peptides, proteins, and thermally labile compounds. Moreover, because of the robustness of modern LC-MS systems, a high sample throughput is normally achievable. The general procedure for analysis of biological samples differs from that for GC-MS, in that aqueous or water-miscible solvents are normally used to introduce the sample onto the HPLC column. Derivatization of the test chemical is not often necessary during initial experiments, and sometimes, it is possible to inject samples of the filtered biological fluid, fortified with internal standard, directly onto the HPLC column. As with GC-MS, the exact procedures used to acquire LC-MS information depend on the nature of the test chemical, the biological matrix, and the concentration of the test chemical and its metabolites in the matrix.

2.5 SINGLE QUADRUPOLE MASS SPECTROMETRY

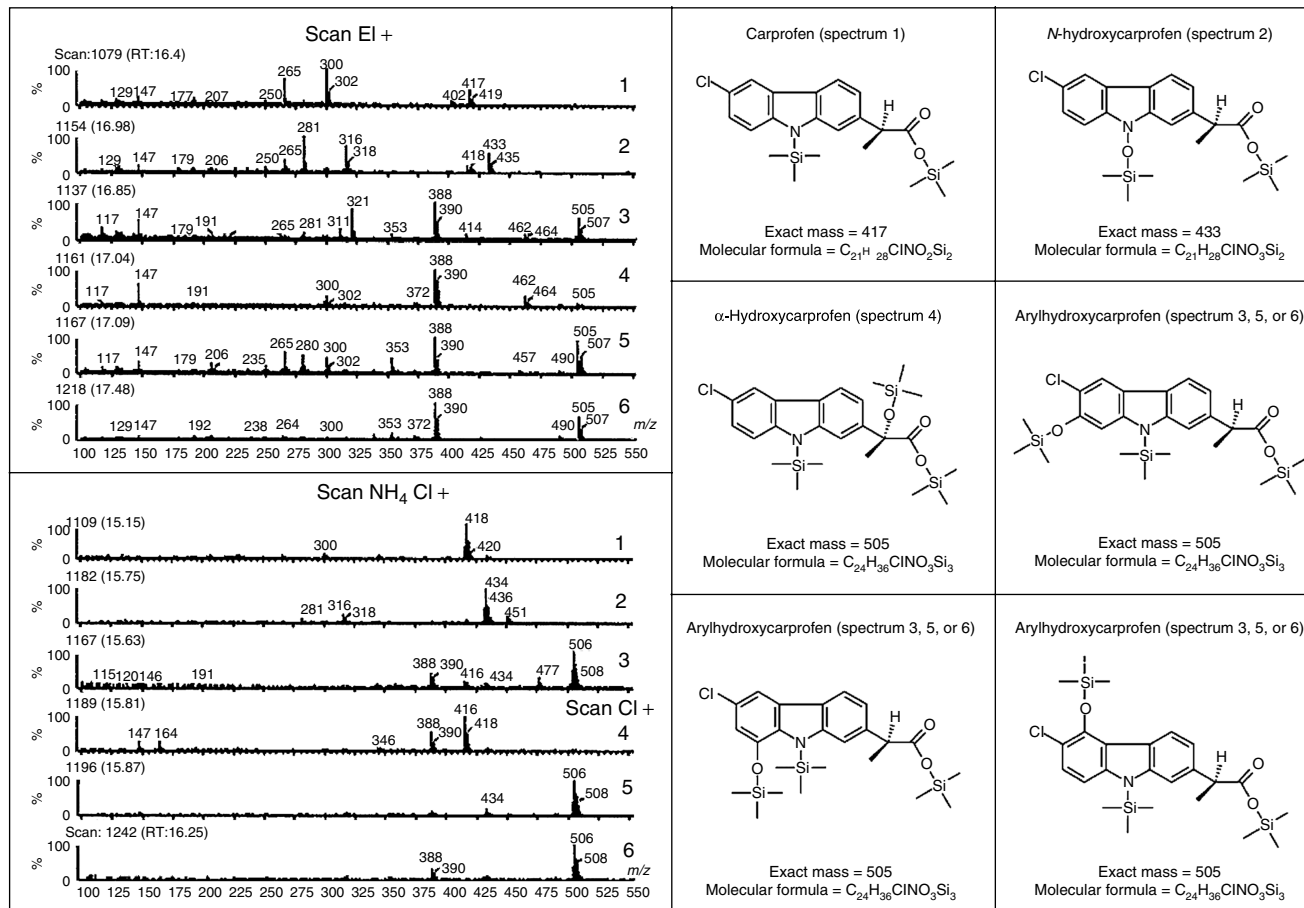
In one of the more common instrumental designs used for GC-MS or LC-MS analysis, ions that are formed in the ion source are accelerated into a single quadrupole mass filter. The mechanism of filtering ions by mass and charge in a quadrupole instrument involves superimposing an alternating radio frequency (RF) current on top of a direct current (DC) on pairs of oppositely charged rods and ramping the amplitudes of these RF and DC voltages at a constant RF/DC ratio. In this way, both a high mass and a low mass filter are formed, which can be used in tandem to filter ions with unit mass resolution across a considerable range of mass-to-charge ratios (m/z). Whether operating in electron ionization or chemical ionization modes, most quadrupole detectors are quite capable of filtering positive or negative ions within an m/z range of, typically,

10–1000 or 10–2000. By filtering masses with unit resolution extremely rapidly (on a millisecond time basis), a mass spectrum over the range of, for example, 10–1000 can be obtained in intervals of time that allow for several complete spectra to be obtained over individual eluting peaks from even fairly rapid chromatographic separations, such as those that occur in capillary GC. In this way, components from complex mixtures can be baseline-resolved chromatographically and spectrometrically, such that full scan spectra can be averaged across the peak, baseline-subtracted, and library-searched if warranted. Full scan spectra recorded using either electron or chemical ionization can give information regarding the potential molecular mass of the analytes (Fig. 2.1) and some indication of the presence of functional groups and structural features, information that can be augmented by information acquired after chemical derivatization (as described later in this chapter). In addition to operating in full scan mode, single quadrupole mass spectrometers are also capable of performing selected ion monitoring (SIM). In this mode, the mass spectrometer is set so that it does not spend cycle time monitoring ions that are not formed by an analyte of interest (as it does in scan mode), but focuses on the base ion and/or some other diagnostic ion(s) formed by the analyte under the conditions being employed for ionization and detection. SIM can thus be used to increase the selectivity and sensitivity of the detection of the analyte that, when paired with retention time (and the relative intensity of detected ions if more than one is acquired), also offers further confidence in the appropriate identification and quantification of the material being detected. The accuracy and sensitivity of the technique are often increased by the use of stable isotope-labeled internal standards, which provides control for recovery and serves as a “carrier” to reduce nonspecific loss to glassware and other potential active sites during sample preparation and analysis. If more than one analyte is of interest, the SIM method can be programmed to change the ion(s) being monitored based on the analyte’s elution time from the gas chromatograph, thereby allowing maximal cycle time to be spent in monitoring the ions of interest.

2.6 TRIPLE QUADRUPOLE/TANDEM MASS SPECTROMETRY (MS/MS)

A triple quadrupole mass spectrometer can be thought of as a two-stage mass spectrometer with a collision cell between the two stages. As in a normal mass spectrometer, ions are accelerated into the first quadrupole (Q1). This quadrupole can either allow all ions through by scanning by mass or act as a mass filter, allowing only those ions with a specific mass-to-charge ratio to pass into the second quadrupole (Q2). If the second and third quadrupole (Q3) regions allow all masses to pass, a normal mass spectrum is recorded. However, if Q2 is pressurized with an inert gas and used as a strongly

Figure 2.1 Positive electron ionization and ammonia chemical ionization scans of carprofen and its metabolites obtained from the derivatized isolate of a hydrolyzed urine sample from treated canines. Note that while the molecular ions are observed in the electron ionization spectra, in most instances, the ammonia chemical ionization provides further support to the assignment of molecular mass because of the presence of intense protonated molecular ions. *Source:* Reprinted from Dumasia MC, Ginn A, Hyde W, *et al.* Detection and identification of carprofen and its *in vivo* metabolites in greyhound urine by capillary gas chromatography–mass spectrometry. *J Chromatogr B* 2003;788(2):297–307, with permission from Elsevier.



focused collision cell, the “parent” ions allowed to pass through Q1 collide with the gas molecules in the collision cells, causing fragmentation to form “daughter” ions. Variation of the modes of operation of the Q1 and Q3 quadrupole thus provides several modes of MS/MS operation. For example, if Q1 scans and Q3 operates in SIM mode, the mode of operation is called *precursor ion scanning* since the mass spectrometer will reveal all ions that produce a specific daughter ion selected by Q3. This common daughter ion can therefore be used to identify molecules whose structures yield a common fragment or structural motif, for example, looking for peptides whose precursor ions give rise to a daughter ion at m/z 79, indicative of phosphorylation. If the opposite approach is taken, where Q1 is operated in SIM mode and Q3 is set to scan, a product ion scan is recorded. This mode of operation is commonly used to examine a molecular ion for its mass spectrometer fragments to provide structural identification. Another useful mode of operation occurs when both Q1 and Q3 scan at a given mass offset, referred to as a *neutral loss scan*. In this instance, primary ions are being detected that lose the same neutral molecule, such as carbon dioxide or a glucuronide, as the mass of the offset between the two scans is set to 44 or 176, respectively. Finally, when both Q1 and Q3 operate in SIM mode, it is referred to as *single reaction monitoring* (SRM) or *multiple reaction monitoring* (MRM). It is in this mode that a significant increase in signal-to-noise ratio is typically observed with GC-MS/MS compared to GC-MS. This is due to the selectivity of the MS/MS process, which removes the background noise contributed by the matrix; that is, the Q1 quadrupole selects a primary ion characteristic of the compound of interest from other ions that may be present in the matrix, passes it into the collision cell Q2, and uses the Q3 quadrupole to detect one of the characteristic daughter ions of the compound of interest. These two stages of selectivity, ignoring compounds that do not produce the sought-after primary ion and fragment to the selected daughter ion, typically result in a considerable increase in the sensitivity of detection through the elimination of noise and the resultant increase in the signal-to-noise ratio of ion detection. Thus, the SRM mode of operation on triple quadrupole mass spectrometers is one of the most common approaches to the quantitative analysis of trace levels of chemicals in complex matrices.

For metabolite identification studies, full scan, precursor ion, and neutral loss scans are often the most effective means of identifying potential metabolites. In precursor ion scans, it is typical for more than a single fragment ion’s precursor ions to be monitored, corresponding to characteristic fragments from a variety of portions of the parent compound. Thus, in metabolite identification studies, precursor ion scanning experiments are used to identify likely metabolites by determining if they contain an unaltered portion of the parent molecule or an expected alteration, such as hydroxylation. In this approach, the operator needs to know only the fragmentation pattern of the parent ion, not the alteration to the parent compound. However, as alluded to earlier, common alterations to these fragments, such as hydroxylation, may be monitored by looking for precursors of the analyte that give rise to fragments corresponding to the parent fragment plus the metabolic hydroxylation, which are 16 amu higher [7]. In this particular example with nefazodone, the mass spectrum of the parent compound reveals prominent fragment ions at m/z 274 and 246 (Fig. 2.2). These ions, and a fragment ion corresponding to the base fragment ion plus 16 amu at m/z 290, are logical candidates to be used as precursor ions to scan for nefazodone metabolites. In addition to precursor ion scans, neutral loss scans at the corresponding m/z fragments

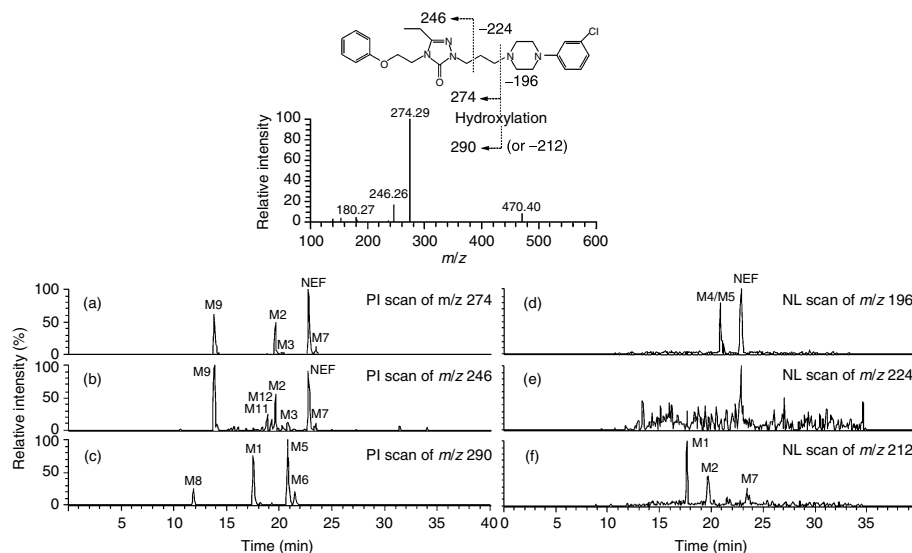


Figure 2.2 Structure and triple quadrupole product ion spectrum of NEF (m/z 470). The analysis of NEF metabolites in human liver microsomes by triple quadrupole LC/MS is shown in panels (a–f). (a–c) Base peak chromatograms of PI scans of m/z 274, 246, and 290, respectively; (d–f) are base peak chromatograms of NL scans of 196, 224, and 212 Da, respectively. NEF, nefazodone; PI, precursor ion; NL, neutral loss. *Source*: Reproduced from Zhu M, Ma L, Zhang D, Ray K, Zhao W, Humphreys WG, Skiles G, Sanders M, Zhang H. Detection and characterization of metabolites in biological matrices using mass defect filtering of liquid chromatography/high resolution mass spectrometry data. *Drug Metab Dispos* 2006;34(10):1722–1733. With permission from the American Society for Pharmacology and Experimental Therapeutics.

of m/z 196, 224, and 212 ($196 + 16$ amu) could also be considered as logical candidates for metabolite identification. As shown in Fig. 2.2, this combined strategy results in several plausible metabolite structures to be detected with a reasonable amount of confidence [8]. Other metabolite specific precursor ion and neutral loss m/z ions can also be useful for metabolite screening. For example, glutathione conjugates are commonly detected under positive ionization conditions by the neutral loss of m/z 129 (loss of the pyroglutamic acid moiety from glutathione) and in negative precursor ion scanning by the loss of m/z 272 (deprotonated γ -glutamyl-dehydroalanyl-glycine from glutathione) and 254 (the dehydrated form of m/z 272) [9].

2.7 ION-TRAP MASS SPECTROMETRY (MSⁿ)

In an ion-trap mass spectrometer, an electrostatic ion gate pulses ions into a 2D or 3D ion trap, as opposed to continuous “beam” instruments such as quadrupoles, where ions continually enter the mass analyzer. For improved performance characteristics, the ion trap is typically filled with helium to a pressure of about 1 mtorr. Collisions between trapped ions and helium molecules dampen the kinetic energy of the ions and contract their trajectories toward the center of the ion trap. The time during which ions are allowed into the trap is critical, in that it should maximize signal while minimizing space-charge effects, which can result in an overall reduction in ion-trap performance.

A conventional (3D) ion trap contains three hyperbolic electrodes, consisting of a ring and two endcaps. Trapped ions are further focused toward the center of the trap by the use of an oscillating potential, called the fundamental RF, applied to the ring electrode. This is accomplished by a parabolic potential with a saddle point, inside the trapping volume, where the strength of the restoring force increases as the ion trajectory deviates from the central axis. An ion will be stably trapped depending on the mass and charge of the ion (m/z), the size of the ion trap (r_0), the oscillating frequency of the fundamental RF (Ω), and the amplitudes of the applied DC (U) and RF (V) voltages. The position of the ion within the stability region in the trap can be moved by changing the amplitude of the applied DC and RF voltages. A mass-selective instability mode is utilized for ion ejection. The mass-selective instability mode of operation utilizes no DC voltage but ramps the RF voltages, and in this mode, ion trajectories become unstable in the axial direction (between the endcap electrodes) but remain stable in the radial direction. Ultimately, ions are ejected through holes in the endcap electrode where they can be detected. Additionally, mass-selective ejection can be facilitated because trapped ions of a given m/z oscillate at a frequency known as the secular frequency that is proportional to the angular frequency of the applied signal, ω . To facilitate ejection of an ion of a given m/z , resonance conditions can be induced by matching the frequency of a supplementary potential applied to the endcap electrodes to the secular frequency of the ion. The ion will then absorb energy from the applied field and the trajectory will linearly increase toward the endcap electrodes until the ion is ejected [10].

Recent advances in ion-trap technology have led to the development of linear (2D) ion traps. In a 2D ion trap, the ring electrode is replaced by a multipole, typically a quadrupole. Ions are cooled by collisions with an inert buffer gas and are trapped radially by a quadrupolar field and axially by an electric field applied to the end electrodes. The position of the ion packed along the axis of the trap can be controlled by varying the repelling voltage applied to the end electrodes. A 2D trap offers many advantages over a 3D trap. The linear geometry of a 2D trap results in a larger trapping capacity than a 3D trap. Many more ions can be trapped without experiencing losses due to space-charge effects of the linear trap's larger internal volume and by focusing along the central axis rather than around a point. The lack of an RF field for entering ions to overcome along the ion-injection axis also gives 2D traps a much improved trapping efficiency compared to 3D traps. Ion ejection from a 2D trap can proceed axially through the endcap electrodes or radially through slits in the center rods.

With triple quadrupole mass spectrometers, a continuous beam of ions is transmitted through the instrument, while mass selection and collision-induced dissociation (CID) of the parent ion continuously occur in separate regions of the instrument (Q1 and Q2, respectively), independent of time. The only time-dependent function is the mass analysis of the daughter ions by the second mass analyzer (Q3). Thus, triple quadrupole mass spectrometers are said to be tandem in space. Ion-trap mass spectrometers, on the other hand, are considered to be tandem in time because parent ion selection, CID, and daughter ion mass analysis are implemented by a timing sequence or scan function [11]. The scan function sets both the amplitude of the fundamental and supplementary potentials and the time taken for each step.

The nominal mass range of an ion-trap mass spectrometer is considerably higher than that of a quadrupole instrument but lower than that achievable on a time-of-flight mass spectrometer (TOF-MS). Quadrupole instruments typically have unit mass

resolution throughout the mass range. Perhaps, the biggest strength of the ion trap lies in its ability to perform multiple stages of mass spectrometry (MS^n), unlike a triple quadrupole instrument or a TOF-MS, dramatically increasing the structural information that can be obtained for a given molecule. Quadrupole ion-trap mass spectrometers can also be extremely sensitive.

While an ion trap has the capability for high mass resolution, mass range, sensitivity, and MS^n that translates into versatile performance as a mass spectrometer, there are also limitations to ion-trap technologies that deserve mention. As noted earlier, the dynamic range of ion traps is limited because when there are too many ions in the trap, space-charge effects lead to diminished performance. Automated scans are used to rapidly count the ions before they go into the trap so that the time ions are allowed to enter the trap is dependent on the ion flux. This ensures only a certain number of ions get in. However, diminished dynamic range can be a problem with ion traps when trace elements in particularly dirty matrices are analyzed because the trap fills with both matrix ions and trace sample ions. Another limitation of the ion trap is that the alternate scan modes of triple quadrupole mass spectrometers, such as precursor ion and neutral loss scans, are currently not possible [10].

LC- MS^n techniques are extremely powerful tools for metabolite identification. With ion-trap mass spectrometers, LC- MS^n experiments can be conducted with extremely high sensitivity, so that structural information can be acquired on extremely small amounts of sample. With this type of instrument, an ion with a particular m/z value can be trapped for a fixed length of time in the ion trap, permitting CID to occur and induce fragment ions, while other ions with different m/z values are ejected [11–13] to occur and induce fragment ions. The trapped fragment ions can then be mass-analyzed to generate a product ion spectrum. The product ion spectrum contains structural information that can be used to determine the structure of the ion selected. The sensitivities for the product ion spectra are excellent because nearly 100% CID efficiency is achieved. Subsequently, a product ion from the first MS/MS experiment can be selected for collisional activation dissociation to produce an MS/MS/MS scan and provide additional structural information about the selected ion. This process can be repeated several times. This technique was crucial to the identification of the metabolites of gemfibrozil (Fig. 2.3) [14] and chromium tripicolinate [15].

2.8 HYBRID AND HIGH RESOLUTION MASS SPECTROMETRY

Metabolite identification and structural elucidation have been greatly facilitated by the development of hybrid and high resolution MS systems [e.g., TOF, Fourier transform (FT), and orbitrap mass spectrometers], which allow for the determination of molecular formula and product ion formula with dramatically increased certainty. If coupled with a tandem approach that allows CID and data-dependent scan activity, the specificity of list-dependent acquisition of MS/MS data for predicted metabolites is improved (as described later).

2.8.1 Quadrupole Ion-Trap Mass Spectrometers

Hybrid instrument systems combine different types of mass analyzers in order to incorporate the best performance characteristics of each into a single instrument. One

common combination consists of a quadrupole mass filter followed by an ion trap (Q-IT). By filtering out unwanted ions using a quadrupole mass filter before they enter the trap, space-charge effects within the trap can be greatly reduced, increasing detection sensitivity for analytes of interest from complex matrix solutions. A particularly useful variation of this combination is the addition of a second quadrupole between the source and the ion trap. An instrument such as this can be thought of as a triple quadrupole instrument in which the final quadrupole is replaced by an ion trap. In this setup, the second quadrupole can be used for normal CID experiments, without experiencing any of the low mass fragment discrimination that results from dissociation in an ion trap, while the final stage of fragment analysis benefits from the increased speed and sensitivity of an ion trap compared to a quadrupole. This type of instrument also retains conventional triple quadrupole modes of operation including neutral loss and parent ion scanning.

2.8.2 LTQ-FT and LTQ Orbitrap

The newest forms of hybrid mass spectrometers consist of two sequential ion traps: a linear ion trap followed by either a Fourier transform-ion cyclotron resonance (FT-ICR) analyzer [such as the LTQ-FT (linear trap quadrupole-Fourier transform)] or an orbitrap analyzer (LTQ Orbitrap). One advantage that comes from having a trap as the first mass analyzer is the ability to fragment parent ions with activation techniques other than CID such as electron capture dissociation (ECD) and electron transfer dissociation (ETD). Another advantage of this setup is increased sensitivity. Much like Q-IT instruments, selected transmission of ions from the linear ion trap reduces space-charge effects by reducing the number of unwanted ions entering the second trap. Sensitivity is also increased by the ability to collect and store ions of interest in the first trap for the amount of time it takes to record the FT-ICR or orbitrap spectrum. LTQ-FT instruments

Figure 2.3 APCI LC/MS spectra recorded in negative ionization mode for metabolites of gemfibrozil (GEM, structure shown). (a) Mass spectrum recorded for the isolated metabolite proposed to represent the acyl glucuronide of the parent compound (1-O-GlcUA-GEM). The MS/MS spectra recorded after isolation and fragmentation of the apparent M-H ion at m/z 425 is shown in the bottom panel along with the structure and fragmentation pathways proposed for this metabolite. (b) Mass spectrum recorded for the isolated metabolite proposed to result from the sulfate conjugate of a hydroxylated metabolite of GEM. The MS/MS spectra recorded after isolation and fragmentation of the apparent M-H ion at m/z 345 is shown in the inset, and the structure and fragmentation pathways proposed for this metabolite are also provided. (c) Mass spectrum recorded for the metabolite proposed to be 2',5'-CH₂OH-GEM. The MS/MS spectra recorded after isolation and fragmentation of the apparent M-H ion at m/z 281 is shown in the inset. The structure and fragmentation pathways proposed for this metabolite are also provided. (d) Mass spectrum recorded for the metabolite proposed to be 2',5'-CH₂OH-GEM. The predominant ion detected with APCI/MS analysis of this metabolite was at m/z 455, which is consistent with a glucuronidated metabolite of GEM that had undergone complete oxidation of one of the ring methyl groups to the carboxylic acid. The detection of fragment ions at m/z 279 and 151 also supports this tentative identification (see proposed structure and fragmentation). It is important to note that the structural elucidation of most of the metabolites of GEM required extended characterizations using MS, MS/MS (and MSⁿ), and numerous studies using NMR and 2D NMR [e.g., heteronuclear multiple bond correlation (HMBC) experiments]. For complete information regarding the analysis and structural assignment, see Ref. 14.

were developed first and have greater resolution and mass accuracy than LTQ Orbitrap instruments, but they are also very expensive and require a cryogenically cooled super-conduction magnet, making the more convenient LTQ Orbitrap instruments a popular choice for this type of hybrid system.

2.8.3 Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometers

A Q-TOF mass spectrometer can be thought of as a quadrupole mass filter and a collision cell (often a second quadrupole) added to the front end of a TOF mass spectrometer. This creates a versatile instrument capable of capturing full high resolution accurate mass spectra in both MS and MS/MS operational modes. In MS mode, the quadrupole operates in RF-only mode, transmitting all ions to the TOF analyzer. In MS/MS mode, the quadrupole is set to transmit a particular m/z value (the parent ion of interest), and the TOF analyzer collects the fragment ion spectrum. The selectivity for quantitation is much better on Q-TOF instruments than on triple quadrupole instruments because of the high resolution with which the TOF analyzer can select fragment ions. Q-TOF instruments have historically suffered from poor dynamic range, but recent advances in technology and design have greatly enhanced the dynamic range of newer Q-TOF instruments. Even with the increased dynamic range, when using a Q-TOF for quantitative analysis, care must be taken to fully assess the signal response of calibration solutions containing the analytes of interest over a suitable concentration range.

2.8.4 Ion Mobility Mass Spectrometry

An additional level of separation can be obtained by combining MS with ion mobility spectrometry (IMS) to create IMS-MS (also commonly referred to as *IM-MS* or *IMMS*). IMS separates ions based on their shape and can therefore directly detect differences in 3D structure among species with the same m/z ratio. The principle behind ion mobility is that ions of different shapes travel at different speeds when pulled by an electric field through a cell containing a relatively high pressure of an inert buffer gas. Owing to increased collisions with the gas, larger more extended ions travel slower than more compact ions. The millisecond timescale for mobility separation interfaces nicely with the microsecond timescale of a TOF detector.

In a conventional ion mobility instrument, ions are pulsed into a mobility cell where they are pulled through by a uniform weak electric field, E , and experience frictional drag because of collisions with the buffer gas. The combination of these two forces results in a constant drift velocity, v_D , which is proportional to E [16]:

$$v_D = K E$$

The proportionality constant, K , is the ion's mobility. Measuring the drift velocity at a series of weak field strengths gives an absolute determination of the mobility. Conventional ion mobility suffers from a lack of sensitivity as many ions are lost due to space-charge effects in the trap in front of the mobility cell and to radial diffusion during transmission through the cell. Commercial instruments are available that implement variations on conventional IMS that have increased sensitivity over conventional IMS, at the expense of not being able to measure absolute mobilities. Two

popular commercial variations are traveling wave (T-wave) IMS and field asymmetric ion mobility spectrometry (FAIMS).

In most ion mobility instruments, including a T-wave ion mobility instrument, mobility separation occurs in the collision cell region. In a T-wave instrument, the mobility cell contains an RF ion guide consisting of a series of ring electrodes. A traveling voltage wave is produced by superimposing a DC potential on the RF of one ring electrode, then switching the potential to an adjacent electrode along the ion guide. Larger ions (lower mobilities) experience more collisions with the buffer gas than more compact ions and thus roll back over the top of the voltage wave more often. As a result, it takes more voltage pulses for larger ions to traverse the cell, and therefore, they exit the cell later than ions with higher mobilities. T-wave mobility separation has significant speed and sensitivity advantages over conventional ion mobility separation, but the path that the ions travel and their collision frequencies are not well defined, making it impossible to measure absolute ion mobilities with this method.

FAIMS differs from most other mobility separation techniques, in that it takes place in the source region and operates at atmospheric pressure. In FAIMS, an asymmetric waveform is applied between two electrode plates and alternates between high and low voltages. Ions move toward the plates at rates that are characteristic and mobility dependent. A DC correction voltage (CV) is applied to alter the path of ions with a specific high field to low field mobility ratio, allowing them to traverse the gap between electrodes without striking the plates. Similar to the operation of a quadrupole mass filter, the CV can be scanned to allow ions with different mobilities to exit. The intricacies of FAIMS separation are complex and not well understood. The separation depends not just on the constant low field mobility but on the ratio of the high field and low field mobilities.

The coupling of IMS to MS provides a rapid separation step before MS analysis, which requires no additional time in either sample preparation or analysis. Most metabolic applications of IM-MS rely on differences in relative mobilities rather than on measurement of absolute mobilities, making the commercially available systems well suited for many of their biological applications. For example, IM-MS can greatly simplify analysis of complex mixtures by filtering out unwanted ions before they enter the mass spectrometer [17], increasing signal-to-noise ratio for analyte identification by separating isobaric compounds in mobility space before mass analysis [18] and separating ions into groups based on m/z mobility ratios to screen for specific modifications [19].

Unbiased LC-MS analysis creates a considerable amount of data, and adding IMS makes the data sets even more complicated. However, the technique is sufficiently sensitive and robust enough to tease out important metabolite information. For example, in its simplest application, consider metabolites that are positional isomers of each other and are impossible to differentiate using MS, as they have identical accurate mass and fragmentation profiles. This particular instance has been studied with ondansetron, where three geometric metabolites of ondansetron were analyzed using an ion mobility LC-MS system, and the metabolites' collisional cross sections were calculated. The cross sections were then compared to molecular modeling and calculated data regarding their conformational cross section in order to putatively identify each of the positional isomers of ondansetron, as they were separated by the ion mobility approach [20]. As a further illustration of the potential application of ion mobility to structural analysis of biomolecules or metabolites, it has also been shown that gas-phase chiral separation

and structural characterization can also be accomplished using ion mobility MS when an appropriate chiral selector, such as a (*S*)-(+)-2-butanol or (*R*)-(-)-2-butanol are added into the nitrogen drift gas. Interestingly, one enantiomer of a racemic mixture interacted more favorably with the (*S*)-(+)-2-butanol chiral selector gas, and when the chiral selector gas was switched to (*R*)-(-)-2-butanol, the order of enantiomer elution from the drift cell was reversed, indicating that some form of the Pirkle rule is being satisfied in the gas phase. Since most studies such as these have involved low molecular weight compounds with only one, or few, chiral centers, it remains to be determined how larger molecules with multiple chiral centers might separate in an ion mobility cell.

2.9 DERIVATIZATION REACTIONS TO ASSIST IN METABOLITE IDENTIFICATION USING MASS SPECTROMETRY

Derivatization reactions can serve several purposes in the isolation and identification of metabolites. First, the chromatographic properties of the metabolite can be altered, such that they may facilitate chromatography. Second, the observation that a derivative is or is not formed in a particular reaction indicates the presence or absence of certain functional groups and thus gives insight into the putative structure of the metabolite. Third, derivatization can produce assignable mass spectral changes that further aid in structural assignments of the unknown metabolite.

In principle, any chemical reaction that converts a compound to a product could be considered a derivatization. In practice, the term generally refers to reactions of a functional group (e.g., an alcohol converted to its ester). Alcohols are most commonly esterified or converted to silyl ethers. Esterification may enhance volatility (e.g., acetates, trifluoroacetates), GC detection (e.g., heptafluorobutyrate, pentafluorobenzyl derivatives), or UV or fluorescence measurement for HPLC (e.g., dansylates). Trimethylsilyl (TMS) ethers and other silyl derivatives are used principally to decrease hydrogen bonding and enhance volatility. By the use of appropriate reagents and conditions, it is often possible to distinguish hindered alcohol groups (e.g., tertiary alcohols) from less hindered ones by their reaction with silylating reagents. Amino groups may be acylated or silylated. Acylation, in addition to usually enhancing volatility, also converts a basic group to a neutral group, thereby changing chromatographic properties drastically. Carboxylic acids may be esterified by the use of diazomethane, boron trifluoride/methanol, or HCl/methanol. Phenolic hydroxyls may be methylated (methyl iodide, dimethyl sulfate, sometimes diazomethane). Ketones may be converted to alkyl oximes, dinitrophenylhydrazones, and so on.

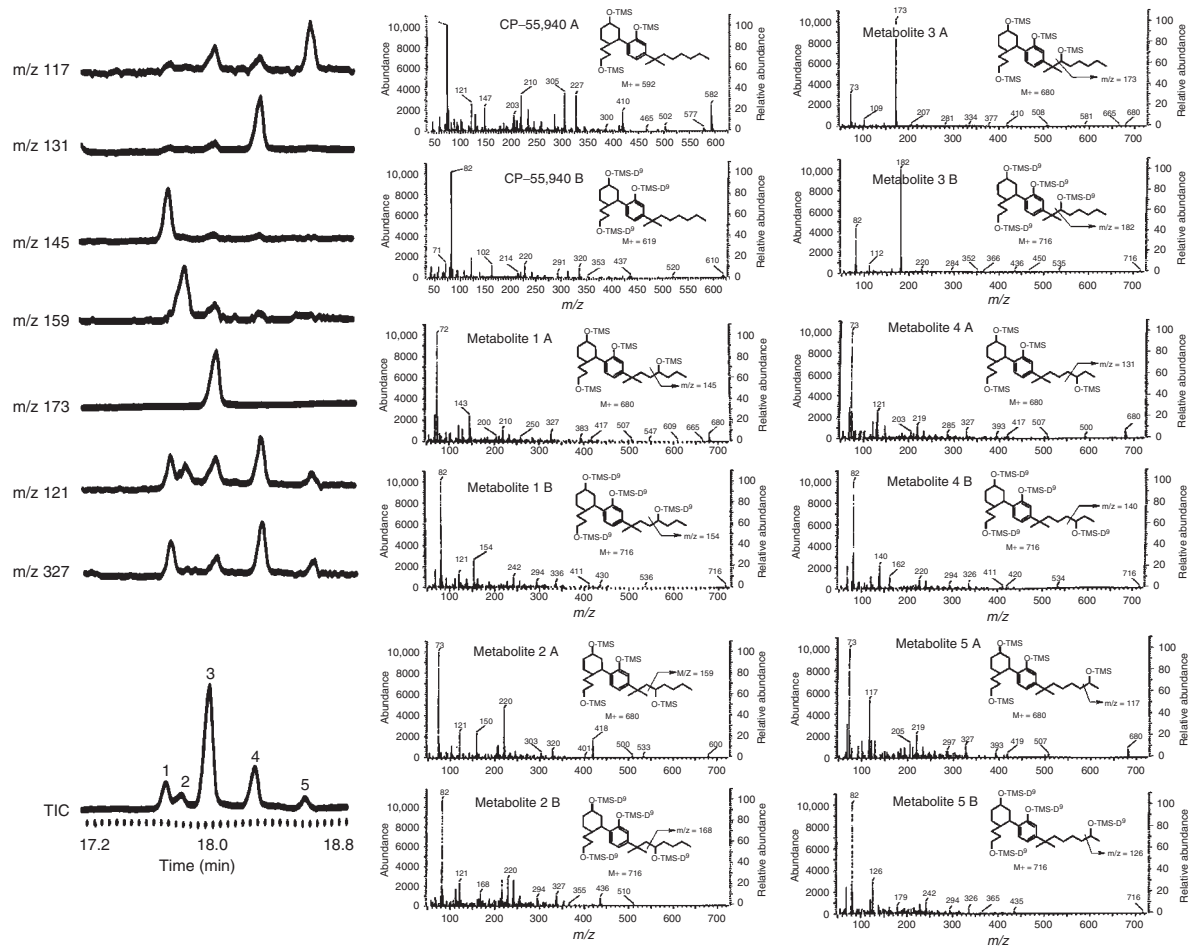
It would require several volumes to review all of the possible derivatization reactions and strategies used to facilitate metabolite identification or structural elucidation. However, it is important to emphasize again that in addition to improving the chromatographic or detection characteristics of analytes of interest, derivatization reagents can be quite useful in structural determination, particularly when combined with mass spectral analysis. Derivatives can provide information regarding the number and nature of functional groups, such as alcohols and acids, and, in some instances, the location or the proximity of certain functional groups. In studies of the metabolism of CP-55,940 (a synthetic cannabinoid agonist), TMS derivatives were prepared using both bis(trimethylsilyl)trifluoroacetamide and the deuterated form of this silylation reagent [21]. Five monohydroxylated metabolites were identified, each with molecular ions

detected at m/z 680 with the TMS derivatives and m/z 716 with the deuterated [$^2\text{H}_9$]-TMS derivative (Fig. 2.4). In this instance, the gain of 36 indicated the presence of 4 hydroxyl groups (i.e., the addition of 1 hydroxyl group to the parent compound). Furthermore, relatively intense ions were present that indicated the position of the hydroxylations and demonstrated that the site of hydroxylation for each metabolite was on a different carbon atom of the dimethylheptyl side chain. Thus, in this case, the derivatization reagent increased the volatility of the metabolites, allowing them to be separated by capillary GC; indicated the number of hydroxyl groups present; and directed fragmentation that provided information regarding the position of the hydroxylations on the molecules' 1,1-(dimethyl)heptyl side chain. Although two dihydroxylated metabolites were also identified in this manner, the position of the hydroxyl groups in these metabolites could not be discerned. It was apparent, however, that both hydroxylations had occurred on the dimethylheptyl side chain.

2.10 METABOLITE IDENTIFICATION STRATEGIES USING LC-MS/MS

One powerful application of LC-MS to metabolite identification is afforded by triple quadrupole instruments that can perform information-dependent MS/MS scan experiments. In a typical metabolite identification experiment, an MS (survey) scan is performed and processed to identify ions of interest as each component elutes from an HPLC column (candidates) during a mobile-phase gradient. Data-dependent product ion (MS/MS) scans are then acquired for the most intense ions present. This is performed, once a mass is selected and allowed to enter the second quadrupole region, by rapidly increasing the collisional energy and performing a scan with the third quadrupole to generate a MS/MS fragmentation pattern. Most metabolite identification software packages available for mass spectrometry include methods for managing the acquisition of data based on information received during a previous scan (e.g., information-dependent acquisition), and ion filtering tools, such as time-based or isotope exclusion lists, to minimize improper candidate ion selection for MS/MS analysis. Selection criteria can be set so that product ion scans are acquired for the n th most intense mass. The number of product ion scans per mass can also be specified. A list of ions to be excluded (e.g., known contaminants) can be specified to reduce the acquisition of irrelevant data. Conversely, masses listed on an inclusion table will always have data-dependent acquisition scans performed. As noted previously,

Figure 2.4 Total ion chromatogram (m/z 50–750) and extracted ion current profiles for m/z 117, 131, 145, 159, 173, 121, and 327 recorded for the TMS derivatives of CP-55,940 metabolites formed in mouse liver S-9 microsomal preparations (at left). The background subtracted mass spectra recorded for the nonmetabolized parent compound, CP-55,940, and the metabolites labeled 1–5 in the total ion chromatogram are shown (at middle and right) for their TMS derivatives (spectra designated A) and their deuterated ($^2\text{H}_9$, or D9) TMS derivatives (spectra designated B), with the diagnostic side chain fragmentation ions highlighted. Note how the increase in the mass between the TMS and the D9-TMS derivative of the apparent molecular ion for CP-55,940 from m/z 592 to 619 is 27 mass units, indicating the addition of 3 TMS groups ($\text{D9} \times 3$). In contrast, the 5 metabolites (1–5) reveal a mass difference between the apparent molecular ions of their TMS derivatives and their D9-TMS derivatives of 36, indicating the addition of 4 TMS groups ($\text{D9} \times 4$). It is also apparent in these figures that the fragmentation of the side chain is directed by the location of the hydroxy-TMS ether derivatives. From Ref. 21.



precursor ion or neutral loss scans can be interspersed with total ion scans and data-dependent product ion scans to increase the likelihood of identifying a potential metabolite. Precursor scans look for molecules that, when fragmented, produce a common mass. This can indicate that an unknown is structurally related to the parent molecule or contains a specified structural element of the parent compound. Neutral loss scans can be especially informative when searching for metabolites. A neutral loss scan searches for ions that, when fragmented, lose a defined mass. This type of phenomena is common to glucuronide- and peptide-conjugated molecules. Thus, the combination of a precursor ion scan common to the parent molecule and a neutral loss scan consistent with a metabolic conjugate would strongly indicate a potential metabolite.

The second component of a commonly used metabolite identification approach is data analysis using software processing programs that search an LC-MS/MS run for metabolic mass gains and losses from a user-defined list and the parent compound molecular weight. With this software, one can compare a sample run with a matrix control and exclude native compounds from consideration and future analysis. Potential metabolites are highlighted, and the transformation name is displayed. Furthermore, a correlation score can be generated by comparing the fragmentation pattern of the parent compound with those generated from unknowns. The correlation score represents the degree to which a fragmentation pattern resembles a reference pattern and is based both on the masses of the fragments and on their relative distribution. Ions of potential interest (based on their plausibility of representing transformation products) for which data dependent product ion scans were not generated in the initial run are typically tagged for reanalysis. Towards that end, most commonly used, commercially available metabolite identification software packages automatically create a new acquisition batch and place the tagged ions on a data-dependent acquisition inclusion list. The samples are then reinjected, and fragmentation data are generated for the tagged masses. The operator may also manually select or exclude masses to be included in the reanalysis.

2.11 METABOLITE IDENTIFICATION STRATEGIES EMPLOYING HIGH RESOLUTION AND ACCURATE MASS MEASUREMENT

The utility of high resolution MS for metabolite identification is based on improved performance characteristics that add to its ability to identify or decrease the number of possible empirical formulas. For instance, for a mass measurement of m/z 118 (where C_{0-100} , H_{3-74} , O_{0-4} , and N_{0-15}), there must be no alternative formulas within 34 ppm for unequivocal assignment of the possible empirical formulas. Increasing the mass measurement to m/z 750 (where C_{0-100} , H_{25-110} , O_{0-15} , and N_{0-15}), there are 626 alternative formulas within 5 ppm. If one narrows down the possibilities in elemental composition, the number of alternative formulas can be decreased dramatically. The most important performance parameters in MS for assigning potential empirical formulas are resolution and mass accuracy. As the resolution and accuracy of the instrumentation are increased, the number of possible empirical formulas can be dramatically decreased, such that with a mass of 516 at 1 ppm or lower, the number of possible formulas is reduced to 4 (given the constraints of C_{35-70} , H_{45-100} , N_{8-16} , O_{9-16} , and S_{0-2}).

The benefits of high resolution and mass accuracy extend well beyond the ability to assign putative empirical formulas to precursor and product ions from collision-induced fragmentation. This becomes particularly evident when a high resolution mass

spectrometer is coupled to a state-of-the-art chromatographic system. When UPLC methods are coupled with high resolution and accurate mass systems, interferences and isobaric species can be resolved based on retention time and mass, which greatly assists in the identification and quantitation of metabolites. With certain hybrid mass spectrometers, extremely useful scan functions for metabolite identification can be performed. For example, with Q-TOF systems, these include the aforementioned product ion, precursor ion, and constant neutral loss scans. With high resolution instruments, the scan of the product ions also occurs at high resolution and mass accuracy. These MS experiments have been demonstrated to be among the most informationally rich techniques for structural characterization of metabolites (for review, see Ref. 22).

For metabolite identification, an initial screening method using rapid MS scans combined with information-dependent MS/MS or MSⁿ scans is performed for treated microsomes/supersomes, cells (e.g., hepatocytes), or animals and their untreated controls. When possible, radiochemical and photodiode array detectors are also used to acquire ancillary information. All data from treated and control systems can be queried by automated data analysis packages to facilitate metabolite identification. Data analysis systems and strategies for metabolite identification typically involve determining the exact mass of the parent. Next, transformation tables are generated and incorporated into the metabolite identification software available on most LC-MS/MS systems. These transformation tables allow exact masses to be calculated for most expected phase I and phase II metabolites (Table 2.1), and the user can then automatically identify and generate ion chromatograms for these hypothesized transformations. The most commonly employed metabolite identification software packages are also able to

TABLE 2.1 Transformation Table (Abbreviated)

Biotransformation	Formula Change	Mass Change (Da)	Mass Defect (mDa)
Oxidative debromination	[M + OH-Br]	-61.9183	81.7
Deethylation (O, N, S)	[M-CH ₃ CH]	-28.0313	-31.3
Oxidative dechlorination	[M + OH-Cl]	-17.9661	33.9
Phosphothionate oxidation	[M-S + O]	15.9772	22.8
Demethylation (O, N, S)	[M-CH ₂]	-14.0157	-15.7
Dehydrogenation	[M-2H]	-2.0156	-15.6
Oxidation (alcohol aldehyde)	[M-2H]	-2.0156	-15.6
Oxidative deamination	[M + O-NH ₃]	-1.0316	-31.6
Reduction	[M + 2H]	2.0156	15.6
Hydroxylation	[M + O]	15.9949	-5.1
Epoxidation	[M + O]	15.9949	-5.1
Oxidation (N, S)	[M + O]	15.9949	-5.1
Oxidation (aldehyde carboxylic acid)	[M + O]	15.9949	-5.1
Dihydroxylation	[M + O ₂]	31.9898	-10.2
Trihydroxylation	[M + O ₃]	47.9847	-15.3
Methylation	[M + CH ₂]	14.0157	15.7
Acetylation	[M + COCH ₂]	42.0101	10.1
Glycine conjugation	[M-OH + C ₂ H ₄ NO ₂]	57.0215	21.5
Sulfation	[M + SO ₃]	79.9568	-43.2
Glucuronidation	[M + C ₆ H ₈ O ₆]	176.0321	32.1
Glutathione conjugation	[M + C ₁₀ H ₁₅ N ₃ O ₆ S]	305.0682	68.2

customize the data analysis parameters with respect to component subtraction, isotope pattern analysis, and mass defect filtering (MDF). The combination of these powerful data mining and spectral correlation processes allows both expected and unpredicted metabolites to be discovered as described in greater detail below.

Several of the available data analysis strategies include methods for component subtraction, which is a relatively simple but powerful method for eliminating chromatographic peaks and ions contained within control samples or solvents from further consideration as metabolites in treated samples. An algorithm compares components found in treated and control samples using retention time, m/z , and composite MS data. Components that are present in both the control and treated samples can automatically be excluded from further analysis, while components that are unique to the treated sample are automatically highlighted for further inspection. This approach can be extended to include UV spectra and radiographic data that can also help confirm MS peaks that are related to the parent drug or present in the control sample. In our laboratory, we have most recently applied this approach to the identification of the metabolites of fluasterone in canines (Fig. 2.5), as part of a research program sponsored by the National Cancer Institute [23].

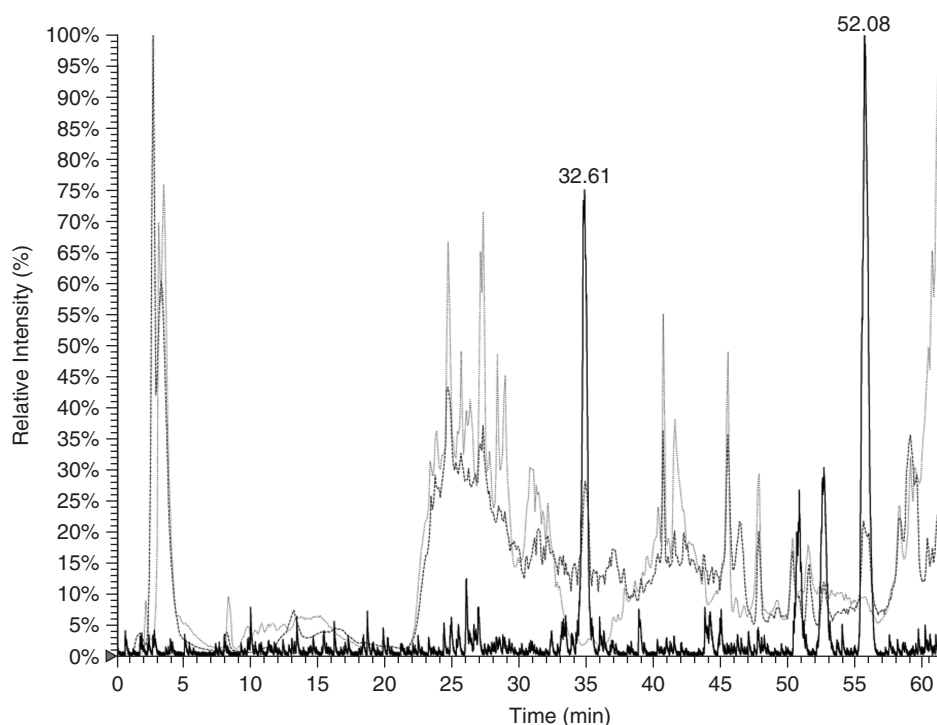


Figure 2.5 Overlay of total ion chromatograms obtained during analysis extracts of canine feces. The total ion chromatogram observed during analysis of a methanolic extract of feces collected before dosing (light grey dotted line) is overlaid with a total ion chromatogram (dark grey dashed line) and radiochromatogram (solid black line) obtained during analysis of a methanolic extract of feces collected between 8 and 24 h following oral dosing with [^{14}C]fluasterone at 15 mg/kg. Several radiolabeled peaks are apparent that are associated with peaks in the total ion chromatogram recorded after dosing and not before dosing. For further information, see Ref. 23.

Isotopic pattern analysis is also useful in metabolite identification with compounds that contain elements such as bromine and chlorine or that have easily distinguishable isotope patterns that are not common in endogenous metabolites found in body fluids. For example, drugs can be labeled using stable isotopes including ^{13}C , ^2H , and ^{15}N or radiolabeled at high specific activities with ^{14}C and other radioisotopes to facilitate metabolism studies. These unique isotope patterns can be used to efficiently detect drug metabolites using available software algorithms that filter full scan mass spectra based on the changes in mass and relative abundance of these isotopes and automatically display extracted ion chromatograms representing those isotope patterns that indicate potential metabolites. Even with nonisotope-labeled compounds, these approaches to isotope pattern assessment can be extremely useful to weigh possible elemental compositions or to refine the potential empirical formula list. While this approach is possible with both low and high resolution instruments, the application of this method to high resolution data sets can increase the power of filtering dramatically. There are, however, well-recognized caveats to this approach. For example, isotopic labeling can be lost if the label is in a metabolically favored or chemically labile position. Thus, one needs to also use the other available filtering methods and metabolite identification strategies to avoid excluding metabolites that have lost their original isotopic signature.

Mass defect filtering is one of the most powerful approaches for removing endogenous interfering ions from the xenobiotic metabolite ions of interest. The term *mass defect* originates from the observation that only the monoisotopic element ^{12}C has an integer value for atomic mass, that is, 12.000000. Thus, mass defect is a term that refers to the difference between the exact mass of an element (or compound) and its closest integer value (Table 2.2). The term *mass defect filtering* refers to a postacquisition data filtering technique based on the range of the calculated mass defects of the parent drug and its metabolites. For example, metabolite identification research performed in our laboratory for the National Cancer Institute involved the identification of both phase I and phase II metabolites of fluasterone (Table 2.3). One can see that the mass defect

TABLE 2.2 Nominal Mass, Exact Mass, and Mass Defect of Common Elements

	Hydrogen	Carbon	Nitrogen	Oxygen	Fluorine	Sulfur	Chlorine
Nuclide	H	^{12}C	^{14}N	^{16}O	^{19}F	^{32}S	^{35}Cl
Nominal mass	1	12	14	16	19	32	35
Exact mass	1.0078	12.0000	14.0031	15.9949	18.9984	31.9721	34.9689
Mass defect	0.0078	0.0000	0.0031	−0.0051	−0.0016	−0.0279	−0.0311

TABLE 2.3 Fluasterone Metabolites and Their Mass Defects (Abbreviated)

Structure	Empirical Formula	Exact Mass	Mass Defect
Fluasterone	$\text{C}_{19}\text{H}_{27}\text{FO}$	290.204593	—
	$\text{C}_{19}\text{H}_{29}\text{FO}$	292.220243	0.015650
	$\text{C}_{19}\text{H}_{29}\text{FO}_2$	308.215158	0.010565
	$\text{C}_{19}\text{H}_{29}\text{FO}_3$	324.210073	0.005480
	$\text{C}_{25}\text{H}_{37}\text{FO}_8$	484.247246	0.042653
	$\text{C}_{25}\text{H}_{37}\text{FO}_9$	500.242161	0.037568

associated with its metabolites was relatively small because a large portion of the parent compound structure remains unchanged during biotransformation. In these cases, the mass defects of these metabolites lie within a relatively narrow range (in this particular instance, between -0.042653 and 0.005085). On the basis of the molecular weight of the parent compound, estimation can also be made regarding the range of molecular weights in which these metabolites will occur. MDF could then be applied to filter out all ions that fall outside the expected range of molecular weights and those ions that are within the expected molecular weight range but exceed the expected mass defect range. This approach using multiple mass defect filters allows the true power of high resolution and accurate mass methods to be leveraged for metabolite identification. In summary, the MDF approach attempts to discriminate metabolite ions from matrix ions based on the similarity of the mass defect values of a drug and its metabolites. An example of this approach, using multiple mass defect filters, is shown in Fig. 2.6.

It is important to compare the mass defect filter approach to alternative approaches, such as the precursor ion scan or neutral loss scans that are useful in triple quadrupole instruments. As shown for the example of nefazodone in Figs. 2 and 6, the ion filtering and enhanced search capability of the MDF technique, together with the empirical formula information associated with the data, make high resolution LC-MS/MS instruments an extremely powerful platform for the screening and identification of both common and uncommon metabolites. MDF is also a postacquisition approach, and the templates are flexible and can be refined over time as more information becomes available. Clearly, multiple MDF is more effective than single MDF to detect and identify both phase I and II metabolites, and it also allows the detection of metabolites from hydrolysis or N-dealkylation, even when the products from such processes have mass defects that differ significantly from the parent. To be used most effectively, the core structures used as filter templates should incorporate fundamental knowledge with respect to biotransformation pathways in order to predict reasonable core structure templates. In addition, MDF can remove interference ions that have mass defects outside the filter windows, thus having the potential to simplify spectra by removing noise or interference ions [8]. Finally, MDF allows users to use low threshold values during data processing so that metabolites at very low levels can be identified easily. In contrast, the precursor ion and neutral loss scan approaches require a detailed examination of the parent compound's MS and MS/MS spectra, and the most appropriate ions need to be selected before this type of MS analysis. Once these ions are selected, the data set that is acquired is highly specific to those experimental design choices, and if the metabolic modification to the parent compound alters the fragmentation pathways, the metabolite may be inadvertently missed until the analyst makes changes to the MS ion acquisition parameters.

Once likely metabolites are identified, some software allows further confirmation to be afforded by spectral correlation. Spectral correlation algorithms allow MSⁿ data for potential metabolites to be automatically compared against authentic reference standards. In most instances, a correlation score is generated by comparing the fragmentation pattern of the parent compound with that generated from unknowns. The correlation score represents the degree to which a fragmentation pattern resembles a reference pattern and is based both on the masses of the fragments and on their relative distribution. In this particular instance, the correlation algorithm searches for product ion spectral components that are the same in metabolized and unmetabolized drugs (common product ions) or different through a consistent mass shift that indicates

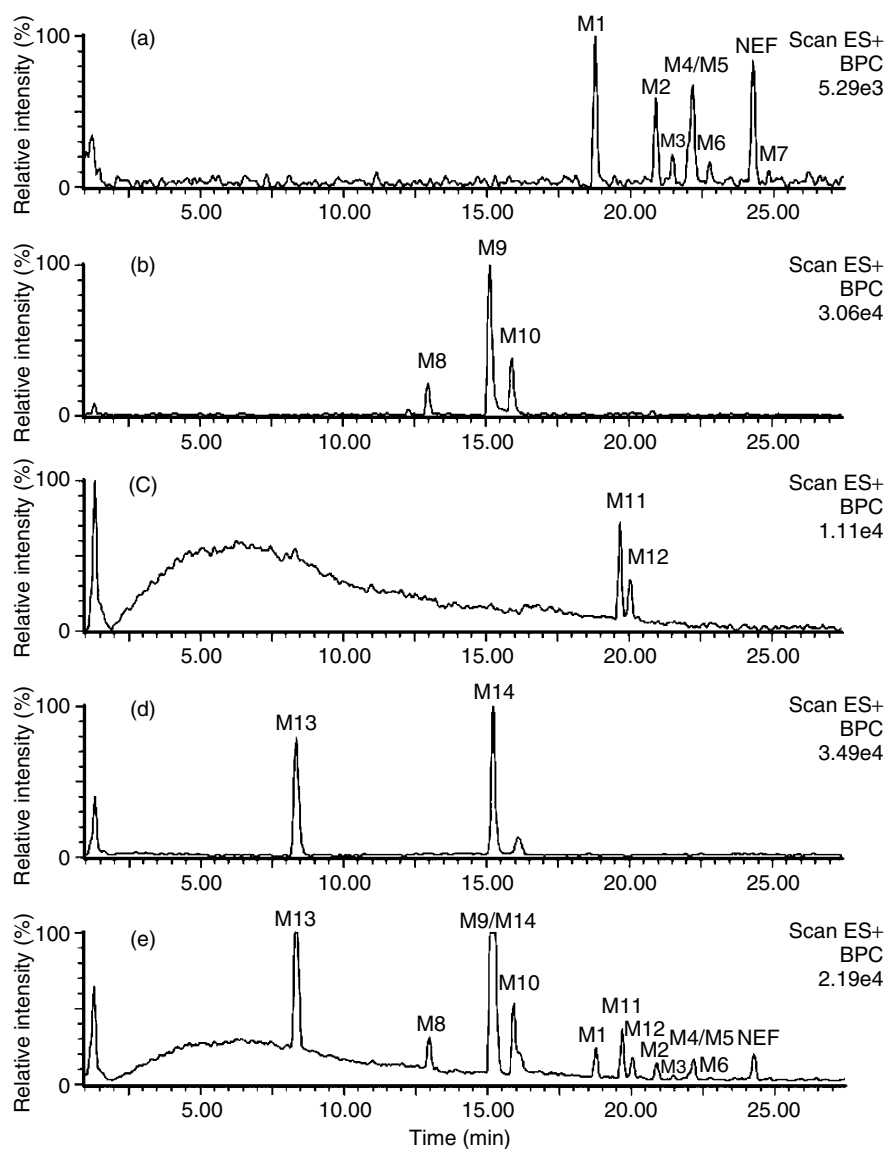


Figure 2.6 Base peak chromatograms of nefazodone metabolites in human liver microsomes determined by LC/FT-MS and mass defect filtering using various filter templates (± 0.040 Da), including (a) nefazodone as a filter template (MDF: 0.1923–0.2723 Da, nominal mass: 420–520 Da), (b) the core structure from a loss of the chlorophenyl group (Fig. 2.2) as a filter template (MDF: 0.1999–0.2799 Da, nominal mass: 310–410 Da), (c) the core structure from the loss of *m*-chlorophenylpiperazine as a filter template (MDF: 0.1105–0.1905 Da, nominal mass: 240–340 Da), (d) the *m*-chlorophenylpiperazine as a filter template (MDF: 0.0445–0.1245 Da, nominal mass: 147–247 Da), and (e) a combination of all the above filter templates. *Source:* Reproduced from Zhu M, Ma L, Zhang D, Ray K, Zhao W, Humphreys WG, Skiles G, Sanders M, Zhang H. Detection and characterization of metabolites in biological matrices using mass defect filtering of liquid chromatography/high resolution mass spectrometry data. *Drug Metab Dispos* 2006;34(10):1722–1733. With permission from the American Society for Pharmacology and Experimental Therapeutics.

a chemical modification such as a glucuronide adduct. The combination of spectral correlation and accurate mass determination of parent and MSⁿ fragment ions often allows certain metabolite structures to be eliminated from consideration, often leaving only one likely possibility. However, most LC-MS/MS systems have software capable of the automated generation of a second set of dedicated MS/MS methods (based on the information gathered during the initial analyses) to further elucidate or confirm potential metabolites in an iterative process.

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3 Infusion Nanoelectrospray Ionization Mass Spectrometry

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3.1	Executive summary	1
3.2	Introduction to electrospray ionization	2
3.3	Modes of liquid introduction for electrospray ionization	6
3.4	Nano-ESI ionization (nano-ESI)	8
3.5	Qualitative analysis	29
3.6	Quantitative analysis	30
3.7	Liquid extraction surface analysis (LESA)	31
3.8	Sampling and nano-ESI MS/MS of live single cells	32
3.9	Conclusions	34
	References	35

3.1 EXECUTIVE SUMMARY

This treatise describes a wide variety of nanoelectrospray ionization (nano-ESI) mass spectrometry experiments that do not employ high performance liquid chromatography (HPLC) or any other separation science on-line before mass spectrometric analysis. This so-called infusion nano-ESI sample introduction would appear to suffer from significant deficiencies in the absence of on-line chromatography, but in fact, rapid, meaningful analytical results may often be obtained from these experiments. As shown with examples herein, there are many cases where a combination of selective preliminary sample preparation can allow complex biological samples to be directly analyzed by infusion nano-ESI mass spectrometry. In certain instances such as with diluted plasma samples, meaningful results may even be obtained without prior sample cleanup.

Although meaningful infusion nano-ESI mass spectrometry (MS) results appear counterintuitive and at odds with modern dogma that HPLC is required with mass spectrometry (LC/MS), there are unique benefits afforded by nano-ESI, where in many cases, infusion sample analysis may provide comparable or faster as well as easier results than that with LC/MS/MS analyses. Nano-ESI performed with liquid flows at or below 100 nL/min exhibits reduced matrix suppression of ionization. In the absence of prior HPLC separation, therefore, endogenous compounds, metabolites, and other potentially interfering compounds can usually contribute to matrix suppression

of ionization for the target analyte(s). However, since nano-ESI suffers less from these suppression issues, one can still obtain meaningful results for all but perhaps trace level components. Nano-ESI performed at these low liquid flows also provides a more uniform response from related compounds such as a parent drug and its metabolites, which can allow semiquantitative analysis in drug discovery without stable isotope or even structural analog internal standards. When infusion analysis of crude, whole samples is possible, there is less risk of discriminating against certain sample components that may be lost in the usual sample preparation procedures. If automated chip-based nano-ESI is performed, sample-to-sample carryover is also eliminated because each sample experiences a virgin flow path en route to the mass spectrometer. For high throughput sample analysis situations such as newborn screening of carnitines as biomarkers for inborn metabolism errors, elimination of the chromatographic process greatly reduces the run time and hence increases the sample throughput. Each of these features offers some benefits for quantitative analysis experiments.

There is also another set of benefits from nano-ESI infusion experiments for qualitative analysis experiments. Extended analysis times provide improved signal-to-noise ratios and higher quality mass spectra from trace level analytes. In particular, when sequential MS/MS experiments are needed, infusion experiments allow ample time for acquiring the requisite data because the sample introduction is continuous rather than dynamic as in the case with the elution of a chromatographic peak. These benefits afford improved data quality for carbohydrate characterization, top-down protein characterization, metabolite characterization, and lipid analysis studies among many others. Finally, the recent application of liquid extraction surface analysis (LESA) of thin tissue samples, for example, provides an automated approach for microsampling of the chemical constituents in selected small regions of tissue organs followed by automated nano-ESI infusion mass spectrometric analysis of the crude extract. This simple, automated experiment can determine the general disposition of a drug and its phase I and II metabolites within the various organs in a thin tissue slice.

This report provides a brief overview of electrospray mass spectrometry with an introduction to nano-ESI along with a variety of applications that suggest some unique analytical merit for this approach in a variety of drug and drug metabolism applications.

3.2 INTRODUCTION TO ELECTROSPRAY IONIZATION

3.2.1 Basic Principles

3.2.1.1 Mechanisms of Electrospray. Electrospray is a process that generates gas-phase ions at atmospheric pressure from chemical entities in solution [1,2]. A compound that is dissolved in a polar or nonpolar solvent may be passed through the capillary path with an applied high voltage (2–5 kV) with either positive or negative polarity to produce gas-phase ions. The liquid will typically be expelled from the exit of the capillary tubing as an aerosol mixture of small charged droplets and vapor. To decrease the initial droplet size, additives that increase the conductivity of the solution (e.g., low molecular weight acids, bases, volatile salts) are customarily added to the solution. Because the eventual gas-phase ion formation requires extensive solvent evaporation, the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds (e.g., methanol, acetonitrile (ACN)). The applied voltage

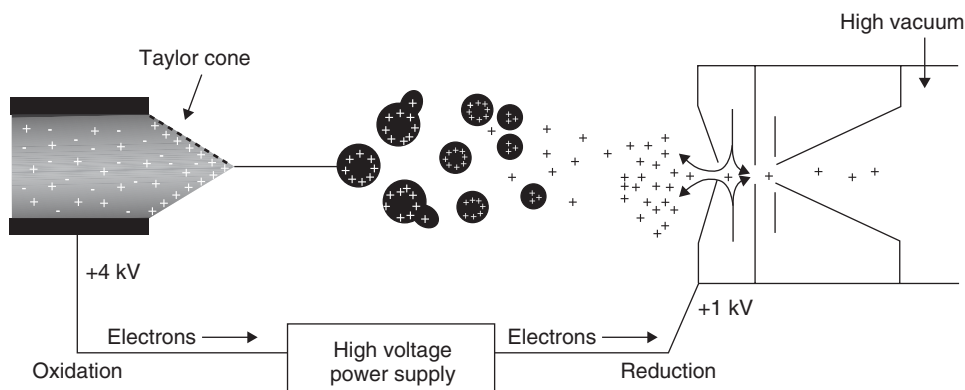


Figure 3.1 Schematic drawing of the electrospray process when a sprayer emitter is coupled with an atmospheric pressure ionization (API) mass spectrometer. *Source:* Reprinted with permission from Advion BioSciences, Inc. Ithaca, NY 14850.

provides a high electric field gradient that produces a charge separation at the surface of the liquid. As a result, the liquid protrudes from the capillary tip in what is known as a *Taylor cone* (Fig. 3.1). The solution that comprises the Taylor cone reaches the Rayleigh limit [3], which is the point at which coulombic repulsion of the surface charge is equal to the surface tension of the solution. At this point, droplets that contain an excess of positive or negative charge detach from the tip of the liquid jet. These droplets move through the atmospheric region occupied by the charged aerosol toward the entrance of the mass spectrometer that is maintained under high vacuum (Fig. 3.1).

These charged droplets generate charged analyte molecules (gas-phase ions) by one of several possible mechanisms [4]. Two competing mechanisms explain how macromolecules become multiply charged in ESI: the charged residue mechanism (CRM) and the ion emission mechanism (IEM) [5]. According to the CRM, the excess charges on ESI droplets are transferred to and remain on macromolecules enclosed within the droplets after solvent evaporation. The charge on a macromolecule is limited by the maximum charge (i.e., the Rayleigh limiting charge) that a droplet similar in size to the macromolecule can contain without undergoing fission. The final gas-phase macromolecular ion results when the last trace of solvent in the microdroplet evaporates leaving a “charged residue.” Conversely, according to IEM theory, analyte ions desorb directly from charged nanodroplets, driven by the high electric field at the droplet surface. The formation of low molecular weight, singly charged ions in ESI has been explained well by the IEM. Whichever mechanism prevails, the ESI process generates gas-phase ions that can be analyzed for mass-to-charge ratio within the mass spectrometer. Amazingly, this process prevails whether the molecules of interest are inorganic ions, small molecule compounds, or macromolecules such as proteins and industrial polymers.

Higher liquid flow electrospray systems can benefit from additional nebulization by an inert gas such as nitrogen, and this process has been called *pneumatically assisted electrospray* or *ion spray* [6]. Additional improvements that facilitate handling higher liquid flows may be achieved with the addition of heat to the sprayer capillary tube and/or the aerosol spray. As the sequence of droplet splitting (fission) and ion evaporation produces gas-phase ions, the flux of ions, gas, and solvent vapor

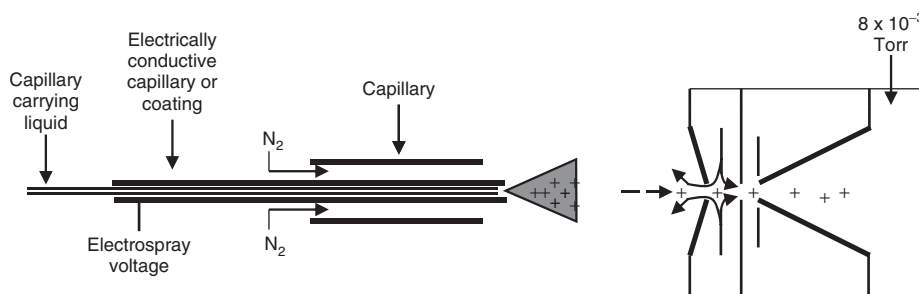


Figure 3.2 Schematic drawing of a pneumatically assisted electrospray (ion spray) LC/MS interface. *Source:* Reprinted with permission from Advion BioSciences, Inc. Ithaca, NY 14850.

is sampled through the inlet of an atmospheric pressure ionization (API) mass spectrometer via an orifice-desolvation region or a heated capillary inlet. The latter stage aids further removal of solvent and declustering of solvent molecules from the analyte ions. This whole process is called *electrospray ionization*, and when it is coupled with mass spectrometry, it provides an extremely useful and versatile analytical tool across many scientific disciplines.

Figure 3.2 shows a schematic drawing of a typical pneumatically assisted electrospray (ion spray) LC/MS interface, which was commonly used for coupling HPLC with mass spectrometry (LC/MS) [7] in the early commercial versions of API LC/MS systems. More recent versions of the common electrospray LC/MS sprayers employ additional heat as in the Turbo V system of AB SCIEX (Concord, Ontario, Canada), the heated electrospray ionization (HESI) system from ThermoFisher (San Jose, CA) or the “JetStream” system from Agilent (Santa Clara, CA). These systems all facilitate use of the higher HPLC mobile-phase flow rates of 0.2–1.0 mL/min common for routine application of modern LC/MS experiments. The central sprayer capillary carries the total HPLC effluent through the LC/MS interface with an applied electrospray voltage of 2–5 kV with a coaxial flow of heated high velocity nitrogen nebulizing gas and/or an auxiliary heated nitrogen gas directed on the spray aerosol mixture. The sprayer voltage produces charged droplets, while the nebulizing gas facilitates reducing the aerosol droplet size through evaporation and dispersion of the aerosol. The use of a nebulizing gas for electrospray experiments provides improved robustness of the interface, better negative ion performance, and the ability to handle higher mobile-phase flow rates than “pure” electrospray [8].

3.2.1.2 Sensitivity. Electrospray ionization can provide very high sensitivity provided the “chemistry” of the analyte is well suited for the process of forming gas-phase ions from solution. The preferred analytes are compounds that exist as ions in solution, such as quaternary ammonium compounds or phosphonium compounds. Alternatively, amines can be converted to quaternary ammonium ions by protonation or strong acid compounds, which may be deprotonated in solution in the presence of weak base to form stable anions and provide very good sensitivity for negative ion electrospray ionization. The ideal situation for sensitivity is the formation of highly charged and hence polar ions in a relatively nonpolar solvent. In this case, the ion may be more stable in the gas phase than the condensed phase and hence produce very good sensitivity. The compounds that often provide the best sensitivity are thus polar compounds that are strong acids or bases.

The opposite will be the case of a neutral compound, which cannot be readily protonated or deprotonated to form a stable ion in solution. Instead, these neutral compounds may form weak ion adducts with additives placed in solution with them. Common examples of these compounds are neutral steroids, sugars, or carbohydrates and related compounds. These compounds may be converted to ions in solution by forming adducts with charged species such as ammonium ions, alkali metal ions, or electronegative species which may adduct to an electron-rich site on the compound of interest. Thus, electrospray sensitivity is very much a function of the chemistry of the compounds of interest and the “spray solvent” composition used to carry out the electrospray experiment. When LC/MS experiments are conducted, there may be a need for a compromise between adjusting the composition of the mobile phase to achieve the necessary chromatographic separation while still establishing the “solution chemistry” needed to obtain a high sensitivity electrospray response.

3.2.1.3 Matrix Suppression of Ionization. A fundamental issue with electrospray analysis of real-world samples is the potential for simultaneous competition for ionization between the analyte of interest with coexisting high levels of other compounds present in solution [9]. Thus, the ion current response from a trace level compound as a pure substance in just the spray solvent may be very high, for example, in the presence of no matrix effects. However, if there is also present in the spray solvent another interfering compound(s), especially if present at much higher levels, there is likely to be a substantial reduction of the target analyte ion current response because of “matrix suppression” of ionization. In other words, the other compound(s) compete for the electrospray ion current and suppress or (less often) enhance the target analyte ion current response [10]. This most often occurs when complex biological or environmental samples are not sufficiently cleaned up by the sample extraction process and/or the analysis is subjected to very fast, short run time HPLC separations such that incomplete chromatographic separation occurs. In the latter instance, coelution of potentially high levels of endogenous compounds such as phospholipids, bile salts, and so on may cause significant suppression of the target analyte(s) response.

Although this topic of matrix suppression remains an important caution for anyone employing electrospray studies, there are a few things one can do to minimize this problem. The best approach is to provide sufficient sample cleanup that the analyzed sample closely resembles an analytical standard. In practice, this is rarely realized although the use of molecular recognition provided by immunoaffinity extraction, for example, approaches this goal. [11,12]. In addition, improved chromatographic separation efficiency such as that provided by ultra performance liquid chromatography (UPLC) with careful method optimization for optimal chromatography also minimizes matrix suppression of ionization for eluted compounds of interest.

3.2.1.4 Range of Compounds Amenable to Electrospray Ionization. Unfortunately, electrospray ionization does not provide universal detection of all compound types. Although it is particularly useful as mentioned above for polar small and large molecules, it is not well suited for the trace detection of polynuclear aromatic hydrocarbon (PAHs) compounds that lack heteroatom functional groups or certain very high molecular weight industrial polymers. There are some tricks that allow improved detection of lipids and other neutral compounds by doping the spray solvent with alkali metal ions such as lithium [13,14], and it is well known that even trace amounts of sodium or potassium from glass solvent containers will produce adduct

ions with oxygen-containing neutral compounds such as steroids. If none of these tricks work, one is forced to employ other ionization techniques that are amenable to LC/MS such as atmospheric pressure chemical ionization (APCI) [15] or atmospheric pressure photo ionization (APPI) [16].

3.2.2 Effects of High Versus Low Liquid Flow Rates on Electrospray Performance

3.2.2.1 Conventional LC/MS Flow Rates. Historically, analytical HPLC users have employed mobile-phase flow rates ranging from 1 mL/min to as high as 6 mL/min when nonmass spectrometer detectors were used. However, when the early LC/MS experiments were carried out, it quickly became apparent that the MS vacuum system could not handle such high liquid flow rates [17]. In contrast, the earliest analytical electrospray-MS experiments were confined to spray solvent flow rates of only about 5 mL/min, which in the early 1980s did not have an HPLC flow regime analog so that electrospray LC/MS experiments were not practical or even possible; for example, micro- and nano-HPLC techniques were very rare at that time. Efforts soon followed to develop LC/MS capabilities that would accommodate higher HPLC flow rates, which have evolved to routinely allow mobile-phase flow rates ranging from 0.2 to 1.0 mL/min. However, even today, it is generally known that electrospray detection limits tend to be higher (poorer sensitivity) at higher spray flow rates than at lower flows. Performance is also diminished if the mobile phase has a high aqueous content as well as high concentrations of mobile-phase additives such as volatile acids, bases, or other additives used for improvement of chromatographic or ionization performance. Thus, the electrospray LC/MS practitioner must honor a compromise in control of these variables to achieve optimal sensitivity and performance of the LC/MS system.

3.2.2.2 Nanoscale Spray Liquid Flow Rates. In contrast to the trend to achieve LC/MS capabilities with higher HPLC flow rates, in the late 1990s, Wilm and Mann [18] revisited the low liquid flow rate regime which they called *nanoelectrospray ionization* (nano-ESI). This technique employed spray liquid flows in the sub-100 nL/min range where the nano-ESI ionization occurred in the spray plume emanating from a pulled-glass capillary emitter with an inside diameter of only 5–10 μm . Because the liquid flow was so low, this technique produced much smaller droplet sizes and ultimately was demonstrated to provide the potential for higher sensitivity determinations. Later studies by Vouros and others [19–22] have reported reduced matrix suppression of ionization when low nanoliters per minute spray flows are employed, and in more recent work, the normalization of different analyte response under nano-ESI conditions has been reported [23,24]. Further examples of nano-ESI are described later in the chip-based nano-ESI section of this report.

3.3 MODES OF LIQUID INTRODUCTION FOR ELECTROSPRAY IONIZATION

3.3.1 LC/MS Sample Introduction

3.3.1.1 Benefits of the Chromatographic Process. The routine coupling of HPLC and more recently UPLC [25] to mass spectrometry (LC/MS) has now become a routine application employed across a large range of scientific disciplines. The additional

analytical power of “LC/MS” includes the unique merits of tandem mass spectrometry (MS/MS) [26]. When the feature of tandem mass spectrometry is combined with HPLC or UPLC, one has LC/MS/MS. This unique analytical tool or combination of techniques was reported in 1982 [27] and has progressed from what was once a curiosity to what is now considered the “gold standard” of, for example, the pharmaceutical industry, which has thousands of systems in routine use. Once the “interface” issues were satisfactorily addressed with the development of pneumatically assisted electrospray [6] and the heated pneumatic nebulizer interface [7], the benefits afforded by LC/MS and certainly by LC/MS/MS have been difficult to ignore. This pertains because each hyphenation “segment” of LC/MS/MS offers an orthogonal component or different mechanism of separation. This provides unequalled analytical capabilities because of the multiplicative nature of these orthogonal separation mechanism techniques.

HPLC sample introduction techniques allow the separation of complex mixtures containing historically intractable compounds. These include very polar and high molecular weight compounds not amenable to, for example, GC/MS techniques. Thus, with the combination of HPLC chromatographic separation of a vast array of challenging compounds and now the ability to detect, identify, and even quantify them via LC/MS/MS, this technology has become widely accepted in many disciplines. More recent applications have appeared in the food industry as well as the clinical diagnostic laboratories of major health care and service industries. In all instances, it is the combination of the chromatographic separation process on-line with MS or MS/MS that has received the major interest. Therefore, the benefits of directly coupling HPLC techniques with MS should be relatively obvious. The HPLC system provides an on-line separation of a complex mixture and introduces the separated components of the mixture sequentially to the mass spectrometer. The better the HPLC separation, the better we expect the expected results to be. The “perfect” LC/MS analysis of a complex sample mixture would efficiently separate or resolve the analyte(s) of interest from each other as well as all endogenous chemical constituents. In practice, this is rarely achieved because real-world samples are chemically very complex and/or the chromatographic process is not sufficiently effective to separate all the mixture components from each other.

To complicate matters, in today’s world, most LC/MS experiments are done with relatively short run times and with incomplete HPLC separations. Thus, much of the time an observed HPLC peak will in fact be composed of two or more chemical components. therefore, we are often doing “mixture analysis” within a mass spectrometric scan acquisition in spite of the fact that we have conducted an HPLC separation. There is a good reason why we can actually do this! One of the major benefits of MS/MS is its “mixture analysis” capabilities. Cooks *et al.* [26] demonstrated this capability in the early 1980s. By selecting the unique mass-to-charge ratio of the ionized component in a mixture that represents the targeted analyte, this “precursor” ion may be focused into the collision cell of the tandem mass spectrometer where it is subjected to collision-induced dissociation (CID) and fragmented to its family of product ions (Fig. 3.3). The selected precursor ion will likely represent only the target analyte even though it was initially in the presence of other nonisobaric compounds. This effectively provides a unique approach to mixture analysis, which can complement the sometimes incomplete HPLC separation process. It is this “mixture analysis” capability that has helped make LC/MS/MS sample analysis techniques so hugely successful and popular. Since this suggests that a sample may not always need to be single component, or pure sample, it

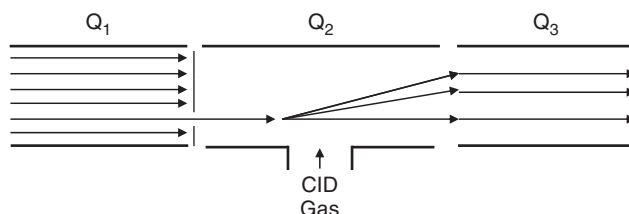


Figure 3.3 Schematic representation of a collision-induced dissociation (CID) precursor–product ion scan or transition in a tandem triple quadrupole mass spectrometer. *Source:* Reprinted with permission from Advion BioSciences, Inc. Ithaca, NY 14850.

is sometimes possible to analyze these samples without the HPLC separation component of the experiment. Thus, “infusion” sample introduction techniques are addressed in the next section.

3.3.2 Infusion Mass Spectrometry Sample Introduction

3.3.2.1 An Alternative to the Chromatographic Process. Samples may be introduced into a mass spectrometer without HPLC. This of course provides no “chromatography” or the considerable attributes provided by HPLC. Thus, the entire HPLC system (solvent bottles, autosampler, pumps, plumbing, and HPLC column) may be completely set aside and replaced by a simple “infusion” pump or other sample solution delivery system, which steadily introduces a continuous supply of the sample solution to the electrospray emitter that produces the electrospray aerosol of charged droplets and gas-phase ions from the sample. This form of sample introduction has been used for many years as part of the “tuning” process to “preflight” and “tune” the mass spectrometer for optimum performance before performing LC/MS experiments (see below).

3.4 NANO-ESI IONIZATION (NANO-ESI)

3.4.1 Early Infusion Using Pulled-Glass Capillaries

In the mid-1990s, the infusion sample introduction approach was extended to a new paradigm that involved an ~ 1000 -fold reduction in the sample solution flow rate through the electrospray emitter. As noted above, Wilm and Mann [18] were the first to report on the relative merits of infusion nano-ESI, and improvements in our understanding of this technology was provided later by Karas and collaborators [21,28]. In these latter experiments, they employed a pulled-glass capillary nano-ESI emitter that had a very small orifice [2–10 μm inside diameter (i.d.)]. This provided a sustained spray with an applied high voltage in the absence of any external hydrodynamic pumping. It had been known for several years that there were challenges with stabilization of the Taylor cone and formation of an aerosol of droplets with just the use of electric fields under higher liquid flow conditions. To improve on the stabilization of analyte ion currents, one must provide additional forces such as from pneumatic nebulization [6] and/or heat [7] to facilitate droplet formation and thus complement the role of the electric field for droplet charging. When very low liquid flows (nanoflow of liquids)

are employed, the situation is much different. Other forms of external energy to the charged droplets are not required because the electric field is adequate to charge the liquid and produce the aerosol.

As noted above, the early reports by Wilm and Mann employed “pulled capillaries” made from small glass tubes similar to lambda pipettes or melting point capillaries which were drawn out to a tapered tip while being heated in a small flame. Often the tapered capillary would become closed by this process but could be reopened by carefully breaking off the extremity of the pulled tip. The sample to be analyzed was added manually via a syringe into the larger, inlet opening of the tapered capillary, and this device would be mounted very close to the vacuum inlet of the mass spectrometer wherein a self-sustained nano-ESI aerosol would commence with an applied sprayer voltage. Impressive results first began to appear in the mid-1990s [18] from this approach, but some early practitioners experienced difficulties with maintaining a sustained spray due to clogging of the tapered capillary and related issues. However, interest began to develop in this approach especially in the field of protein and peptide characterization where the continuous infusion of limited, small sample volumes provided extended analysis times to interrogate the sample via multiple MS/MS experiments [29]. In addition, improved sensitivity, increased ion current stability, so-called normalized response, and an apparent decrease in matrix effects [19–24, 30] appeared to offer worthwhile benefits because of nano-ESI. An excellent review of nano-ESI developments was reported recently by Covey and Pinto [31] and Marginean *et al.* [32], while a more general review of electrospray fundamentals has been reported by Cech and Enke [33].

Owing to the increasing interest in these nano-ESI techniques, more reliable pulled capillaries became commercially available first by New Objective, Inc. and later by other suppliers (Proxeon BioSystems, Denmark). However, there was still a considerable degree of manual skill associated with using these devices. The tapered flow path followed by the sample from the inlet of the pulled capillary to the exit tip could cause clogging, which sometimes would be irreversible and require repeating the experiment with the installation of a new pulled-capillary emitter. The small spray orifice dimensions of these devices (10–30 μm i.d.) require particle-free samples that can be difficult to achieve with real-world biological samples. Thus, there was relatively slow acceptance of nano-ESI for some time because it was relatively challenging to successfully carry out routine experiments.

More recent developments, however, have provided commercially available tools that help the new user adapt to these. A few years ago, Agilent introduced its “ChipCube” hardware which integrates a microfabricated HPLC column with a nano-ESI emitter. This “chip” is inserted into the ChipCube wherein all electrical and microfluidic connections are made inside the instrument. The user simply uses a relatively conventional autosampler to make sample injections followed by nanoscale LC/MS techniques (Agilent Technologies, Santa Clara, CA). More recently, Waters has introduced its Trizaic microfabricated chip device which also provides an integrated nanoscale HPLC column with a (removable) nano-ESI tip for on-line nano-LC/MS analysis of, for example, dried blood spots (Waters, Milford, MA). Finally, there is also a new glass chip reported by scientists at Eksigent Technologies which has been coupled via a fused silica capillary directly to a pulled-capillary nano-ESI emitter for on-line nano-LC/MS applications (Eksigent Technologies, Dublin, CA). Each of these recent commercial introductions suggests that in time nanoscale techniques

including nano-ESI will grow as the analytical benefits of high sensitivity and related opportunities become more routine.

3.4.2 Chip-Based Nano-ESI

3.4.2.1 The ESI Chip™. In the late 1990s, there was a growing awareness that nano-ESI had some worthwhile merits, but only the more determined scientists were willing to further explore these. At this time, two scientists from Advion working within the Cornell Nanofabrication Facility at Cornell University (Ithaca, NY) developed a microfabricated chip-based device as an alternative to the pulled capillary for nano-ESI [34]. The first-generation ESI Chip™ was a 10×10 array of approximately $5\text{ }\mu\text{m}$ i.d. microfluidic nozzle emitters. The “ESI Chip” was fabricated from a polished $500\text{-}\mu\text{m}$ -thick silicon wafer. This substrate enabled the formation of an inlet feature that could be etched opposite the nozzle exit to form a cylindrical through channel. This allowed for the communication of liquid introduced from the back or inlet side of the chip through the substrate, which could then be sprayed toward the inlet of an API inlet of a mass spectrometer. The introduction of sample to the ESI Chip through channel is accomplished by the seal formed by a polymer-based, conductive pipette tip close coupled around the inlet region of the chip-nozzle region. Figure 3.4 shows a close-up view of the ESI Chip with increasing magnification allowing visualization of the actual nozzle emitter features. Although the first-generation ESI Chip consisted of a 10×10 array of 100 nozzle emitters, this device was soon expanded to a 20×20 array of 400 nozzle emitters. The current version employs sample introduction via a disposable pipette tip which is depicted in Fig. 3.4. The nozzle dimensions are controlled to within a micron such that changes in the i.d. of the nozzle are used to limit the flow rate of liquid for nano-ESI conditions. There are three general categories of ESI chips that differ with respect to nozzle i.d.s. The common dimensions are 2.5, 4.1, and $5.5\text{ }\mu\text{m}$ i.d. which provide liquid flow rates in ranges of 20–40, 60–100, and 100–500 nL/min, respectively.

A unique feature of the ESI Chip is the presence of the nano-ESI counterelectrode integrated into the nozzle structure. This is accomplished using the semiconductor properties of the silicon substrate and a dielectric coating on all surfaces of the chip [35]. This dielectric allows the application of voltage to liquids delivered to the nozzle structure while also applying a separate voltage or a ground to the silicon underlying the dielectric. The application of a voltage difference between the applied fluid and the silicon creates an electric field across the dielectric and the surface surrounding the nozzle when the fluid exits the nozzle. The high field generated by this close geometry produces a Taylor cone that ultimately produces the nano-ESI plume of charged droplets and thence gas-phase ions that are necessary for electrospray mass spectrometry.

3.4.2.2 The TriVersa NanoMate Robot. A critical requirement for employing the ESI Chip for automated nano-ESI applications is the precise alignment of the conductive pipette tip containing the sample with the inlet of the ESI Chip emitter channel that resides within the array of ESI chip nozzles as well as providing proper alignment of the emitter with respect to the API inlet of the mass spectrometer. The NanoMate was the first-generation robot to accomplish this task. This device positioned the chip appropriately near the mass spectrometer inlet while also providing a real-world-to-chip interface for sample introduction. It has been noted that lab-on-a-chip devices would

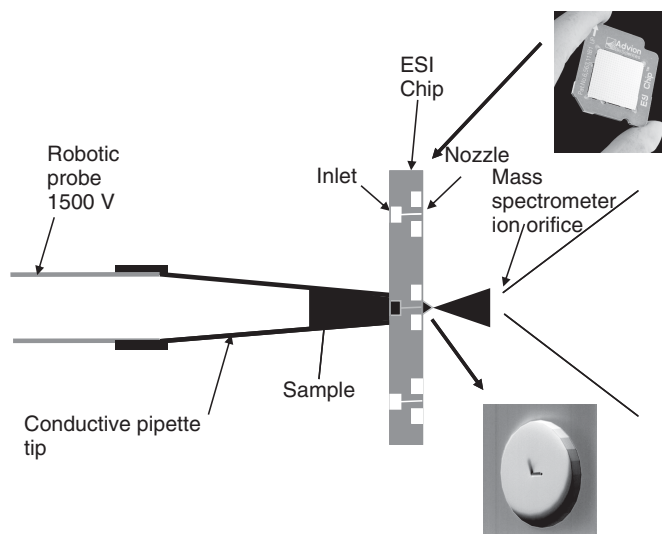


Figure 3.4 Schematic drawing of how the robotic probe from the TriVersa NanoMate delivers a conductive pipette tip to the inlet of the ESI chip. The upper inset shows the entire ESI chip, while the lower inset shows a magnification of a single microfabricated nano-ESI spray emitter. *Source:* Reprinted with permission from Advion BioSciences, Inc. Ithaca, NY 14850.

seem to have a bright future, but only if we have convenient and effective means of transferring real-world samples to the chip [36]. The NanoMate robotic system accomplished this in a rather simple way by employing a single-use, disposable pipette tip to aliquot individual samples from sequential wells in a multiwell plate followed by delivering that sample solution directly to the rear, inlet feature of an individual nozzle emitter in the ESI chip (Fig. 3.4). The robot holds a 96 or 384 rack of electrically conductive pipette tips and either a 96-well or 384-well plate of samples in solution. The pipette tips are coated with a proprietary material to preclude oxidation of analytes and to facilitate a leak-tight seal between the tip of the pipette and the ESI chip. The robot operates by picking up a new pipette tip with its metal mandrel which communicates with a gastight syringe for sample aspiration from the multiwell sample plate. The device is computer controlled such that it will go to a selected sample well(s) to aspirate some sample and then deliver the sample solution to a particular ESI chip-nozzle inlet. A schematic drawing of this pipette tip–ESI chip interface is shown in Fig. 3.4. When the pipette tip has mated to form a leak-tight seal at the rear, inlet side of the ESI chip, a combination of nitrogen gas pressure (0–0.5 psi) and high voltage (1.2–1.6 kV) are applied to the pipette tip whereupon fluid delivery and nano-ESI commences. The sprayer voltage and gas pressure may be optimized via the Chipsoft software to form a stable nano-ESI spray current. The robot uses a new pipette tip and nozzle emitter for each sample which eliminates any possibility of sample-to-sample carryover or cross contamination. When operated in this manner, this robotic system provides automated nano-ESI infusion of sample solutions, allowing ample acquisition and analysis time for multiple MS or MS/MSⁿ experiments, but in this format, this is done without any on-line chromatographic separation (see below). A later version of this robotic system was developed and called *the TriVersa NanoMate*, which had the additional

features of automated on-line LC-fraction collection-MS as well as on-line LC/MS via an LC coupler to the chip in addition to infusion experiments [37–45].

3.4.3 Attributes and Challenges of Automated of Nano-ESI Infusion Experiments

3.4.3.1 Sensitivity. There are a number of benefits afforded by nanoflow techniques when the liquid flow rate of the sprayed sample solution is reduced substantially below the usual microliter per minute regime. Nanoflow electrospray has taught us that the efficiency of the ionization process improves approximately in proportion to the flow rate reduction. Although the flux of sample molecules entering the API inlet of the mass spectrometer is much lower under nanoflow conditions, the signal per unit time remains constant and detection limits are improved. Enke [46] has also taught us that the improvement in sensitivity under very low liquid flow rate conditions must be due to a greater efficiency in the conversion of the surface excess charge to vapor-phase ions. An additional benefit of this approach is the use of signal-averaging techniques especially in the tandem mass spectrometry mode where the selection of a precursor ion, for example, greatly reduces the chemical noise in these experiments such that ions from chemical noise are not included in the measurement. Thus, multicomponent mixtures of peptides from enzymatic digests of proteins can be analyzed by nano-ESI infusion MS/MS with plenty of “time” to produce high quality mass spectra [31]. In addition, when the available sample is limited, nanoflow infusion of very small volumes (1 μ L) at 50 nL/min can last for 20 min, allowing plenty of time for interrogating the many different components of the sample mixture with multiple MS/MS experiments, and so on. It is worth noting that although “separation” of the sample components is not being afforded by chromatography in such an infusion experiment, another important “separation science,” tandem mass spectrometry, is providing a mass-to-charge separation in the microsecond timescale. This is accomplished by sequentially selecting nonisobaric precursor ions of individual chemical components of a mixture, for example, and subjecting them to CID independent of the other components in the mixture. This “mixture analysis” capability of MS/MS is a very important benefit, which either reduces the need for chromatography or, in some cases, replaces it.

3.4.3.2 Matrix Suppression of Ionization. The importance of matrix effects in electrospray was briefly discussed above. Certainly, it is well known that the presence of other chemical constituents in the electrospray solvent concurrent with the analyte of interest can produce an abnormal effect on the ion current response of the latter [9,10,19, 47–50]. However, there are indicative reports in the literature which suggest that under the lower flow rate conditions of the spray solvent, under nano-ESI conditions, these matrix effects are reduced [19,22]. Thus, Karas *et al.* showed that when a solution of sodiated turanose at 10×10^{-5} M and *n*-octylglucoside at 10×10^{-6} M was sprayed through a pulled-capillary needle at flows ranging from 1000 nL/min to as low as 20 nL/min, a significant reduction of ionization suppression was observed [22]. Thus, the ratio of turanose/octylglucoside increased from about 2 to 13 as the spray solvent flow was reduced to below 50 nL/min. Similarly, Vouros *et al.* [19] reported a reduction in matrix suppression of ionization under LC/MS conditions as the HPLC effluent was split postcolumn such that the spray to the API mass spectrometer was

operated under nano-ESI conditions. In an early report, Enke [46] described a predictive model for matrix and analyte effects in electrospray ionization of single charged ionic analytes. In this paper, Enke [46] suggests that in low flow electrospray experiments, the solution flow rates are up to 1000 times less than conventional electrospray, but the needle spray currents are comparable. Under these conditions, the excess charge (Q) will be as much as 1000 times greater than that for normal electrospray. One would thus predict that the linear operating range would extend to much higher concentrations and greatly reduce the depressive effect of higher electrolyte concentrations; for example, one should observe reduced matrix suppression to ionization.

3.4.3.3 Ion Current Stability. A common deficiency of electrospray ionization that most users do not fully appreciate is the instability of the ion current generated by this technique. Anyone who has ever worked with electron ionization (EI) mass spectrometry knows what a very stable ion current really is. The ion current from a rhenium filament operated in a vacuum of 2×10^{-5} , which is common for most EI GC/MS experiments, is significantly more stable than that from a conventional ion spray or turboionspray LC/MS experiment. This becomes especially relevant under trace quantitative analysis conditions where the precision and accuracy of the chromatographic peak area (or height) measurement is important.

One of the reasons for a more unstable ion current under higher liquid flow electrospray experiments is the challenge associated with rapidly desolvating the relatively large liquid droplets in the spray aerosol. Since we do not observe ion current in electrospray ionization until or unless we generate gas-phase ions, the mechanism(s) of the electrospray process must be known to truly understand why electrospray ion current can display considerable instability under different conditions. An excellent description of the relevant factors has been reported by Marginean *et al.* [32]. In this treatise, these researchers teach us that the charged liquid is ejected from the cone apex as a liquid jet which breaks down into small charged droplets due to varicose wave instabilities developed along its surface. This cone-jet electrospray regime has been shown to be the optimum for many applications [51]. The transitions between “regimes” of operation, induced by changing operating parameters such as voltage, flow rate, spray solvent composition, distance between the spray emitter and the counterelectrode, and others can be sudden and chaotic. As an example, by increasing the spray voltage of a dripping electrospray, chaotic transitions to the pulsating and then to the cone-jet regimes have been observed and labeled a burst and a stable modes, respectively [32]. These and other microscopic features within the electrospray process contribute to ion current instability. Such instability is most prevalent in higher liquid flow rate, gradient LC/MS applications where there are ongoing changes in the spray conditions where it is difficult to remain within a given, optimal spray regime, for example, the cone-jet regime. However, when infusion nano-ESI conditions are maintained, it is a bit more straightforward to maintain constant, low flow spray conditions without changing the spray solvent chemistry. When the cone-jet regime is maintained, one can obtain truly stable ion current conditions employing infusion nano-ESI conditions.

Figure 3.5 compares the ion current signal stability between infusion employing Turboionspray (TIS) and the ESI Chip using a 10-ms selected reaction monitoring (SRM) dwell time for venlafaxine (m/z 278.2 > 58.1) [35]. The ion current signal stabilities measured from 1 min of spray for TIS and the ESI Chip were 15% and 3.4% relative standard deviation (RSD), respectively. These data were obtained with

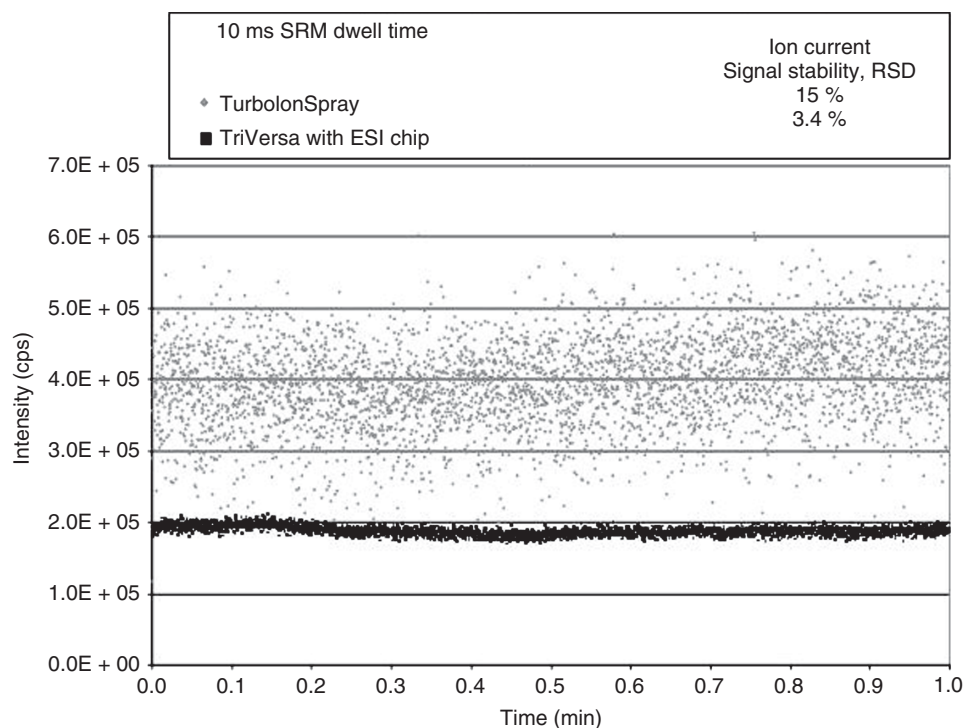


Figure 3.5 Plot of SRM data points collected every 10 ms using Turbo ion spray (TIS) and the ESI Chip for venlafaxine. See text for experimental details.

a 5 μ M venlafaxine solution at 5 μ L/min mixed with 400 μ L/min of 75% ACN, 27% water, and 0.2% formic acid. This solution was sprayed using the TIS at 450° C while monitoring the venlafaxine SRM transition from m/z 278.2 > 58.1. The nano-ESI data were obtained with a 5 μ M venlafaxine solution at 5 μ L/min mixed with 600 μ L/min of 75% ACN, 25% water, and 0.2% formic acid. This solution was split to deliver 200 nL/min to the ESI Chip while monitoring the venlafaxine SRM transition from m/z 278.2 > 58.1. Data collected every 10 ms resulted in a 15% RSD with TIS and 3.4% RSD with the ESI. From these data, it is clear that when short dwell times are employed, the ESI Chip ion current stability remains much more stable (lower trace in Fig. 3.5) than that of the TIS ion current stability (upper trace in Fig. 3.5). These data demonstrate a beneficial aspect of infusion nano-ESI conditions, in that often the spray ion current can be significantly more stable than it is at much higher spray solvent flow rates. The benefits of this include improved signal-to-noise levels at very low analyte concentrations and improved accuracy and precision for quantitative analyses.

3.4.3.4 Normalized Response. One of the challenges in early drug discovery is acquiring *in vitro* and *in vivo* quantitative information on the parent drug and its possible metabolites from new chemical entities by electrospray LC/MS/MS techniques. In the early stages of drug discovery, stable isotope internal standards are not available, and yet reliable quantitative information is still needed. One approach to this dilemma would be to assume that the electrospray response from the metabolites is the same

as the parent compound [23,30]. If this were true, one might assume that the area (or heights) of the LC/MS chromatographic peak(s) for metabolites are comparable to the parent drug when referenced to a chemical analog or homolog internal standard used to produce a calibration curve based on the parent compound. Ramanathan *et al.* [24] have shown that by maintaining the same spray solvent conditions across a gradient LC/MS run, “response-normalized” results may be obtained under nano-ESI conditions. In their work, one HPLC unit performs the analytical separation, while the other pump adds solvent postcolumn with an exact reverse of the mobile-phase composition such that the final composition entering the nano-ESI source is isocratic throughout the entire HPLC run. The data obtained from four different structural classes of compounds [vicriviroc (VCV), desloratadine (DL), tolbutamide, and cocaine] and their metabolites indicate that by maintaining the solvent composition unchanged across the HPLC run, the influence of the solvent environment on the ionization efficiency is minimized.

This is reinforced by some observations we have made in related infusion nano-ESI studies. Figure 3.6 shows results from an infusion experiment employing the ESI Chip for an equimolar solution containing 10 exp-5M concentrations for each of aspartame, cortisone, and reserpine (unpublished results, Advion BioSciences, Inc). In the first panel, the protonated molecules for these compounds at m/z 295, 361, and 609, respectively, are shown to display unequal relative abundances when sprayed at 50 μ L/min. For example, the protonated molecules for aspartame and cortisone are less than half the abundance of reserpine present at the same concentration. However, when the spray solvent flow is reduced to nano-ESI conditions at 100 nL/min, the protonated molecule ions at m/z 295 and 361 for aspartame and cortisone, respectively, are increased to be nearly the same as for the protonated molecule of reserpine at m/z 609. These results are consistent with the published results of others which suggest that at least in some instances, nano-ESI flow conditions can provide a normalized response for dissimilar compounds when presented at equimolar concentrations.

3.4.3.5 Dynamic Range. Another important aspect of quantitative analysis experiments or the bioanalytical determination of drugs and their metabolites in either drug discovery or drug development with biological samples is the dynamic range of the quantitative SRM LC/MS experiments [52]. Sometimes, the concentration of the drug and/or its metabolites can range from low picograms per milliliter to several micrograms per milliliter [53]. Thus, it would be helpful if the linear dynamic range could span five to six orders of magnitude or more. However, in conventional SRM LC/MS experiments, it is often a struggle to achieve a linear dynamic range of concentrations that span even three orders of magnitude.

A recent report describes the use of chip-based automated nano-ESI bioanalytical determination of reboxetine with a 500,000-fold linear dynamic range in dog plasma using an analog internal standard [54]. The optimal nano-ESI response for reboxetine was observed with 90% ACN/water without mobile-phase modifiers. Infusion SRM data were collected for all samples to generate ion current profiles with a base “infusogram” ion current profile width of ~20 s. Matrix suppression was evaluated and ranged from ~30% to 60% across the different lots and species which were found to be suppressed by similar amounts, suggesting the similarity in ionization properties between the two. A comparison of analyses by nano-ESI-MS/MS and liquid chromatography

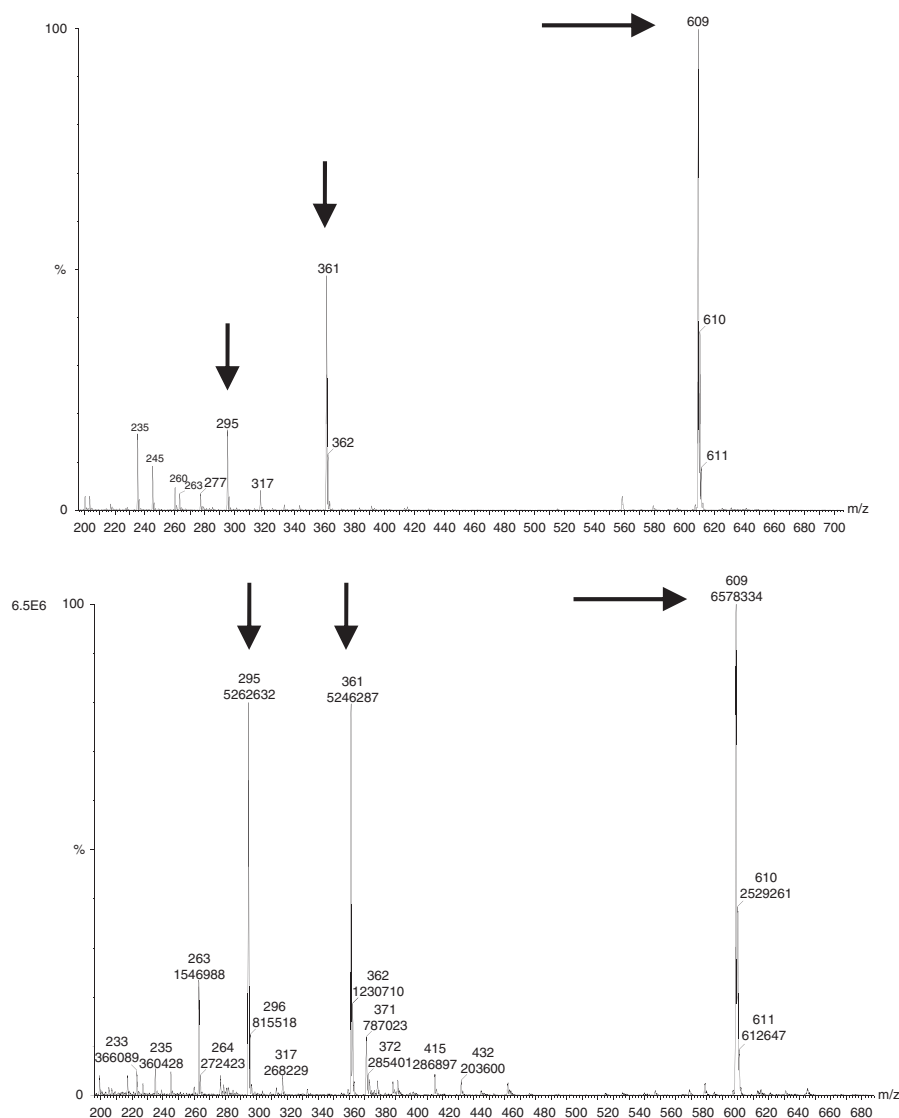


Figure 3.6 Infusion of an equimolar 10^{-5} M solution of aspartame, cortisone, and reserpine under two different solution flow rate conditions. The protonated molecules in each experiment are denoted with their respective arrows. See text for experimental details. *Source*: Reprinted with permission from Advion BioSciences, Inc. Ithaca, NY 14850.

coupled to tandem mass spectrometry (LC/MS/MS) showed that nano-ESI-MS/MS had a greater slope for the calibration standard curve compared to LC/MS/MS, indicating greater sensitivity for the former technique. These authors also indicated that the amount of sample infused during nano-ESI-MS/MS was ~ 80 -fold less compared to the amount of sample injected during LC/MS/MS. The absence of carryover (attributed to the lack of a common fluid path) in the nano-ESI infusion technique enabled the

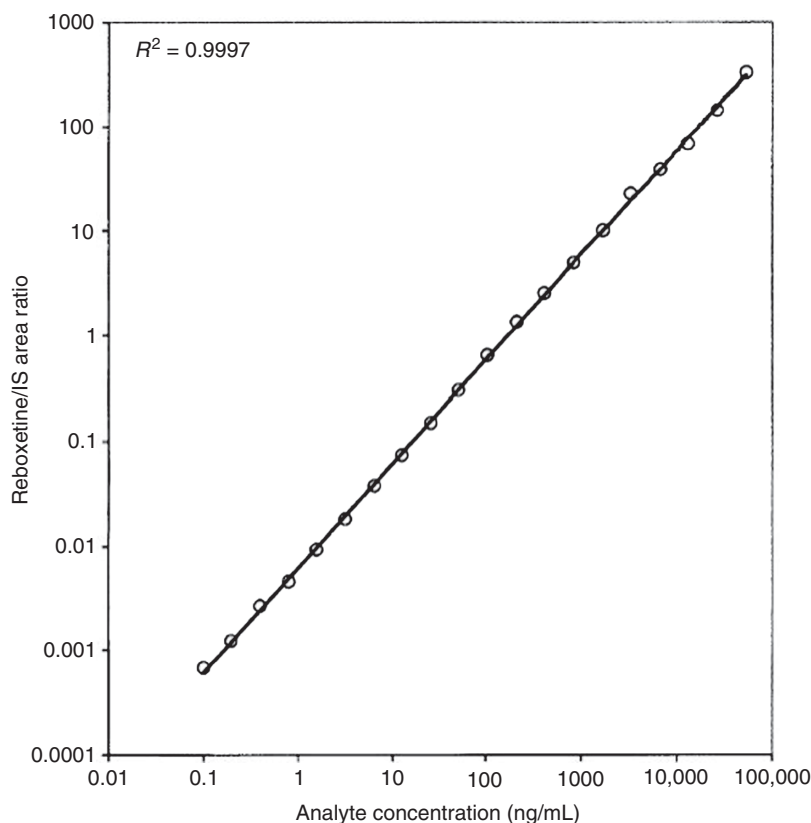


Figure 3.7 Calibration standard curve for reboxetine in dog plasma ranging from 100 pg/mL to 50,000 ng/mL (500,000-fold range). The plot is displayed in log–log scale using power regression with no weighting. *Source:* Reprinted with permission from Wickremsinhe ER, Ackermann BL, Chaudhary A. Validating regulatory-compliant wide dynamic range bioanalytical assays using chip-based nanoelectrospray tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2005;19:47–56.

extension of the assay linear dynamic range to 500,000-fold, and the possibility of analyzing samples in a single batch without the need for reanalysis of samples with high concentrations.

The amount of carryover in typical LC/MS/MS analyses limited the range of the reboxetine assay to 200-fold, thus requiring a second dynamic range assay or analysis following dilution to quantify samples with concentrations above the range. This not only delays the reporting of data but also invariably increases the cost of analyses as well as the amount of documentation required, especially when operating under GLP compliance. In contrast, this report presents data using robotic instrumentation that demonstrates total elimination of system carryover with a dynamic range spanning 30,000-fold for reboxetine, and extendable up to 500,000-fold. A standard curve ranging from 0.1 to 50,000 ng/mL was prepared and analyzed demonstrating the possibility of analyzing samples spanning a dynamic range of 500,000-fold (Fig. 3.7).

3.4.3.6 Conservation of Limited Sample Quantities. Infusion nano-ESI analyses of precious samples provides the opportunity to acquire data from multiple sequential experiments such as MS/MS, MS^n , or K_d studies [55] while consuming very little of the sample. For example, during the course of studying biospecific noncovalent host–guest interactions between a precious target of limited availability with a series of drug candidates, an infusion nano-ESI analysis experiment conducted at 100 nL/min will consume only 2 microliters of sample over an experimental acquisition time of 20 min. Thus, conservation of precious or limited sample quantities may be achieved by this analytical strategy.

3.4.3.7 Signal Averaging. In some instances, LC/MS/MS experiments are conducted in order to identify a trace level analyte. To accomplish this task, a high quality full-scan CID mass spectrum is often required so the analyst can interpret the spectrum for structural characterization. In many cases, the quality of a CID mass spectrum may be compromised due to the low level of the analyte that provides a weak LC/MS/MS signal. This fact coupled with a relatively fast scan rate needed to acquire data across the HPLC peak produces mass spectra with sufficiently weak ion current signals that it may be uncertain which CID mass spectral peaks are real. This problem may be mitigated if the sample analyte can be continuously infused while the mass spectrometer is scanned in a slower, repetitive manner and the ion current signal is “averaged” as is customarily done in extended NMR experiments. This “signal-averaging” approach can produce much higher quality full-scan CID mass spectra for structural characterization.

An example of these described benefits is shown in Fig. 3.8a–c, which shows the infusion full-scan MS/MS mass spectra for a very weak chromatographic peak from the carboxy-ibuprofen-diglucuronide metabolite from the LC/MS/MS analysis and fraction collection of a human urine extract after the administration of ibuprofen [35]. This weak metabolite was fraction collected (see below) during the gradient LC/MS/MS run which provided a CID mass spectrum of insufficient quality for metabolite identification. The collected fraction was subsequently analyzed by nano-ESI infusion using the TriVersa NanoMate and the ESI Chip coupled to a QTOF mass spectrometer system. Figure 3.8a–c shows the infusion MS/MS mass spectra of the protonated molecule at m/z 589.3 of the trace LC fraction for the carboxy-ibuprofen-diglucuronide peak described above. A sequence of full-scan CID acquisitions for 5 s (Fig. 3.8c), 1.8 min (Fig. 3.8b), and 8 min (Fig. 3.8a) shows a dramatic improvement in the signal-to-noise ratio with extended acquisition time as well as mass spectral data quality from these signal-averaging experiments, which may be readily accomplished by nano-ESI infusion of trace fractions collected from an LC/MS/MS experiment. Infusion of this trace LC-collected metabolite enables the acquisition of a high quality MS/MS spectrum by signal averaging the product ion mass spectrum of the precursor ion at m/z 589.3. Infusion of LC fractions can easily improve the signal-to-noise ratio by a factor of 10 when compared to that obtained using on-line LC/MS/MS, and up to 50-fold signal enhancement has been reported [56].

3.4.3.8 Extended Analysis Time for Multiple MS/MS Experiments. Today’s modern mass spectrometer systems provide a wide variety of possible experiments that can be performed while an analyte is being ionized and introduced into the mass analyzers. This includes changing voltages and potentials which are helpful for establishing the

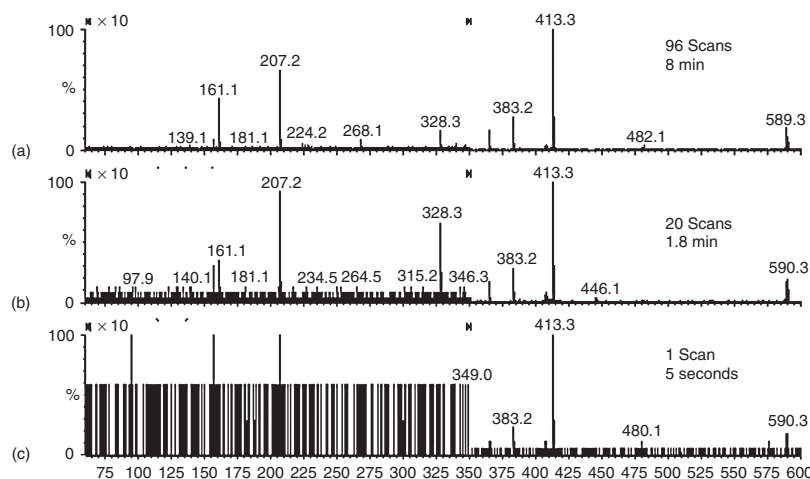


Figure 3.8 Infusion MS/MS of m/z 589.3 of an LC fraction for the carboxy-ibuprofen-diglucuronide peak from an extended analysis time LC separation. (a) sum of 96 scans; (b) sum of 20 scans; (c) single scan. *Source:* Reprinted with permission from Schultz GA, editor. A silicon-based ESI chip with integrated counter electrode and its applications combined with mass spectrometry. Miniaturization and Mass Spectrometry, ed. S. Le Gac and A. van den Berg. 2009, The Royal Society of Chemistry.

optimum sensitivity performance of the system as well as multiple MS/MS experiments to derive additional structural information from a particular chemical compound (see below). As an example, a modern QTRAP system is a triple quadrupole mass spectrometer with a linear ion trap (LIT) feature in Q3. Thus, the conventional tandem quadrupole scan modes are available as well as several high sensitivity ion trap mass spectrometer scans using the final quadrupole as a LIT mass spectrometer. These include an enhanced product ion (EPI) mode, enhanced resolution (ER) mode, and an enhanced MS (EMS) mode, which all benefit from the third quadrupole operating as a LIT. A wide variety of useful experiments may be programmed with this system, but sometimes, it is difficult to complete all of them during the rapid elution of a narrow HPLC peak in the LC/MS/MS mode. When infusion MS is performed, however, there is ample time to perform all the experiments necessary to serve a given need. There are similar benefits when extended analysis time is available for MSⁿ experiments with three-dimensional ion traps and FT-ICR systems. Therefore, infusion experiments are particularly useful to an analyst who wants sufficient extra time to employ the many additional analytical capabilities of a modern mass spectrometer while examining the mass spectral behavior of a particular chemical compound.

3.4.3.9 Selectivity Afforded by High Resolution Mass Spectrometry. Within just the past few years, there has been a renaissance of interest and analytical capabilities with the advent of modern time-of-flight (TOF) mass analyzers[57] as well as orbitrap technology [58]. This capability is a renaissance because high resolution mass spectrometry was available and employed over 40 years ago, albeit not with the facility of modern condensed-phase sample inlet systems [59]. Specifically, this capability provides high resolution mass separation such that isobaric compounds with even very

small mass differences may be resolved and distinguished from each other. In addition, this capability also provides detailed elemental composition information for not only the molecular species but also the series of product ions resulting from MS/MS or other decomposition processes. As such, this complement or possible alternative to MS/MS can provide valuable selectivity for both the detection as well as the identification of unknown compounds. This latter information may be acquired either coupled with on-line separations such as HPLC or via nano-ESI infusion of suitably cleaned up samples as described above. In the latter case, the added selectivity of high resolution mass measurement can make up for the lack of chromatographic separation provided matrix suppression of ionization or other issues do not preclude satisfactory detection of important analytes. A recent review of the merits of high resolution mass spectrometry and accurate mass measurement was recently reported by Brenton and Godfrey [60].

3.4.4 Applications of Nano-ESI Infusion

3.4.4.1 Infusion of Analytical Standards. One of the initial experiments any expert analytical mass spectrometer system operator should perform is to “tune” the ion optics and calibrate the mass axis of the instrument before analyzing samples of interest or those containing unknown analytes. This has been done for many years with GC/MS instruments by opening a valve which permits the continuous introduction of a volatile reference compound into the ion source mass analyzer. This provides a stable, continuous introduction of a sample that has ions of known mass-to-charge ratios and relative abundances that may be used to tune and optimize the mass spectrometer ion source parameters and the ion optics of the system. For volatile samples, a similar approach was used historically for high resolution MS where perfluorokerosene (PFK) was “leaked” into a heated batch inlet as a gaseous reference compound for exact mass measurements[61].

An API mass spectrometer also requires tuning optimization and mass axis calibration which may be similarly done via the infusion of a reference compound solution or mixture of compounds. For the equivalent experiment in today’s electrospray-MS environment, a solution of a reference standard or mixture of chemical compounds is “infused” continuously through an electrospray sprayer device to produce a continuous source of ions from the reference standards. This allows the operator or the “auto-tune” software program to “tune” and optimize the ion optics of the mass spectrometer and calibrate the mass axis with reference to these standard reference compounds. In these experiments, the sample is usually a standard mixture of known compounds as a relatively clean solution free of any levels of endogenous materials. Today’s modern API MS systems are routinely tuned and calibrated with this sample introduction approach wherein an aqueous/organic solvent mixture of the tuning standard sample is introduced to the mass spectrometer via infusion at flows of perhaps 5–10 mL/min. Of course, this approach would be expected to perform optimally with simple mixtures containing appropriate reference compounds whose molecular weights span the mass range of interest.

The chip-based nano-ESI system described above is ideally suited for automation of these experiments. Thus, a known reference sample containing a selected reference compound or mixture of compounds is infused continuously via nano-ESI in either the positive or negative ion mode while the ion source and ion optics are optimized for

sensitivity, mass resolution, and accurate mass assignment under computer-controlled conditions. This may be done manually by a skilled operator or in an automated mode using the appropriate vendor-supplied “autotune” software. Modern mass spectrometer systems are much more stable and dependable than those of the past, but it is still important to monitor the performance of an API MS system on an ongoing basis as the performance may deteriorate due to exposure to dirty samples, high voltage power supply drift, changes in collision gas pressures in the collision cell, humidity in the laboratory environment, and other variables which can affect the performance of these systems. A special application of this capability is described in the next section.

3.4.4.2 Automated Compound Tuning Optimization. A special case of compound tuning described as a semiautomated procedure for this tuning routine was reported in a recent report by King *et al.* This report [62] describes a semiautomated parameter optimization for high throughput MS/MS detection workflows, which include many different chemical compounds of interest. Although it is generally known that each chemical compound may have a slightly different set of tuning parameters, especially the collision energy (CE) for MS/MS experiments, most MS/MS experiments these days are set up with a fixed set of “average” MS/MS experimental conditions, which may in fact discriminate against some of the compounds. In an ideal experiment, one would optimize the instrument operational parameters for each individual compound so as to obtain the best performance for each compound. However, this is very difficult and typically impractical to do during LC/MS/MS experiments since the timescale as chromatographic peaks elute one after another is only a few seconds. This does not leave enough time for optimizing the tuning parameters.

King *et al.* described a semiautomated procedure that involves placing solutions of many different new chemical entities (future drug candidates) into individual wells of a multiwell plate followed by automated nano-ESI infusion of each compound [62]. Each infusion experiment may last several minutes if necessary, which allows plenty of time to optimize the tuning and MS/MS operational parameters for each compound. These tuning parameters may then be stored in the vendor acquisition software and employed in subsequent on-line LC/MS/MS bioanalytical experiments used for the quantitative determination of these compounds in biological samples. Figure 3.9a–d shows the fragment–precursor intensities as a function of CE for different chemical compounds, which include urapidil, reserpine, acetaminophen, and 6- β -hydroxytestosterone. While the ion current intensities do change with CE, the curves for the fragments are relatively broad. A noted exception to this general trend observed in Fig. 3.9a–d is acetaminophen where the CE voltage required for this compound is much lower (Fig. 3.9c). These results show that a small change in CE can significantly affect the response of the m/z 110 fragment ion for this compound. Thus, the ability to automatically infuse a large number of different chemical compounds in a sequential manner allows sufficient time to determine the optimum CE and other tuning parameters for each compound and thus obtain the best mass spectral results for subsequent pharmacokinetic and toxicological studies[62]. These researchers also reported that the optimized tuning conditions provided nearly identical mass spectral results across the three different triple quadrupole models provided by one major vendor.

3.4.4.3 LC/MS/MS with Simultaneous Fraction Collection. In the early days of HPLC and analytical mass spectrometry, researchers “collected fractions” from their

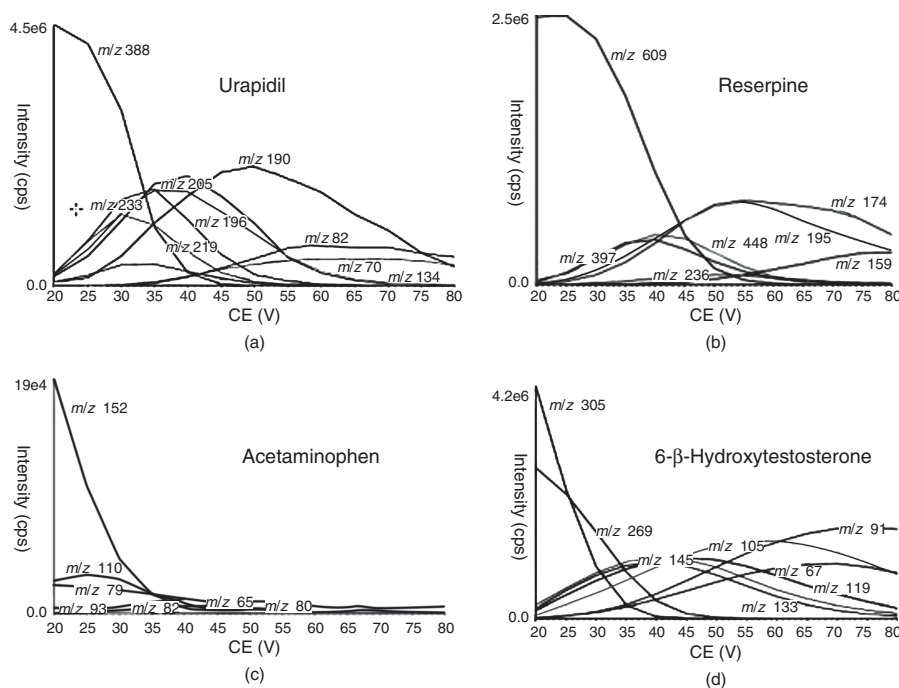


Figure 3.9 Fragment and precursor ion intensity as a function of collision energy (CE). (a) Breakdown curve for urapidil. (b) Breakdown curve for reserpine. (c) Breakdown curve for acetaminophen. (d) Breakdown curve for 6- β -hydroxytestosterone. See text for experimental details. *Source:* Reprinted with permission from Geddes K, *et al.* Semi-automated tandem mass spectrometric (MS/MS) triple quadrupole operating parameter optimization for high-throughput MS/MS detection workflows. *Rapid Commun Mass Spectrom* 2009;23:1303–1312.

HPLC separations and analyzed the individual fractions of collected peaks by direct insertion probe mass spectrometry. This was a tedious procedure, but the valuable information from mass spectrometry made it worth doing. The merits of this information motivated early investigators to explore various ways to accomplish on-line LC/MS and LC/MS/MS techniques. Today, these techniques are ubiquitous and offer a tremendous source of analytical horsepower to researchers across many disciplines. Modern quantitative analyses are performed by LC/MS/MS via SRM experiments that can sample many times across even very narrow UPLC chromatographic peaks, which may be only a few seconds wide at the base. These SRM experiments, however, monitor only one or two precursor–product ion transitions, which can be performed in 5–50 ms. However, for qualitative mass spectral identification of an unknown metabolite or compound, one needs a full-scan CID mass spectrum, which on a triple quadrupole mass spectrometer usually requires one or more seconds to acquire. Also, there are additional meaningful MS/MS experiments such as MS^{*n*} or the summation of multiple scans, which require more time than is often available during the elution of a narrow HPLC or UPLC chromatographic peak.

Today, the mass spectrometric identification of drug metabolites is a common need in the pharmaceutical industry. Although in some cases the full-scan LC/MS/MS analysis

of a biological sample or extract can provide the necessary data for metabolite identification, often the quality of the CID mass spectra is insufficient to provide compound identification. In these instances, it is helpful to have an automated procedure for HPLC or UPLC fraction collection followed by an automated means of analyzing the collected fractions. Hopfgartner *et al.* have reported on the use of the TriVersa NanoMate for providing both of these capabilities [56]. Figure 3.10 shows a schematic drawing of the system. HPLC column dimensions ranging from 4.6 to 0.320 mm i.d. may be used with the effluent split appropriately between the flow needed for chip-based nano-ESI to the mass spectrometer (100–200 nL/min) and the portion split to a multiwell plate for on-line fraction collection (see above). These researchers reported that the longer time frame available, because of the low flow rates of chip-based infusion, allows for the performance of many different types of experiments such as neutral loss, EPI studies, and MS³ and even polarity switching which is possible on the QTRAP mass spectrometer system. Thus, the most meaningful experiments for the relevant fractions can be selected in real time during infusion, which would ordinarily require several reinjections when using only LC/MS techniques. These experiments allow for the post-LC/MS/MS infusion analysis of selected fractions, which may contain metabolites whose structural characterization may be required. The ability to continuously infuse selected fractions while performing a variety of MS/MS experiments with a steady analyte ion current signal can provide improved quality mass spectral data from which structural characterization may be facilitated.

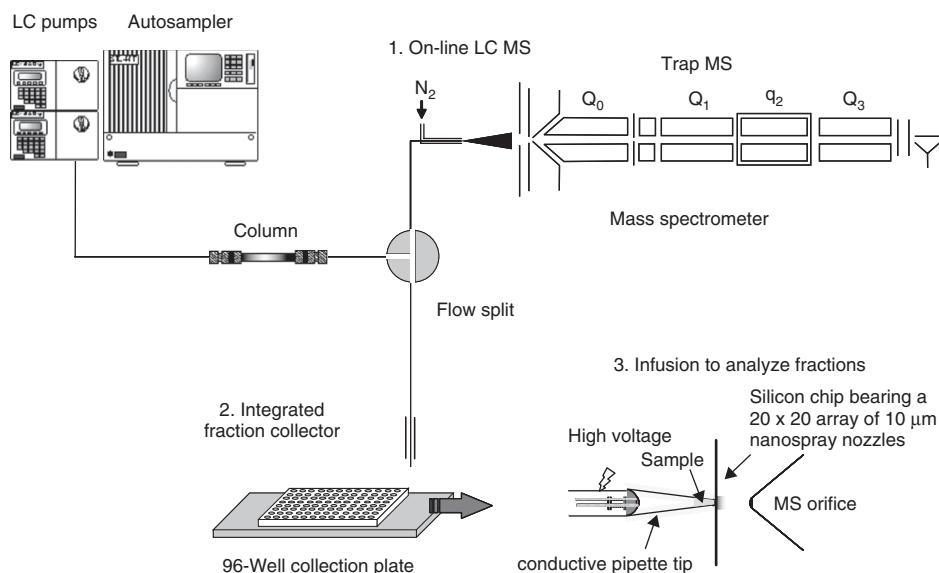


Figure 3.10 Schematic drawing showing the configuration for on-line LC/MS with fraction collection and off-line chip-based nano-ESI infusion of the collected fraction. *Source:* Reprinted with permission from Staack RF, Hopfgartner G, Varesio E. The combination of liquid chromatography/tandem mass spectrometry and chip-based infusion for improved screening and characterization of drug metabolites. *Rapid Commun Mass Spectrom* 2005;19:618–626.

3.4.4.4 Proteomics. There is little doubt that the study of an organism's complete complement of proteins, which is the basis of proteomics, will have a significant impact in all areas of the life sciences in the years to come. This results because to really understand biological processes, we need to understand how proteins function in and around cells since they are the functioning units. In recent years, mass spectrometry has become an important tool for dissecting protein structure and function. Using electrospray ionization and matrix-assisted laser desorption ionization (MALDI)-based approaches, it has been possible to monitor protein folding, characterize noncovalent protein complexes, and assess the contribution of individual amino acid residues to a protein's function.

The task of studying the proteome has its share of challenges [63]. One involves the very high number of proteins that need to be identified. The ~35,000 genes in the human genome can code for at least 10 times as many proteins; in extreme cases, a single gene alone can code for more than 1000. Another challenge is that amino acids, the base units of the peptides that make up proteins, are so small. The analytical challenges involved with fully characterizing the sequence of proteins and very importantly the three-dimensional structure and most importantly the function of proteins are the holy grail of modern molecular chemistry.

In proteomics-based mass spectrometry, the scientist is faced with detecting and identifying minute quantities of one or more potentially unknown biologically important macromolecular compounds. Not only is this a challenge in itself, but it is often necessary to detect minor differences between an individual's healthy versus disease state, differences between male and female sexes, or differences among ethnic groups, and so on. Often, the biological dynamic range can be as high as 10^{12} , while the dynamic range of our measurement tools may be only 10^3 . Thus, a careful combination of sample preparation and high separation efficiency is needed to isolate and characterize potential biological species of interest from the vast array of other biological matrix components.

As an example of the complexity and analytical challenges that modern scientists face, Boernsen *et al.* [64] recently reported on a controlled protein precipitation method combined with chip-based nano-ESI infusion mass spectrometry for profiling human plasma samples. It was reported that plasma is known as a very complex mixture with high albumin concentrations that make the analysis even more of a challenge. If one wishes to study the plasma metabolome, the sample pretreatment procedure should not affect this aspect of the sample. These researchers reported that when the plasma is slowly and carefully diluted in 50% organic solvent, slow protein precipitation occurs without small molecule loss. In Fig. 3.11a, a typical mass spectrum of infused fresh diluted plasma is shown. This sample is a representative of a typical "protein crash" where the proteins are rapidly denatured and the sample centrifuged to produce a biological supernatant sample for subsequent analysis. The multiple charged albumin peaks are very broad in such a sample, and almost no individual peaks are visible in this mass range as observed in Fig. 3.11a. Also, in the low mass range between m/z 100 and 900, the mass spectrum does not exhibit good spectral quality. In contrast, if one allows extended incubation times for a slow partial protein precipitation of the same sample, the resulting mass spectrum will change for the better. Figure 3.11b shows the same diluted plasma but after 16-h incubation time at 4°C. The total ion count has increased by a factor of 20. Also, in the low mass region between m/z 100

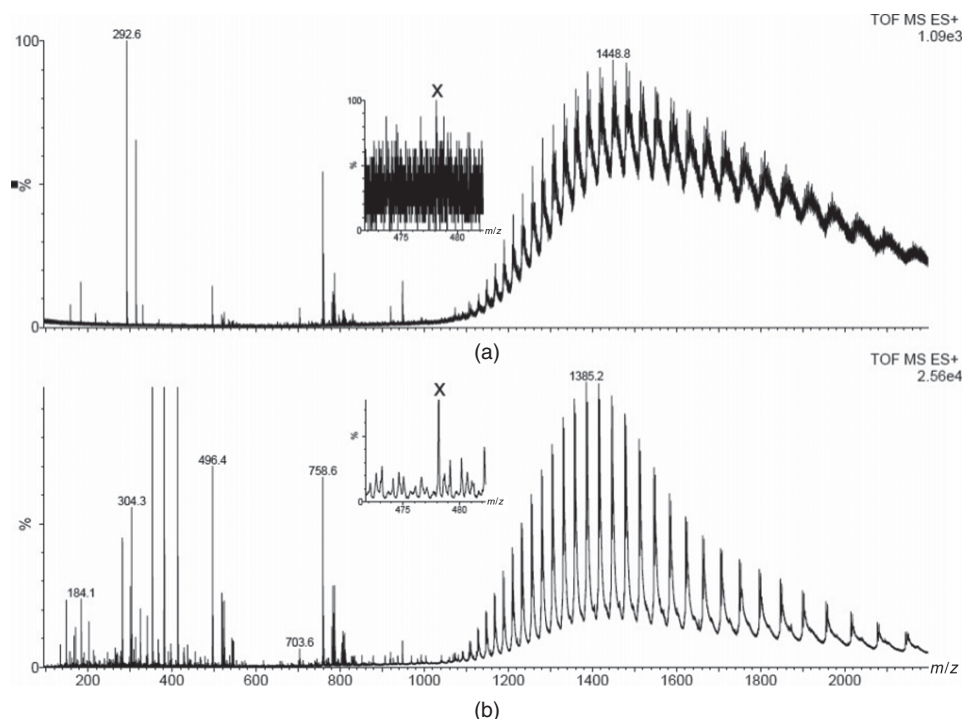


Figure 3.11 Typical mass spectrum after 10-min infusion of diluted human plasma (1:250) by 50% acetonitrile/water with 0.1% formic acid. (a) Fresh diluted plasma. (b) Typical mass spectrum under the same conditions but measured 16 h after dilution (method a). See text for experimental details. *Source:* Reprinted with permission from Boernsen KO, Gatzek S, Imbert G. Controlled protein precipitation in combination with chip-based nanospray infusion mass spectrometry. An approach for metabolomics profiling of plasma. *Anal Chem* 2005;77(22):7255–7264.

and 900, there is improved spectrum quality. The typical signal increase is shown again in the embedded figure where the x-marked peak has increased dramatically.

This example highlights a benefit of optimized sample pretreatment coupled with infusion chip-based nano-ESI mass spectrometry. In this approach, unwanted discrimination of sample components inherent in many sample extraction procedures as well as concomitant discrimination that sometimes occurs as part of the chromatographic process (changes in three-dimensional protein structure, etc.) are avoided. Thus, the infusion mass spectrum shown in Fig. 3.11b represents the composition of the entire sample, and this chip-based infusion approach offers the ability to easily measure hundreds of compounds in parallel with excellent reproducibility. Although ion suppression no doubt exists, it is reduced due to the benefits of nano-ESI [64].

3.4.4.5 Noncovalent Interactions. It has long been known that biospecific noncovalent interactions play a key role in life processes as well as the effectiveness of drug host–guest interactions. Important biological functions are also known to be mediated through noncovalent interactions between biopolymers and other components existing in the cell. Although certain spectroscopic techniques have been used to shed some

light on studies involving both qualitative and quantitative aspects of these noncovalent interactions, it was not until the early 1990s that electrospray mass spectrometry was shown to be a uniquely useful tool for studying these biomolecular structures and noncovalent interactions [65–75]. In some of these reported studies, the quantity of receptor and related chemicals was very limited and very costly. These experiments were typically done by infusing the sample via a syringe pump at 5–50 microliters/min when often only a small quantity of sample was available. In addition, often these experiments involved spraying totally aqueous solutions at neutral pH, which can be rather challenging under ordinary electrospray conditions where sometimes the limited sample was consumed before meaningful results were obtained. What was needed was an approach which provided very mild nano-ESI-MS conditions such that reliable results could be obtained while minimizing the quantity of sample consumed during the experiments.

A good example of a step forward in this approach was reported by Zhang *et al.* [55]. This report described using a chip-based nano-ESI system to perform a quantitative study for competitive binding noncovalent interactions between ribonuclease A (RNase A) and cytidylic acid ligands (2'-CMP, CTP), a well-characterized model protein–ligand complex, and between an inactive endocellulase mutant and four oligosaccharide ligands. Both titration and competitive binding approaches were performed before automated nano-ESI-MS analysis with a QTOF mass spectrometer. The latter provided a selective measurement of the actual noncovalent complexes and unbound species immediately following these traditional binding experiments. Dissociation constants for each complex were calculated from the sum of ion peak areas of free and complexed proteins during the titration and competition experiments. The measured K_d values for the RNase A–CMP and other complexes were found to be in excellent agreement with the available published values obtained by standard spectroscopic titration techniques. These experiments were performed in an automated manner and in replicates of three, thus providing a rigorous and thorough evaluation of the competitive binding results. In the wake of this report, a number of pharmaceutical researchers have employed this automated nano-ESI-MS approach to screen their combinatorial products or new chemical entities against selected protein targets.

3.4.4.6 Lipid Characterization. Lipidomics is defined as the large-scale study of the pathways and networks of cellular lipids in biological systems and has emerged to become an important new field of scientific endeavor. The study of lipids by mass spectrometry has increased markedly in the past 10 years. Both electrospray ionization [76] and more recently MALDI [77] coupled with mass spectrometry have been shown to provide very important analytical characterization capabilities for this complex class of biological molecules. Lipids in general span a wide range of polarities with many close similarities including isomers with important subtle unsaturation and other structural differences. Consequently, even modern chromatographic separation techniques including UPLC do not provide sufficient separation efficiency when combined with MS to adequately characterize these compounds. As a result, some researchers have explored the potential to simply infuse whole lipid extracts to a mass spectrometer using electrospray ionization. Although Murphy *et al.* were among the first to explore this route [76], there have been a number of other pioneers in lipid mass spectrometry studies to follow suite [14,76,78,79].

A nice example of the relative benefits of sample infusion of the entire sample extract containing lipids was reported by Han *et al.* [13]. These workers reported an increased differential ionization of multiple phospholipid classes through infusion microfluidics with chip-based ionization resulting in substantial enhancement of intrasource separation/selective ionization of phospholipid classes in comparison to the conventional ion source. Earlier, Han *et al.* [14] had demonstrated the differential ionization efficiency of lipid classes based on their inherent electrical properties, and they subsequently referred to this as “intrasource separation.” These researchers described these phenomena as benefiting from the achievement of a minimal flow rate of infusion to flow rates of <500 nL/min. This behavior was prescribed as benefiting from the efficient ionization provided by an optimized chip-based Taylor cone under very low flow, microfluidic nanospray conditions.

With reference to Fig. 3.12a and b, several fundamental observations were made. First, the infusion nanospray mass spectrum of a diluted solution of mouse myocardial lipid extract acquired in the negative ion mode showed abundant ions corresponding to anionic phospholipids. Thus, the intensities of m/z 678.5 and 834.5 observed in Fig. 3.12a with the conventional syringe-based infusion were substantially enhanced

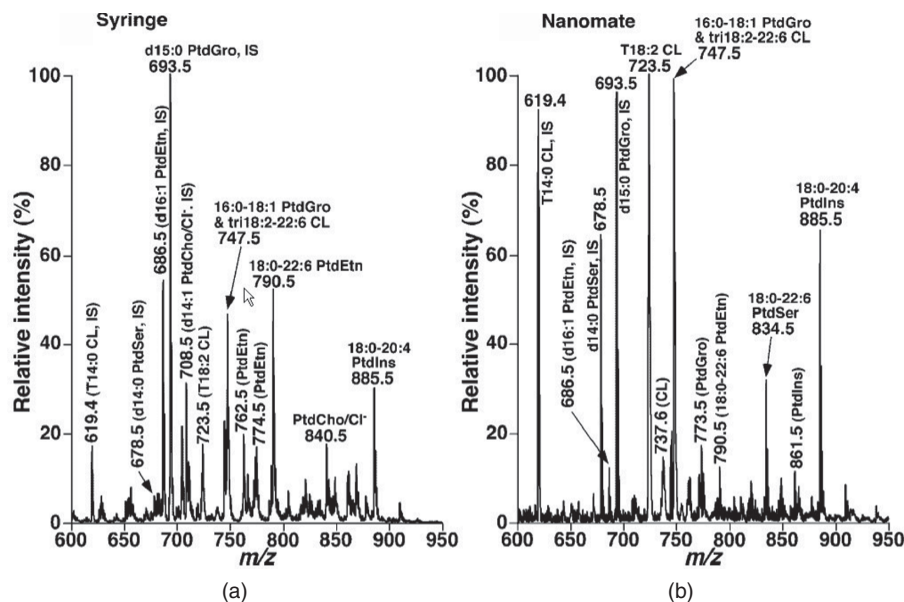


Figure 3.12 Comparisons of ESI mass spectra of mouse myocardial lipids acquired from an ion source coupled to a syringe-pump-driven interface to that obtained using a nanomate nano-ESI interface. Mass spectrum in (a) was acquired in the negative ion mode directly from a diluted lipid extract infused with a syringe pump-driven interface. The mass spectrum in (b) was acquired in the negative ion mode directly from a diluted mouse myocardial lipid extract. See Text for experimental details. *Source:* Reprinted with permission from Han X, Yang K, Gross RW. Microfluidics-based electrospray ionization enhances the intrasource separation of lipid classes and extends identification of individual molecular species through multidimensional mass spectrometry: development of an automated high-throughput platform for shotgun lipidomics. *Rapid Commun Mass Spectrom* 2006;22:2115–2124.

in Fig. 3.12b which employed chip-based nano-ESI infusion. Second, the ionization efficiency of the ions corresponding to the doubly charged cardiolipin molecular species were also greatly increased in the microfluidic-based ionization system relative to those ionized in an ion spray source coupled with a typical syringe pump-driven interface. In addition, the peak intensity ratio of ions at m/z 693.5 and 686.5, corresponding to 15:0–15:0 phosphatidylglycerol (PtdGro) and 16:1–16:1 phosphatidylethanolamine (PtdEtn), respectively, was approximately three times higher in the mass spectrum acquired via the chip-based microfluidic approach in comparison to that acquired with the syringe pump-driven capillary device. Finally, interfering ions corresponding to chlorine adducts of some components (e.g., those at m/z 708.5 and 850.5) were in very low abundance in the mass spectra acquired using the microfluidic approach thereby resulting in a much clearer spectrum for anionic phospholipid characterization (Fig. 3.12b) [14]. These results corroborate the contention that microfluidics applied to nanospray mass spectrometry facilitates the intrasource separation/selective ionization of lipid classes and particularly improves ionization with doubly charged molecular species (e.g., cardiolipin in this case).

3.4.4.7 Carbohydrates. The comprehensive characterization of a complex carbohydrate sample is a challenging endeavor. Although comprising but a small number of different monomers (hexose, *N*-acetylhexosamine, deoxyhexose, etc.), the variable positions of linkage, coupled with branching; the stereochemistry of monomers; and linkage anomericity quickly inflate to a huge number for even a small set of residues. With the added complication of branching, a neutral MS loss may originate from multiple antennae, and as is common, analytes in low abundance can be obscured by more prominent ions.

A comprehensive carbohydrate sequence is particularly challenging when considering the constitutional and stereoisomers that are abundant in glycan arrays. Not only are these compounds somewhat challenging to ionize via electrospray but also they are structurally very similar and complex with many closely related isomers. Constitutional isomers, also referred to as *structural isomers*, are defined as an identical atomic composition arranged in a different structure. Stereoisomers are exemplified with those frequently encountered in the hexoses, mannose, galactose, and glucose.

In a recent report [80], Reinhold *et al.* demonstrated the use of sequential mass spectrometry (MS^n) to define previously unreported structural isomers and qualitative aspects in complex mixtures without prior chromatographic separation. Although the analytical potential of various on-line chromatographic approaches is acknowledged, they emphasize the benefits of continuous infusion for providing the needed time for MS^n experiments as well as the merits of signal averaging when the increasingly weak MS^n ion current signal is observed. The principles involved have been previously considered [81], and complex biological applications recently reported [82]. The overall focus has been to better define MS^n as a universal sequencing strategy consistent with the goals of automation and high-throughput analysis.

In Reinhold's report, mass spectra were obtained from a LIT equipped with a nano-ESI ion source [80]. The mass spectra were collected automatically using the software of the combined systems. Ten scan events were programmed with the first event, scan event 1, being a profile analysis. Subsequent ion scans were automatically selected as the most abundant or from a preprogrammed neutral loss list. In situations where more than one product mass satisfied the neutral loss list, the more intense product was

selected as the precursor for a subsequent scan. Signal averaging was accomplished by increasing the number of microscans for each scan and was generally between 5 and 50. This improved spectral quality was necessary at the higher orders of MS^n due to diminishing ion current signal, particularly beyond MS^5 or MS^6 .

This analytical approach was demonstrated using a sample previously identified to contain a variety of structural isomers. In ovalbumin, several structural isomers have been reported with the composition $Man_3GlcNac_5$ and observed as the methylated, reduced ion at m/z 1923. Sequential fragmentation of this precursor, m/z 1923.1–1663.8, 130.6, 111.5, and 852.4, provided the subsequent spectra, MS^2 – MS^6 . The most abundant ions in the spectra (MS^{2-4}) result from a facile loss of nonreducing terminal HexNacs or the reducing-end GlcNAcol. In the MS^5 spectrum, a fragment ion m/z 893.5 was consistent with loss of a terminal hexose (i.e., fully methylated at all nonanomeric positions). These authors conclude that a distinct pattern of fragments could be discerned for each isomer thus providing a unique and viable approach for glycan structure elucidation [80]. It was also concluded that the extended analysis time and minimum sample consumption of nano-ESI-based infusion of these samples is the key for providing the quality and level of mass spectral described in these studies.

3.5 QUALITATIVE ANALYSIS

Mass spectrometry has long been recognized as a very useful analytical tool for the qualitative determination of compound structures. Coupled with NMR techniques, complete structural characterization including stereochemistry can often be accomplished for previously unknown structures ranging from natural products to biopolymers. However, even modern micro-NMR techniques do not possess the very high sensitivity that may be routinely accomplished by mass spectrometry. Therefore, the latter is the primary technique for structural elucidation of unknown compounds.

Although LC/MS/MS and related techniques are possible with a separation science technique coupled with mass spectrometry, often the high quality, full-scan mass spectral data often required for structural elucidation is not possible in the “on-line” mode (see above). This is because of the need for a relatively slow scan rate of the mass spectrometer to acquire these data, which is not easy to accomplish while a chromatographic peak elutes within a few seconds. In most instances, the highest quality mass spectral data are obtained by collecting an HPLC or UPLC peak followed by the continuous infusion of this now “clean” sample to the mass spectrometer. This experiment provides much more time to acquire the full-scan mass spectral data with concomitant signal averaging for an improved signal-to-noise ratio as well as allowing for a sequence of MS/MS experiments to be performed which are readily available from modern MS systems. Most modern LC/MS/MS systems do not provide sufficient time during the chromatographic peak elution to perform multiple full-scan MS/MS experiments. This is especially true for FT-ICR and ion mobility systems [83,84].

Several examples for the qualitative characterization of chemical compounds by infusion nano-ESI have been discussed above. In an earlier drug metabolism study, sequential on-line fraction collection of an entire chromatographic run over short time segments followed by robotic infusion of selected fractions which correspond to chromatographic peaks of interest was discussed [56]. A very different approach is to use the infusion of a host–guest complex to study the qualitative aspects of biospecific

noncovalent complex interactions [66]. An alternative elegant approach is described above in Section 3.4.4.7 where sequential MSⁿ experiments are performed on complex glycans, which can provide unique qualitative insight into the detailed structure of these challenging yet biologically important compounds [80].

3.6 QUANTITATIVE ANALYSIS

3.6.1 Small Molecule GLP Bioanalysis

Historically, over the past 20 years, the quantitative determination of drugs and their metabolites has been developed, matured, and accepted using HPLC coupled with tandem mass spectrometry (MS/MS) [85]. In the vast majority of cases, triple quadrupole mass spectrometers have been shown to be the preferred MS/MS tool for performing these experiments. Although ion trap [86] and TOF [87] mass spectrometers have been shown capable of providing quantitative LC/MS results, the triple quadrupole system has become the accepted gold standard within the pharmaceutical industry as well as in many other applications demanding high quality quantitative results. The benefits of LC/MS/MS include a final “separation” of chemical and matrix components before MS analysis, concentration of the analytes of interest in the HPLC (or UPLC) peak for improved sensitivity, a reduction in matrix suppression of ionization as a result of the chromatographic separation, and a retention time for the targeted components as an additional element of selectivity. In addition, at least two modes of ionization, electrospray and APCI, are available to handle the wide diversity of chemical compound classes. In contrast, the time needed for adequate chromatography leads to a reduction in sample throughput when large batches of samples must be analyzed. As a result, there is growing interest in sample analysis with little or no on-line chromatography [88,89]. One of these approaches is flow injection analysis (FIA), which has been used quite successfully for carnitine biomarkers in newborn screening [90], while another is infusion of a suitably cleaned up sample [88]. In each of these approaches, there is no concentration of analyte from the extract as in a chromatographic peak or presence of a chromatographic peak retention time. However, there have been some reports of successfully performing quantitative analyses with an appropriate degree of prior sample preparation followed by rapid, sequential infusion of the sample extract containing a suitable internal standard.

Corkery *et al.* [91] reported on a chip-based quantitative method for the determination of gemcitabine. To focus on one aspect of this report, the authors described the combination of a novel particle discriminator interface (PDI) coupled with the ESI Chip (Advion BioSystems, Ithaca, NY) for the rapid (15 s), sequential, automated infusion of solid-phase extraction (SPE) extracts of biological samples following rigorous GLP workflow and guidelines for gemcitabine in human plasma. Specifically, a plasma calibration curve ranging from 0.5 to 250 ng/mL was prepared in duplicate with quality control (QC) samples analyzed in replicates of six at a low, mid, and high level of 0.5, 125, and 250 ng/mL, respectively. The isotopically labeled internal standard was fortified in each sample at 6 ng/mL. The linear calibration curve was calculated for regression analysis and gave an $R^2 > 0.998$. The accuracy and precision of the method was <20% for the lower limit of quantitation (LLOQ) and less than 15% for all other data points on the calibration curve. In general, all the usual GLP acceptance criteria were met with this infusion MS/MS method of analysis.

The authors summarize that the chip-based format coupled with the particle discriminator interface API inlet was sufficiently accurate, precise, and reproducible to meet standard GLP validation acceptance criteria. They concluded that this approach demonstrated a three times lower LLOQ than LC/APCI/MS/MS and that the analysis time and sample consumption of the former technique was reduced in comparison with the latter. With respect to matrix suppression of ionization in the absence of on-line HPLC, the authors report a matrix effect of 51% or a factor of 2 suppression. However, even though suppression was observed, the analyte and internal standard were suppressed to a similar extent, and there was no change in the variance of the blank (background), leading to little change in the LLOQ [91].

3.7 LIQUID EXTRACTION SURFACE ANALYSIS (LESA)

3.7.1 Profiling Thin Tissue Slices for Drugs and Their Metabolites

A modern trend in mass spectrometry is ambient ionization, which has demonstrated that direct surface sampling and mass spectrometric analysis without sample preparation is possible in many instances. Among the early entries to this field were direct analysis in real-time (DART), desorption electrospray ionization (DESI), extractive electrospray ionization (EESI), secondary electrospray ionization (SESI), and many combinations of desorption with postionization methods working at atmospheric pressure [92]. Two key components of these approaches are surface analysis without sample preparation with a common theme of forming ions at ambient pressure and temperature.

A recent twist to this approach has been reported by Kertesz and Van Berkel [93], which involves fully automated microliquid extraction-based surface sampling and ionization using a chip-based robotic nanoelectrospray system. In one sense, these reports are related to the other forms of surface sampling and ambient ionization, but in this latter instance, the difference is that analytes undergo a short, robotic microextraction process using a suitable extraction/spray solvent contained in a pipette tip followed by immediate nano-ESI infusion of the sample. This process is an extension of an earlier report which describes the direct ion spray MS analysis of samples from card-based SPE material [94].

This technique has been described as liquid extraction surface analysis with an acronym of LESA. This concept was first described by Kertesz and Van Berkel [93], where they adapted the above-described TriVersa NanoMate to form a microliquid droplet of extraction/nano-ESI spray solvent between the sample surface and the pipette tip typically used with this device (Fig. 3.13). In general, a new pipette picks up a solvent aliquot from the solvent reservoir (Fig. 3.13a) and then moves to place the pipette tip within close proximity to the sample surface to be analyzed (Fig. 3.13b). The microdroplet accomplishes a microextraction of chemical constituents present on or within the sample surface and then transfers this extracted sample directly to the ESI Chip described above for automated infusion nano-ESI-MS analysis of the extract (Fig. 3.13c). Extraction of soluble analytes occurs at the interface between the microliquid droplet and the sample. The resulting extract solution is withdrawn into the pipette and delivered directly to the ESI Chip for infusion nano-ESI-MS or MS/MS analysis of the extract mixture.

Figure 3.14a–d shows an example where a tissue from a mouse dosed with sulforaphane was examined using the LESA technique. The SRM ion current signal levels

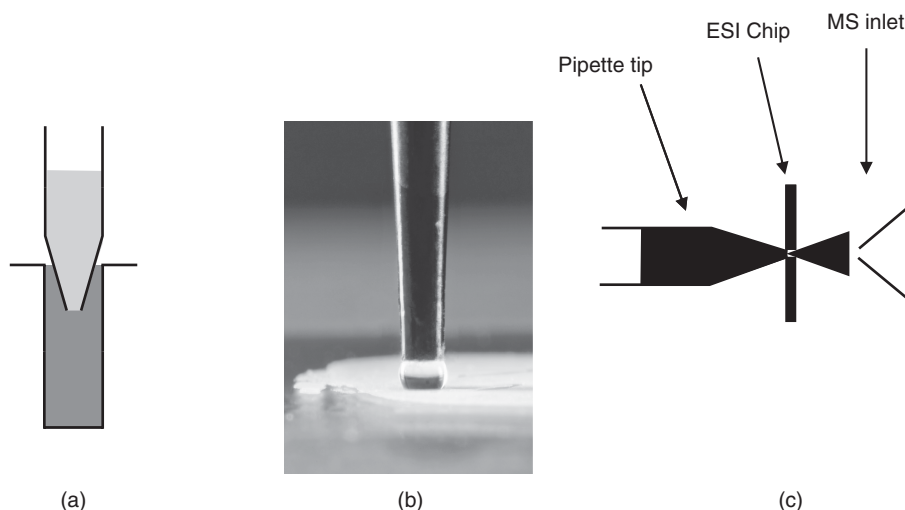


Figure 3.13 (a–c) Schematic representation of liquid extraction surface analysis (LESA). (a) Pipette tip aliquots from a solvent reservoir. (b). Close-up photo of the microdroplet liquid junction between the pipette tip and the surface to be extracted. (c) Depiction of pipette tip delivering the microextract from the sample surface to the inlet of the ESI Chip for nano-ESI infusion MS or MS/MS analysis. *Source*: Reprinted with permission from Advion BioSciences, Inc., Ithaca, NY.

for (b) sulforaphane (m/z 178 $\rightarrow$ 114) (c) the GSH conjugate (m/z 485 $\rightarrow$ 308) and (d) the NAC conjugate (m/z 341 $\rightarrow$ 178) were recorded during 1-min spraying of samples taken at each point. Dwell time was 50 ms for each transition monitored. The extraction solvent was 80/20/0.1 (v/v/v) ACN/water/FA while the aspirated and dispensed solvent volumes were 3 and 2 μ L, respectively, and aspirated sample volume was 2 μ L. The areas sampled by LESA are annotated in the photograph of the tissue shown in Fig. 3.14a. The SRM “infusagrams” obtained for sulforaphane and its glutathione (SFN-GSH) and *N*-acetylcysteine (SFN-NAC) metabolites from the sequential sampling of the different organs are shown in Fig. 3.14b–d, respectively. The two compounds were not detected in the kidney or liver tissues (spots 2 and 5, respectively). On the other hand, NAC conjugate of sulforaphane was readily detected in the liver and kidney (spots 2 and 5, respectively), and only background level signals were recorded for the stomach and stomach walls (spots 1, 3, and 4, respectively) [95].

3.8 SAMPLING AND NANO-ESI MS/MS OF LIVE SINGLE CELLS

A final example of what may offer insight into future possibilities of infusion nano-ESI mass spectrometry is a report on live single-cell video mass spectrometry for cellular and subcellular molecular detection and cell classification [96]. These authors describe employing a modern pulled-capillary nano-ESI sprayer, which is used first as a sampling device for the cell contents followed by its use as a nano-ESI spray emitter. In general, a 2–10 μ m i.d. pulled-capillary is carefully inserted through the cell wall of a single live cell while observing the process under a suitable microscope. A small

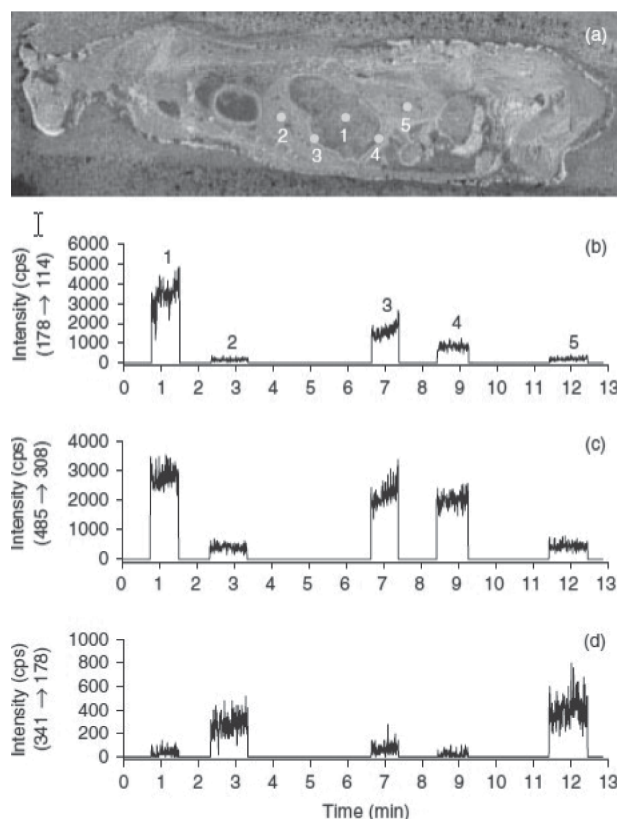


Figure 3.14 (a–d) LESA tissue example. (a) Photograph of a sulfuraphane-dosed mouse. Sampling locations: 1 = stomach/contents; 2 = liver; 3 and 4 = wall of stomach; and 5 = kidney. SRM ion current signal levels for (b) sulfuraphane (m/z 178 $\rightarrow$ 114) (c) the GSH conjugate (m/z 485 $\rightarrow$ 308) and (d) the NAC conjugate (m/z 341 $\rightarrow$ 178) were recorded during 1-min spraying of samples taken at each point. *Source:* Reprinted with permission from Kertesz V, Van Berkel GJ. Fully automated liquid extraction-based surface sampling and ionization using a chip-based robotic nanoelectrospray platform. *J Mass Spectrom* 2010;45:252–260.

portion (one picoliter) of the cell contents is aspirated into the tapered pulled-capillary sampling device and withdrawn from the cell medium. A 1 μ L portion of appropriate aqueous organic spray solvent is manually introduced through the opposite, larger opening of the pulled capillary via syringe while avoiding the formation of an air bubble gap between the introduced solvent and the cell contents. The pulled-capillary spray emitter is then placed at the inlet position of an API tandem mass spectrometer, and the nano-ESI high voltage turned on to commence static nano-ESI analysis of the cellular contents.

Figure 3.15a–g shows a representative set of nano-ESI-MS/MS data for a series of experiments, which sampled cellular cytoplasm, granules, and extracellular contents from a single live cell. These data show molecular identification of cell-specific peaks by MS/MS analysis. Cytoplasm (a) or a granule (b) in a single cell was microsucked into nanospray tips. The positive ion mass spectra of the cytoplasm (a) and a granule

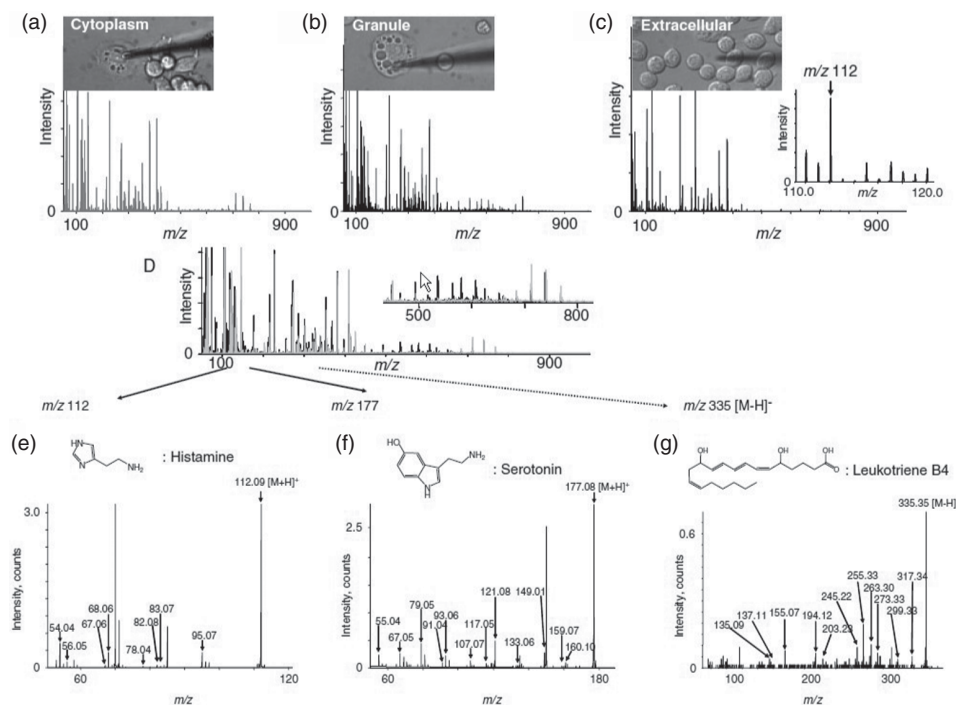


Figure 3.15 (a–g) Molecular identification of cell-specific peaks by MS/MS analysis. See text for experimental description and details. *Source*: Reprinted with permission from Mizuno H, *et al.* Live single-cell video mass spectrometry for cellular and subcellular molecular detection and cell classification. *J Mass Spectrom* 2008;43:1692–1700.

(b) of a cell were superimposed to show the overall difference (d). The site-specific peaks were analyzed by MS/MS, while granule-specific peaks at m/z 112 (e) and m/z 335 (negative ion) (g) were identified as due to histamine and leukotriene B4, respectively. The nonsite-specific molecular ion peak at m/z 177 was identified as that of serotonin (f). The mass spectrum inset (in c) was a positive ion mass spectrum of the medium sucked from a point near a reacting cell after stimulation. The released histamine from RBL-2H3 granules was detected at m/z 112.

3.9 CONCLUSIONS

In summary, LC/MS and LC/MS/MS techniques are here to stay and will continue to be applied to many important applications in the future. One of its key benefits is the “compression” or concentration of the entire analyte into a chromatographic peak which can provide good sensitivity. Continuous nano-ESI infusion does not provide this concentration effect, but it does allow for signal averaging and improved sensitivity due to the formation of very small droplets in the electrospray aerosol which can help improve sensitivity. In many instances, LC/MS run times are so short (1–2 min) that minimal chromatography is occurring, and many of the HPLC peaks are complex mixtures themselves. In these and certain other instances, a judicious choice and application of little or no sample preparation coupled with nano-ESI infusion of the

cleaned up extract can often provide the qualitative and sometimes the quantitative data quality required. In these latter applications, all the issues associated with operating and maintaining an HPLC system are replaced by a simple, cheaper, faster, and automated infusion nano-ESI experiment. As with any choice of techniques for solving a problem, a careful decision for the appropriate tools and techniques employed should be considered to best solve the problem at hand and hence obtain the optimal data quality required.

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4 A Perspective on the Evolving Field of Ambient Ionization Mass Spectrometry

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4.1	Summary	1
4.2	Introduction—ionization in mass spectrometry	2
4.3	Spray-based ionization techniques (DESI, DESSI, DAPPI)	4
4.4	Electrical discharge ionization techniques (DART, ASAP, DAPCI, PADI, DBDI)	7
4.5	Ambient laser-assisted desorption electrospray ionization techniques (ELDI, MALDESI, LAESI, IR-LADESI)	11
4.6	Remote sampling	15
4.7	Applications	17
4.8	Future outlook	26
	References	27

4.1 SUMMARY

Since the introduction of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) in the 1980s and the continued advancements in instrumentation, mass spectrometry (MS) has become the premiere method for scientific analyses because of its high sensitivity and molecular specificity. ESI and MALDI have played key roles in the development of MS for biological applications. However, in order to continue to expand the number of applications that can be addressed using MS, recent research has focused heavily on the development of several novel “ambient” ionization techniques.

Development of ambient ionization techniques is arguably one of the fastest growing fields in MS. Interest exists in the ability to conduct ionization in the open air with no vacuum constraints, thus increasing the number of applications amenable to MS. In addition, these techniques allow for faster analysis times because of limited sample preparation. A plethora of ambient ionization techniques, along with their associated abbreviations, have been introduced in the past decade. To provide a perspective to the reader, this chapter provides a brief review of ionization in MS; however, the focus is

on techniques introduced over the last several years. These techniques are divided into three main categories:

1. Spray-based ambient ionization techniques
 - a. Desorption electrospray ionization (DESI)
 - b. Desorption sonic spray ionization (DeSSI) or easy ambient spray ionization (EASI)
 - c. Desorption atmospheric pressure photoionization (DAPPI)
2. Electrical discharge ambient ionization techniques
 - a. Direct analysis in real time (DART)
 - b. Atmospheric pressure solids analysis probe (ASAP)
 - c. Desorption atmospheric pressure chemical ionization (DAPCI)
 - d. Dielectric barrier discharge ionization (DBDI)
 - e. Plasma-assisted desorption ionization (PADI)
3. Laser-assisted desorption electrospray ionization techniques
 - a. Electrospray-assisted laser desorption ionization (ELDI)
 - b. Matrix-assisted laser desorption electrospray ionization (MALDESI)
 - c. Laser ablation electrospray ionization (LAESI) and infrared laser-assisted desorption electrospray ionization (IR-LADESI)

The specific techniques listed (see above) were chosen on the basis of their varying ionization mechanisms and currently are the more reported methods in the literature. Each technique is described in detail utilizing accompanying schematics. In addition, other ambient techniques that are similar to the ones above, in regard to mechanisms and setups, are briefly described where appropriate. Little emphasis is placed on the exact mechanism of each technique, since most are still under investigation; however, relevant references are provided to direct the interested reader. Reported studies involving applications encompassing both small (e.g., metabolites) and large (e.g., peptides and proteins) molecules are summarized where possible for each technique.

The conclusion section comments on the current state of ambient ionization and discusses future advances that are warranted before the realization of the full capabilities of these methods.

4.2 INTRODUCTION—IONIZATION IN MASS SPECTROMETRY

While the art, science, and practice of MS is over a century old, significant advances in technology have allowed it to more effectively address contemporary questions related to human health and disease, national security, environmental issues, and drug discovery, among many others. Since ionization is essential for mass spectral analysis (i.e., neutrals are not detected) and this chapter is centered on the development of novel ambient ionization methods, it is instructive to briefly discuss the history of ionization methods in MS.

Seminal work by A. J. Dempster led to the development of the first mass spectrometer and the electron impact ionization (EI) method around 1920 [1]. In EI, molecules

are bombarded with high energy electrons (~ 70 eV) leading to ejection of an electron from the analyte and ionization. While EI has undergone significant improvements, nearly 100 years later it remains a major workhorse for MS. Introduced several decades later in the 1950s, chemical ionization (CI) was reported by Munson and Field [2,3]. This technique makes use of ion–molecule reactions and allows for the measurement of intact molecular ions as opposed to EI, where the molecular ion tends to undergo extensive fragmentation. In 1969, Beckey introduced field desorption (FD) for the analysis of organic molecules [4,5]. MacFarlane and Torgerson later presented plasma desorption (PD) [6] in 1974, which, for the first time, allowed ionization of large, polar, and nonvolatile biological molecules. Nearly a decade later, Barber *et al.* introduced fast atom bombardment (FAB) [7], which largely shadowed PD as it did not require a time-of-flight (TOF) mass analyzer and was not a radioactive ionization source. In 1984, Fenn and coworkers demonstrated ESI [8] for the analysis of macromolecules by imparting multiple charges on the analyte. In 1987, through independent efforts, Tanaka [9] and Karas and Hillenkamp [10,11] introduced MALDI, which generates singly charged ions of large biopolymers. It is important to note that ionization methods such as EI, CI, FD, and FAB are still in use today although ESI and MALDI have revolutionized MS. This is recognized in part by the awarding of the Nobel Prize in 2002 to Fenn and Tanaka for their inventions [12].

In summary, the rate of introduction of new ionization sources to MS spanning a period of almost 70 years (EI—1918 to MALDI—*ca.* 1987) is ~ 0.15 sources/year. This includes other ionization sources not specifically mentioned above such as thermospray (TS) [13], secondary ion mass spectrometry (SIMS) [14,15], inductively coupled plasma (ICP), [16] and its predecessors for elemental analysis: thermal ionization [17] and spark source [18]. This modest introduction rate of new ionization sources is interesting and quite surprising because ionization of a neutral molecule is a prerequisite for subsequent mass analysis. In the past decade, there has been significant acceleration in the development of ionization technology as over 25 ambient techniques have been introduced [19].

4.2.1 Ambient Ionization Techniques

A general definition of an ambient ionization technique is any method that can directly analyze species either deposited or naturally occurring on a surface (e.g., metabolites in a leaf) at atmospheric pressure with limited to no sample preparation. These methods differ from ESI, which is performed at atmospheric pressure but is not capable of direct analysis since significant sample preparation is required. These techniques are also different from MALDI and SIMS, which are capable of direct analysis of surfaces; however, they are performed *in vacuo*. These capabilities significantly reduce analysis times for two main reasons: (i) no lengthy sample extraction, preparation, and chromatography is required as material can be analyzed directly from the surface and (ii) no time-consuming vacuum pump downs as analysis occurs in open air. In addition, performing ionization outside the instrument expands the breadth and geometry of samples amenable to MS detection. This ultimately opens new avenues of research that only a short time ago would not have been applicable to MS.

The purpose of this chapter is not to provide an exhaustive review of every technique and its applications; this would be quite complex and may require a whole book unto itself, but rather to provide an overview to the reader on this new, exciting,

and promising arena in MS. As recently described by Fernandez and coworkers [20], the techniques discussed in this chapter are organized into three subgroups based on their mechanisms and include (i) spray-based techniques, (ii) electrical discharge techniques, and (iii) laser assisted desorption electrospray ionization techniques. Setups and mechanisms are briefly discussed while commenting on relevant applications. The chapter concludes with a detailed discussion on some of the widely reported applications and current limitations. Techniques introduced in the past decade but not explicitly mentioned in this chapter include laser-induced acoustic desorption ionization (LIAD) [21,22], radio acoustic desorption and ionization (RADIO) [23], infrared laser ablation metastable-induced chemical ionization (IR-LAMICI) [24], and atmosphere pressure glow discharge (APGD) [25,26]. For the interested reader and to supplement the information described herein, several reviews have recently been written on ambient ionization [19,20,27].

4.3 SPRAY-BASED IONIZATION TECHNIQUES (DESI, DESSI, DAPPI)

Reported by Takats *et al.* in 2004 [28], desorption electrospray ionization (DESI) arguably initiated the “gold rush” in the development of novel ionization methods. The report was significant for two main reasons: (i) demonstration of direct mass spectral analysis of species from a variety of surfaces (e.g., conducting, insulating, *in vivo*) and (ii) this was the first method aside from ESI that could produce multiply charged species from high molecular weight analytes. In general, multiple charging of species is advantageous for several reasons, including (i) increases in the detectable mass range, (ii) multiply charged species are more conducive to typical dissociation methods [e.g., collision-induced dissociation (CID), electron capture dissociation (ECD), and electron transfer dissociation (ETD)], (iii) and offers several advantages for FTMS-based instruments (e.g., increased resolving power and mass accuracy).

The DESI source essentially consists of a three-way tee in which nitrogen is supplied to one port while the liquid is infused through a silica capillary. The liquid is charged through application of a high voltage via a liquid junction. The pneumatically assisted charged mist is directed at an analyte-bearing surface where species are desorbed and transferred to the atmospheric interface of the mass spectrometer. Figure 4.1 displays a typical schematic of the DESI process and introduces several geometric parameters whose optimizations are essential for sensitive DESI-MS analysis [29]. Typical values for these parameters include $\sim 50^\circ$ for α (angle of incidence), $0\text{--}10^\circ$ for β (angle of collection), $2\text{--}3\text{ mm}$ for d_1 (distance between emitter tip and surface), and $0\text{--}1\text{ mm}$ for d_2 (distance between MS capillary and surface). It is important to stress that these values are representative of literature reports, and further exploration may be warranted to achieve optimal sensitivity and depend on several factors related to specific experiments (e.g., solvent flow rate, nebulizer gas velocity, and surface characteristics).

Early reports emphasized at least two different mechanisms of ionization in DESI [28–30], depending on the analyte and geometric parameters utilized; however, further research seems to have come to a consensus on a two-step process referred to as the *droplet pick-up mechanism* for desorption and ionization. Species are first dissolved by a thin liquid film created via the accumulation of solvent on the surface. Next, small analyte-containing secondary droplets are desorbed from the thin solvent film on the surface via a combination of incoming charged droplets and nitrogen

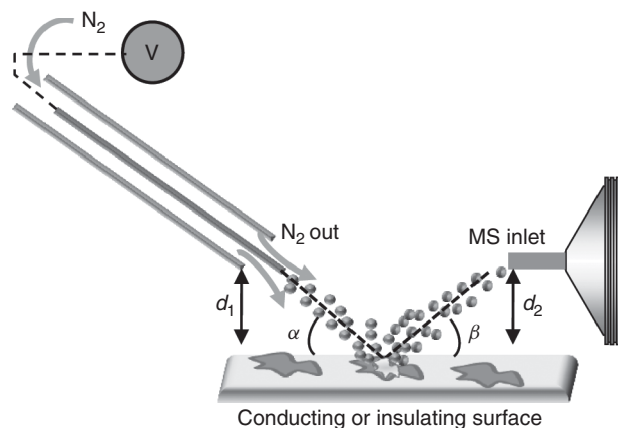


Figure 4.1 The DESI source consists of two concentric capillaries in which nitrogen gas is supplied to the outside capillary and solvent is infused in the inner capillary. The charged pneumatically assisted spray is directed at an analyte-bearing surface where material is solubilized and secondary analyte-containing droplets are sampled by the atmospheric interface of the mass spectrometer. Optimizing the geometry of the source is critical in achieving high signal intensity. See text for a full description of geometric parameters. (See color insert.)

gas. After liberation from the surface, secondary analyte-containing droplets are transferred to the atmospheric interface of the mass spectrometer via a combination of factors including the dynamic drag of the vacuum interface, coulombic repulsion, and flow of nitrogen gas reflecting off the surface. Ionization is analogous to ESI following well-established charge residue [31,32] or FD theories [33,34]. Reports utilizing orthogonal measurement methods [35–37], computational fluid dynamic modeling [38,39], and thermometer ions [40] have contributed to the development of the current droplet-pickup theory and the interested reader is directed to these reports for further information. DESI has been utilized to analyze a range of species from small molecules (e.g., lipids [41,42], amino acids [43], explosives [44–47], carbohydrates [48], nucleobases [49], metabolites [50–54], pharmaceuticals [55–57] to industrial polymers [58,59] and intact proteins [60–63] from both conducting and insulating surfaces. The technique has been coupled to most mass spectrometers, including Fourier transform ion cyclotron resonance (FT-ICR) [48,60,64], orbitrap [65], two-dimensional ion trap [28], triple quadrupole [66,67] and other hybrid MS platforms [55,68,69].

While significantly understudied, the type of surface plays a major role in DESI. For samples in aqueous solutions, the deposition of these samples onto a hydrophobic surface forms a smaller diameter spot than a corresponding hydrophilic surface (e.g., plain glass) [70]. Consequently, samples are more concentrated (material per unit area) when using a hydrophobic surface leading to more material desorbed by DESI and higher signal intensity. However, not all analytes give an adequate signal from hydrophobic surfaces. For example, carbohydrates, intrinsically very hydrophilic, have little interaction with hydrophobic surfaces (e.g., polytetrafluoroethylene (PTFE)) and as a consequence of turning on the high pressure gas are completely removed from the surface [48]. For carbohydrates, more hydrophilic surfaces are warranted. For method development in DESI, it is advantageous to survey several types of surfaces with varying characteristics (e.g., roughness, hydrophobicity, and conductivity) before beginning experiments.

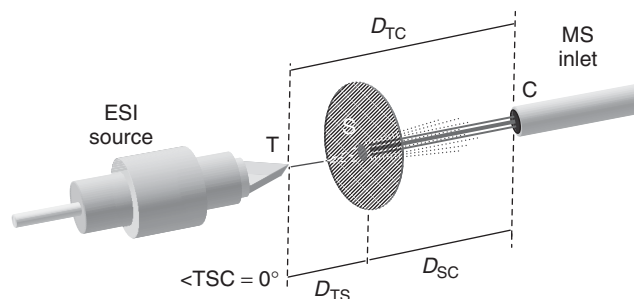


Figure 4.2 A schematic of transmission mode DESI. A variant of conventional DESI, sample (S) is deposited onto a mesh screen, which is then placed on-axis in between the ESI emitter and inlet of the mass spectrometer. D_{TC} is the distance from electrospray tip to capillary and D_{SC} is distance from sample to capillary. *Source:* Used with permission from Ref. 72.

In its most common form, the DESI source resembles the schematic in Fig. 4.1; however, variants of this design have been recently introduced to minimize the effects of geometry (see above) on signal intensity. As a result, these new configurations have effectively increased the robustness and therefore the reproducibility of DESI. Cooks and coworkers have performed DESI in a small pressure-tight stainless steel enclosure where the spray and MS capillary are parallel to each other [71]. They report a reduced dependence of geometry on signal intensity and similar sensitivities when compared to conventional DESI. In addition, the enclosed DESI source protects the analyst from exposure to organic solvents or other toxic/infectious materials that may be under investigation. In another variation, termed *transmission mode desorption electrospray ionization* (TM-DESI), Brodbelt and coworkers described the application of analyte onto a mesh screen, which is then placed between the ESI emitter and MS capillary [72]. This in-line arrangement of components (Fig. 4.2) significantly reduces the geometric degrees of freedom and according to these authors leads to more efficient optimization; however, this technique is less suited for bulk materials and *in vivo* analyses. More recent studies, using TM-DESI, have investigated the importance of surface and mesh characteristics on analyte desorption [73] and have shown increased selectivity using a mesh-bound photocleavable agent to selectively capture thiol-containing compounds from a mixture [74]. The continued refinement of DESI will aid in achieving the most robust configuration.

By merely turning off the voltage of a typical DESI setup and increasing the gas velocity, one can perform desorption sonic spray ionization prior (DeSSI) [75]. DeSSI, synonymously referred to as *easy ambient sonic spray ionization* (EASI) [76–78], is a hybrid technique taking characteristics from both DESI [28] and from sonic spray ionization [79]. Since no potential bias is applied, the spray plume in DeSSI is essentially neutral and the charging of molecules is a result of the statistical fluctuations of the distribution of cations and anions within the solvent droplets. Reports indicate similar sensitivities for small molecules when compared to DESI (with high voltage) for the analysis of drug tablets [75]. This technique may be well suited for small molecule analyses because of the absence of solvent clusters in the low m/z range that occur when performing conventional DESI. In addition, DeSSI could be well suited for field

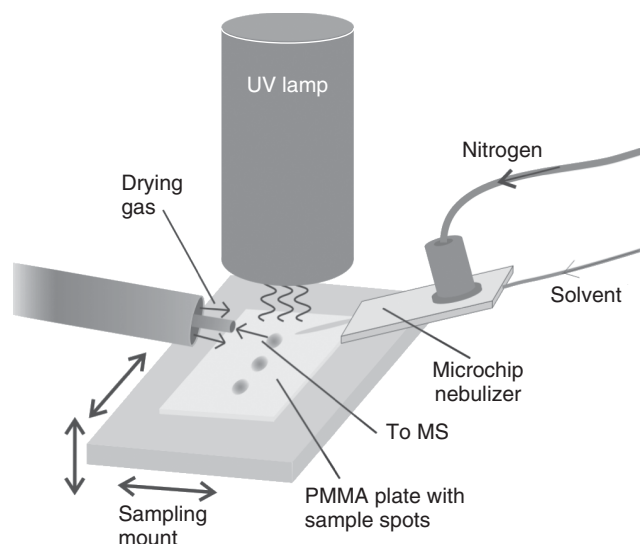


Figure 4.3 A schematic of the DAPPI source. The interaction of heated solvent molecules and photons leads to desorption and ionization of analyte. Ionization is based off APPI mechanisms and as a result the choice of dopants (solvents) is critical for achieving optimal ionization. *Source:* Used with permission from Ref. 89.

analyses (e.g., pesticides, herbicides, and explosives) using portable mass spectrometers [80–88], where access to a high voltage power supply is less practical.

Instead of a jet of charged liquid droplets, DAPPI employs a jet of heated gas solvent molecules (e.g., toluene) and photons as the driving forces for analyte desorption and ionization [89]. A schematic of the setup is shown in Fig. 4.3, where a solvent jet is created at an angle of 45° to the surface by a microchip nebulizer and photons are emitted from a krypton lamp (10 eV) perpendicular to the plane of the surface. Since desorption of analyte is attributed to thermal processes, DAPPI is mainly limited to smaller molecules that are semivolatile. Ionization is analogous to atmospheric pressure photoionization (APPI) [90] and, as a result, the choice of dopants (i.e., solvents) plays a critical role in the type of ionic species observed. Preliminary data show promise for the analysis of metabolites and pharmaceuticals with limits-of-detection (LODs) ranging from 56–670 fmol for selected analytes. A clear advantage exists for the analysis of neutral and less polar species by DAPPI over the more widely reported DESI technique [89].

4.4 ELECTRICAL DISCHARGE IONIZATION TECHNIQUES (DART, ASAP, DAPCI, PADI, DBDI)

DART is currently one of the most reported ambient ionization techniques in the literature and was introduced shortly after the first report of DESI by Robert Cody *et al.* in 2005 [91]. A schematic of a basic DART source is shown in Fig. 4.4 and mainly consists of a tube divided into three different chambers in which a gas, typically helium or nitrogen, flows at $\sim 1\text{--}5\text{ L/min}$ [91]. Ions and metastable atoms or molecules

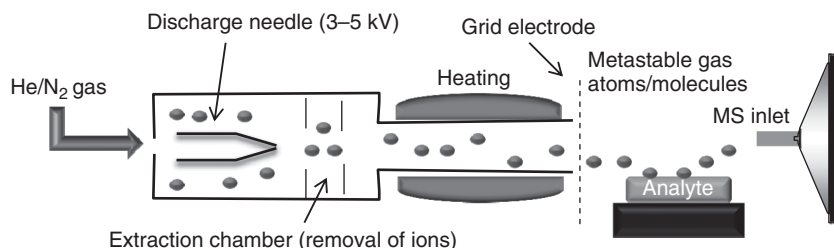


Figure 4.4 A schematic of the DART source. Plasma is generated as a result of an electrical discharge. Ions are then separated from the plasma and the metastable gas molecules are directed at the surface.

(depending on the gas used) are created through an electrical discharge via application of a high voltage (3–5 kV) between the needle electrode and grounded counter electrode [92]. Ions are then removed from the plasma through a second region that is electrically biased and the gas is subsequently heated in the third compartment before exiting the source. The stream of excited gas molecules is then directed toward an analyte-bearing surface located on or off axis between the source and atmospheric interface of the mass spectrometer.

The driving force for desorption is reportedly a result of thermal processes that limit the technique to relatively low molecular weight semivolatile species [27]. The ionization mechanism(s) of DART involves the production of protonated water clusters via Penning ionization of excited He molecules (2^3S 19.8 eV) [91]. These gas-phase reagent ions quickly react with the desorbed analyte molecules to predominantly produce protonated species via proton transfer. This proton transfer mechanism is dependent on analyte molecules having higher proton affinities than the ionized water clusters [92]. Other ionization pathways are possible and are often analyte dependent [92,93].

Although the ionization and desorption processes in DART are still under investigation [92,94,95], the technique has been used to analyze a diverse set of analytes from various surface types. These applications include the analysis of self-assembled monolayers on gold surfaces [93], analysis of fatty acids from bacterial cells [96], analysis of counterfeit drugs [97], species separated on planar chromatographic plates [98], melamine detection in contaminated pet food [99], metabolomic fingerprinting [100], the determination of acrylamide in drinking water [101], and the analysis of alkaloids from plant tissue cultures [102,103]. In recent research, Fernandez and coworkers reported desorption electrospray metastable-induced ionization (DEMI) [104], a hybrid of DART and DESI. This technique is aimed at circumventing the limitations of ionization in DESI (i.e., nonpolar molecules) and DART (i.e., molecular weight limited).

Another electrical discharge technique, termed *atmospheric pressure solids analysis probe*, uses a heated nitrogen gas stream (100–500°C) to desorb semivolatile species existing in liquid or solid form from a melting point capillary that is placed into the source region, as shown in Fig. 4.5. Spearheaded by Larsen and coworkers, the main advantage of ASAP is that the method is easily implemented, requiring only minor modifications, on existing or commercial ESI/APCI (atmospheric pressure chemical ionization) sources [105]. This simplicity reduces the cost associated with machining or purchasing a commercial source. Following thermal desorption via the heated gas, gas-phase analyte ions are then ionized via a corona discharge within the source and

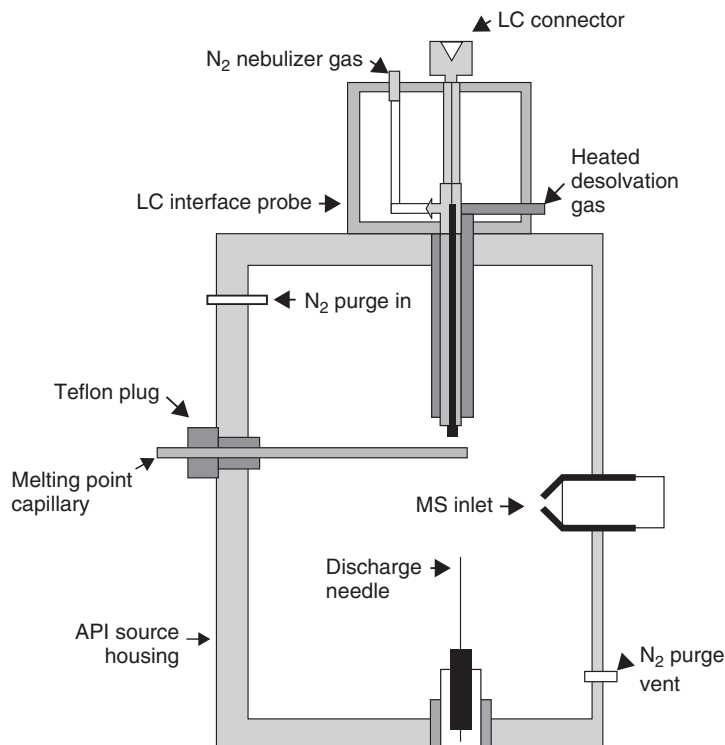


Figure 4.5 A cross-sectional view of an ESI/APCI source modified for ASAP analysis. *Source:* Used with permission from Ref. 105.

sampled by the atmospheric inlet of the mass spectrometer. Mechanisms of ionization are similar to traditional discharge APCI techniques. While DART and ASAP both utilize heated gas jets and electrical discharges for desorption and ionization, respectively, ASAP does not selectively remove ions from the plasma as in DART. However, both DART and ASAP have been found to produce similar spectra in negative ion mode, suggesting similar modes of ionization [95].

Besides the ease of implementing the ASAP technique, another noteworthy advantage is that desorption and ionization are performed in an enclosed area from where the waste can be vented, thereby preventing exposure to potential toxic materials that may be under interrogation [105]. The versatility and rapid analysis of the technique was presented in the original publication, where mass spectral analysis of plant tissues, plastics, and paper currencies were obtained in <10 s [105]. More recently, ASAP has been used for the determination of the effects of various inhibitors on the ergosterol pathway in fungal cells [106].

Developed by Cooks and coworkers, DAPCI was first used to investigate the potential mechanisms of DESI [29,107]. The technique, a surface sampling variation of APCI and mechanistically similar to both DART and ASAP, uses a tapered stainless steel needle in place of the ESI emitter in DESI. As a result, the setup of the DAPCI source is quite similar to DESI with minor geometric differences. Vapors of various solvents (e.g., toluene, acetone, and methanol) are mixed into the annular nitrogen gas

flow via a T-junction while a voltage of 3–6 kV is applied to the stainless steel needle, which then initiates a corona discharge. Gaseous ions and molecules desorb species from the surface, and ionization occurs through charge transfer and gas-phase ionization. DAPCI has shown advantages over DESI for ionization of less polar analytes [29]. DAPCI has been used for the nonproximate analysis of various small molecule species [108], detection of melamine in milk [109], and analysis of various explosives [107].

The last two techniques in this group of electrical discharge ionization methods are DBDI [110] and PADI [111]. Both are relatively new to the field and limited reports on exact mechanisms and applications currently exist. These two specific techniques differ from DART, ASAP, and DAPCI in that they utilize an alternating voltage to create a low temperature discharge to drive desorption and ionization as opposed to a high voltage electrical discharge. DBDI utilizes a dielectric material placed between a needle and a counter electrode in which an alternating voltage is applied (3500 V), creating a dielectric barrier discharge [112]. The dielectric material limits the amount of current between the electrodes and as a result a stable low temperature plasma is produced. A schematic of the original source design is shown in Fig. 4.6. Gas flows (12–48 m/s) through the needle electrode and supplies the media in which the discharge occurs. A glass plate is placed between the needle and counter electrodes and serves both as the dielectric material and sample substrate. The technique has been used for amino acid analysis, where mostly protonated species were observed with an LOD of 3.5 pmol. A high reproducibility was observed for the analysis of alanine (relative standard deviation (RSD) <6%) [110]. An alternative design, but similar in that it uses

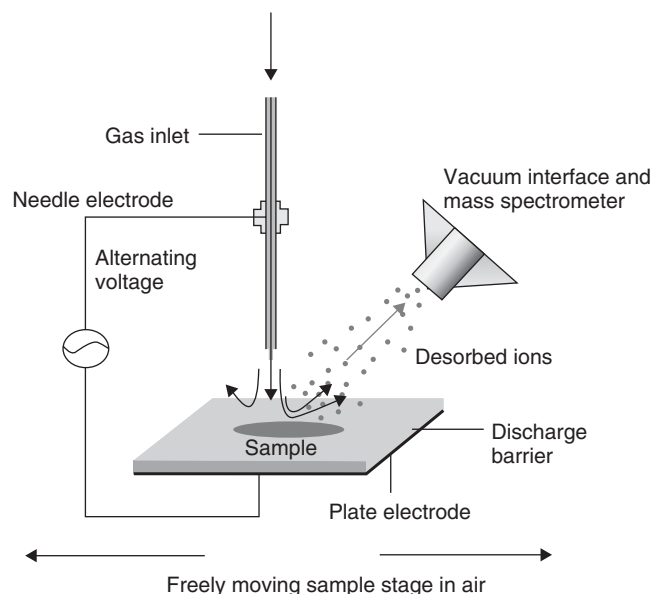


Figure 4.6 A schematic of the DBDI source in which a dielectric material is placed between a counter electrode and a needle electrode. An alternating voltage is applied to the needle electrode, creating a low electrical discharge that drives desorption and ionization of analyte. *Source:* Used with permission from Ref. 110.

a dielectric material to create a low temperature discharge, has recently been reported and termed *low temperature plasma* (LTP) probe [113].

PADI utilizes a lower voltage peak to peak (300 V) than DBDI to create a stable glow discharge, resulting in a cold plasma with relatively low ion energies (5–20 eV) [111]. As a result, PADI has distinct advantages for analysis of thermally labile analytes when compared to higher energy corona discharge techniques. The glow discharge is easily visible to the eye and aids in alignment of the source, sample, and inlet of the mass spectrometer. A schematic of the source and a picture of the discharge are shown in Fig. 4.7. The nonthermal plasma is around 1 mm in diameter and extends up to 10 mm from the source. Desorption of analyte is thought to occur through a combination of energy transfer from metastable helium and ion impact, while ionization is attributed to proton transfer reactions from ionized water clusters. PADI has shown similar results when compared to DESI for the analysis of a variety of pharmaceuticals; however, a lower degree of fragmentation and higher signal intensity were observed from PADI-generated mass spectra [111].

4.5 AMBIENT LASER-ASSISTED DESORPTION ELECTROSPRAY IONIZATION TECHNIQUES (ELDI, MALDESI, LAESI, IR-LADESI)

The final group of ambient techniques discussed in this chapter combines the favorable characteristics from both MALDI and ESI into one-source design and are often referred to as *hybrid methods*. For example, MALDI produces singly charged ions, while ESI produces multiply charged ions. Multiple charging affords more efficient fragmentation of large biological molecules; thus, ESI-produced ions are readily amenable to top-down [114,115] and bottom-up characterization [116,117] methods, including CID [118], ECD [119] and ETD [120]. However, MALDI allows for direct analysis [121,122] and imaging applications [123,124], while ESI does to a far lesser degree. As a result, a source design that embodies both characteristics of MALDI and ESI would have a significant impact spanning the realm of the biological sciences.

A representative setup of the techniques discussed in this section is shown in Fig. 4.8. First, a laser ablates material deposited onto a stainless steel plate and a mixture of neutrals, ions, and other charged particles are created. This process is analogous to atmospheric pressure matrix-assisted laser desorption ionization (AP-MALDI) [125,126]. As shown in Fig. 4.8, the sample plate is placed between and slightly lower than the inlet of the mass spectrometer and the ESI emitter. Following laser ablation, neutral gas molecules are post ionized through fusion with ESI-charged droplets. Once the gas molecules are solvated, the mechanism(s) of ionization are believed to be highly similar to ESI [127].

Laser ablation followed by post-ESI of neutrals was first reported by Shiea and coworkers in 2005, in which they observed multiply charged cytochrome *c* species [128]. They named this process electrospray-assisted laser desorption ionization (ELDI). This technique was in part based on the same group's developments of fused droplet electrospray ionization (FD-ESI), where they interacted with aerosols containing nonvolatile species with an ESI beam [129,130]. In the seminal reports of ELDI, it was demonstrated that an optimal protein signal was obtained without the addition of any organic matrix [131]. However, shortly after the first reports of ELDI, Muddiman and coworkers introduced MALDESI and stated that in their experiments an organic

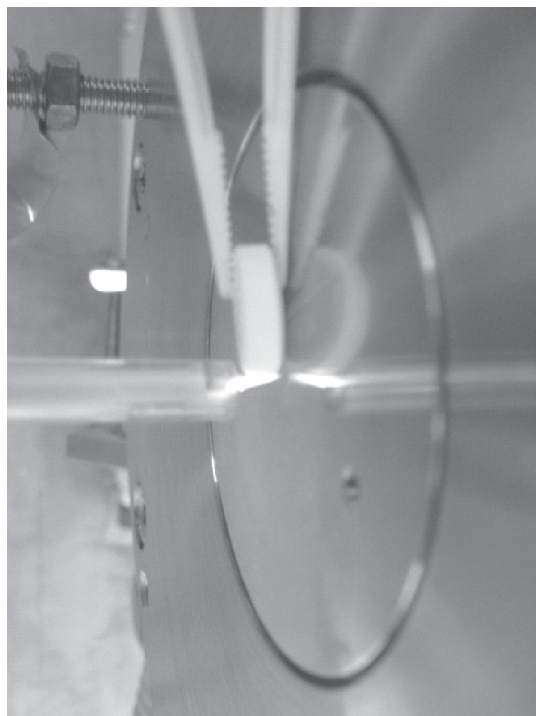
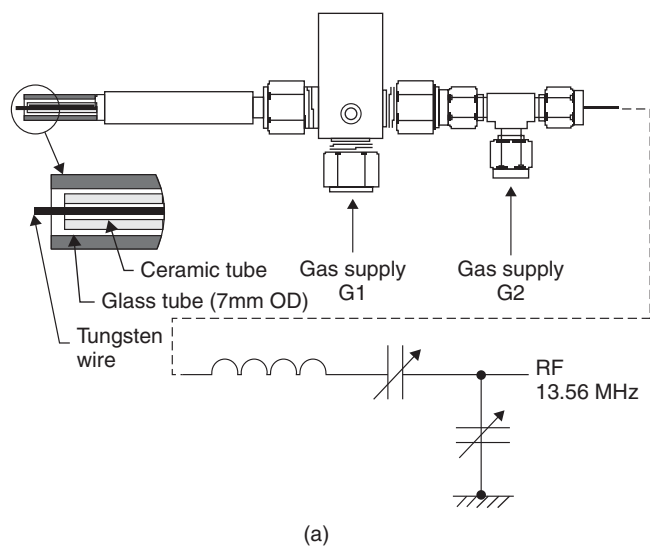


Figure 4.7 (a) Schematic of the PADI source. (b) PADI analysis of a pharmaceutical tablet. The low temperature discharge is shown. *Source:* Used with permission from Ref. 111. (See color insert.)

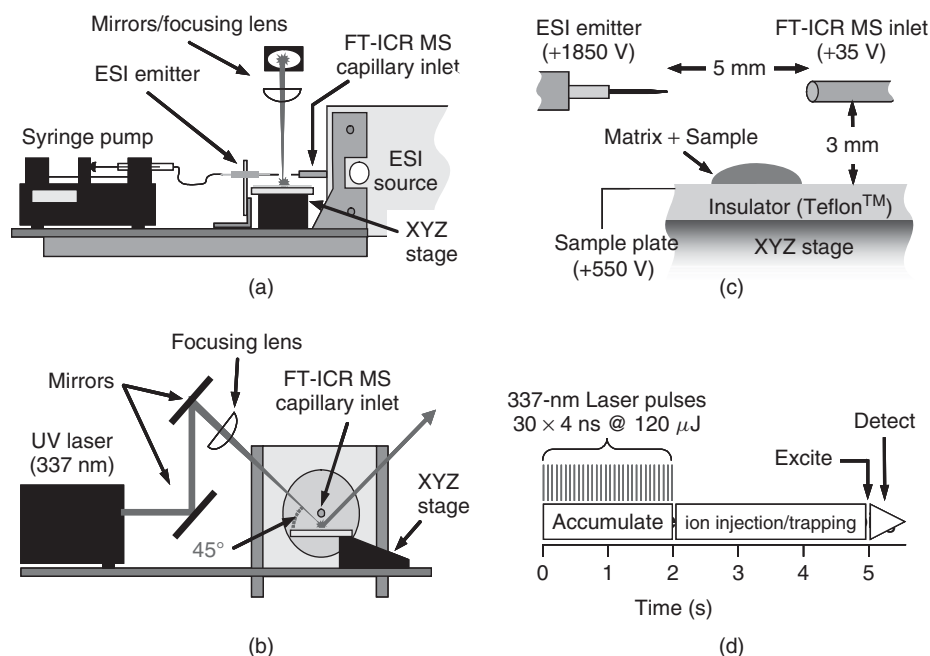


Figure 4.8 (a-b) The setup of a typical laser-assisted desorption electrospray ionization experiment. The laser is focused via a series of lens and ablates material from a surface. Gas-phase molecules interact with charged ESI droplets and are ionized. (c) A close-up view of the setup. The sample is typically placed a few millimeters below the inlet of the MS capillary and ESI emitter. (d) The series of events used by Muddiman and coworkers for their MALDESI FT-ICR setup [127]. The ELDI source uses a UV wavelength laser with no matrix. The MALDESI source uses a UV laser with an exogenous organic matrix. Both LAESI and IR-LADESI utilize an infrared wavelength laser and for samples with sufficient water content no exogenous matrix is required. *Source:* Used with permission from Ref. 127.

matrix was required for a protein signal and as a result argued the technique's name should reflect the importance of the matrix [127]. More recent studies have reported similar findings that the addition of an organic matrix significantly increases signal quality [132]. Regardless, ELDI and MALDESI have been used to analyze both condensed and liquid samples [132,133]. The implementation of liquid MALDESI requires a slightly different configuration in which no electrospray post ionization is used and interestingly the charging of the sample containing the liquid droplet occurs through an application of a high voltage to the stainless steel sample plate. Several smaller or daughter droplets are created on laser ablation and then are sampled by the mass spectrometer [133]. Ionization is believed to then follow similar ESI pathways. Muddiman and coworkers also demonstrated that one can electrochemically oxidize analytes using the liquid MALDESI setup [133].

Several applications of both the ELDI and MALDESI techniques have been reported. ELDI has been used for the direct detection of intact proteins in blood, tears, saliva, and serum [131]; analysis of illicit drugs [128]; direct interrogation of organic compounds separated by thin layer chromatography [134]; real-time analysis of organic reactions [135]; detection of inks in documents [136]; and various other species from

solid surfaces [137]. Similar to reactive DESI, reactive ELDI has been reported for real-time reduction of disulfide bonds by the addition of dithiothreitol (DTT) into the electrospray solvent. In addition, as observed in ESI [138,139], the addition of nitrobenzyl alcohol (NBA) to the spray solution afforded an increase in protein charge state for ELDI-generated mass spectra [140]. These experiments provide further support for the fusion of gas-phase neutrals/particulates with charged droplets as the underlying mechanism in ELDI/MALDESI. MALDESI has shown utility for the analysis of intact proteins, especially when coupled to high resolving power platforms where the monoisotopic mass of moderately high molecular weight species can be determined unambiguously [127,141]. Muddiman and coworkers have increased this accuracy by doping known species into the spray solution and utilizing these masses for internal calibration, affording mass measurement accuracies of intact proteins of <1 ppm. MALDESI has been shown to be relatively sensitive with detection of subfemtomole quantities determined using stable isotope-labeled and naturally occurring peptides [141]. Top-down sequencing of apomyoglobin has been demonstrated utilizing a hybrid LTQ-FT-ICR mass spectrometer [142]. In addition, MALDESI has been reported for the direct analysis of lipids from egg yolk, carbohydrates from milk, and O-linked glycans released via chemical methods from heavily glycosylated mucin, effectively demonstrating direct analysis of three out of the four classes of biological molecules (i.e., proteins, lipids, carbohydrates) [142]. A report describes the most recent version of the MALDESI source, which is equipped with a high repetition rate laser affixed to a vibrationally damped breadboard. The sample target is placed on a computer-controlled motorized stage, allowing full interrogation of the sample plate with 35 nm resolution. A complete list of parts, vendors, and in-house fabricated components are described for anyone interested in replicating the source [143].

Both MALDESI and ELDI setups were developed using a UV laser; however, Vertes and coworkers introduced LAESI in 2007 [144], which, in principle, is similar to ELDI and MALDESI but utilizes an Er:YAG infrared wavelength laser. Through independent investigations and concurrent to the development of LAESI, Rezenom *et al.* reported an analogous technique utilizing an infrared laser and coined the name infrared laser-assisted desorption electrospray ionization (IR-LADESI [145]. Muddiman and coworkers coupled both 2.94- μm [142] and 10.6- μm infrared lasers [146] to the MALDESI platform and called the technique *infrared matrix-assisted laser desorption electrospray ionization* (IR-MALDESI) [142]. More recently, Loo and coworkers reported the coupling of an optical parametric oscillator (OPO) infrared laser with their ELDI setup and referred to the technique as *infrared electrospray laser desorption ionization* (IR-ELDI) [147]. Regardless of the overabundance of acronyms describing the similar techniques, several unique advantages exist for implementing an IR laser. First, infrared radiation has a sampling depth of $\sim 1\ \mu\text{m}$, which is several orders of magnitude larger than a UV laser, and as result a more substantial amount of material is removed from the sample plate on laser ablation [148,149]. The ionization efficiency of infrared radiation is low, which explains the low sensitivities of IR-MALDI compared to UV-MALDI. This phenomenon should be directly applicable to these postionization techniques [149]. Also and perhaps the most significant advantage for biological analyses (e.g., tissues, plasma, *in vivo*) is that water exhibits a strong infrared absorption between 2.8 and 3.2 μm and thus no exogenous matrix is required for sensitive analysis. IR laser ablation with ESI post ionization has been used for analysis of proteins [142,144,145,147], lipids [142,144], carbohydrates [142], pharmaceuticals

[145], metabolites [145,150], and profiling of single cells [151]. LAESI has shown its potential for imaging MS [150,152–154].

4.6 REMOTE SAMPLING

These ambient ionization sources are generally designed such that the sample is in close proximity to the atmospheric interface of the mass spectrometer. This closeness undoubtedly aids in the collection and subsequent detection of ions; however, it limits the location and geometry of the sample. One can envision using these techniques to generate ions several feet from the mass spectrometer and then transporting them to the instrument for subsequent detection. This decoupling of the ion source and mass spectrometer in space would mitigate the need for any sample manipulation (i.e., transporting the sample). Areas of applications include national security (e.g., screening of explosives), pharmaceuticals, and environmental. This remote sampling of the analyte has been accomplished via different but effective ways in the literature. Cooks and coworkers increased the inlet capillary length and were able to detect ions from as far as ~ 10 ft away from the mass spectrometer [108,155]. This setup, shown in Fig. 4.9, was used for detection of explosives, pharmaceuticals, illicit drugs, and peptides [108,155]. An alternative design, shown in Fig. 4.10, utilized a commercial air ejector to transport DESI-generated ions from the point of formation to the MS inlet via a flexible polyethylene tube [156]. A vacuum, created by the flow of nitrogen gas through the air ejector, enabled the detection of dyes, polymers, and peptides from as far as 3 feet away from the inlet. Both setups were relatively sensitive, with detection limits reported in the low nanogram range for both designs. The setup shown in Fig. 4.10 required the purchase of an inexpensive device and was coupled to a flexible polypropylene tube with no modification to the MS inlet, whereas the setup illustrated in Fig. 4.9 incorporated a rigid stainless steel capillary and required minor modifications to the MS interface. Further comparisons are not available at this time because of insufficient information.

Instead of remotely transporting *ions*, Dixon *et al.* described the use of an air ejector for transporting either solid *particulate* or *neutrals* created by laser desorption and interacting them with an ESI beam and called this process remote analyte sampling, transport and ionization relay (RASTIR) [157]. Small proteins (<10 kDa) were laser desorbed from a stainless steel plate and transported to the mass spectrometer for

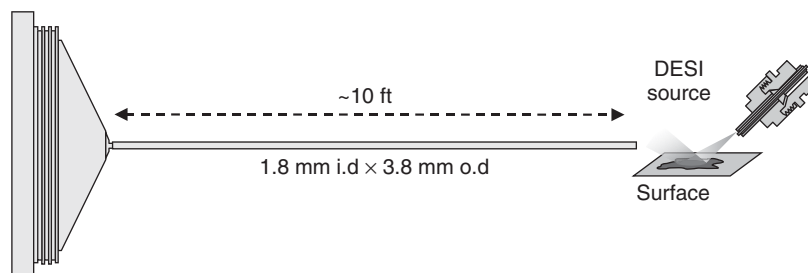


Figure 4.9 The MS capillary can be simply extended up to ~ 10 feet to remotely analyze samples.

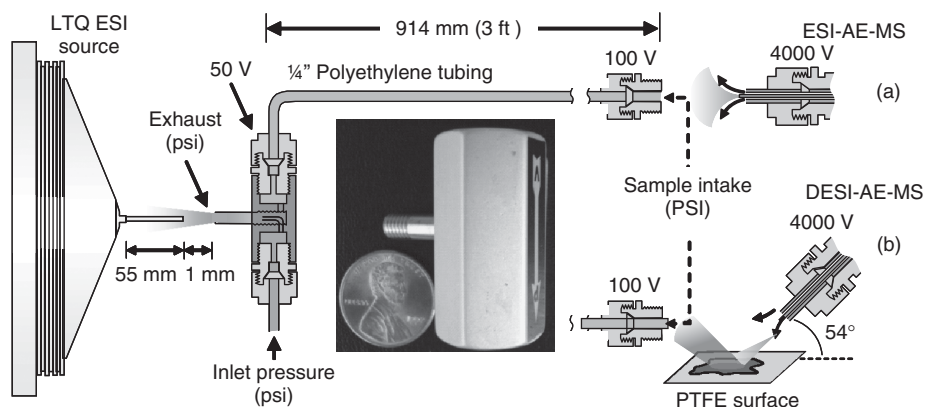


Figure 4.10 Analytes are transferred to the mass spectrometer via the use of an air ejector. Nitrogen flow through the device creates a negative pressure (i.e., vacuum) and transfers DESI- or ESI-generated ions to the mass spectrometer. The relative size of the device in relation to a penny is displayed (center). *Source:* Used with permission from Ref. 156. (See color insert.)

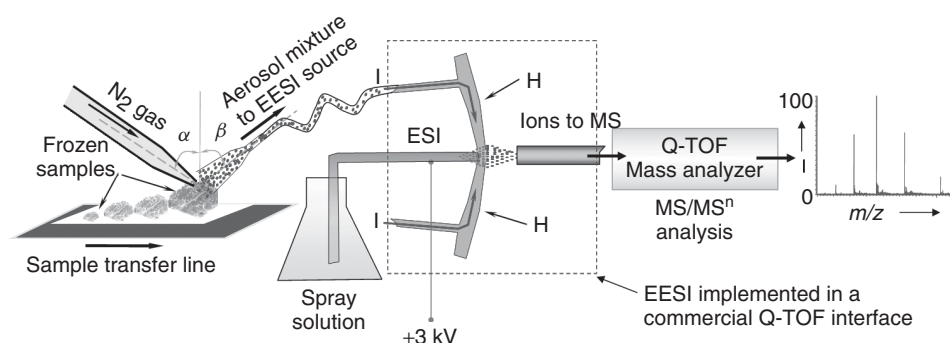


Figure 4.11 A schematic of ND-EESI setup. High pressure nitrogen gas desorbs volatile species from the surface and the analyte-containing gas stream is then mixed with the desolvation gas for ESI. Ionization occurs via the fusion of gas molecules with charge ESI droplets. *Source:* Used with permission from Ref. 158.

subsequent ionization by fusion with ESI beam and detection by LTQ-FTI-CR-MS. Multiply charged spectra were observed and mechanisms of ionization were believed to be similar to ELDI/MALDESI. Zenobi and coworkers simply used high pressure nitrogen gas to desorb volatile metabolites from the surface and then merged the analyte-containing gas stream with the normal desolvation line of ESI and termed this neutral desorption extractive electrospray ionization (ND-EESI) [158]. Using principal component analysis (PCA) analysis, it was demonstrated that one could readily differentiate frozen meat by the metabolomic profiles using ND-EESI. In these studies, metabolites were desorbed and then transported to the mass spectrometer across a distance of ~ 4 feet (Fig. 4.11). The ultimate goal of these studies is the development of an analytically robust method to decouple the sample and mass spectrometer in space for any number of applications ranging from national security, tissue imaging, *in vivo* analysis, pharmaceuticals, and environmental analysis.

4.7 APPLICATIONS

This chapter, thus far, has discussed the mechanisms, setups, and limitations while briefly summarizing the applications of several of the more widely used ionization techniques for ambient MS. Table 4.1 summarizes the attributes of each technique and provides a quick reference guide for the reader. In this table, the techniques are grouped together in their respective class as described (see above). The conclusion of this chapter consists of an in-depth discussion of some of the more pertinent applications of these ambient techniques including explosives, absolute quantification, pharmaceutical analysis, and imaging MS.

4.7.1 Explosives

Trace detection of explosives is a growing area of interest because of the increasing concern surrounding national security. Ion mobility spectrometry (IMS) is one of the more widely used detection methods for explosives [159,160] and is currently utilized in airport checkpoints around the world. Although IMS detection is rapid, relatively sensitive, and performed at atmospheric pressure, the technique suffers from poor linearity and poor selectivity, and hence difficulties exist in the identification of species in complex mixtures. Several reports exist on the rapid analysis of explosives from different surfaces by ambient ionization MS. This is most likely due to the combination of several factors including (i) increased (post 9/11) security concerns at airports and other highly populated venues, (ii) the unparalleled molecular specificity and sensitivity of MS, and (iii) the capability to conduct open air ionization from a variety of surfaces with limited to no sample preparation leading to fast analysis times (<10s) [44].

Explosive analysis was demonstrated in the first report of DESI when 1,3,5-trinitro-1,3,5-triazinane, commonly referred to as *RDX*, was detected in the negative ion mode from a leather surface [28]. The leather reproduced a real-life surface (e.g., luggage) at an airport. The ability to analyze various nonconducting surfaces is critical for explosive analysis because of the diverse nature of the surfaces from which these analytes are potentially derived. Several other explosives including octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4,6-trinitrotoluene (TNT), pentaerythritol tetranitrate (PETN), triacetone triperoxide (TATP) [46] and composition 4 (C4) have been detected by DESI and DAPCI-MS from paper, glass, plastics, skin [47], and even prints derived from a finger that had come into contact with C4 [44]. Owing to the high electron affinities of the nitro or nitrate functional groups characteristic of many explosive compounds, most are more effectively ionized in the negative ion mode via electron capture or by doping in salts to form stable adducts. By doping HCl into the DESI spray, referred to as *reactive DESI*, Cooks and coworkers observed a 10-fold improvement of the LOD of RDX, allowing the detection of 10 pg from several surfaces [44]. Other explosives have shown similar detection limits (100 fg–10 pg) when analyzed as pure compounds via reactive DESI mode [87,107]. These sensitivities should be adequate for real-world explosive applications, as a recent study indicated that as great as microgram amounts of explosive are present in fingerprints after someone recently handled the plastic-bonded explosive C4 [161]. Besides DESI and DAPCI, several other ambient techniques including DBDI [162] and DART [163] have shown the ability to detect explosives from real-world surfaces with similar analytical performance to DESI.

TABLE 4.1 A Summary of Ionization Techniques

Technique	Ionization Mechanism(s)	Comments	Molecular Weight Range	Multiple Charging	Reported Applications	Seminal Work
Desorption electrospray ionization (DESI)	Desorption through solvation of analyte ESI-like ionization	Initiated the development of novel sources	<66 kDa ^a	Yes	Pharmaceuticals, explosives, metabolite, peptide, and proteins	2004 [28]
Desorption sonic spray ionization (DeSSI) or easy ambient spray ionization (EASI)	Statistical fluctuations of charged species in solvent droplets–proton transfer	No voltage is applied	<1500 Da	No	Pharmaceuticals, fatty acids, dyes, fuels	2006 [75]
Desorption atmospheric pressure photoionization (DAPPI)	Interaction through dopant ionized molecules	Ionization of low proton affinity analytes	<1000 Da	No	Pharmaceuticals and small molecules	2007 [89]
Direct analysis in real time (DART)	Protonated water clusters	Ionization of both polar and nonpolar species	<1500 Da	No	Pharmaceuticals, counterfeit drugs, explosives, metabolites	2005 [91]
Atmospheric pressure solids analysis probe (ASAP)	Thermal desorption corona discharge ionization	Modification made to commercially available sources	<1000 Da	No	Polymers, metabolites	2005 [105]
Desorption atmospheric pressure chemical ionization (DAPCI)	Corona discharge of volatile solvent vapors	Complimentary to DESI	<1000 Da	No	Explosives, metabolites	2005 [29,107]

Plasma-assisted desorption ionization (PADI)	Low temperature discharge	Softer ionization than corona discharge methods	1000 Da	No	Pharmaceuticals and metabolites	2007 [111]
Dielectric barrier discharge ionization (DBDI)	Low temperature discharge	Uses a dielectric material to create a low temperature discharge	<1000 Da	No	Amino acid analysis, explosives	2007 [110]
Electrospray-assisted laser desorption ionization (ELDI)	Laser desorption followed by secondary electrospray ionization	No matrix is used (UV laser)	<66 kDa	Yes	Proteomics, pharmaceuticals, small molecules	2005 [128]
Matrix-assisted laser desorption electrospray ionization (MALDESI)	Laser desorption followed by secondary electrospray ionization	An organic matrix is used (UV laser)	<66 kDa	Yes	Proteomics, glycomics, lipids	2006 [127]
Laser ablation electrospray ionization (LAESI)/IR LADESI	Laser desorption followed by secondary electrospray ionization	IR laser for desorption (water rich sample)	<66 kDa	Yes	Proteomics, tissue imaging, metabolomics, lipids	2008 [144,145]

^aDESI becomes significantly less sensitive at MW > 20 kDa

The majority of these reports have been qualitative in nature; for a rapid screening method, the simple, fast, unambiguous identification of an explosive will normally suffice. However, there are some applications where determining the absolute amount of an explosive present would be useful. Obtaining quantitative information from these ambient ionization techniques is difficult for numerous reasons and as a result is less often reported in the literature. These difficulties stem mainly from the varying degrees of ionization efficiencies of different analytes, morphology of the sample, and the synthesis and incorporation of a stable isotope-labeled (SIL) species into the sample deposited on the surface. Label-free methods (e.g., absolute intensity) could circumvent this limitation; however, analyte suppression and the nonuniform distribution of analyte on a surface [36], similar to the sweet spot effect in MALDI, makes this method less robust as well. Nevertheless, under careful optimization Cooks and coworkers have performed absolute quantification of RDX via the construction of a 5-point calibration curve. Triplicate measurements of the calibration points showed a relative standard deviation of <3% and analysis of an unknown sample gave an ~2% error in the correct amount of material. These results are encouraging for simple mixtures, but in experiments where the complexities differ between the calibration standards and actual samples suppression of analyte may occur and would affect the accuracy of the results.

The area of the sampling in DESI is typically <2–3 mm² and the effective ionization region is ~0.1 mm² [70]. A major limitation in screening of large objects is the relatively low amount of surface area that is actually sampled compared to the large amount of surface area that needed to be screened (e.g., suitcase, clothes, etc). The area sampled in DESI is largely a function of flow rate, surface characteristics, tip-to-surface distance, and the diameter of the spray capillary [29]; by optimizing these parameters one can significantly increase the sampled spot area, but this may lead to less than optimal desorption and ionization performance. Some have developed large-area desorption DESI devices by implementation of three DESI sprayers [29,108] or by utilizing a large-area DESI source [164]. These devices have increased the sampling area of DESI to ~3 cm²; however, still further advancements are warranted for streamlining the interrogation of samples with large surface areas.

4.7.2 Pharmaceuticals and Counterfeit Drugs

Another impact area for these ambient ionization sources is in the pharmaceutical industry, where increasing competition and regulatory pressure have required companies to enhance throughput efficiency. Currently, for quality control and drug discovery experiments, active pharmaceutical ingredients (APIs) from drug formulations are extracted, purified via chromatographic methods and analyzed using UV-vis or MS detection. These steps are time consuming, and increase the amount of time to mature products from bench top to bedside. Methods to obtain both qualitative and quantitative information from drug formulations with limited to no sample preparation would be of significant importance in an environment where throughput and profit are often directly related.

Several ambient techniques have been used for analysis drug tablets. Cooks and coworkers reported the use of a home-built variable speed moving belt in which samples were placed on the belt and analyzed by DESI [57]. This conveyor belt apparatus simulated a real pharmaceutical production line with the last step in production being

DESI-MS analysis. Utilizing this setup both qualitative and semiquantitative information were obtained from tablets, liquids, and ointments at a rate as high as three samples per second. Acetaminophen, acetylsalicylic acid, and caffeine were all detected from an Excedrin tablet and various components were detected from a Centrum multivitamin. Williams *et al.* demonstrated the ability to obtain high mass accuracy DESI-MS spectra using a novel dual ion source for polarity switching accurate mass detection of ingredients [165]. A reference ion used as a lock mass was infused into a separate MS inlet, while DESI was performed and afforded mass errors of active ingredient <2 mTh. In addition, the study showed the utility of polarity switching in obtaining information from pharmaceutical ingredients that preferentially ionized in either positive or negative ion mode. This setup increased throughput further, since the need to analyze the sample twice in both positive and negative modes was negated. DeSSI demonstrated increased abundance of low molecular weight analytes derived from pharmaceutical formulations when compared to DESI. This was attributed to suppression of analyte signal by various water clusters/matrix ions that are observed in the low molecular range when performing DESI [166].

The high throughput capabilities of these techniques were exemplified in a study of liquid formulations by DART using a 96-well plate. The process was fully automated and the ends of glass melting point capillaries were dipped into the analyte solution and then placed between the DART source and mass spectrometer by an autosampler. This setup afforded a high linearity; however, deviations were observed at high concentrations and were attributed to dimer formation. DART affords a complimentary mode of ionization compared to DESI and ESI, a clear advantage in the analysis of low polarity analytes in formulations [166].

An interesting application of these techniques is in the high throughput screening of counterfeit drugs, which is a growing problem in both developing and developed countries. Fernandez and coworkers have performed extensive research in the development of ambient methods for the qualitative and quantitative analysis of pharmaceuticals that fight malaria; a disease that claims over 1 million lives each year [167]. Often, these drugs are counterfeited and the fraudulent types mimic both the tablets and packaging of the genuine drugs, making it difficult to distinguish between the two and require more sophisticated measurement methods to identify the counterfeit tablet. Both DART [97] and DESI [56,97] have been reported for the qualitative identification of the active ingredient artesunate, one of the more commonly counterfeited antimalarials. Unexpected ingredients such as acetaminophen [167] are often found in these counterfeit antimalarials, possibly to deceive less specific analytical methods and/or provide temporary relief of symptoms associated with the disease. More recently, counterfeits containing low sub-therapeutic levels of the active ingredient artesunate have been discovered. These findings are significant as they warrant robust quantitative screening methods to determine the quality of tablets, and the widespread distribution of these drugs could render oral artesunate ineffective because of the development of genetically resistant strains.

Obtaining absolute quantification information from these ambient techniques is difficult for reasons discussed (see above). However, Fernandez and coworkers developed a method using a SIL version of artesunate and reactive DESI for the absolute quantification of artesunate in counterfeit antimalarials [168]. One of the major problems associated with using SIL labels of target analytes is obtaining an evenly distributed

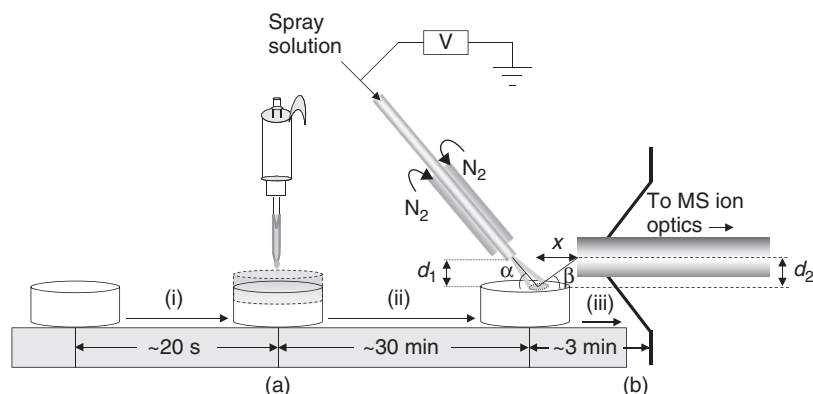


Figure 4.12 Method used for absolute quantification of antimalarials by reactive DESI-MS. *Source:* Used with permission from Ref. 168.

amount of analyte on the surface of a solid tablet. This is dependent on surface porosity, roughness, and solvent used for deposition. Several methods of SIL deposition were explored in the initial stages of method development, including doping the SIL into the ESI solvent and pneumatically spraying it onto the tablet. It was found that by simply pipetting the SIL on top of the tablet's surface and allowing it to dry led to the most robust results. After allowing the solution to dry on the tablet's surface, it was subjected to DESI-MS analysis. A high precision (7.9% RSD) for the ratio of heavy versus light artesunate was determined across various areas of the tablets, indicating an evenly distributed stable isotope label form of artesunate. The ratio was found to be independent of flow rates, nebulizer gas pressure, and geometric variables; however, the hardness of the tablet was found to have a dramatic effect. This procedure for SIL deposition and DESI-MS analysis is summarized in Fig. 4.12.

A calibration curve of various concentrations of artesunate tablets (0.02–0.35 mg artesunate/mg of tablet) demonstrated excellent linearity ($r_2 = 0.9985$). Test samples were used for cross validation of the method and a correlation of 0.9971 was observed. These results indicate the potential of DESI-MS for absolute quantification of artesunate in antimalarials. However, a problem arose when attempting to obtain quantitative information from tablets with different hardness than from which the calibration curve was created. Table 4.2 shows the absolute amounts of three different tablets that were obtained in the field. A large error in absolute amount was observed and attributed to the various degrees of hardness of the tablet. After correcting for this variable, an approximate 6% error in the accuracy was achieved on average. Exact concentrations of artesunate in these tablets were determined by traditional high performance liquid chromatography (HPLC) methods and these values are also shown in Table 4.2. These encouraging results emphasize the importance of surface properties on obtaining reproducible quantitative results and indicate the potential for high throughput quantitative analyses from solid dosage products.

4.7.3 Imaging Mass Spectrometry

Imaging MS is a promising arena because of the ability to determine the identity, abundance, and spatial arrangement of species on surfaces, specifically biological

TABLE 4.2 A Summary of the Absolute Quantification of Artesunate in Antimalarials by DESI-MS. The Second Column is Corrected for Tablet Hardness

Sample	Artesunic Acid (mg/tablet)		HPLC
	Predicted with no Correction	Predicted with Correction	
Tablet 1	73 ± 4	58 ± 3	53 ± 2
Tablet 2	73 ± 5	58 ± 4	56 ± 2
Tablet 3	68 ± 4	53 ± 3	50 ± 2

Source: Used with permission from Ref. 168

tissues. The potential of imaging MS has long been recognized and dates back to the early 1970s when the first molecular imaging experiments using laser desorption were reported [169]. Almost 30 years later, tissue imaging of proteins by MALDI evolved from the direct analysis of biological specimens was reported by Caprioli [170,171]. Currently, most imaging experiments utilize vacuum techniques SIMS or MALDI and each offer different capabilities [172–174]. SIMS affords the highest attainable resolution of any ionization technique with submicron readily achievable [175]; however, the technique is limited to small molecules/peptides and instrumentation is expensive. MALDI is capable of imaging large molecules, such as proteins, with low micron resolution attainable (i.e., 25–100 μm). The ability to conduct open air ionization, as opposed to *in vacuo*, facilitates the analysis of samples in their native state without detrimental sample preparations/manipulations while also increasing the breadth of samples amenable to interrogation. In addition, for protein imaging studies, MALDI produces only singly charged species, which limits these experiments to relatively low resolving power TOF instruments.

Some of the early investigations of direct molecular profiling using these ambient techniques were reported by Cooks and coworkers. In these preliminary studies, they showed the utility of DESI to interrogate plant tissue with absolutely no sample preparation. Various alkaloids were detected from the leaves, seeds, stems, roots, and flowers derived from jimsonweed, poison hemlock, and deadly nightshade [30]. Similar mass spectra and signal intensities of alkaloids were observed for DESI-MS when compared to LC-ESI-MS, albeit the latter requiring significant sample extraction, preparation, and separation of species. In 2005, the same group reported direct molecular profiling of lipids from rat brain, mouse liver, and metastatic human liver adenocarcinoma tissue. A notable finding from these studies was that the normalized abundance of various lipids could be used to distinguish normal versus tumor regions of liver tissue [176]. More recently, other ambient ionization techniques have shown potential for direct biological profiling. DART has been used to detect alkaloids from hairy root plant. Spectra of various alkaloids species were readily obtained by simply holding the tissue in between the source and inlet of the mass spectrometer [102,103]. In addition, DART was utilized to profile whole bacterial cells [96] and volatile organic compounds from eucalypts [177]. Larsen and coworkers investigated the inhibition of the ergosterol pathway in fungal cells via the ASAP method [106]. MALDESI has been utilized for the direct profiling of lipids in egg yolk [142], while LAESI has shown the potential for profiling of plant tissues and whole cells. All these examples demonstrate

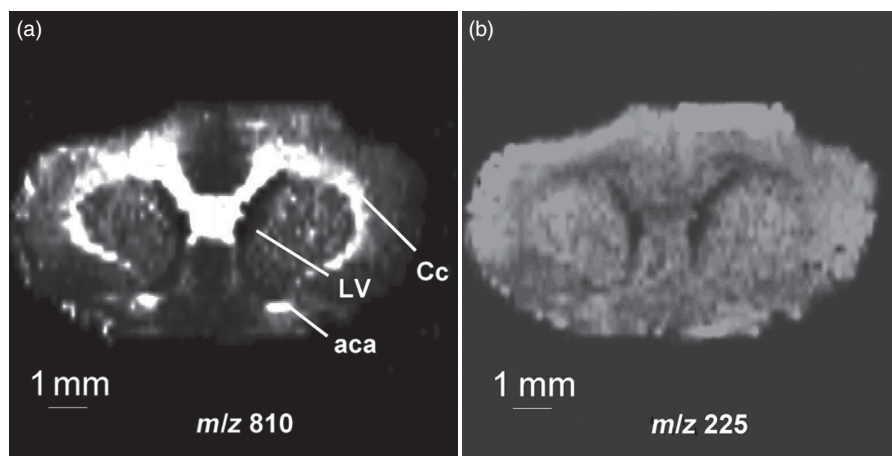


Figure 4.13 Atmospheric pressure imaging of lipids in a coronal section of rat brain tissue by DESI-MS. *Source:* Used with permission from Ref. 178. cc, corpus callosum; LV, lateral ventricles; ac, anterior commissure. (See color insert.)

the great potential of these exciting new ambient ionization methods for direct profiling of tissue.

Although several techniques have been reported for tissue profiling, imaging applications are limited to techniques in which there is a finite area of desorption on the surface. For example, ASAP is not well suited for imaging experiments since the area of desorption and thus mass spectra analysis are not well defined. In addition, for imaging of peptides and proteins, a technique capable of desorption and ionization of nonvolatiles is required.

Figure 4.13a and b shows the mass spectral images of phosphatidyl serine (m/z 810) and myristoleic acid (m/z 225) obtained from a 4- μm coronal section of rat brain tissue in negative ion mode using DESI [178]. Phosphatidyl serine is shown to be concentrated in the region corresponding to the white matter in the brain and significantly less distributed in other parts, while the selected ion image of myristoleic acid shows this species much more evenly distributed throughout all regions of the tissue. Other structural features were readily discerned from these images, including the lateral ventricles and the anterior commissure. In these experiments, the lateral resolution was $\sim 400\ \mu\text{m}$, which is typical for an optimized DESI experiment [179]. Recently, the three-dimensional image of lipid distributions in rat brain tissue was reported by DESI. In these experiments, the 3D images were constructed via the careful acquisition of several 2D images [180].

LAESI has also been used to image rat brain tissue [154]. Figure 4.14 displays distributions of several molecular species corresponding to metabolites and lipids across a 100- μm coronal section of tissue. Mass spectra were obtained by ablating the tissue with mid-infrared pulses (2940 nm) at a repetition rate of 20 Hz. Using this setup, Vertes and coworkers were able to achieve a 200- μm lateral resolution. It is important to note that no exogenous matrix was required since water absorbs IR radiation and is intrinsically found in tissue. To prevent drying of the sample and/or analyte mobilization, the tissue was kept frozen during the experiments by placing it on a cooling stage. As with DESI imaging of brain tissue, LAESI revealed several species

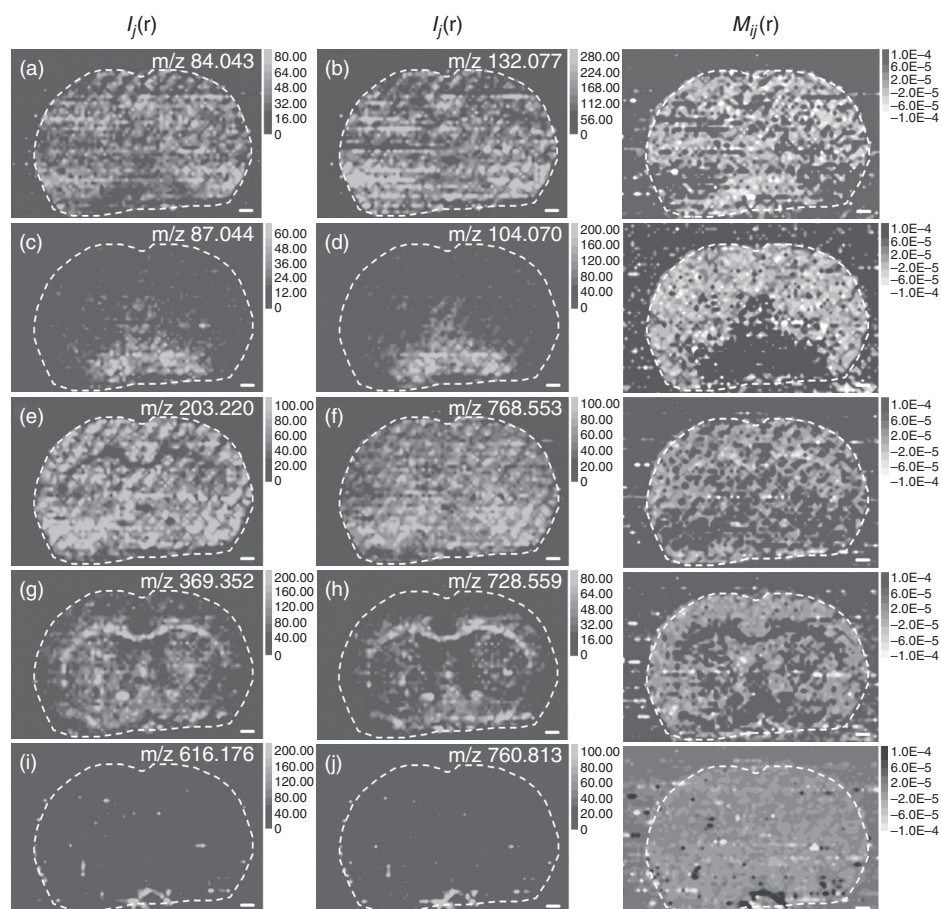


Figure 4.14 Atmospheric pressure imaging of lipids and metabolites in a coronal section of rat brain tissue by LAESI-MS. Species identified with high correlations are as follows (a) ethanolamine with (b) creatine; (c) γ -butyrolactone with (d) γ -aminobutyric acid and/or 2-methylalanine; (e) spermine with (f) PC(35:4) and PE(38:4); (g) cholesterol- H_2O with (h) plasmalogens PC (O-33:3) and/or PE(O-36:3); and (i) heme with (j) α chain of hemoglobin. The plots of the highly correlated pairs are shown in the third column and colocalization is indicated by dark areas. *Source*: Used with permission from Ref. 154. (See color insert.)

that were localized in specific parts of the matrix. For example, Fig. 4.14c reveals that γ -butyrolactone is highly concentrated in the septal complex, while Fig. 4.14e shows the spermine ion to be more evenly distributed through the whole tissue except the corpus callosum. Using simple cross correlation statistics of all possible pairs of ion intensities distributions, Vertes showed the ability to correlate species based on their localization within the tissue. Not all analytes correlated well to one another; however, some showed a high correlation as shown in Fig. 4.14. The colocalization images of the paired species are shown in the last column of Fig. 4.14. These plots are validated by investigating the relationship of both the heme group and the α -chain of hemoglobin. Since both are derived from the same species, a high correlation is expected and is

shown in Fig. 4.14i and j. The identities of all species in these images are given in the caption of Fig. 4.14.

It is difficult to compare DESI and LAESI for imaging applications because of the relatively early developmental stages of both ionization techniques to imaging MS; however, the use of a laser for desorption instead of a solvent jet provides apparent advantages. Although, currently DESI is commercially available [181] and simpler to implement than LAESI, DESI is molecular weight limited and analysis of proteins larger than 20 kDa has proven difficult [61]. This phenomenon limits the technique's capabilities for protein imaging applications. The radius of the desorption area of the DESI plume on the surface is considerably larger than that which can be achieved with a laser [143] and often ill defined [70]. In addition, careful optimization of DESI spray on the surface is required to avoid excessive splashing [70], which theoretically could lead to mobilization of analyte on the tissue and inaccurate mass spectral images. Regardless, numerous reports exist for both DESI [41,54,178,180,182,183] and LAESI [150,152–154] for imaging applications, and only future investigations will decipher the full potential and limitations of each technique for these type of experiments.

4.8 FUTURE OUTLOOK

There has always been an active area of interest in the development of ionization methods for MS; however, in the past decade the intensity of this pursuit has significantly increased. This growing field of ambient ionization is largely a result of the technological advances in instrumentation, which has led to increased sensitivity and specificity and the emerging number of scientific problems that can be addressed using MS. In addition, the majority of vendors now offer commercial instrumentation with API interfaces as opposed to vacuum, which are easily modified to accommodate the different ambient techniques. Applications of these techniques have dominated the literature with far less studies covering mechanistic characteristics. A more in-depth understanding of desorption and ionization mechanisms is needed and will likely direct and shape future applications of each technique. For pharmaceutical applications, where analytes of interest are at a high overall concentration, these techniques are near ready to make an impact at a qualitative level. For studies involving absolute quantification, further research is needed in several fundamental areas before data can be determined in a high throughput manner with any degree of confidence. In biological applications and biomarker discovery experiments where physiologically relevant species are at a low level of concentration, the sensitivity of these techniques must be improved. This could partially be circumvented by increasing the collection efficiency of ions or charged droplets at the inlet of the mass spectrometer via the implementation of an aerodynamic device [184–187].

Despite these yet-to-be-achieved goals, the future of ambient ionization is quite encouraging and exciting. This can simply be shown by the large advancements that have been made in this arena in a relatively short period of time. Most of these techniques are in their mere infancy of existence. Fig. 4.15 displays a plot of the number of papers published as a function of year for FAB, MALDI, ESI, and the ambient techniques discussed herein. Interestingly, the number of reports of all of these techniques is similar to the number of reports of FAB in 2009 and reminiscent of ESI and MALDI in the early 1990s. As a result and similar to the developmental

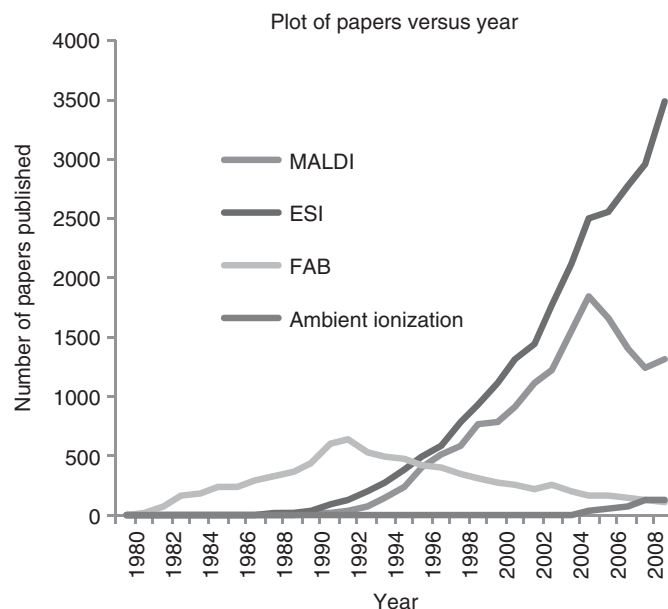


Figure 4.15 A plot of papers published as a function of year using ESI, MALDI, FAB, and new ambient ionization techniques. This graph was created using ISI Web of Knowledge and the search terms: ESI-MS or electrospray ionization; MALDI-MS or matrix-assisted laser desorption ionization; FAB-MS or fast atom bombardment; and all of the ambient technique described herein. (See color insert.)

stages of ESI and MALDI, the ultimate impact of these techniques will only be realized through extensive research.

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5 Matrix-Assisted Laser Desorption/Ionization

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5.1	Introduction	1
5.2	Historical development of MALDI-MS	2
5.3	Overview of MALDI-MS	5
5.4	Analyte/matrix combinations of pharmaceutical interest	9
5.5	Second-generation MALDI approaches	13
5.6	Using MALDI-MS for structural characterization of biomolecules	16
5.7	Methodology and applications	19
5.8	Summary	23
	Acknowledgments	24
	References	24

5.1 INTRODUCTION

Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) burst on the scene in the late 1980s and has since revolutionized the field of MS. MALDI-MS is a viable MS approach for the characterization of biomolecules, synthetic polymers, petroleum and coal products, and inorganic and organic materials. The simplicity, speed, and performance features of MALDI-MS permit the characterization of compounds previously intractable to MS analysis.

While MALDI-MS has not been a traditional “first-choice” for pharmaceutical research [because of the widespread utilization of liquid-chromatography (LC)-based MS methods], one goal of this chapter is to introduce and highlight several areas where MALDI-MS provides clear and compelling advantages in the pharmaceutical analytical toolbox. To be sure, MALDI-MS is not considered here as a replacement for LC-based MS approaches, but instead is viewed as a powerful complement, especially in applications requiring high throughput analysis or the characterization of high molecular weight compounds.

This chapter is constructed to guide the reader through a brief history of MALDI-MS, recognizing its fundamental role in transforming not only modern MS, but a wide number of related disciplines, including those of interest in the pharmaceutical field. Next, the basics of MALDI-based instrumentation are covered with an emphasis on

the laser source and mass analyzer combination. The chapter continues by reviewing the basics of the MALDI matrix, describing necessary matrix properties and reviewing various matrix/sample preparation strategies. A discussion of matrix/analyte combinations of particular interest in the pharmaceutical field is also supplemented by an introduction into new approaches to MALDI-MS, including matrix-free approaches and the use of room temperature ionic liquids as matrices. The latter third of the chapter discusses approaches for obtaining structural information using a MALDI-based mass spectrometer, followed by selected applications of MALDI-MS in fields of particular interest to biopharmaceutical research.

5.2 HISTORICAL DEVELOPMENT OF MALDI-MS

Since the development of the laser as a high intensity source of light, there have been many investigations of the evaporation and ionization of solid materials by focused laser light [1]. Laser desorption (LD) became an effective method for producing gas-phase ions from nonvolatile low mass thermally labile organic salts during the 1970s and 1980s [2–6]. In LD, the probability of obtaining a useful mass spectrum depends critically on the various physical properties of the analyte (e.g., photoabsorption, volatility, etc.). Furthermore, molecules with masses over ca. 1000 Da almost always produce only fragment ions.

LD-based approaches were not regarded as a viable technique for examining high molecular mass compounds until 1987 [7]. At that time, the research laboratories of Tanaka and Hillenkamp independently discovered a new approach that could couple LD with efficient sample ionization. Their key advance was the introduction of an excess of a light-absorbing compound, the matrix, that could be combined with the analyte of interest to yield intact molecular ions on laser irradiation. Specifically, the addition of ultrafine metal powder (UFMP) to analyte solutions in glycerol [8] and the cocrystallization of the analyte with an organic small molecule solution [9] revolutionized the area of MS by producing mass spectra of proteins of ca. 100,000 Da.

Tanaka *et al.* [8] changed the perception that LD cannot be used for high mass biomolecules without fragmentation, an achievement recognized by the Nobel Prize in Chemistry in 2002 [10]. A sample was made into a solution and dripped onto a sample holder. UFMP (cobalt powder of about 300 Å diameter) and glycerol were dissolved with organic solvents and also dripped onto the sample holder. This mixture was then vacuum dried for a short time to remove volatile compounds of the solution. The sample holder, with the sample, was then introduced into the mass spectrometer for analysis. A nitrogen laser was used for sample ionization. By this method, Tanaka and coworkers [8,11] were able to detect several high mass molecular ions (Fig. 5.1), for example, lysozyme (molar mass 14.3 kDa), chymotrypsinogen (molar mass 25.7 kDa), poly(propylene glycol) (PPG) (average molar mass 4 kDa), carboxypeptidase A (molar mass 34.4 kDa), bovine insulin (molar mass 5.7 kDa), cytochrome *c* (molar mass 12.3 kDa), and PEG20K (average molar mass ca. 20 kDa).

The method of sample preparation chosen by Karas and Hillenkamp was fundamentally different. Their long experience with LD led them to believe that often it was the substrate under a layer of organic material on a surface that absorbed the laser light, not the organic molecules themselves. In 1985, Hillenkamp and coworkers began to

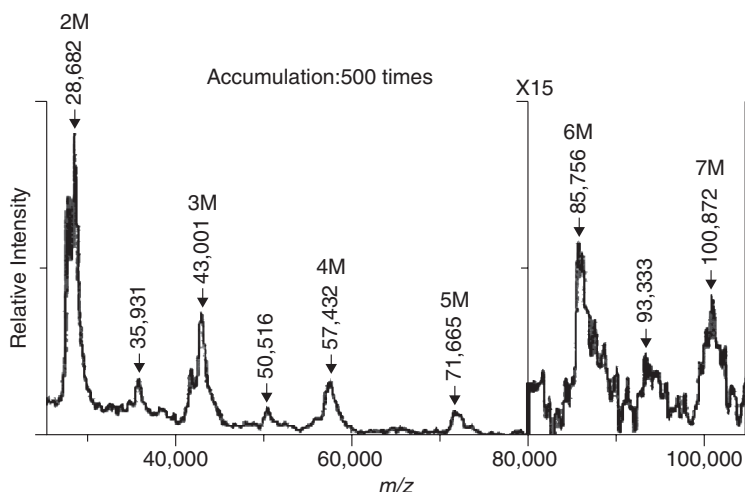


Figure 5.1 Laser ionization mass spectrum of lysozyme from chicken egg white, molecular weight 14,306 Da. Source: Figure reproduced with permission from “Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry.” Tanaka K, Waki H, Ido Y, *et al.* Rapid Commun Mass Spectrom 1988;2:151–153. Copyright John Wiley & Sons Limited.

publish papers containing the hypothesis that large molecule ions could be produced by mixing the analyte with a matrix material that was chosen for its ability to absorb light [12,13]. This matrix material would absorb light, resulting in its ablation and, hopefully, to a coupled desorption of analyte molecule ions. The profound role of the matrix in driving the desorption process led to the designation of this approach as matrix-assisted laser desorption/ionization (MALDI). A variety of matrices were investigated, including tryptophan, nicotinic acid, 3-nitrobenzyl alcohol, and 2-nitrophenyloctylether, and the results published were promising, but not spectacular [7].

The breakthrough for MALDI as applied to polypeptides came with the discovery that nicotinic acid had special properties for large polypeptides [9,13,14]. A Nd:YAG laser at 266 nm was used. Nicotinic acid is a solid at room temperature, with a low vapor pressure (but a tendency to sublime) and a high absorption coefficient at 266 nm. When aqueous solutions of a protein and nicotinic acid were mixed and a droplet of this mixture was dried, the deposit formed had the properties they had sought. They reported the ability of MALDI-MS to generate a large number of intact molecular ions as well as dimers and doubly charged molecular ions of proteins in the mass range above 10,000 Da, stable at least up to time lengths of about a millisecond. In all cases, singly charged molecular ions were the base peaks and no fragmentation was observed above 1000 Da (Fig. 5.2) [9].

The Hillenkamp–Karas approach rapidly underwent essential developments over the subsequent years, resulting mainly in the change of the preferred laser wavelength from the originally used 266 nm to 337 nm, as well as the discovery of at least a dozen new good “chemical matrices.” Hillenkamp defined the “chemical matrix” as a preferentially organic compound that is either a liquid or that dissolves homogeneously in a suitable solvent and that *simultaneously* acts as the light absorber and interacts

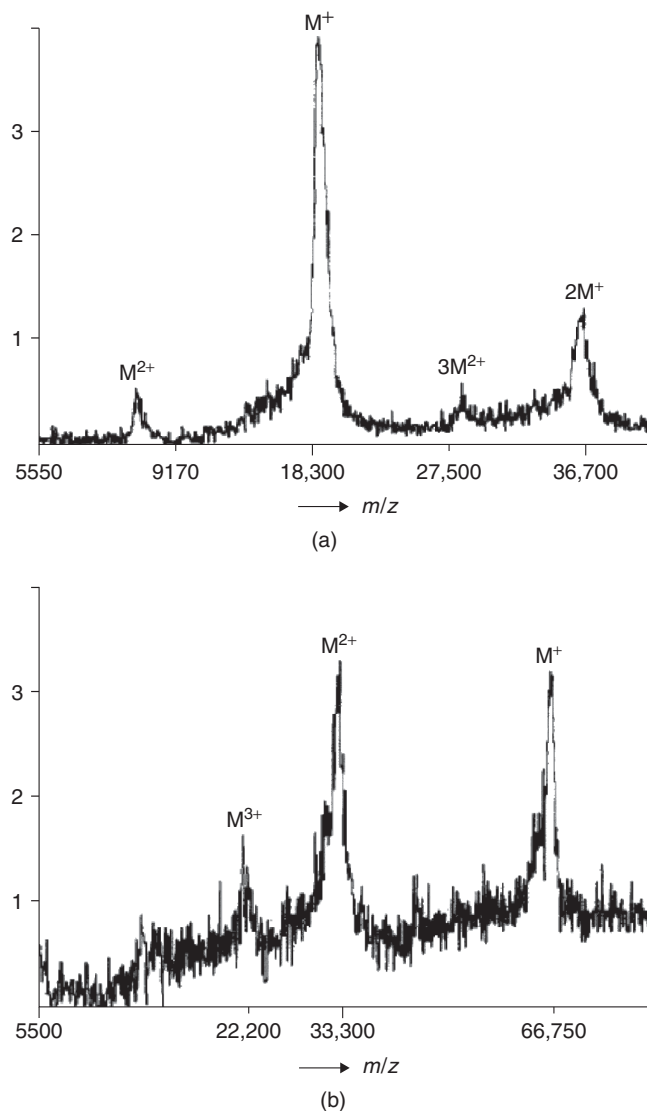


Figure 5.2 (a) Matrix-UVLD spectrum of β -lactoglobulin A, mass range of molecular ion region, 50 accumulated individual spectra. (b) Matrix-UVLD spectrum of bovine albumin, mass range of molecular ion region, 100 accumulated individual spectra. *Source:* Figures reproduced with permission from "Laser desorption ionization of proteins with molecular masses exceeding 10000 Da." Karas M, Hillenkamp F. *Anal Chem* 1988;60:2299–2301. Copyright 1988 American Chemical Society.

with the analyte in the ion-formation process [15]. A large variety of compounds has been empirically tested in the past for their suitability as a matrix. Today, analysts commonly make their choice from a relatively small number of established "chemical matrices," for example, from benzoic or cinnamic acid derivatives. The actual choice depends on the type of the sample being analyzed with only a few matrix compounds

[e.g., 2,5-dihydroxybenzoic acid (2,5-DHB)] proving useful for a number of different compound classes [16].

5.3 OVERVIEW OF MALDI-MS

It has nearly been 25 years since those seminal demonstrations of soft ionization by LD, and the intervening years have generated a substantial literature surrounding the fundamentals of the MALDI process, instrumentation developments (both in lasers and mass spectrometers), sample preparation methods, matrix discovery, and applications in numerous fields. For more comprehensive reviews of that literature, the reader is invited to seek out additional publications in the area [17–28].

Biomolecule analysis by MALDI-MS requires a MALDI mass spectrometer, typically a time-of-flight mass spectrometer (TOF-MS), a suitable matrix that depends on the analyte class of interest, and appropriate sample preparation. Below, each of these requirements are briefly examined, with a focus toward those items or approaches most commonly used in a bioanalytical laboratory.

5.3.1 MALDI-MS Instrumentation

5.3.1.1 MALDI Laser Source. Among the various commercial vendors of MALDI-based mass spectrometers, systems are differentiated by laser wavelength, repetition rate, and laser spot size. The pulsed nitrogen laser ($\lambda = 337$ nm) and the frequency-tripled Nd:YAG laser ($\lambda = 355$ nm) are the most common lasers used in UV-MALDI-MS [29]. IR lasers were used in some early studies of MALDI-MS, and have been explored as alternatives to UV lasers [30–32], primarily because of the larger option for matrices and/or sample desorption in the IR range. However, while IR lasers are more successful in characterizing higher molecular weight samples than are UV lasers for specific applications, such as nucleic acids [33,34], in general the majority of MALDI-based applications are conducted using UV lasers.

Operationally, laser fluences on the order of 10^6 to 10^7 W/cm² are common in MALDI-MS. The best results are achieved by working at or just above the threshold irradiance necessary to generate analyte signal. To achieve the appropriate laser irradiance, some means of attenuating the laser output are typically employed and are common accessories on commercial instruments. Lasers are available in a wide range of repetition rates, ranging from the Hz range for nitrogen lasers up to modern Nd:YAG lasers with rates >1000 Hz. Spot sizes from 5 up to 150 μ m are available, depending on the beam focusing technology used.

5.3.1.2 MALDI Mass Analyzers. The most common instrument configuration is the MALDI ion source coupled to a TOF mass analyzer. The sensitivity, resolution, and mass accuracy depend on the particular TOF used. A detailed discussion of TOF-MS can be found in the chapter titled of this volume. Here, we briefly discuss the varieties and operational characteristics of these mass analyzers, highlighting their coupling to MALDI ion sources.

Standard TOF mass analyzers are available in two configurations, a linear time-of-flight (L-TOF) or a reflectron TOF (re-TOF). In L-TOF, ions are accelerated for less than a few hundred nanoseconds. Once ions leave the acceleration region and

enter the field-free region, any fragmentation that occurs will not be detected. In this case, less fragmentation is observed in L-TOF as this instrumental configuration cannot differentiate the parent and fragment ions. A significant disadvantage of the L-TOF is that it cannot reduce peak broadening occurring from the initial velocity distribution of ions. Typically, poor resolution and mass accuracy are the result.

A common instrumental approach for increasing the resolution and mass accuracy in TOF is the addition of an electrostatic mirror at the end of the flight tube that reverses the ion's direction and refocuses the ion toward the detector. The re-TOF mass analyzer effectively doubles the path length of the ion, yielding longer flight times and, hence, higher mass spectral resolution. In addition, the electrostatic ion mirror compensates for the initial energy spread of the ions, thereby improving focusing of ions of a single m/z more effectively at the detector, which improves instrumental mass accuracy.

All commercial MALDI-TOF systems are equipped with time-lag focusing, commonly referred to as *delayed extraction* (DE). This instrumental development was coupled to TOF as a means of reducing the initial velocity spread of ions while they are still in the accelerating region of the mass spectrometer, yielding improved resolution and lower mass measurement errors [35–37]. In the DE mode of operation, a second electrostatic gate is included in the ion source region. During the laser pulse, this electrode is held at a potential sufficient to prevent the acceleration of the laser-desorbed ions into the field-free region of the mass spectrometer. After a suitable delay period, which is mass dependent, the ions are then accelerated into the field-free region and analyzed as usual. In addition, to avoid detector saturation due to the matrix ions present in large excess in the sample, most commercial instruments are equipped with a low mass ion gate. Low mass matrix ions are deflected from the detector or the detector is turned on immediately after these low mass ions reach the detector.

The choice among TOF mass analyzers, linear versus reflectron, ultimately depends on the types of analytes to be characterized, and on the available budget. Without question, re-TOF mass analyzers offer higher performance (resolution, mass accuracy) when analyzing lower molecular weight compounds such as peptides, lipids, simple carbohydrates, and oligonucleotides. While in some cases the use of a re-TOF mass analyzer may result in a loss of sensitivity, as the ions must travel through an electrostatic mirror for refocusing, the gains in mass accuracy and resolution usually more than compensate for any sensitivity losses. L-TOF mass analyzers are more effective when high molecular weight ($M_r > 10\text{--}20\text{ kDa}$) are the primary focus of analysis. Examples include intact proteins, glycoproteins, nucleic acids, and polymer complexes.

In addition to traditional TOF mass analyzers, a number of hybrid systems can be used with a MALDI ion source. Hybrid systems tend to be most useful if one needs to obtain structural information on the MALDI-generated ions via collision-induced dissociation (CID) methods. The most common hybrid systems are the tandem time-of-flight (TOF-TOF) and quadrupole time-of-flight (Q-TOF) configurations. The TOF-TOF configuration enables structural studies of MALDI-generated ions at high collision energies. Ion formation occurs under vacuum, similar to standard MALDI-TOF configurations. In contrast, most Q-TOF instruments with a MALDI ion source form ions at atmospheric (or somewhat lower) pressures and focus those ions into the mass analyzer through a quadrupole mass analyzer. In addition to the differences arising by forming ions at atmospheric pressure [28], Q-TOF instruments also have limitations on the mass range that can be analyzed. These differences arise due to the focusing properties of the quadrupole. However, like TOF-TOF systems, the Q-TOF

configuration does enable structural studies of MALDI-generated ions in a low energy quadrupole (or similar) collision cell.

5.3.2 MALDI Matrix Properties and Preparations

5.3.2.1 Matrix Properties. The MALDI technique involves the process of desorption, dissociation, and ionization of the analyte and the matrix under high laser energy. The essence of MALDI-MS is the matrix. Generally, analyte compounds are embedded in a surplus of matrix (ca. 1000-fold molar excess) and are codesorbed on laser excitation. The general features of effective MALDI matrices are already known: they must dissolve (liquid matrix) or cocrystallize (solid matrix) with the sample, strongly absorb the laser light, remain in the condensed phase under high vacuum conditions, stifle both chemical and thermal degradation of the sample, and promote the ionization of the sample via any of a number of mechanisms [7,38–44]. More explicitly, MALDI matrices must have the following properties:

1. **Solubility**, this condition can be expanded to include any solvent system in which the analyte of interest will codissolve with the matrix.
2. **Absorptivity**, which allows the energy to be deposited in the matrix, not the analyte. In UV-MALDI, the matrix is typically an organic compound that has a relatively high molar absorptivity (ϵ_λ) at the wavelength of the laser, and whose structure is based on an aromatic core (a chromophore) suitably functionalized to achieve the desired properties. In the case of IR-MALDI, fewer restrictions apply because wavelengths around 3 μm are effectively absorbed by O–H and N–H stretch vibrations, while wavelengths around 10 μm cause excitation of C–O stretch and O–H bending vibrations. Therefore, nonaromatic compounds can perform well as matrices in IR-MALDI.
3. **Reactivity**, the matrix itself is chemically inert toward the analyte. Matrices that covalently modify analytes cannot be used. A matrix can serve as a protonating or deprotonating agent or as electron-donating or electron-accepting agent.
4. **Volatility**, the matrix should have a sufficiently low vapor pressure to minimize volatilization in the ion source vacuum, yet should sublime relatively easily.
5. **Desorption**, the matrix should facilitate codesorption of the analyte on laser irradiation.

5.3.2.2 Matrix Preparations. Sample preparation/handling is often considered the most important step in MALDI-MS. The quality of the preparation has a direct impact on the quality of the analytical results. Different types of sample preparations are available for various types of analytes and purposes [45,46]. Moreover, most instrument vendors now provide premade MALDI sample targets to reduce both the labor involved in sample preparation and to increase the reproducibility of analysis. Despite these advances, the techniques involved in matrix preparation are worth reviewing. Thus, here we present a brief overview of the three major sample preparation categories: solid matrix, liquid matrix, and special preparations. The interested reader is referred to the following expanded discussion of MALDI matrix preparations (both historical and modern) for more details [47].

5.3.2.2.1 Solid Matrices. The oldest and original sample preparation method introduced in 1988 by Hillenkamp and Karas [9], which has remained, with minor modifications, in place for nearly two decades is the *dried-droplet method*. The approach is simple—a freshly prepared saturated solution of matrix compound is mixed with analyte solution and dried at ambient temperature—but aggregation of higher amounts of analyte/matrix crystals in a ring around the edge of the drop yield inhomogeneous and irregularly distributed crystals on the MALDI target. This inhomogeneity requires the search for “hot” spots to generate good quality spectra [48–50].

Various methods were subsequently developed in attempts to reduce the preparation inhomogeneity. The *crushed crystal* [51], *fast evaporation* [52], *overlayer* [49] and *sandwich* [45,53] methods are the more common examples. Among these, the two-layer (*overlayer*, *sandwich*) approaches wherein the matrix is first spotted on the sample target followed by the analyte and additional matrix or analyte-matrix mixture are most commonly used for solid matrix preparations. These approaches provide high detection sensitivity and excellent spot-to-spot reproducibility, which are mainly due to the increase of the matrix-to-analyte ratio and improved isolation between analyte molecules as a result of analyte/matrix deposition features.

As an alternative approach for reducing analyte/matrix inhomogeneity, the *electrospray* technique was investigated. In the electrospray technique, the sample-analyte mixture is electrosprayed from a high voltage (3–5 kV) stainless steel capillary onto a grounded metal sample plate, mounted 0.5–3 cm away from the tip of the capillary (Fig. 5.3) [54,55]. It creates homogeneous layers of equally sized microcrystals, wherein the analytes are evenly distributed. Later, Caprioli [56] used this sample preparation technique for MALDI imaging of tissue samples, and Clench [57] used it to spray matrix-only solutions on thin layer chromatography plates.

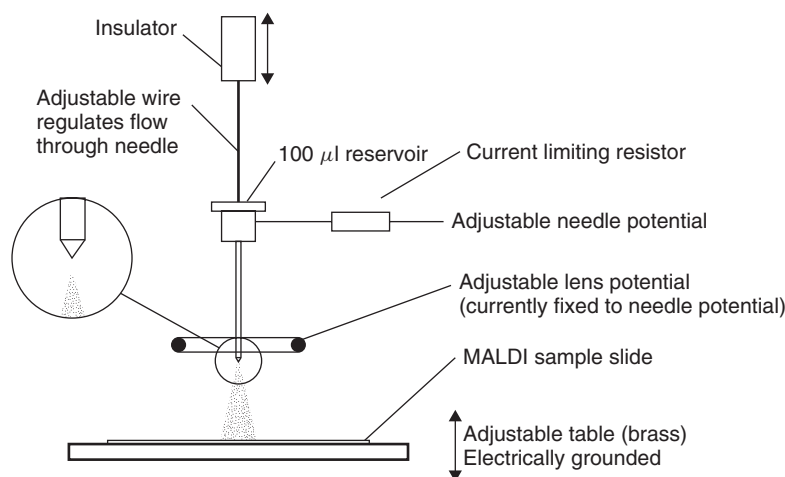


Figure 5.3 Electrospray sample deposition setup employed for preparation of MALDI sample slides. *Source:* Figure reproduced with permission from “Improved reproducibility and increased signal intensity in MALDI as a result of electrospray sample preparation.” Axelsson J, Hoberg A-M, Waterson C, *et al.* Rapid Commun Mass Spectrom 1997;11:209–213. Copyright John Wiley & Sons Limited.

5.3.2.2.2 Liquid Matrices. Owing to difficulties in preparing homogeneous samples using the solid matrix techniques, alternatives based on the use of liquid matrices have also been investigated. Historically, the first technique investigated was the *chemical-doped* liquid technique of Tanaka and coworkers. That approach was subsequently modified by adding an appropriate solubilizing reagent to promote the dissolution of the matrix materials into the liquid support. Selection of the solubilizing reagent is empirically related to an acid–base relationship. This technique has wider scope and can convert most of the solid matrices to the corresponding solution forms to suit different classes of analytes [58].

The more straightforward *chemical liquid* technique simply involves dripping 0.5 μL of liquid matrix (a molar ratio of chemical liquids) onto a 1-mm² area of fibrous paper (Kimwipe™) followed by 0.5 μL of the sample solution. The paper is attached to the sample holder with double-sided sticky tape [59]. In the case of two-phase matrices, fine metal powder or graphite particles are suspended on a low volatility solvent, where the fine particles absorb most of the energy from the laser beam and the liquid molecules provide the charge for ionization. The analyte is mixed with the matrix suspension. After solvent evaporation, the remaining paste on the sample holder is introduced into the ion source [15,60,61]. Other successful chemical liquid approaches involve the addition of solvents that promote ionization or reduce adduct peak formation during MALDI-MS analysis [62].

5.3.2.2.3 Special Preparations. For insoluble samples, it is almost impossible to embed them in a matrix environment by any of the traditional methods. It has been found, however, that by pressing a mixture of finely ground sample and analyte, it is possible to obtain MALDI data from insoluble compounds, for example, insoluble or high molecular weight synthetic polymers. A thick sample is produced by this method, resulting in long-lived samples that yield signals for thousands of laser shots impinging on one location [63].

Among the most unique early developments in MALDI-MS sample preparation was the discovery that solid supports could function as a matrix. Best known is the use of porous Si as a sample support, without the need for any other matrix (desorption/ionization on silicon or DIOS). The concept of an “active” MALDI support is very promising for practical applications, but is not without limitations including a limited mass range, poor reproducibility of the fabrication process, and effect of surface contamination from earlier experiments [64].

5.4 ANALYTE/MATRIX COMBINATIONS OF PHARMACEUTICAL INTEREST

5.4.1 Peptides and Proteins

MALDI-MS owes much of its popularity to its usefulness as an analytical tool for the characterization of peptides and proteins. While an extremely large number of matrix candidates have been investigated for their applicability in peptide and protein analysis, a relatively small group has proved reliable over the past 15+ years. The members of this small group include 2,5-dihydroxybenzoic acid (2,5-DHB) [65], α -cyanohydroxycinnamic acid (CHCA or HCCA) and sinapinic acid (SA)

[66–68]. In addition to these more general purpose matrices, other matrices such as 1,5-diaminonaphthalene (DAN) have found favor in specific applications involving peptides and proteins.

Arguably, the most effective class of matrices for peptide and protein analysis is the class derived from cinnamic acid [66–68]. The most effective of these derivatives are 3,5-dimethoxy-4-hydroxycinnamic acid (SA) and CHCA or HCCA. SA can selectively ionize proteins in the presence of high concentrations of contaminating materials, such as lipids, carbohydrates, and salts. It is relatively nonselective in its behavior toward proteins of significantly different primary structures and modifications (such as phosphorylation and glycosylation). The general affinity of this matrix for proteins, coupled with its ability to tolerate high concentrations of various contaminants (buffering agents, acidification agents, and denaturing agents) allows the application of MS to mixtures of crude biological extracts, including egg white, human blood cell lysates, *Escherichia coli* cell lysates, crude seed extracts, enzyme digest mixtures, and commercial preparations of proteins containing many impurity proteins and peptides. CHCA was found to be a highly effective and efficient matrix for peptides and glycopeptides in the molecular mass range 500–5000. The MALDI mass spectra of proteins obtained from CHCA showed an increased tendency for multiple protonation compared with other widely used matrices.

SA does have two undesirable properties: (i) the formation of an adduct of a photodehydration product of SA with proteins, having a mass 206 Da higher than the protonated protein and (ii) the production of an intense background of ions below m/z 1000, limiting the utility of the technique for the measurement of low mass peptides. With CHCA, certain analytes have been found to undergo fragmentation during MALDI to a degree that complicated their analysis [69]. The excessive fragmentation occurred usually as a post-source-phenomenon; however, in-source fragmentation was also observed.

With the advent of improved mass analyzers, recently there has been significant interest in using MALDI-MS for “top-down” sequencing of biomolecules. In MALDI-TOF-MS, this sequencing approach can be enabled through matrix-driven fragmentation of the biomolecule, either during the desorption/ionization step (in-source) or from metastable fragmentation (post-source). While the matrices described above have been used for in-source decay (ISD) and post-source decay (PSD) sequencing of proteins, DAN [70] has been shown to yield preferred ISD fragmentation from proteins [71].

5.4.2 Glycopeptides and Glycoproteins

Glycopeptides and glycoproteins contain free amino groups that can be protonated efficiently and they exhibit frequently more abundant mass spectral ions than saccharides that are ionized by sodium addition. The ratio of the protonated to sodium adduct for glycopeptides depends on the percent of peptides in the molecule [72]. 2,5-DHB was found to be a preferred matrix for glycopeptides and glycoproteins compared to CHCA and SA by the dried-droplet sample preparation technique, although CHCA with the sandwich sample preparation technique can yield better results for the analysis of glycopeptides [45]. 2,6-Dihydroxyacetophenone (DHAP) containing diammonium hydrogen citrate (DAHC) exhibited superior spectra of sialylated glycopeptides with

better resolution and less fragmentation compared to conventional matrices [73]. Protonated molecular ions were the prominent products and minimal cationic adducts were present during analysis.

5.4.3 Carbohydrates

MALDI-MS is a particularly valuable analysis tool for carbohydrates because it enables underivatized, as well as derivatized, compounds to be examined. MALDI has been applied to the analysis of carbohydrates since the earliest reports of this technique [48]. 2,5-DHB is one of the most successful matrices for carbohydrate analysis. Additives may affect crystallization, embedding the analyte more homogeneously [74]. Using 2-hydroxy-5-methoxybenzoic acid as a comatrix in the 10% range with 2,5-DHB, producing a mixture known as “super-DHB,” resulted in improved ion yields and a two- to threefold increase of signal-to-noise ratio for the analysis of a dextran standard (Dextran 1000) presumably through a disordering in the 2,5-DHB crystal lattice allowing for “softer” desorption [75]. A mixture of 2,5-DHB and 1-hydroxy isoquinoline (HIQ) in a weight ratio of 3:1 was best suited for the analysis of β -cyclodextrin, maltoheptaose, sucrose octaacetate, chitotetraose, and raffinose-5-hydrate, providing enhanced molecular ion abundances, mass resolution, and suppression of unwanted matrix peaks [74].

Acidic oligosaccharides are more difficult to analyze using conventional matrices. 6-aza-2-thiothymine (ATT) and 2,4,6-trihydroxyacetophenone (THAP) were found to be particularly useful for acidic oligosaccharides, with THAP preferred because it gave less fragmentation [76]. In addition, THAP offered improved sensitivity for detection of acidic glycopeptides over CHCA. Among the different substituted acetophenones, 2,5-DHAP produced relatively strong signals from neutral carbohydrates in positive ion mode, which was comparable to 2,5-DHB [72]. Esculetin (6,7-dihydroxycoumarin) efficiently ionized neutral glycans with better resolution than 2,5-DHB in both MALDI and AP-MALDI [72]. 5-Chloro-2-mercaptobenzothiazole (CMBT), an analog of MBT (2-mercapto-benzothiazole), has been found to be not only effective for the analyses of glycolipids but also superior to conventional matrices for the analysis of some oligosaccharides [77].

5.4.4 Lipids

As lipids are low molecular weight compounds, MALDI analysis can be limited because of matrix background products. Thus, although a significant number of studies have been conducted, by far 2,5-DHB has been found to be the most effective matrix for various types of lipids and phospholipids [78–87].

5.4.5 Glycolipids

Underivatized and permethylated gangliosides from commercial sources and highly purified gangliosides from the human brain containing up to five sialic acid residues have been analyzed by MALDI [88]. A variety of matrix and wavelength combinations were tested in both the positive and negative ion modes. The best results were obtained with the matrices 2,5-DHB, 4-hydrazinobenzoic acid, DAN, and ATT. Negative ion mass spectra of the underivatized gangliosides exhibited spectra of better quality than

the positive ion mass spectra with higher signal-to-noise ratios, improved resolution, reduced fragmentation, and as expected, fewer sodium or potassium adducts.

Sphingolipids and both neutral and acidic glycosphingolipids exhibited good signals in the positive ion mode but with the nature and abundance of the molecular ion and extent of fragmentation strongly dependent on the matrix [87]. 2,5-DHB, CHCA, and esculetin were found to provide the best signals. All of the compounds predominantly gave sodium adduct ions, whereas only sphingosine gave abundant protonated molecules. Fragmentation of the oligosaccharide chain of glycolipids containing HexNAc tended to be more random than that seen when HexNAc was absent, and it provided considerable sequence information.

5.4.6 Nucleic Acids

As with other biomolecule compound classes, a large number of candidate matrices have been investigated for the MALDI-MS analysis of nucleic acids. Over the years, only a handful has found widespread use within the field: picolinic acid (PA), 3-hydroxypicolinic acid (3-HPA), ATT and THAP. The *de facto* standard for small oligonucleotides is THAP, together with di- and triammonium salts of organic or inorganic acids [89]. A mixture of THAP in acetonitrile and aqueous triammonium citrate in a 1:1 molar proportion was found to be a good matrix for the detection of synthetic oligodeoxynucleotide samples [90]. A high proportion of volatile solvent as well as the high salt content ensured fast cocrystallization of the matrix, co-matrix and analyte molecules. ATT, co-crystallized with ammonium citrate, has been shown to be a suitable matrix for the analysis of oligonucleotides and short DNA fragments [91]. The major advantages of ATT over other conventional matrices, e.g., THAP and 3-HPA, were improved resolution and mass accuracy, easy sample preparation, and applicability to crude or partially purified samples.

Although 3,4-diaminobenzophenone (DABP) was originally introduced as a novel matrix for the analysis of peptides and proteins by MALDI-MS, it was also demonstrated to be useful in the analysis of oligonucleotides [92]. Intact oligonucleotide ions with this matrix can be readily produced with lower laser powers, resulting in better detection limits, less fragmentation, and fewer alkali metal ion adducts compared to conventional matrices, for example, 3-HPA and THAP. This matrix alone was particularly useful for the analysis of small oligonucleotides (<30-mers) without any co-matrices. Besides, minimal fragmentation and fewer alkali metal ion adducts were seen even at low concentrations of oligonucleotides. It was also found that samples prepared with DABP are highly homogeneous, which resulted in excellent shot-to-shot reproducibility, better resolution and a higher signal-to-noise ratio.

A unique aspect of MALDI-MS analysis of nucleic acid components is the necessity of a co-matrix to minimize cation adduction and improve oligonucleotide signals. The effect of ammonium salt in the detection of oligonucleotides was systematically investigated using several matrices with ammonium salt additives [93]. The results showed that the presence of ammonium salt in the matrix had a beneficial effect on protonation and deprotonation of oligonucleotides in addition to suppressing alkali-ion adducts. The addition of a co-matrix of sufficiently high proton affinity can serve as a proton sink to reduce oligonucleotide fragmentation in MALDI-MS [94,95]. A direct correlation between the comatrix proton affinity and the oligonucleotide molecular ion stability was found. The polyamine co-matrices—spermine tetrahydrochloride,

spermine, spermidine trihydrochloride, and spermidine, were evaluated for their effectiveness in enhancing the mass spectral quality of oligonucleotides [96,97]. In general, polyamine co-matrices were found to be more effective than monofunctional amines for improved mass spectral data.

5.5 SECOND-GENERATION MALDI APPROACHES

The prior section primarily described the use of solid, UV-absorbing organic compounds as matrices for a variety of biomolecular compound classes. As such, those matrix/analyte combinations arise from the Hillenkamp and Karas approach to MALDI-MS. However, while matrices such as 2,5-DHB, CHCA, DABP, and THAP have proved their utility in numerous MALDI-based analyses and publications, investigations into alternative approaches to MALDI-MS have resulted in a significantly different type of matrix—one that is not a low molecular weight organic salt. Three such second-generation MALDI approaches are briefly described below. Because these approaches are either newer or more broadly applicable than those matrices described above, this section focuses on the initial development of these approaches along with representative examples of how such approaches may be used for future MALDI-based investigations.

5.5.1 DIOS (Desorption/Ionization on Silicon)

Just as the matrix serves to trap analyte molecules and absorb UV radiation, nanoporous materials such as porous silicon were found by Siuzdak and coworkers to be an effective medium for desorbing compounds as well as generating intact ions in the gas phase [64,98–101]. This is due to the ability of its nanometer-sized pores to trap analyte molecules, and to the silicon surface, with its photoactive and photoluminescent characteristics, that effectively absorbs UV light. This approach, DIOS has demonstrated characteristics similar to MALDI in that intact molecules are observed at the femtomole to attomole levels with little or no fragmentation (Fig. 5.4) [64,99]. The structure and physiochemical properties of the porous silicon surfaces are vital for DIOS-MS performance and are controlled by the selection of silicon and the electrochemical etching conditions [100]. DIOS surfaces are produced through a simple galvanostatic etching procedure which yields a UV-absorbing porous silicon exterior with high surface area and thermal insulating properties.

The observation of a variety of intact ions directly desorbed from these surfaces suggests that porous silicon has properties such as high surface area and strong ultraviolet absorption that may enhance pulsed LD and ionization. The energy for analyte release from the surface may be transferred from silicon to the trapped analyte through vibrational pathways, or as a result of the rapid heating of porous silicon, producing hydrogen that releases the analyte, which was defended by a study of Ogata *et al.* [102] on possible changes in the environment of hydrogen in porous silicon with thermal annealing. It has also been noted that porous silicon absorbs hydrocarbons from outside air, or while under vacuum, thus rapid heating/vaporization of trapped hydrocarbon contaminants or solvent molecules may increase the vaporization and ionization of the analyte embedded in the porous silicon. DIOS-MS offers good sensitivity, high

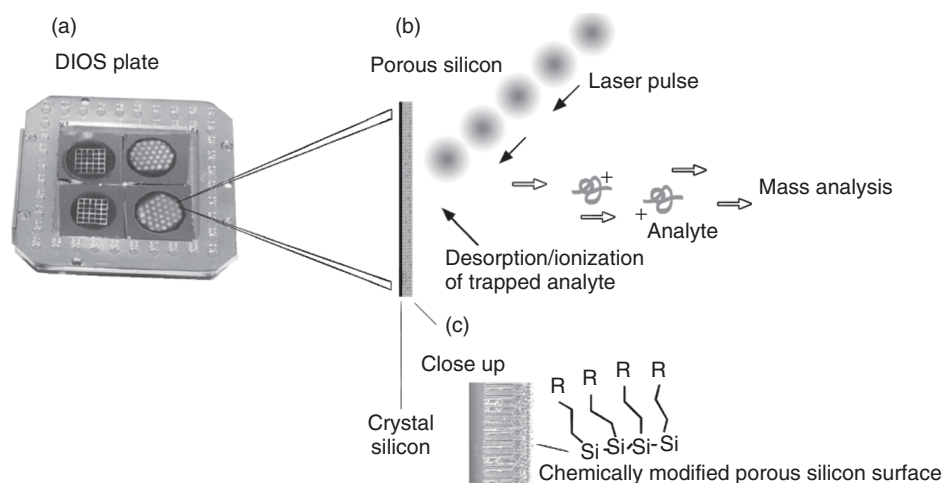


Figure 5.4 Experimental configuration for the DIOS-MS experiments. (a) Four porous silicon plates are placed on a MALDI plate. Each of the porous silicon plates contains photopatterned spots or grids prepared through illumination of *n*-type silicon with a 300-W tungsten filament through a mask and an *f*/50 reducing lens. (b) The silicon-based laser desorption/ionization process, in which the sample is placed on the porous silicon plate and allowed to dry, followed by laser-induced desorption/ionization mass spectrometry. (c) Cross section of porous silicon, and the surface functionalities after hydrosilylation; R represents phenyl or alkyl chains. *Source*: Figure reprinted by permission from Macmillan Publishers Ltd: Nature. “Desorption/ionization mass spectrometry on porous silicon.” Wei J, Buriak J, Siuzdak G. *Nature* 1999;399:243–246. Copyright 1999. (See color insert.)

tolerance to contaminants, no matrix interference and more control over biological MS as the surface properties of porous silicon can easily be customized [64].

5.5.2 Carbon Nanotubes

Carbon nanotubes (CNTs), prepared from coal by an arc discharge method, were investigated as the matrix for analyses of small molecules by MALDI-MS [103]. It was observed that the CNT layer on the probe before deposition of the sample solution appears to have web morphology, which was masked by formation of analyte micro-crystals after deposition of the sample solution. However, there is almost no change in configuration and shape for a single piece of CNT before and after the deposition of sample solution on the CNT layer (Fig. 5.5). Nanotubes provide a lower laser power threshold for desorption/ionization and higher detection sensitivity and mass resolution of small molecules than organic matrices. CNTs may greatly simplify sample preparation, eliminate interference of matrix background ions, and improve shot-to-shot reproducibility. A type of polyurethane adhesive, NIPPOLAN-DC 205, was employed to improve the applicability of CNTs as matrices for analyses of low mass weight compounds [104].

A functionalized CNT, CNT 2,5-dihydroxybenzyl hydrazine derivative, was synthesized and used as both a pH adjustable enriching reagent and as a matrix in MALDI-MS analysis of trace peptides [105]. Functionalized CNT provided two-fold advantages

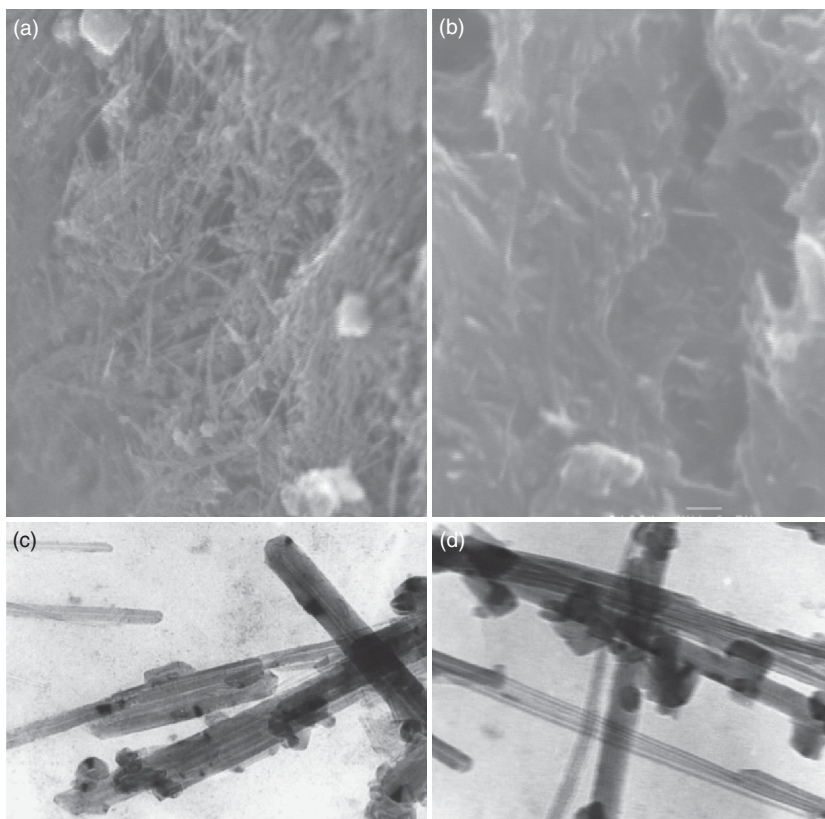


Figure 5.5 (a) Morphology of carbon nanotube layer on probe well before and after dropping sample solution and (b) scanning electron microscopy (SEM) image ($\times 5000$ -fold) of carbon nanotube layer before and after dropping sample solution. (c) and (d) Transmission electron microscopy (TEM) image ($\times 100,000$ -fold) of carbon nanotube layer before and after being deposited by sample solution. *Source:* Figures reproduced with permission from “Carbon nanotubes as assisted matrix for laser desorption/ionization time-of-flight mass spectrometry.” Xu S, Li Y, Zou H, *et al.* *Anal Chem* 2003;75:6191–6195. Copyright 2003 American Chemical Society.

over pristine CNT. The efficiency of enrichment of trace peptides was greatly enhanced as a result of increased surface area to volume ratio for adsorption in basic conditions. It also provided a convenient method for quick isolation. Compared with CHCA without enrichment, the detection limit for analytes can be enhanced about 10 to 100 times after enrichment by the functionalized CNT.

5.5.3 Room Temperature Ionic Liquid Matrices

Room temperature ionic liquids (RTILs) are salts with melting points close to or below room temperature. They look like a classical liquid but they do not contain any molecules: they are made of ions. Typically, an RTIL consists of nitrogen- or phosphorus-containing organic cations and large organic or inorganic anions [106]. They have good thermal stabilities, remain liquid over a range of 200 to 300°C and

have practically no vapor pressure [107]. They produce homogeneous solutions with great vacuum stabilities and are good solvents for a variety of organic, inorganic, and polymeric substances [108].

It has been demonstrated that ionic liquids and solids make useful MALDI matrices [109–115]. With ionic matrices, it is possible to combine the beneficial qualities of liquid and solid matrices. Ionic liquids produce a much more homogeneous sample solution (as do all liquid matrices), yet they have greater vacuum stability than most solid matrices. In most cases, an ionic matrix that produces greater spectral peak intensities and lower limits of detection than comparable solid matrices can be found. Most ionic liquids readily dissolve biological oligomers, proteins, and polymers. However, ionic liquids can vary tremendously in their ability to promote analyte ionization. Both the cationic and anionic portion of the ionic matrix must be chosen with a consideration for the special requirements of UV-MALDI detection. The ionic matrix must not only have significant absorbance at the desired wavelength but also available protons. Most conventional ionic liquids that lack these properties are ineffective as MALDI matrices (Fig. 5.6).

Zabet-Moghaddam *et al.* [114] characterized five different ionic liquids by laser desorption ionization (LDI) and by MALDI-MS. Signals of both anions and cations of the ionic liquids could be observed both in LDI- and in MALDI-MS without any metal adducts. Low molecular mass compounds and peptides could be analyzed best in the presence of water-immiscible ionic liquids, whereas proteins gave the best results in water-miscible ionic liquids. Optimal analytical conditions depend on the molar ratio of matrix-to-analyte and ionic liquid-to-matrix, although the homogeneity of samples in the presence of ionic liquids was reduced compared with classical MALDI preparations.

Recently, direct tissue profiling of peptides with ionic liquids made of matrix mixtures (CHCA/2-amino-4-methyl-5-nitropyridine (CHCA/2A4M5NP) and CHCA/*N*, *N*-dimethylaniline (CHCA/DANI)) was reported [116]. The ionic matrices were found to have several advantageous features for direct tissue analysis, especially CHCA/aniline as compared to CHCA, 2,5-DHB, and SA. These advantages included improved signal intensity and sensitivity, homogeneous crystallization, the possibility of *in situ* partial fragmentations, a high resistance to laser ablation especially using a high repetition rate laser, and the possibility to analyze compounds in positive and negative modes using the same slice for MALDI-MS high quality imaging.

5.6 USING MALDI-MS FOR STRUCTURAL CHARACTERIZATION OF BIOMOLECULES

Structural characterization of biomolecules or other analytes can be performed using MALDI-MS. MALDI-MS is particularly well suited for the analysis of enzymatic or chemical digestions of larger biomolecules including proteins, nucleic acids, and polysaccharides [117–119]. As with many other categories of MS, gas-phase sequencing of the analyte can be performed in MALDI-TOF-MS. In MALDI-MS, the desorption/ionization step itself is sufficiently energetic to cause fragmentation, and this fragmentation process depends on the chemical behavior of both analyte and matrix, as well as the type of instrumentation utilized. Fragmentation initiated by the desorption/ionization process is a continuous process, which begins immediately as the ions

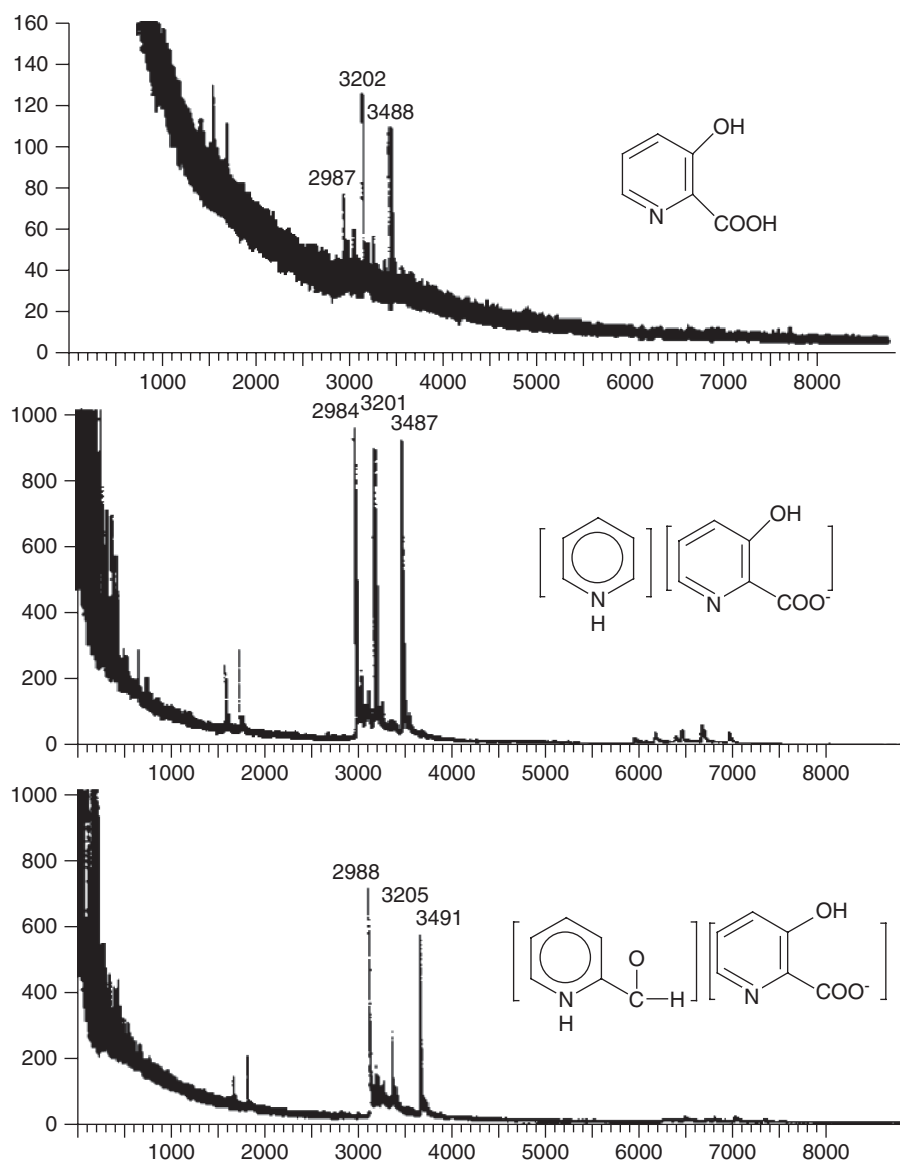


Figure 5.6 MALDI mass spectra of the three oligonucleotides d(pT)₁₀, d(pC)₁₁, and d(pC)₁₂ in different matrices: (a) 3-HPA-10% ammonium citrate, (b) ionic solid 21, and (c) ionic solid 26. Spectra obtained cumulating 100 UV 237-nm laser shots. For the three experiments, the oligonucleotide-to-matrix molar ratio was 1:500,000 and the laser fluence was the same (attenuation 10). The signal strength is expressed in arbitrary units corresponding to the accumulation of 100 shots on a good spot. The 3-HPA scale (top spectrum) differs eight times from that for the two salts (bottom spectra). *Source:* Figure reproduced with permission from "Ionic matrices for matrix-assisted laser desorption/ionization time-of-flight detection of DNA oligomers." Carda-Broch S, Berthod A, Armstrong D. *Rapid Commun Mass Spectrom* 2003;17:553-560. Copyright John Wiley & Sons Limited.

are formed and continues until they are detected. There are essentially four differing time scales for desorption/ionization-induced dissociations in MALDI-TOF-MS: prompt, fast, fast metastable, and metastable.

1. Prompt dissociations occur on a time scale equal to or less than the desorption event.
2. Fast dissociations occur after the desorption event, but before or at the beginning of the acceleration event.
3. Fast metastable decays occur on the time scale of the acceleration event.
4. Metastable decays occur after the acceleration event, during the field-free flight time of the ion.

From these various dissociation pathways, two analytical methods have been developed for the structural characterization of biomolecules: PSD and ISD sequencing techniques.

5.6.1 Post-Source Decay

Many samples undergo metastable decompositions during their travel in the field-free region of a TOF mass spectrometer. This metastable decay process is dependent on many variables but is primarily influenced by the matrix. PSD requires the use of a re-TOF mass spectrometer for the accurate characterization of these metastable ions [120]. By adjusting the retarding voltages on the reflectron ion mirror, the metastable ions can be brought into focus at the detector. PSD is most applicable for the sequence characterization of small to moderate length peptides, and when combined with other enzymatic or chemical digestion approaches, can be used for protein characterization [121,122]. PSD has also been demonstrated for the structural characterization of oligosaccharides (Fig. 5.7), some synthetic polymers, and oligonucleotides [123–125]. The drawbacks of PSD-based methods for structural analysis are the typically longer analysis times (as compared to ISD, below) and the relatively large precursor mass selection window that limits detection or hinders assignment of some fragment ions.

5.6.2 In-Source Decay

ISD was first reported in 1995, where backbone fragmentation of proteins was shown to yield resolvable fragment ions [126]. With the advent of “top-down” methods for biomolecular sequencing, wherein the analyst focuses on obtaining structural information from the intact biomolecule of interest, MALDI-based ISD sequencing has proved both efficient and effective in obtaining wide-sequence coverage from a minimal amount of sample. ISD has proved to be more useful and effective for the structural characterization of a good range of biomolecules, including carbohydrates [127–129] peptides (Fig. 5.8) [130,131] and proteins [132,133]. As mentioned earlier, matrices such as 1,5-DAN promote radical-initiated fragmentation of protein backbones, providing a relatively facile route for high sequence coverage for moderate (up to tens of kilodaltons) proteins [70,71].

Implementation of ISD on modern MALDI-TOF mass spectrometers is routine. Unlike “conventional” MALDI-MS, where one operates at a laser fluence below

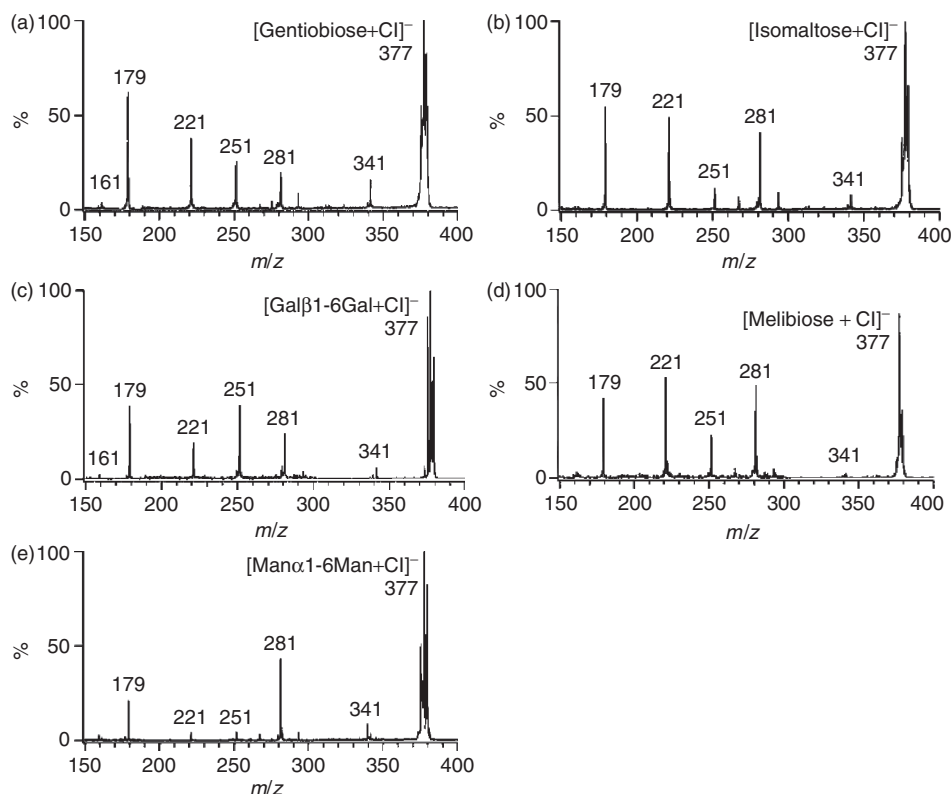


Figure 5.7 Negative ion PSD of chloride adducts of 1–6 linked disaccharides. (a) gentiobiose($\text{Glc}\beta 1\text{--}6\text{Glc}$); (b) isomaltose ($\text{Glc}\alpha 1\text{--}6\text{Glc}$); (c) $\text{Gal}\beta 1\text{--}6\text{Gal}$; (d) melibiose ($\text{Gal}\alpha 1\text{--}6\text{Glc}$); and (e) $\text{Man}\alpha 1\text{--}6\text{Man}$. The α -(isomaltose) and β -configuration (gentiobiose) in 1–6 linked glucopyranosyl disaccharides can be readily differentiated by examining whether the relative abundance ratio of m/z 251:281 is larger than unity (β isomer) (Fig. 5.7a) or smaller than unity (α isomer) (Fig. 5.7b). This peak intensity difference is also applicable to other 1–6 linked disaccharides. *Source*: Figure reproduced with permission from Guan B, Cole R.B. MALDI linear-field reflectron TOF post-source decay analysis of underivatized oligosaccharides: determination of glycosidic linkages and anomeric configurations using anion attachment. *J Am Soc Mass Spectrom* 2008;19:1119–1131. © 2008 American Society for Mass Spectrometry.

threshold, to promote ISD one operates at higher laser fluences, which are empirically determined. As fragmentation occurs within the source region of the mass spectrometer, the use of time-lag focusing or DE techniques (described above) are necessary to fully resolve and accurately characterize these fragment ions. Details of the ISD process and its applications in the structural characterization of biomolecules are available in a recent review [134].

5.7 METHODOLOGY AND APPLICATIONS

Molar mass is an intrinsic molecular property, which, when measured with sufficient accuracy, becomes a unique and unusually effective parameter for characterization

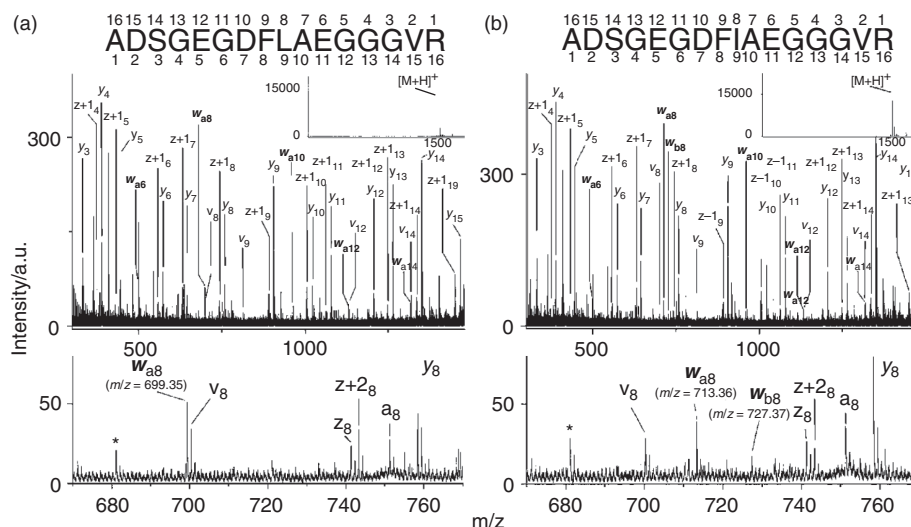


Figure 5.8 MALDI orthogonal-TOF mass spectra of (a) fibrinopeptide A (human, MW 1536.57 u) and (b) its (Leu/Ile) 9-substituted isomer recorded at a buffer gas pressure of 1.2 mbar. The small insets show the full mass spectra including the molecular ion regions, the expanded regions at the bottom the m/z range from 670 to 770. Asterisks denote matrix cluster derived ion species. *Source:* Figure reprinted with permission from Soltwisch J, Dreisewerd K. Discrimination of isobaric leucine and isoleucine residues and analysis of post-translational modifications in peptides by MALDI in-source decay mass spectrometry combined with collisional cooling. *Anal Chem* 2010;82:5628–5635. copyright 2010 American Chemical Society.

of chemical compounds. MALDI-MS experiments include conventional determination of molecular weights, structural characterization via analysis of sequence-informative product ions, and, in some cases, quantitative determination of an analyte from a sample. The level of mass accuracy and the overall usefulness of molecular weight determination will depend on the capabilities of the mass analyzer and the quality of the mass spectral results. Depending on the instrument configuration and type of sample, mass measurement accuracies are on the order of 0.01–1% with sensitivities at the pmol–subfmol level. One clear advantage of MALDI-MS over spray-based methods which generate multiply charged ions (such as ESI-MS) is the tendency of MALDI-MS to yield mass spectra characterized by singly charged ions. Mass assignments are readily made, which can often improve compound identification or allow for the analysis of simple mixtures of analytes without requiring prior separation of the mixture.

MALDI-MS has been used in a variety of applications. One of the more recent and promising applications of MALDI-MS in the field of drug metabolism is MALDI-based mass spectrometry imaging (MSI). MALDI-MSI is the subject of the chapter titled *Gas-Phase Ion Mobility-Mass Spectrometry (IM-MS) and Tandem IM-MS/MS Strategies for Metabolism Studies and Metabolomics* in this volume; thus, it is not be discussed here. A significant number of timely reviews on other applications of MALDI-MS in the pharmaceutical field have been published [17,135–146], and the interested reader is referred there for more comprehensive coverage of these applications. Below, several applications of particular importance to the pharmaceutical industry are described.

5.7.1 Bacterial Identification

One application where the characteristics of MALDI-MS provide clear advantages over ESI-based methods is in the field of bacterial identification. Lay and coworkers first demonstrated the direct identification of bacterial strains by MALDI-TOF-MS in 1996 [147]. Since then, numerous publications and dedicated MALDI-based systems have arisen for this specific application [142]. Bacterial identification via MALDI-MS is possible because it has been shown that, more often than not, each bacterial species generates a unique MALDI mass spectrum fingerprint, which can be used for identification (Fig. 5.9). When sample handling and preparation is standardized, then such fingerprints can be queried against a database of organism MALDI-MS spectra allowing for routine and rapid identification. While analytical challenges remain present when attempting to differentiate pathogenic from nonpathogenic strains of certain bacteria, this MALDI-based approach cannot be duplicated by any ESI-based method in terms of speed, simplicity, and sensitivity. Of particular interest at the present time is the ability to use such identifications to identify infection loads within patients via identification of bacteria in serum and plasma [140]. While there are obvious clinical implications to such studies, this approach can also prove useful in studying new drug efficacy and potency in clearing infections. Further, when combined with recent advances in MALDI-MS analysis of small molecules (described below), MALDI-MS can be considered as a complementary workhorse for the analytical laboratory supporting the development of new drug entities (NDEs) as well as a useful tool during clinical studies.

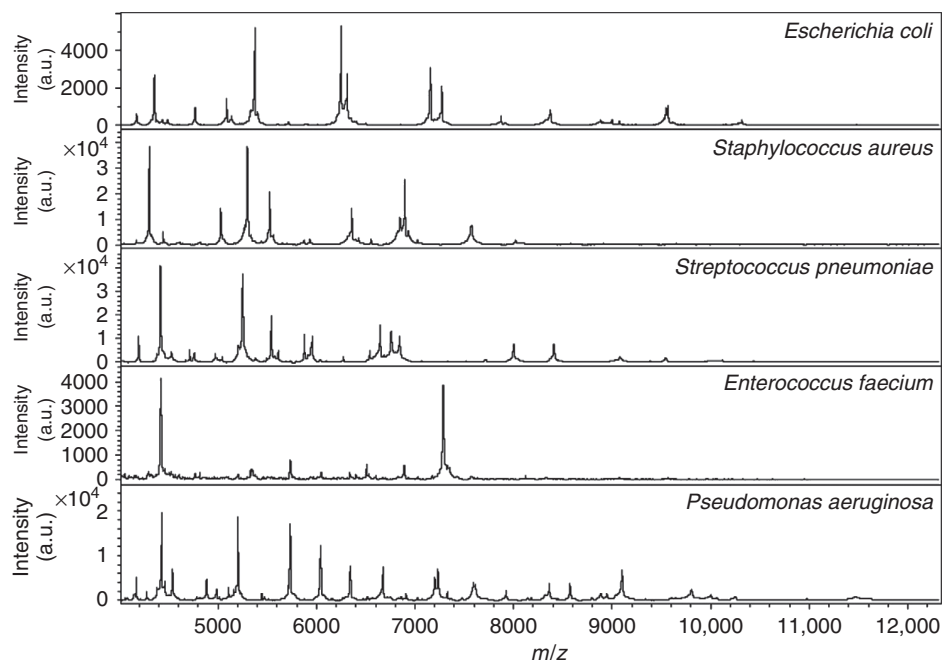


Figure 5.9 Spectral fingerprints obtained from whole colonies of five different bacterial species. The matrix used is HCCA. *Source:* Figure reprinted with permission from Carbone E, Mesquita C, Bille E, *et al.* MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem 2011;44:104–109. copyright 2011 Elsevier.

5.7.2 Small Molecule Analysis

While MALDI-MS has traditionally been overlooked as an analytical method for the identification and quantification of small molecules ($M_r < 750$ Da), several research groups have been developing new techniques and modifications to conventional MALDI-MS that would enable its use in this field. As noted earlier in this chapter, one of the historical limitations of MALDI-MS is that the requirement of a matrix tends to result in a high background below $m/z 1000$ because of matrix-related ions including matrix cluster ions and matrix adduct ions. Further, sample preparation challenges can lead to inhomogeneous sample/matrix mixtures, which hinders accurate and precise quantitative analysis by MALDI-MS.

However, the advantages of MALDI-MS (speed, simplicity, sensitivity, and minimal sample consumption) provide a clear motivation to seek out alternative approaches in the type of matrices used as well as sample preparation that would enable the use of MALDI-MS for high throughput analysis of small molecules. Although the various approaches investigated are too numerous to discuss here, the most promising include the use of high molecular weight matrices and automation of sample preparation techniques [139]. Because the high background in the low mass region of a MALDI mass spectrum arises from matrix-related ions [148], investigations into the use of matrices whose molecular mass is above 1000 Da were pursued with reasonable success [149,150]. While porphyrins have been a popular choice for high molecular weight matrices, certain polymers and pure carbon materials (such as CNTs described earlier) also show promise. It deserves mention here that matrix-free MALDI preparations, such as the DIOS approach that was discussed earlier, are also suitable techniques for characterizing small molecules by MALDI.

In addition to the numerous refinements in sample preparation that were covered earlier in this chapter, approaches that automate sample preparation using robotically controlled electrospray-like deposition and approaches that can (pre-)concentrate the sample or sample/matrix solution are useful when attempting to perform quantitative analysis of small molecules by MALDI-MS. Among the latter, the use of hydrophobic sample targets [151] has proved to be both effective and easily implemented, either commercially (AnchorChip™ technology) or by simple modification of existing sample target plates [152].

5.7.3 Applications in Drug Delivery

The final application highlighted here, where MALDI-MS demonstrates clear utility and advantages over ESI-based methods, is the characterization of drug delivery systems. In the majority of the instances where MALDI-MS has been used, the drug delivery vehicle has been a high(er) molecular weight polymer. A detailed review of this application area has recently been published [138], but from the MS perspective the advantages of MALDI-MS for polymer analysis are several. First, because—by definition—polymeric systems contain multiple components, the use of an ionization method that can handle such “mixtures” without necessitating on-line separation is a clear advantage in terms of analysis simplicity and speed. Further, the analysis of polymers by MALDI-MS has a long and rich history [153], which allows investigators interested in biocompatible polymers easy entry into the field as standard matrix/analyte compositions and preparations have already been established. Another advantage for

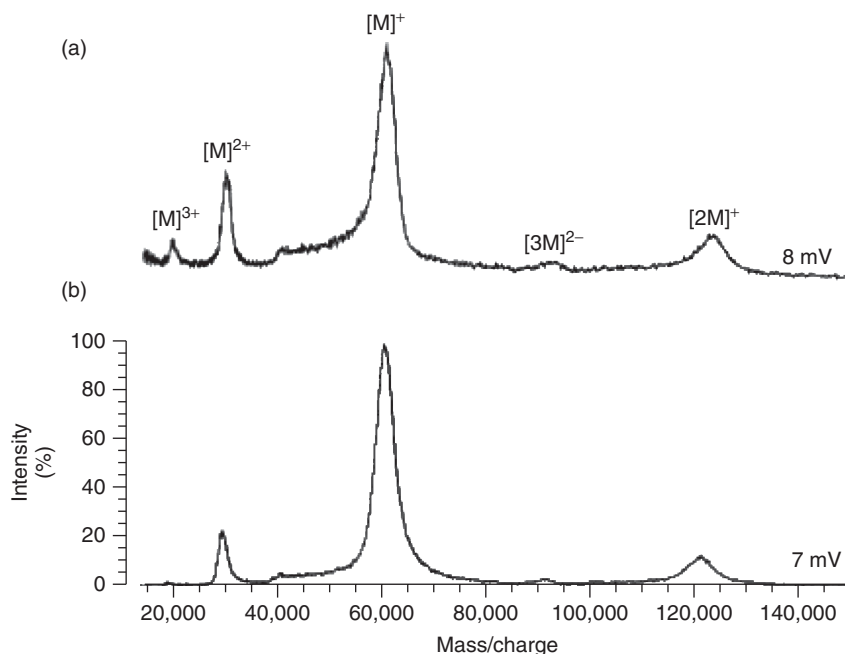


Figure 5.10 Positive ion MALDI linear TOF-MS of interferon $\alpha 2a$ (IFN $\alpha 2a$) monoPEGylated with a 40-kDa branched polyethylene glycol (PEGrIFN): (a) with a standard secondary electron multiplier detector and (b) with a high mass detector. *Source:* Figure reprinted with permission from Seyfried B, Siekmann J, Belgacem O, *et al.* MALDI linear TOF mass spectrometry of PEGylated (glyco)proteins. *J Mass Spectrom* 2010;45:612–617. copyright 2010 John Wiley & Sons.

MALDI-MS, especially when used with TOF mass analyzers, is the ability to characterize high molecular weight distributions at low charge state (high m/z values), thereby reducing the need for spectral deconvolution prior to data interpretation (Fig. 5.10). As improvements in MALDI-based approaches for quantitative analysis increase, the use of this analytical method to identify stoichiometries in polyplexes (drug–polymer noncovalent complexes) should not be far behind.

5.8 SUMMARY

MALDI-MS has become a very powerful tool for the analytical characterization of a wide variety of samples. MALDI-MS owes its beginnings to early work performed on LD/ionization MS. However, it was not until the introduction of the strongly UV-absorbing matrix that this technique became a widely acclaimed and accepted instrumental method for chemical characterization. MALDI-MS was independently reported by Hillenkamp and coworkers [13] and Tanaka and coworkers [8]. Hillenkamp and coworkers described the use of a solid crystalline matrix (nicotinic acid), which has become the standard approach to MALDI-MS. Interestingly, both groups reported

initial success with liquid matrices, an approach that has seen a recent resurgence [58,114].

MALDI-MS is, without question, one of the developments in ionization methods for MS (the other being ESI) that have significantly advanced the applications of MS to high molecular weight, nonvolatile, and thermally labile samples. MALDI-MS is particularly well suited for the characterization of polar compounds, a trait that is, more than any other, responsible for the success of this ionization technique.

Conceptually, sample analysis using MALDI-MS is a relatively straightforward procedure. As described in detail earlier, the analyte of interest is mixed with an appropriate matrix, spotted on a sample plate and analyzed. The analyte is detected in the mass spectrum predominantly as a singly charged ion, simplifying its identification. MALDI-MS is a simple, versatile, and powerful analytical tool that is now found in all types of research laboratories. In fact, the performance characteristics of MALDI-MS have made it an almost indispensable method for the modern bioanalytical laboratory, and, as illustrated in the three representative applications in this chapter, MALDI-MS should be considered a necessary and advantageous analytical tool in pharmaceutical research.

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6 Triple Quadrupole and Quadrupole Ion Trap Mass Spectrometers

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6.1	Summary	1
6.2	Introduction	2
6.3	Triple quadrupole mass spectrometers	8
6.4	Quadrupole ion trap mass spectrometers	11
6.5	Concluding remarks	22
	References	23

6.1 SUMMARY

Mass spectrometers based on ion separation using electrodynamic quadrupolar fields have been dominant in the evolution of the field of mass spectrometry. This chapter discusses triple quadrupole mass spectrometers and quadrupole ion trap mass spectrometers, two of the most important and commercially successful instruments in the history of mass spectrometry. An overview of the principles of operation is discussed in terms of the Mathieu stability diagram, a well-known graphical representation to determine whether ions will have stable or unstable trajectories in a radio frequency (RF) electric field. A brief introduction of tandem mass spectrometry (MS/MS) is also presented.

After the general introduction, each type of instrument is discussed individually. It should be noted that while both types of instruments are based on the same theory of ion motion in electrodynamic fields, there are very important differences in the way experiments are implemented. This is in large part because the triple quadrupole mass spectrometer is the so-called beam instrument where the different stages of an experiment are performed “in space” as the ions travel from the ion source to the detector. Conversely, quadrupole ion traps are “trapping” instruments and the stages of the experiment are performed sequentially “in time,” with the ions being manipulated in the electric field.

A brief historical background is provided for each type of instrument, and then the method of operation is covered with an emphasis on the MS/MS capabilities. For the triple quadrupole mass spectrometer, its key MS/MS feature is the variety of MS/MS

modes in which it can be operated: product ion scan, parent ion scan, neutral loss scan, and single and multiple reaction monitoring.

The discussion of quadrupole ion traps first covers how ions are manipulated in the device. The ability to manipulate ions (“ion taming”) is one factor that makes the quadrupole ion trap a very useful instrument. The ion manipulation is critical in MS/MS operation. Differences between the operation of 2D and 3D instruments are also covered. Finally, there is an overview of the wide array of methods to perform MS/MS experiments: collisional activation using resonance or nonresonance techniques, infrared multiphoton dissociation (IRMPD), and gas-phase chemical reactions, namely, ion–molecule and ion–ion reactions.

6.2 INTRODUCTION

There are two types of quadrupole mass analyzers that use rf electric fields to confine ions in two or three dimensions: the beam-type quadrupole mass filter and the trapping quadrupole ion trap mass spectrometer. The quadrupole mass filter and two-dimensional (2D) quadrupole ion trap are physically composed of four parallel rods, which are typically hyperbolic (ideal) or round [1]. While the quadrupole name is widely applied to rods themselves, it is the electric fields produced between the electrodes (shown in Fig. 6.1) that are of analytical value, hence the three-dimensional (3D) ion traps consisting of three electrodes are referred to as *quadrupole ion traps*, although sometime also referred to as *Paul traps*, after the inventor Wolfgang Paul [1]. Because

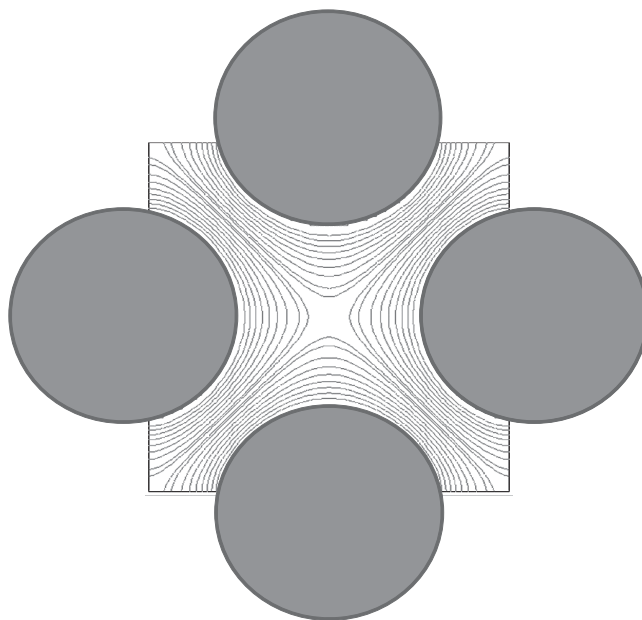


Figure 6.1 Axial view of a quadrupole mass filter rod assembled with an equipotential electric field plot. In an ideal system, the electric field is quadratic. The plot for a three-dimensional quadrupole ion trap would look very similar, with differences mainly in electrode spacing.

the electric field is created with an rf potential, the quadrupole field is dynamic. At any given point in time, the ions experience a field that forces them toward the center of the device in one dimension (trapping) and a corresponding field that forces them away from the center in the other dimension (repelling). As the phase of the rf voltage changes, the dimension in which the ions are trapped and the dimension in which they are repelled alternate back and forth at the frequency of the applied rf voltage. If this frequency is in the appropriate range, typically in the hundreds of kilohertz to a couple of megahertz, the ions have a stable trajectory that periodically goes through the center of the device, or as is commonly stated, the ions are trapped.

Of the mass spectrometers most commonly used today, those containing quadrupole mass filters are the oldest and most mature. The most common configurations for mass spectrometers using the quadrupole mass filter are the single quadrupole instrument (typically coupled to some form of chromatography), the triple quadrupole instrument (which for current instruments may instead consists of two quadrupoles and a higher order multipole), or a quadrupole used in conjunction with another type of mass analyzer to create a hybrid instrument. By grouping quadrupoles in tandem or coupling with another mass analyzer MS/MS experiments can be performed. Most of the modern day MS/MS instruments using quadrupole fields found their origin in work 25–30 years ago.

6.2.1 General Theory of Operation

The physics of ion motion in a quadrupolar field is the same whether the ions are analyzed by a single quadrupole mass filter or multiple quadrupole mass filters in series. In addition, the principles of a quadrupole mass filter can be modified slightly to produce 2D and 3D ion traps. All quadrupoles require the use of rf waveforms to confine ions to stable trajectories allowing them to be either transmitted from entrance to exit or trapped within the volume of the device. At a given value of rf voltage, a dc voltage can be added to define the range of ion mass-to-charge ratios that will have stable trajectories. In the case of beam-type quadrupole mass filters, rf and dc voltages of the same phase or polarity are applied to pairs of rods directly opposite each other, with adjacent rods being 180° out of phase for rf voltages and of opposite polarity for dc potentials. The stability of ions in a linear quadrupole is achieved when the ion motion in the radial dimensions of the quadrupole never exceeds the inscribed radius, r_0 , of the rods. The motion of the ions can be described by a second-order differential equation. The details of the equation of motion are not critical to the general understanding of how a quadrupole works; it is sufficient to know that a general solution to the problem can be derived from the Mathieu equations developed in the late 1800s [2]. Ion trajectories fall into one of the two following classes: stable, where the motion periodically passes back through the origin, and unstable, where the motion increases to infinity. Determination of whether an ion has a stable trajectory or unstable trajectory depends on what is known as the Mathieu parameters q_u and a_u (Eqs. 6.1 and 6.2).

$$q_u = \frac{4eV}{m\Omega^2 r_0^2} \quad (6.1)$$

$$a_u = \frac{8eU}{m\Omega^2 r_0^2} \quad (6.2)$$

In these equations, m is the mass of the ion, Ω is the frequency of the rf voltage applied to the rods (which is called hereafter as the *drive voltage* to differentiate it from other rf voltages that can be applied to manipulate ions), r_0 is the radius of the circle inscribed in the rod assembly, V is the amplitude (0-to-peak) of the rf drive voltage, U is the amplitude of the dc voltage, e is the charge, and u is the dimension to which the equation applies (x or y —the z -dimension is along the length of the rods). The set of solutions calculated by the Mathieu equation for stable trajectories of ions is often plotted in what is referred to as the *Mathieu stability diagram*. In Fig. 6.2, the stability diagram for a quadrupole mass filter is plotted. Ions having a and q values within the dashed lines have stable trajectories and those outside the dashed lines are unstable. It should be emphasized that this treatment does not provide information about the actual physical x - and y -positions of a given ion, only that the values of x and y are always less than the distance from the origin to the rods (stable) or whether the ion trajectory will at some point in time exceed the radius of the device in either the x - or y -dimension.

When a quadrupole mass filter operates with just rf drive voltages applied to the rods ($U = 0$), it behaves as a high pass filter, allowing the transmission of all ions with mass-to-charge ratios greater than the low mass cutoff i.e. those mass-to-charge ratios

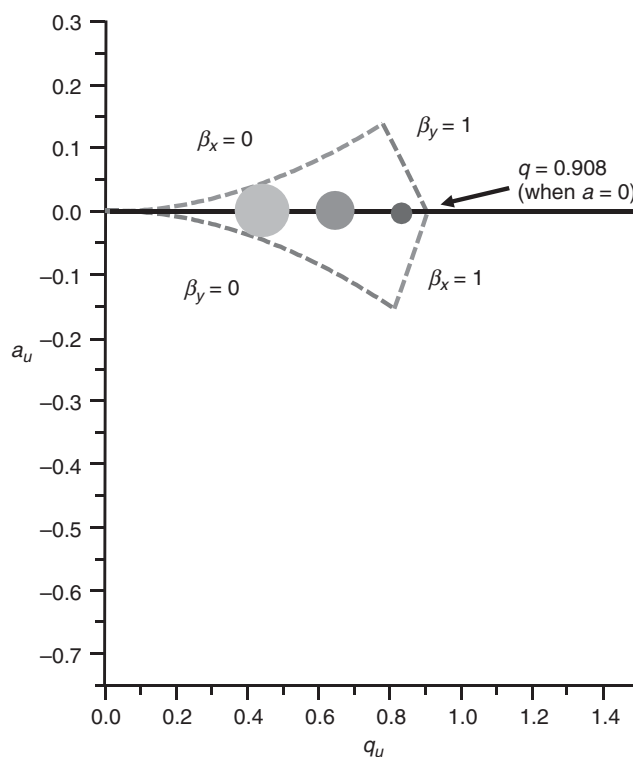


Figure 6.2 The Mathieu stability diagram for a quadrupole mass filter and a 2D quadrupole ion trap. The stability region is symmetrical about the q -axis. In this plot, the a and q values are shown for ions of different mass-to-charge ratios when there is no mass resolving dc voltage applied (rf-only mode). Mass-to-charge ratios are represented by the diameter of the dots.

that have a q_u value less than 0.908 (Fig. 6.2). When operated in such a manner, the quadrupole mass filter functions as an ion guide. This mode of operation has played a major role in the development of quadrupole systems for MS/MS.

Mass analysis in quadrupole mass filters is accomplished by the application of both rf and dc voltages to the rod sets. For a given value of V and U , each mass-to-charge value will lie along a “scan line.” The ratio of the rf and dc voltages is selected such that the line passes through a small area at the apex of the stability region (Fig. 6.3). In this case, ideally ions of only a single mass-to-charge value will have stable trajectories in the device. As the rf and dc amplitudes are increased, keeping the ratio constant, successively larger mass-to-charge ratios are stable and will pass through the device to the detector.

For the 3D quadrupole ion trap, the same Mathieu equation applies, but in three dimensions. The x - and y -dimensions, which are the plane of the ring electrode, are combined in polar coordinates to give a radial dimension (r), and now, there is also an rf field in the z -dimension. To meet the conditions of the Laplace equation, there is an asymmetry between the r - and z -dimensions ($r^2 = 2z^2$), so the Mathieu

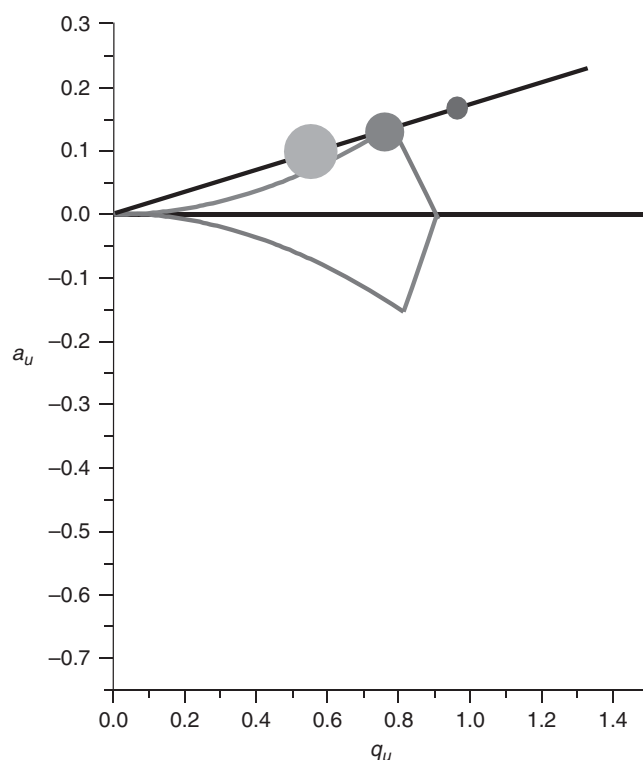


Figure 6.3 The Mathieu stability diagram for the operation of a quadrupole mass filter. The ratio of the rf to dc voltage determines the slope of the line on which lie the ions' a and q values. The values are selected such that only one mass-to-charge ratio lies within the stability region. That mass-to-charge ratio will be transmitted through the quadrupole to the detector. All other ions have a and q values that lie outside the stability diagram resulting in unstable trajectories through the mass filter.

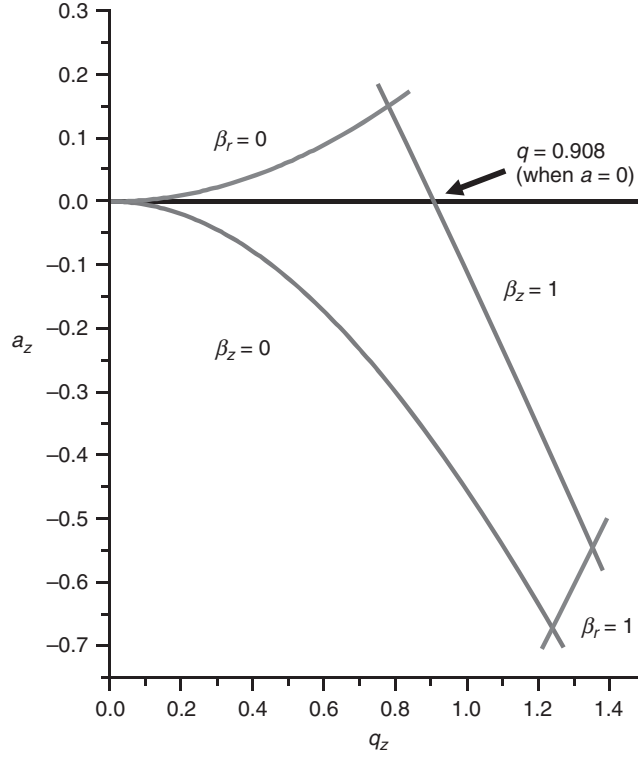


Figure 6.4 The Mathieu stability diagram for a three-dimensional quadrupole ion trap. The stability region is not symmetrical, but the intersection with the q -axis for $a = 0$ (rf-only mode) is still $q = 0.908$.

stability diagram for the three-dimensional ion trap (Fig. 6.4) is not symmetric like the quadrupole mass filter. The a_u and q_u equations are shown below.

$$a_z = -2a_r = \frac{16eU}{m\Omega^2 r_0^2} \quad (6.3)$$

$$q_z = -2q_r = \frac{8eV}{m\Omega^2 r_0^2} \quad (6.4)$$

It is important to note that while stable ions pass through the quadrupole mass filter and hit a detector, stable ions in a 3D quadrupole ion trap remain in the device and thus they need to be further manipulated to be detected (see later discussion). This added operational complexity hindered the development into a mass spectrometer of the 3D quadrupole ion trap for several decades after the quadrupole mass filter was commercialized.

A key feature of quadrupole-based instruments is the ease with which they can be interfaced to chromatography methods. Gas chromatography/MS and liquid chromatography/MS using single quadrupole devices are routine methods. However, the feature that is probably most important in the success of quadrupole-based devices is the ability to perform MS/MS [3]. In fact, the triple quadrupole was developed expressly for MS/MS. The MS/MS experiment can be divided into three distinct steps.

In the first step, an ion formed in the ion source is isolated in the first stage of MS. This ion, called the *parent ion* or *precursor ion*, is then reacted in some manner (step two). The ions thus produced from the reaction of the parent ions, called *product ions*, are analyzed in the second stage of MS (step three). This is often referred to as taking the mass spectrum of an ion in a mass spectrum. It also has analogies to chromatography/mass spectrometry experiments where the chromatography separation is replaced by the first stage of MS.

The reaction of the parent ions to generate product ions is a key component to the MS/MS experiment. Before quadrupole-based instruments, collision-induced dissociation [CID, also called *collision-activated dissociation (CAD)*] was the only reaction used. The development of quadrupole-based instruments expanded the types of reactions that could be used for MS/MS. Current reactions used include ion–molecule reactions, ion–ion reactions, ion–electron reactions, and photodissociation. However, CID is still by far the most widely used reaction.

The CID reaction involves colliding the parent ion with a target (collision) gas. To avoid chemically reactive collisions, a nonreactive gas is typically used, such as He, Ar, or N₂. The collision between the parent ion and the target can convert kinetic energy of the parent ion into internal energy of the parent ion, internal energy of the target gas, or kinetic energy of the target gas. Only the first process is useful in the MS/MS experiment. If the parent ion acquires sufficient internal energy, it will dissociate to give product ions that can be used to identify the parent ion structure. Conversion of the parent ion's kinetic energy to target gas internal energy can be minimized using a monoatomic target gas to eliminate the possibility of vibrational excitation of the target gas. N₂ is often used as a compromise target gas because of its low cost, and while not monoatomic, it has a limited number of degrees of freedom.

While the dynamics and kinematics of the ion-neutral collisions are quite complex, at a basic level, fragmentation of the parent ion is dependent on the amount of ion kinetic energy that can be converted into ion internal energy. The maximum amount of internal energy that can be obtained in a collision with a target gas is the center-of-mass collision energy (Eq. 6.5)

$$E_{\text{com}} = E_{\text{lab}} \left(\frac{m_t}{m_t + m_i} \right) \quad (6.5)$$

where E_{com} is the center-of-mass collision energy, E_{lab} is the parent ion laboratory kinetic energy, m_t is the mass of the target gas, and m_i is the mass of the parent ion. Given that the most commonly used collision gases have masses from 4 to 40 Da and parent ions have masses in the hundreds of daltons, only a small fraction of the ion kinetic energy can be converted to internal energy, even in the best case when all the center-of-mass collision energy is converted to internal energy. For quadrupole-based MS/MS instruments, the ion kinetic energy usually is in the range of 5–20 eV. Thus, multiple collisions are necessary to produce dissociation.

Both triple quadrupole mass spectrometers and ion trap mass spectrometers are powerful MS/MS instruments, but there are very substantial differences in their capabilities and methods of operation. The rest of this chapter is divided into two major sections, one dealing with the quadrupole mass filters in MS/MS and the other, quadrupole ion traps, both 3D (the original quadrupole ion trap) and 2D (a common component of mass spectrometers at present).

6.3 TRIPLE QUADRUPOLE MASS SPECTROMETERS

6.3.1 Origin

The linear quadrupole mass analyzer was first described in the scientific literature in 1958 [4] and has been used since as a mass filter, ion guide, and ion trap. The first commercial single quadrupole mass analyzer instrument, used as a residual gas analyzer, became available in 1961 [5]. In 1974, the first arrangement to be used for MS/MS, three quadrupoles in series, was published [6]. This instrument used a mass-analyzing quadrupole (Q1) to isolate parent ions for transmission into the second quadrupole operated as an rf-only ion guide. As these ions passed through the second quadrupole (q2), they were photodissociated. The products of the photodissociation and any remaining parent ions were then mass analyzed with the third mass quadrupole (Q3). It was the work of Yost and Enke in 1977 that produced the basic design for all the so-called triple quadrupole instruments to come. The key aspect of the QqQ geometry is the middle quadrupole (q) being operated in the rf-only mode and with a collision gas introduced to effect CID [7]. From an instrumental perspective, CID is much simpler to implement than photodissociation and is also much more efficient in a beam-type experiment, so the development of CID in a quadrupole ion guide made possible a commercially viable instrument.

A generic schematic of a triple quadrupole is shown in Fig. 6.5. One of the attractive features of triple quadrupoles is the simplicity of operation. The mass-analyzing quadrupoles (Q1 and Q3) are operated as shown in Fig. 6.3; that is, a scan line determined by the ratio of the rf and dc voltages intersects the apex of the stability diagram and ions of only a single mass-to-charge ratio are transmitted through the quadrupole. To obtain a mass spectrum, Q1 can be mass-selectively scanned while q2 and Q3 are operated in rf-only mode, producing a mass spectrum identical to the operation of a single quadrupole analyzer. Conversely, the same can be achieved by operating Q1 and q2 in the rf-only mode while mass scanning Q3 to produce a single stage of mass analysis.

6.3.2 Tandem Mass Spectrometry

Each stage of the MS/MS experiment in a triple quadrupole is performed in a different quadrupole, and thus, the experiment is sometimes referred to as *tandem-in-space*. The quadrupole in which the dissociation is effected (q2) functions as an ion guide

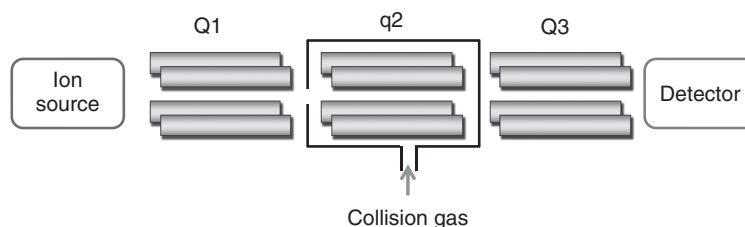


Figure 6.5 A schematic of a triple quadrupole mass spectrometer. Q1 and Q3 can be operated in either a mass filter mode (Fig. 6.3) or the rf-only mode (Fig. 6.2). q2 is operated in the rf-only mode at an elevated pressure to effect collision-induced dissociation for MS/MS experiments.

and operates with just rf drive voltages applied (Fig. 6.2), and thus, ions of all mass-to-charge ratios above a certain value are transmitted. There have been few changes to the triple quadrupole since they were first described in the 1970s and essentially all the changes have involved modifications of q2. The present triple quadrupole mass spectrometers often use a hexapole or octapole as the ion guide. Although these higher order fields are not suitable for mass analysis because of the coupling in the ion motion in the x - and y -dimensions, while each dimension is independent in a quadrupole field, they have a wider range of transmission in the rf-only mode [8,9]. The other change in q2 that has become common is a mechanically nonlinear rod set, so that there is not a line of sight from the ion source to the detector. Because the ions follow the centerline of the field, it is possible to do anything from having a small bend [10] in the ion guide to making a 180° curve.

6.3.2.1 MS/MS Efficiency. A key performance feature that made the triple quadrupole a success compared to the sector instruments used at that time was the increase in MS/MS efficiency. MS/MS efficiency (Eq. 6.6) is defined as the ratio of summed product ion abundance (ΣD) to the initial parent ion abundance before activation (P_0).

$$E_{\text{MS/MS}} = \frac{\Sigma D}{P_0} \quad (6.6)$$

The triple quadrupole has MS/MS efficiencies of 1–10%, which are typically 10–100 times greater than those of the sector instruments. There are several reasons for the increased MS/MS efficiency in triple quadrupoles compared to the sector instruments, which were the principal MS/MS instruments at the time of the development of the triple quadrupole. The major factor leading to improved MS/MS efficiency is the rf-only ion guide (q2) in which the CID reaction takes place providing strong focusing for ions after collision with a neutral gas. Thus, even if the ions are scattered substantially in the collision with the target gas (typically N₂ or Ar), product ions or undissociated parent ions are refocused back to the center axis and efficiently transmitted to Q3 for mass analysis. Because of the focusing characteristics of the rf-only ion guide, higher pressures of collision gas can be used than in older non-rf-confined collision cells, allowing a higher number of collisions between the parent ion and target gas, which further improves the MS/MS efficiency. Finally, an often overlooked effect is the time frame of the experiment. The transit time through q2 is on the order of hundreds of microseconds. This is in contrast to sector instruments where the CID process occurs in a time frame closer to 10 μs. Thus, kinetically slower reactions occur in the triple quadrupole that are not observed in sector instruments, which can further increase the MS/MS efficiency.

6.3.2.2 Modes of Operation. One of the key advantages of triple quadrupole mass spectrometers is the multiple types of MS/MS experiments that can be readily implemented. By far, the most common MS/MS experiment is the product ion scan. This scan is used to obtain structural information on ions in the mass spectrum with the goal of identifying the structure of the neutral compound from which the ion was formed. In a product ion scan, an ion formed in the ion source (parent ion) is selected in Q1

by fixing the values of the rf and dc voltages applied to the Q1 rods; the parent ion is reacted in q2, typically using CID; and then the product ions from the reaction are detected by scanning Q3 in the normal mode.

While any tandem mass spectrometer is capable of readily performing such product ion scans, triple quadrupole instruments are unique in their capability to perform other types of MS/MS experiments. In addition to product ion scans, triple quadrupoles can perform parent ion scans and neutral loss scans. The parent ion scan and neutral loss scans are used to screen for targeted compound types in a mass spectrum. For example, m/z 149 is a characteristic product ion of phthalates. By just reversing the modes of operation of Q1 and Q3, a parent ion scan is performed that will detect all the parent ions that dissociate to produce a product ion at m/z 149. This means that Q3 has fixed values of rf and dc voltages and Q1 is scanned in the normal manner.

The neutral loss scan, is useful when a compound type loses a common neutral fragment, such as phosphorylated peptides losing 98 Da (H_2PO_4). In a neutral loss scan, both Q1 and Q3 are scanned in the normal manner, but the scans are offset by the neutral mass of interest (e.g., 98 for singly charged and 49 for doubly charged phosphorylated peptides). This offset means that if the rf and dc voltages are at values to transmit m/z 656 through Q1, the Q3 rf and dc voltages would be set to transmit m/z 558 (singly charged ions) or m/z 607 (doubly charged ions). Thus, if m/z 656 dissociates by the loss of 98 Da, the product ion will be transmitted through Q3 and detected. If it loses any other mass (e.g., 120 to give an ion at m/z 536), that ion will not be transmitted and there will be no output signal from the detector. Because these scans are performed using identical hardware and need only specific software written to control the electronics via computer, all these scans can be implemented on any commercial triple quadrupole mass spectrometer.

In addition to the above scanning modes of operation, another very useful mode of operation is reaction monitoring. In reaction monitoring experiments, Q1 and Q3 are operated in a static mode to filter all ions but one parent ion and one product ion. This mode of operation is termed as *selected reaction monitoring (SRM)*. SRM scans maximize the sensitivity for the detection of known or targeted compounds in complex mixtures. Multiple SRM experiments can be performed where Q1 and/or Q3 vary, but do not scan. One form of MRM provides increased specificity. Q1 is fixed to transmit a given parent ion, while Q3 “hops” between selected mass-to-charge ratios of known product ions. The specificity is increased not only due to the detection of multiple product ions but also by the ratio of the intensity of the product ions. Another form of MRM involves both Q1 and Q3 hopping between predetermined parent ion and product ion mass-to-charge ratios. This approach can be implemented to screen for multiple targeted compounds in a single sample. This is often used for quantification where one parent ion is the target analyte (e.g., drug metabolite) and the other compound is an internal standard. Commonly, the internal standard will be an isotopically labeled version of the target analyte. In this case, if the isotope label is lost as a neutral, only Q1 will need to be changed, by the mass of the isotopic label, while Q3 will be held constant because the product ion will have the same mass-to-charge ratio for both parent ions. If the isotopic label or part of the label is retained in the product ion, both Q1 and Q3 will be hopped.

An important but subtle consideration in performing these various MS/MS experiments is the transit time of the ions through each quadrupole. As an example in the case of an MRM where Q1 is rapidly switched between the parent ions m_1 and m_2 ,

the rate at which Q1 can be switched is limited by the time it takes product ions to transit through q2 and Q3. In the event that Q1 is switched too rapidly from parent ion m1 to parent ion m2, product ions of m1 could be identified as having come from m2 if m1 and m2 share common product ions. In the case where m1 and m2 have different product ions, the scan speed will be limited by the time it takes for ions to be transmitted through to the detector, as any product ions present in Q3 when the mass is switched will be lost and sensitivity will be reduced.

6.4 QUADRUPOLE ION TRAP MASS SPECTROMETERS

6.4.1 Origin

Although the 3D quadrupole ion trap was first described at the same time as the quadrupole mass filter and used for trapping charged particles in physics experiments and to study ion–molecule reactions, it took several decades before it became a useful mass spectrometer. The transition from a quadrupole mass filter to a 3D quadrupole ion trap can be visualized as taking the quadrupole rod assembly and bending it into a vertical circle, joining the entrance and exit ends of the rods. The two rods in the vertical plane will form a donut-shaped ring, while the two rods in the horizontal plane will each form an “endcap” that is solid with a hyperbolic surface. Figure 6.6b shows the electrodes taken apart, and Fig. 6.6a is a cross-sectional drawing. It should be noted that to transfer ions into and out of the quadrupole ion trap, holes have to be drilled in the endcap electrodes. These holes, along with the fact that the electrodes do not extend to infinity, cause the electric field to deviate slightly from an ideal quadrupole field. These deviations can be expressed in terms of higher order fields, which can have positive and negative effects on the operation of the ion trap [11].

The key challenge to the development of the quadrupole ion trap as a mass spectrometer was an efficient way to detect mass-selected ions. Ions with stable trajectories in a quadrupole mass filter pass through the quadrupole to a typical mass spectrometry detector, an electron multiplier. Conversely, ions with stable trajectories in a quadrupole ion trap remain in the ion trap, and it is necessary to make them unstable to eject them to a detector. A simple but elegant solution to this problem was developed by Stafford and colleagues [12] in the early 1980s and led to the commercialization of the quadrupole ion trap by the Finnigan Corp. Operating the quadrupole ion trap with only an rf drive voltage means ions of increasing mass-to-charge ratio can be made sequentially unstable and ejected by increasing (ramping) the amplitude of the rf drive voltage. Because there is no dc voltage, the Mathieu a_u parameter is zero, and whether an ion has a stable trajectory or not is determined by the Mathieu q_z parameter. As noted in Fig. 6.4, ions with a $q_z < 0.908$ have stable trajectories. From Equation 6.3, q_z is proportional to the amplitude of the voltage (V) and inversely proportional to mass-to-charge ratio. For a given V, the mass-to-charge ratio that has a q_z of 0.908 is known as the *low mass cutoff*. As V is increased, the low mass cutoff increases and ions of increasing mass-to-charge value are ejected in the z -dimension. Placing an electron multiplier outside the electrode assembly on the z -axis allows the ions to be detected. This mode of operation is termed as *mass-selective instability*. While commercial quadrupole ion traps no longer use mass-selective instability, this invention catalyzed the development of the quadrupole ion trap mass spectrometer.

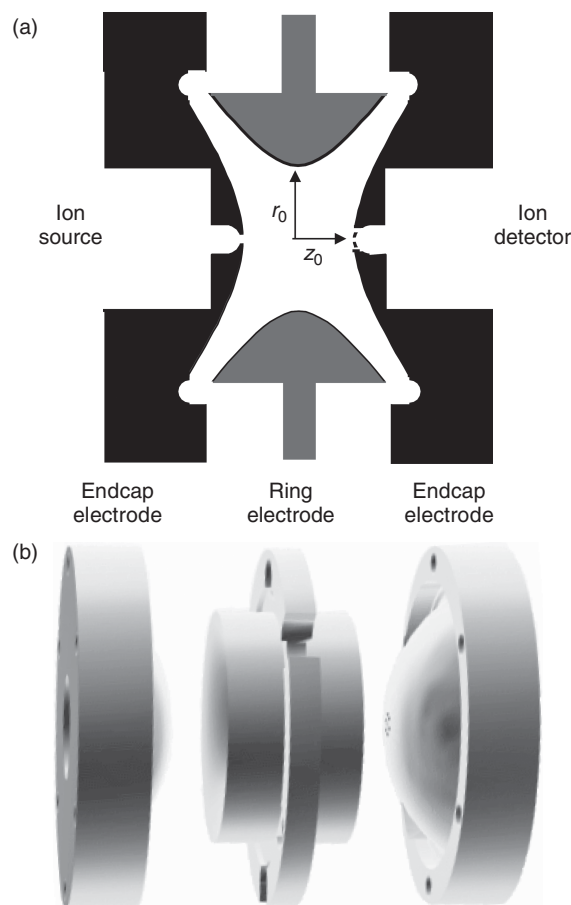


Figure 6.6 A cutaway schematic of a 3D quadrupole ion trap mass spectrometer. (a) Ions are injected into the ion trap through one of the endcaps and ejected through the other endcap to an electron multiplier for detection. (b) A three-dimensional model of the hyperbolic trapping electrodes commonly used in commercial 3D traps.

The original quadrupole ion traps were not even called mass spectrometers. They were marketed as Ion Trap Detectors™ and were used for gas chromatography/mass spectrometry. A rather unique operating feature of the quadrupole ion trap is the improvement of both resolution and sensitivity when operating with helium bath gas pressures in the millibar range [12]. The improvement in performance is a result of collisionally cooling the ions to the center of the electrodes that leads to more efficient ejection of the ions to the detector.

6.4.2 Ion Manipulation in Quadrupole Ion Traps

The power of the quadrupole ion trap lies in the ability to manipulate the trapped ions in time. The parameter that allows the ions to be controlled is what is termed the *secular frequency*. At a given set of operating parameters, V and U , ions of each mass-to-charge have a unique frequency at which they move in the z - and r -dimensions. For

a given drive frequency, Ω , the secular frequency is determined by Equation 6.7

$$\omega_u = \frac{\beta_u \Omega}{2} \quad (6.7)$$

where β_u is given in Equation 6.8:

$$\begin{aligned} \beta_u^2 = a_u + & \frac{q_u^2}{(\beta_u + 2)^2 - a_u - \frac{q_u^2}{(\beta_u + 4)^2 - a_u - \frac{q_u^2}{(\beta_u + 6)^2 - a_u - \dots}}} \\ & + \frac{q_u^2}{(\beta_u - 2)^2 - a_u - \frac{q_u^2}{(\beta_u - 4)^2 - a_u - \frac{q_u^2}{(\beta_u - 6)^2 - a_u - \dots}}} \end{aligned} \quad (6.8)$$

which for $q_z < 0.4$ and $q_r < 0.2$ can be approximated by Equation 6.9:

$$\beta_u = \sqrt{a_u + \frac{q_u^2}{2}} \quad (6.9)$$

The plot in Fig. 6.7 shows the iso- β lines, that is, sets of values of a and q that give a constant β and thus a constant secular frequency of ions of a given mass-to-charge ratio. The limits of the stable trajectories are actually bounded by $\beta_u = 0$ and $\beta_u = 1$ (as noted in Figs. 6.2 and 6.4), so ions will have a secular frequency up to one half the drive frequency, Ω . From a practical perspective, only the secular frequency in the z -dimension is useful. Because in the typical operating mode of quadrupole ion traps $U = 0$, Equation 6.9 reduces to Equation 6.10.

$$\beta_z = \frac{q_z}{\sqrt{2}} \quad (6.10)$$

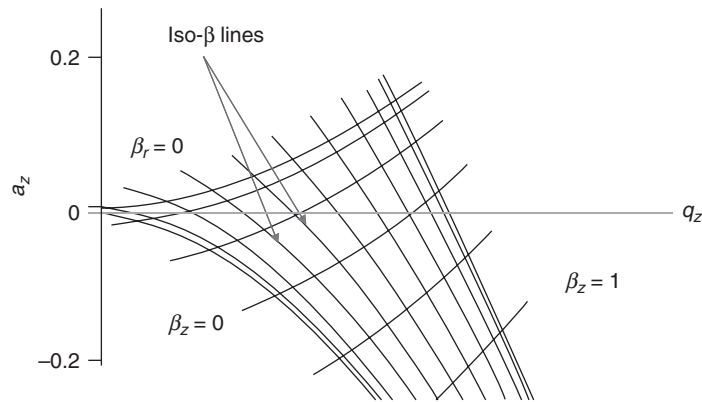


Figure 6.7 Upper part of the Mathieu stability diagram for a quadrupole ion trap showing the iso- β lines. These lines are plots of a and q values that correspond to a given secular frequency. The secular frequency is the main parameter used to manipulate ions in a quadrupole ion trap.

The ions in a quadrupole ion trap are manipulated by applying a supplemental rf voltage at the secular frequency of the ions to the endcaps (z -dimension).

When the frequency of the supplemental rf voltage is the same as the secular frequency of a given mass-to-charge ratio, the ions that are in resonance will absorb power. This is termed as *resonant excitation* and increases the kinetic energy of the resonant ions. Resonance excitation is the key to almost every aspect of experiments performed in quadrupole ion traps. When resonance excitation is large enough, the ions will gain sufficient kinetic energy to be ejected from the trapping region; this process is often called *resonant ejection*.

Resonant ejection is how all commercial quadrupole ion trap systems obtain mass spectra, as it provides several advantages over mass-selective instability. First, it allows a larger range of mass analysis. With mass-selective instability, the ions have to reach a q_z value of 0.908 to be ejected and detected. With standard-sized electrodes (e.g., $r_0 = 1.0$ cm) this leads to a practical upper mass-to-charge ratio limit of around 1000. By performing resonant ejection at a lower q_z value, the mass range is extended proportionally. This allows the mass range to be extended to the 2000–4000 range, although much higher is possible [13]. The actual scan is analogous to the mass-selective instability scan, ramping up the rf drive amplitude so that the ions sequentially come into resonance and are mass-selectively ejected, from low to high mass-to-charge ratio, with the applied supplemental rf voltage. Another advantage of resonant ejection for mass analysis is increased resolution. A somewhat unique capability of the quadrupole ion trap is the ability to change resolution by changing the scan speed. By reducing the rate at which the rf drive voltage is ramped, the relative width of the peak is decreased, increasing the resolution [14]. For significant gains in resolution, the ramp rate must be drastically reduced, making impractical the simultaneous collection of a wide mass range and high resolution mass spectra. However, for narrow ranges, such as the isotopic distribution of a multiply charged ion, the slower scan is useful to obtain resolution allowing charge-state determination.

6.4.3 Linear Quadrupole Ion Traps

The linear ion trap, or 2D ion trap, is a quadrupole mass filter that traps the ions in two dimensions, x and y (or radial), using the rf drive voltage and traps ions in the axial dimension (z) with a dc voltage. The major advantage of the 2D ion trap versus the 3D ion trap (quadrupole ion trap) is the efficiency of ion injection. In the quadrupole ion trap, because there is an rf field in all three dimensions, the ions must be injected through an rf electric field. Only during a small phase angle of the rf cycle are the voltages compatible with efficient injection. Also, the injection is very sensitive to the amplitude of the rf potential. However, with the 2D ion trap, there is no rf electric field in the axial dimension, and as a result, ions can be injected over all 360° of the rf cycle. This leads to substantial reduction in the time required for ion injection.

Once in the trapping field, there is very little difference in the way ions are trapped in a 2D ion trap and a quadrupole ion trap. The Mathieu stability diagram also applies for 2D ion traps, and in the absence of dc potentials applied to the quadrupole rods, ions with a $q_z < 0.908$ are trapped radially. Ions will be prevented from exiting axially by applying to an electrode at the exit of the 2D trap a dc voltage slightly greater than the entering ions' kinetic energy. These blocked ions then turn around and travel back toward the lower dc potential applied at the entrance of the 2D trap. This dc potential

is lower than the ions' kinetic energy when they are entering the 2D trap. However, collisions with the bath gas will have reduced the ions' kinetic energy, so by the time ions return to the entrance, too little kinetic energy remains to overcome the entrance barrier and they will be trapped axially.

There are two means of implementing dc axial trapping. The most common method involves using plates/lenses with apertures at the front and back of the quadrupole rod assembly. This truncates the rf electric field, introducing higher order fields near the entrance and exit of the rods. Alternatively, short sets of quadrupole rods can be used in front of the entrance and at the exit of the main trapping rod assembly. The rf voltage can be capacitively coupled to the end segments, yielding minimum distortion of the rf electric field in the center segment, which is where the ions typically reside. Simulations of the electric fields obtained when applying a supplementary resonance excitation voltage to a pair of rods resulting from these two designs are presented in Fig. 6.8 [15]. Although the resulting electric fields generated by these two approaches result in only minor differences for trapping ions, they are significant for mass analysis.

One approach to mass analysis with the 2D ion trap is very similar to resonant ejection used in the quadrupole ion trap [15]. In this case, a supplementary rf voltage is applied to a pair of rods opposite to each other to resonantly eject the ions radially. Slots must be machined into the rods to allow the ions to exit to external electron multiplier(s). Homogeneity of the field is essential in this design, and thus, the use of end segments and precise alignment of the rods are critical. This design is the basis for the Thermo linear ion trap systems [15]. Although ions are ejected through both opposing rods to which the resonant frequency is applied, this is a highly efficient form of mass-selective ion ejection. In some cases, two electron multipliers are used to increase detection efficiency.

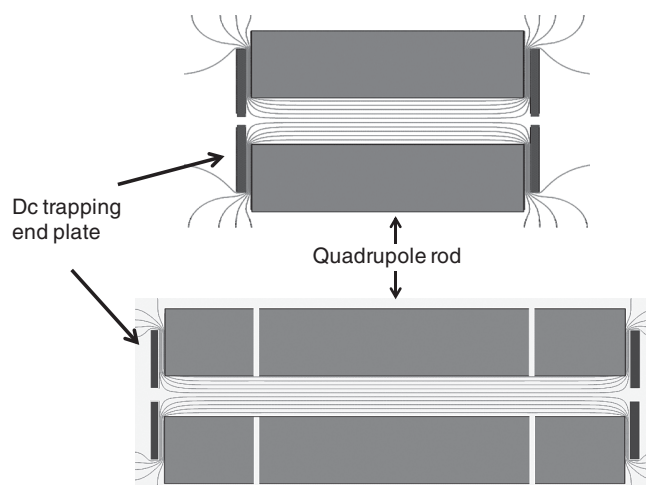


Figure 6.8 Simulation results showing the dipolar electric field in a single-segment 2D ion trap and in a three-segment 2D ion trap. The nonlinearity of the field where the end plate truncates the electric field can cause axial ejection. In a three-segment ion trap when the ions are trapped in the center segment, this is not a problem. In a single-segment ion trap, the axial ejection can be used to mass-selectively eject the ions axially.

An alternate mass analysis method takes advantage of the higher order fields that result from truncating the rf electric field with an aperture plate to perform axial ejection [16]. As noted earlier, ion motion in higher order fields is coupled; that is, when an ion is excited in one dimension, the initial kinetic energy gain is in that dimension, but it can then couple into other dimensions. This is in contrast to a pure quadrupole field where an ion increases its kinetic energy (and amplitude of motion) only in the dimension of the resonance excitation voltage. In the axial ejection 2D traps, ions are bunched toward the exit of the 2D ion trap and resonantly excited in the radial dimension. The increase in radial motion is coupled into axial motion, and the ions gain enough axial kinetic energy to overcome the dc potential at the trap end plate, resulting in ejection and detection by the electron multiplier. The coupling of radial excitation to axial ejection is not as efficient a process as radial excitation and ejection but more than 20% of the ions can be detected. This method of mass analysis is used in the AB Sciex Q-Trap[®] systems.

Another form of axial ejection has been introduced by researchers from Hitachi [17]. In this implementation, vanes are added between the quadrupole rods that produce a field that is approximately harmonic along the central axis. Ions can then be axially resonated and ejected. This is a more efficient ejection process than coupling between radial and axial motions but is still not as efficient as radial resonant ejection.

6.4.4 Tandem Mass Spectrometry

In the quadrupole ion trap, the different stages of the MS/MS experiment are performed in the same analyzer volume but at different points in time. Thus, MS/MS in trapping instruments is sometimes referred to as being *tandem-in-time*. A key feature of tandem-in-time experiments is that multiple stages of MS/MS (MS^n) can be performed. MS^n is not going to be specifically discussed here, but it can be a very powerful experiment to obtain ion structure/differentiation of isomers.

6.4.4.1 Ion Isolation. There are a number of approaches to isolating the parent ion in a quadrupole ion trap. To isolate parent ions of a given mass-to-charge ratio, ions of both lower and higher mass-to-charge ratios must be ejected. Lower mass-to-charge ions that have a $q_z < 0.908$ at the maximum amplitude of the rf drive voltage can be ejected by simply ramping the rf drive to a sufficient amplitude such that low mass-to-charge ions are ejected at $q_z = 0.908$. Higher mass ions can be simultaneously ejected during the ramp to ejection ions with mass-to-charge ratios less than that of the parent ion by applying a supplemental rf voltage at a frequency corresponding to a mass-to-charge ratio just higher than that of the parent ion at the start of the ramp. As the rf drive amplitude increases, sequentially higher mass-to-charge ions will be resonantly ejected, while those masses lower than the parent ion are ejected via mass-selective instability.

To this point, only single frequency resonance excitation waveforms have been considered. However, there is no reason that waveforms with multiple frequencies cannot be used. A standard means for manipulating ions with a waveform containing multiple frequencies is known as *SWIFT*, which is the acronym for Stored Waveform Inverse Fourier Transform [18]. In SWIFT, a frequency domain waveform is generated to achieve the desired purpose of the ion manipulation, for example, to eject all ions except parent ions for an MS/MS experiment. Such a waveform includes all secular

frequencies except those of the parent ion. Then, an inverse Fourier transform of the frequency domain spectrum is performed to obtain a time domain supplemental rf voltage signal that can be applied to the quadrupole ion trap. This time domain signal will cause all the unwanted ions in the quadrupole ion trap to be ejected. Very complex ion manipulation can readily be performed by this method, but one has to be careful in how the waveform is calculated, that is, the phases of the time domain signal must be such that a physically unattainable amplitude is not produced mathematically.

A simple way to approximate a SWIFT waveform is to add a series of sine waves separated by 500 Hz over the range of the resonant frequencies of ions (0 to one half the rf drive frequency). Then the frequencies of the parent ion can be “notched” out. Because the parent ion q_z value is controlled by the rf drive amplitude, a constant value of q_z for parent ion isolation can be used by changing the rf drive amplitude to set the parent ion at the desired q_z for isolation, and thus, the same waveform is applicable to all parent ions.

6.4.4.2 Ion Activation.

6.4.4.2.1 Collisional Activation. Several methods can be used for ion activation, each with different advantages and limitations. The most common form of activation is by collision with a neutral target gas as part of the overall process known as *CID*. There are several methods to induce energetic collisions between ions and neutral gas molecules where kinetic energy can be converted into internal energy. As mentioned earlier, the deposition of internal energy into parent ions leads to dissociation and formation of product ions, from which structural information can be deduced.

The type of dissociation products observed in collisional activation methods used in ion traps can be different from those observed in beam-type mass analyzers. It should be noted that the collision energy in a quadrupole ion trap is ill-defined. The ions' kinetic energy is constantly changing due to the dynamic nature of the electric trapping field, the acceleration of ions due to supplemental voltages, and the deceleration of ions due to collisions with the He bath gas. While the collision energies in triple quadrupole instruments can be similar in magnitude to those in the quadrupole ion trap, two fundamental differences often lead to different MS/MS spectra being observed. First, helium is typically the collision gas in the quadrupole ion trap, whereas nitrogen or argon is more commonly used in triple quadrupoles. With heavier gases as the collision gas, much more kinetic energy can be converted to internal energy per collision (Eq. 6.5), leading to a different internal energy distribution. The second difference between the quadrupole ion trap and triple quadrupole is the time frame for the reaction. Ions must dissociate (react) in 100 μ s or less in the triple quadrupole instrument to be observed in the next stage of analysis. However, in ion traps, the times are two or three orders of magnitude longer. This longer time frame allows low internal energy, but kinetically slow reactions to occur, and in fact can significantly favor such reactions.

6.4.4.2.2 Resonance Excitation. The first and by far the most common method for collisional activation is resonance excitation [19–22]. This method employs the same phenomenon of resonance as the resonance ejection method discussed previously. As noted above, when a supplemental rf voltage is applied to the endcap electrodes at an ion's secular frequency, that ion's kinetic energy increases. The difference between excitation and ejection is one of degree. By judiciously choosing the amplitude of the supplementary rf voltage and the time it is applied, the ion's kinetic energy can be

increased without supplying sufficient energy for ejection because kinetic energy is also lost from collisions with the He bath gas. A typical excitation voltage would be a few hundred millivolts (peak-to-peak) and 10–40 ms long.

Unfortunately, determining the frequency needed for resonance excitation is not always straightforward. The equation for determining the secular frequency of an ion (Eq. 6.8) is not exact. The resonant frequency is affected by higher order fields and can change as the ions gain kinetic energy [23]. The number of trapped ions also affects the secular frequencies of those ions [24]. The sum of the charge within the trapping volume or the “space charge” of the ions acts as a dc potential. The effective a_z value no longer equals zero, and the secular frequency shifts (Eq. 6.9). To address this problem, most commercial quadrupole ion traps are designed to control the number of ions trapped. Alternatively, the rf drive amplitude can be scanned over a narrow range.

Once the appropriate frequency is determined, the ion’s kinetic energy can be increased. However, one must be cautious of the competition between ejection and excitation [22]. If too much energy is supplied, a significant fraction of the parent ion population can be lost through ejection. The MS/MS efficiency is then decreased. This not only hampers the identification of the product ions but also limits subsequent stages of MS/MS due to an insufficient number of ions.

To favor excitation over ejection, the parent ion should be trapped as strongly as possible. The ions can be thought of as being held in an energy well, called the *pseudopotential well*. The “depth” of this well is the maximum kinetic energy the ion can have and still remain trapped. The deeper the well, the more kinetic energy the ion can gain before ejection. Ions in deeper wells can undergo more energetic collisions as well as a greater number of collisions. This allows the ion to gain enough internal energy to dissociate before it is ejected. The depth of the well, D , is related to the q_z parameter and can be approximated by the Dehmelt pseudopotential well model for q_z values less than 0.4 (Eq. 6.11).

$$D = \frac{q_z^2 m r_0^2 \Omega^2}{32e} \quad (6.11)$$

This equation shows that ions trapped with higher q_z values reside in a deeper well and can be excited to higher kinetic energies without being ejected. By increasing the rf drive amplitude, an ion’s q_z value can be increased (Eq. 6.3). However, at higher q_z values, the q_z values of lower mass-to-charge ions may exceed 0.908 becoming unstable, so the smaller mass-to-charge product ions are not trapped if they are formed. To balance between the well depth and trapping of the product ions, a q_z of 0.2–0.4 for the parent ion is generally chosen. As a result, product ions less than about one-third the mass-to-charge of the parent ion are generally not observed in CID experiments performed on trapping mass analyzers.

A variant of resonance excitation that allows lower mass product ions to be trapped is called *HASTE* (*high amplitude short time excitation*) [25] or *PQD* (*pulsed q dissociation*) [26]. The implementation of these techniques involves using a much larger amplitude resonant supplementary rf voltage (>1 V), but for a very short time (1–2 ms). The parent ion q_z value during the resonance excitation is similar or greater than that used in conventional resonance excitation, but as soon as the excitation step is complete, the rf drive amplitude is decreased to put the parent ion q_z at around 0.1, thus allowing the slow-forming low mass product ions to be trapped.

Another variation of typical resonance excitation CID involves the use of heavy gases (Ar, Xe, Kr) as the collision gas. Heavy gases offer the benefit of higher energy deposition per collision. As discussed earlier, the maximum amount of laboratory frame kinetic energy (E_{lab}) that can be converted to internal energy through a collision, E_{com} , is given by Equation 6.5. For a parent ion with a given E_{lab} , a heavy gas provides a higher m_t and a greater percentage of energy deposition than helium. The addition of heavy gas molecules to the quadrupole ion trap allows higher energy deposition and a greater degree of dissociation via CID. Also, the frequency range for efficient excitation is wider with heavy gases, reducing the required precision of the secular frequency [27]. With heavy collision gases, q_z values as low as 0.05 can be used, allowing low mass-to-charge products to be detected [28]. However, the heavy gas also deteriorates the mass spectral resolution and sensitivity of the quadrupole ion trap because of the greater scattering on collision during ejection. Pulsing in a heavy gas for CID and allowing it to pump away before detection can alleviate these problems at the cost of increased time per scan (reduced duty cycle).

6.4.4.2.3 Nonresonance Excitation. Frequency shifts due to the number of trapped ions are part of the motivation for finding other means of dissociation, although resonance excitation CID is still by far the most common method for ion dissociation. Controlling the number of trapped ions may be problematic if the ion source output fluctuates significantly, as can be the case with matrix-assisted laser desorption ionization (MALDI). This maybe one reason why no commercial quadrupole ion traps currently offer MALDI as an ionization option. One nonresonance technique less affected by the number of ions is boundary-activated dissociation (BAD) [29–31]. In this technique, a dc voltage pulse is applied to the endcaps instead of an ac voltage, causing a change in the ions' a_z values. As the a_z value become large enough, the ion's a and q points approach the boundary of the stability diagram. The a_z value is chosen so that the ion is not ejected, but because it is near the boundary, its trajectory increases in amplitude. As the ions' motions become larger in amplitude, there is greater force acting on the ions, causing their kinetic energy to increase. Collisions with the bath gas molecules can then lead to dissociation just as with resonance excitation. Only the magnitude of the dc must be adjusted to optimize the dissociation. As in resonance excitation, the ion must reside in a sufficiently deep well that the large-amplitude oscillations do not result in ejection. So, again, the q_z values used are typically between 0.2 and 0.4, and low *mass-to-charge* product ions are not observed. However, the secular frequency is irrelevant in BAD, so no tuning is required and any fluctuations in frequencies are inconsequential. Therefore, BAD is particularly useful for MS/MS when the ion intensity fluctuates significantly from scan to scan, as is common with MALDI.

Another activation technique using CID also involves a nonresonant approach [32]. Nonresonance excitation is capable of increasing an ion's kinetic energy to 40 eV and can do so in only a few microseconds. Like HASTE, the ions remain trapped with a higher kinetic energy than that that can be achieved via resonance excitation. Thus, the internal energy deposition can also be greater. This nonresonance excitation involves applying a low frequency square wave (50–500 Hz) to the endcaps. Because this frequency is so low in comparison to the secular frequencies of the ions ($\sim 100,000$ Hz), this square wave can be more reasonably thought of as a series of dc pulses. The square wave causes the trapping field to change instantaneously. The ions will accelerate quickly to compensate for this change. While doing so, collisions with the bath

gas convert the kinetic energy to internal energy. This process is repeated as the square wave cycles. Nonresonance excitation does not result in ejection because the kinetic energy added to the ions does not continuously increase the ions' periodic, stable motions as each of the previously discussed methods do. Instead, the ions change from one periodic motion to another very quickly. The kinetic energy is gained as the ions adjust from one motion to the other, not as a periodic motion is increased in amplitude.

Nonresonance excitation differs from resonance excitation in that it is not selective for any one species of ions. All trapped ions will be excited simultaneously. This can be desirable or not, depending on the application. Also, because more energy can be deposited quickly by nonresonance techniques, higher energy dissociation channels can be accessed. Thus, nonresonance excitation can form a different group of product ions from those seen with resonance excitation.

6.4.4.2.4 Infrared Multiphoton Dissociation (IRMPD). Another method for inducing dissociation differs from all the previously discussed techniques, in that it does not make use of collisions. Instead of increasing the kinetic energy of ions and then using collisions to convert that to internal energy, IRMPD uses photons to deposit energy into the ions [33–38]. IR-wavelength photons are sent into the trapping volume where they can be absorbed by the trapped ions. An IR-transparent window in the vacuum housing allows access to the trap. A laser beam can then be directed into a hole drilled through the ring electrode.

IRMPD offers several advantages over other dissociation techniques. First, it is not a resonance technique, and any ion in the path of the laser will be excited. This can be used, for example, to dissociate multiple analytes simultaneously. The laser beam will also excite product ions. These ions will subsequently dissociate and can provide a richer product ion spectrum. However, the spectrum can also be more complicated and hide the genealogy of the ions. The order in which product ions are formed in this type of MS/MS is not as clear as in stepwise MSⁿ.

One very important advantage of IRMPD is that low q_z values can be used. All other excitation methods described rely on increasing the kinetic energy to increase the internal energy of ions. Therefore, the ions must be able to absorb that kinetic energy without being ejected, which requires higher q_z values. IRMPD has no such restrictions, and low mass-to-charge product ions are easily trapped and observed. However, a complication with IRMPD in the quadrupole ion trap is that collisions with the bath gas molecules between absorption events can remove energy from the ions. If collisions occur quickly enough compared to the rate of photon absorption, the ions will not reach the critical energy for dissociation. All the early IRMPD experiments either reduced the bath gas pressure, which compromises sensitivity and resolution, or had a supplemental activation method concurrent with the IRMPD. However, recently, it has been demonstrated that appropriate focusing of the laser beam allows IRMPD to be performed at normal operating conditions without any supplemental ion activation [38].

6.4.4.3 Gas-Phase Reactions. Another type of MS/MS reaction for which trapping instruments are particularly useful involves gas-phase ion–molecule and ion–ion reactions. The quadrupole ion trap is particularly versatile for these types of analyses due to the combination of controlled timing of the reactions, mass-selecting capabilities for both reactants and products, and MSⁿ for both the formation of the reactants [39] and examination of the products [40]. Often, these reactions are between the trapped

ion and a volatile neutral introduced into the vacuum chamber. However, reactions between two ions have also been reported [41–45]. Ion traps allow the reaction to be controlled by holding the reactants together for a variable time before ejection and detection. Therefore, studying the kinetics is straightforward, and the extent of the reaction can be well controlled.

6.4.4.3.1 Ion–Molecule Reactions. Ion–molecule reactions in which a chemical reaction occurs rather than just the transfer of energy (CID) can be useful as a means of interrogating ions in a quadrupole ion trap. Reactions that are specific for a certain functional group or structure can be used to provide information about an analyte [46]. While ion–molecule reactions can be performed in a triple quadrupole instrument [47,48], the ability to easily control and vary the reaction time in a quadrupole ion trap allows additional information, such as reaction rate constants, to be determined.

Two simple ion–molecule reactions are hydrogen/deuterium (H/D) exchange and proton transfer. The gas-phase H/D exchange can be used to probe structures of ions, in particular, larger ions that may have multiple conformations [49]. H/D exchange is also useful in distinguishing functional groups [50]. While H/D exchange just comprises a change in mass of the analyte ion, proton transfer reactions involve transferring charge from the analyte ion to the neutral molecule. For singly charged ions, this is not a useful reaction, as a loss of charge results in the formation of a neutral that cannot be detected by the mass analyzer, but for multiply charged ions, deprotonation can be a very useful reaction [39]. [51]. There are thermodynamic limits to proton transfer ion–molecule reactions, which lead to the development of ion–ion reactions.

6.4.4.3.2 Ion–Ion Reactions. Reactions between two oppositely charged species can also be used. A quadrupole ion trap is particularly useful for these types of reactions, as both positively and negatively charged ions can be trapped simultaneously under normal operating conditions. Ion–ion reactions provide greater control in that ions are injected as needed, while neutral reactant molecules are usually present at a constant pressure. As with the ion–molecule reactions, deprotonation can also be achieved via ion–ion reactions. Ion–ion reactions have an advantage over deprotonating ion–molecule reactions, in that anions are much stronger bases than neutral compounds. Anions can completely deprotonate multiply charged proteins and peptides in ion–ion reactions, whereas neutral compounds used in ion–molecule reactions typically cannot. Ion–ion deprotonation reactions have been useful to determine charge states and to declutter charge-state convoluted spectra. By decreasing the charge on trapped ions, the mass-to-charge values shift. Where multiple charge states of proteins overlap, shifting to a lower charge state for each protein can separate the overlapping signals. Ion–ion deprotonation of multiply charged proteins can be done using fluorocarbon anions [43].

The ion–ion proton transfer reactions lead to the development of ion–ion electron transfer reaction [41,52,53]. Electron transfer dissociation (ETD) is becoming an increasingly popular method for the analysis of peptides and proteins. ETD is closely related to electron capture dissociation (ECD) [54] where a multiply protonated ion captures an electron to reduce the charge state by one. This reaction is exothermic and thus, after capture (or transfer) of the electron to the ion, dissociation can occur. When an electron is added to a multiply protonated ion, the resulting ion is a radical. The dissociation chemistry of radical ions is significantly different than that of even electron ions and thus ETD is often complementary to CID. A particular advantage

of ETD is that the dissociation chemistry has the ability to retain and identify sites of posttranslational modification [55–57]. In CID, functional groups added as posttranslational modifications to peptides and proteins are often facilely lost as neutral fragments and limited sequence information is obtained. With ETD, the radical dissociation chemistry is such that the posttranslational modification remains attached to the peptide/protein and typical sequence ions are produced. This allows identification of the site of posttranslational modification.

6.5 CONCLUDING REMARKS

The triple quadrupole mass spectrometer and the quadrupole ion trap mass spectrometer are probably the two most widely used instruments for MS/MS. Both are quite mature technologies, having been in existence for over 25 years. There are still improvements being made, but, in general, they are incremental and often more related to peripheral aspects of the mass spectrometry experiment rather than the actual mass analysis step.

The triple quadrupole instruments excel in the area of quantification and targeted compound analysis. The single reaction monitoring and MRM modes of operation of triple quadrupoles provide high duty cycles, maximizing sampling efficiency. Also, the quadrupole ion traps have some limitations in the area of quantification because of ion statistics. A limited number of ions can be trapped before space charge effects impact performance of ion traps. Development of 2D ion traps and newer 3D ion traps taking advantage of higher order fields have increased the ion capacity, but the triple quadrupole is still usually the instrument of choice for quantification.

The quadrupole ion traps excel in compound identification using MS/MS. The high sensitivity of ion traps combined with the fast scan rates and very high MS/MS efficiency make the ion trap uniquely compatible with chromatography/MS/MS experiments. quadrupole ion traps are also very powerful research instruments due to the variety and ease of implementation of ion activation methods.

Of course, triple quadrupoles and quadrupole ion traps do have limitations. In particular, neither the mass resolution nor mass accuracy is particularly high. While both these performance metrics have been improved over the years, in the near future, at least, quadrupole-based instruments will not compete in terms of resolution or mass accuracy with time-of-flight (TOF), ion cyclotron resonance (often referred to as *FTMS*), or orbitraps. However, one can get the best of all performance metrics by using hybrid instruments, that is, quadrupole-based analyzers combined with these other high performance analyzers. The hybrid QTOF instrument is similar in popularity to triple quadrupoles and quadrupole ion traps and the technology has been around about as long [58]. Quadrupoles interfaced with *FTMS* was also first reported over 25 years ago [59]. More recently, quadrupole ion traps combined with TOF [60,61], *FTMS* [62], and orbitraps [63] have become commercially available. Another hybrid instrument of importance is the hybrid triple quadrupole 2D ion trap. This is a multipurpose instrument that provides performance capabilities of a stand-alone triple quadrupole mass spectrometer along novel modes of operation that take advantage of a 2D ion trap [64]. As the instruments become more sophisticated and data systems more powerful, if cost is not a concern, only imagination will limit the possibilities. However, because of their MS/MS capabilities, it is likely that quadrupole-based analyzers will almost always have a role in mass spectrometry instrumentation.

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7 Hybrid Mass Analyzers in Drug Metabolism Applications

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7.1 Summary	1
7.2 Introduction	2
7.3 Drug metabolism studies in support of drug discovery and development	2
7.4 Mass analyzers in support of drug metabolism studies	5
References	29

7.1 SUMMARY

In the complex and expensive drug discovery and development process, characterizing metabolism and pharmacokinetic (PK) properties of new molecular entities (NMEs) is important to develop efficacious, safe, and rewarding drugs for patients. Among the analytical techniques used in the biopharmaceutical industry, assays based on liquid chromatography mass spectrometry (LC-MS) are highly sought out by the drug metabolism and pharmacokinetic (DMPK) scientists due to their sensitivity, selectivity, specificity, and speed. Several mass analyzer types have been used in DMPK studies, including quadrupole mass filters (QMFs), linear and three-dimensional ion traps (ITs), time-of-flights (TOFs), and Fourier transform mass spectrometers (FTMSs). Some of the desired qualities of the individual mass analyzer types and ion manipulation techniques are combined into one instrument to configure hybrid mass spectrometers. Such hybrid mass spectrometers become even more selective and specific when either a TOF- or a FTMS-based high resolution mass spectrometer (HRMS) is combined with either ITs or QMFs. HRMS-based hybrid mass analyzers, including Q-TOFs, linear ion trap-orbitrap (LIT-OT), and QMF-OT, have begun to become frontline mass spectrometers across the biopharmaceutical industry, especially in the DMPK laboratories. The focus of this chapter is on strategies based on hybrid mass spectrometers and procedures available for detection and characterization of xenobiotics at various stages of drug discovery and development.

7.2 INTRODUCTION

The study of metabolism is an integral part of drug discovery and development for understanding absorption, distribution, metabolism, excretion, and toxicological (ADMET) properties of an NME. Identification and characterization of structurally diverse metabolites or drug-derived materials can be challenging because they are usually present in low concentrations (nM to μ M) within complex *in vivo* or *in vitro* matrices that consist of lipids, proteins, or other endogenous materials. In recent years, liquid chromatography coupled with mass spectrometry (LC-MS) [1–4] has emerged as an important analytical tool for the separation, detection, characterization, and quantification of drug-derived materials from drug metabolism studies [5].

7.3 DRUG METABOLISM STUDIES IN SUPPORT OF DRUG DISCOVERY AND DEVELOPMENT

7.3.1 Discovery Stage Studies

The study of drug metabolism is a very important part of drug discovery, which helps in the optimization of metabolism properties of NMEs, in parallel with the optimization of their potency and efficacy. One of the earliest high throughput assays in the discovery stage determines the metabolic stability of NMEs, using human and/or animal liver microsomes or hepatocytes. Metabolic stability of an NME can be measured by means of quantitation assays that monitor the rate of disappearance of the NME (over a window of time at fixed time points) by utilizing a mass spectrometer [2,3,6]. These types of assays are often referred to as *high throughput-ADME (HT-ADME) screening assays* [7]. Higher throughput, robust, cost-effective screening assays have been developed in the recent years to cater to the increasing amount of NMEs in early discovery [6,8–13]. These assays use rapid, generic, fast gradient LC-MS/MS methods for the analysis of samples of CYP inhibition, Caco-2, permeability, efflux transport evaluation, protein binding, hepatocyte clearance, and isozyme profiling [14,15].

Although identification of metabolites may not be required for all compounds in early discovery, it may be useful to utilize highly sensitive mass spectrometers [LIT, QIT (quadrupole ion trap), TOF, Q-TOF, LTQ-Orbitrap (LTQ-OT)] that can be operated in full scan mode, data-dependent MS/MS mode, or targeted MS/MS mode with great selectivity and speed, which are very essential features to calculate the rate of disappearance of an NME and possibly metabolite formation. Since biotransformation of drugs usually leads to a change in molecular weight of the NME, metabolites formed can be determined by shifts in mass-to-charge (m/z) ratios as shown in Table 7.1.

Following HT-ADME screening assays, NMEs selected for further developments are filtered using *in vivo* PK screening assays. In order to accelerate the PK screening processes, automated sample preparation methods [solid phase extraction (SPE) and liquid–liquid extraction (LLE)], cassette dosing [16,17], cassette analysis [18–21], N-in-one dosing [22], and cassette-accelerated rapid rat screening (CARRS) [23] have been explored. Possibilities for obtaining metabolite data within PK screening assays have also been explored [19,20,24].

TABLE 7.1 Common Biotransformation Reactions and Associated Changes in Exact Mass and Elemental Composition

Biotransformation Reactions	Exact Mass Change (Da)	Elemental Composition Change
Hydroxylation/N-/S-oxidation/epoxidation	+15.9949	+O
Demethylation	−14.0157	−CH ₂
Deethylation	−28.0312	−C ₂ H ₄
Oxidative defluorination	−1.9957	−F + OH
Oxidative dechlorination	−17.9662	−Cl + OH
Dehydration	−18.0106	−H ₂ O
Dehydrogenation	−2.0157	−H ₂
Decarboxylation	−43.9898	−CO ₂
Ketone formation	+13.9792	−H ₂ + O
Methyl to carboxylic acid	+29.9741	−H ₂ + O ₂
Glucuronide conjugation	+176.0321	+C ₆ H ₈ O ₆
Sulfate conjugation	+79.9568	+SO ₃
Glycine conjugation	+57.0215	+C ₂ H ₃ NO
Cysteine conjugation	+119.0041	+C ₃ H ₅ NO ₂ S
N-Acetylcysteine conjugation	+161.0147	+C ₅ H ₇ NO ₃ S
Glutathione conjugation	+305.0682	+C ₁₀ H ₁₅ N ₃ O ₆ S

In late discovery, *in vitro* incubations in microsomes and hepatocytes are carried out for a cross-species comparison using both rodents and nonrodent species for full characterization of metabolites and determination of short- and long-term toxicological evaluation. It is advantageous to have HRMS-based instruments such as the LTQ-OT or Q-TOF or fast-scanning instruments such as the QTRAP to obtain both quantitative and qualitative data from metabolite profiling experiments [25–28]. An integrated streamlined process (METPRO) has been developed in our laboratories, which allows the identification of metabolites and the determination of kinetic parameters as well as profiles from a single analytical platform [29,30]. An alternative approach is to use the radiolabeled form of the NME (³H or ¹⁴C) [31,32] to conduct *in vitro* or *in vivo* experiments. Resulting samples are analyzed in parallel, using a split flow method, by a radioactivity detector (RAD) (online or off-line) and a mass spectrometer. This simultaneous detection of all drug-derived materials by an LC-MS and a RAD provides confirmation of the molecular weight and structural information of all radioactive peaks in a sample. Concurrently, the RAD provides quantitative estimates of all drug-derived materials in the absence of reference standards for quantitation using LC-MS.

Preclinical screening for reactive metabolites is an important tool to predict potential toxicological liability of an NME before progression into development. Characterization of glutathione conjugates and acyl glucuronides in discovery assists in early avoidance of structural liabilities that may lead to adverse effects. Experimental and mass spectrometric methods used in such reactive metabolite screen are the subject of recent publications [33–38] and are discussed in the chapter titled **Matrix-Assisted Laser Desorption/Ionization**. Another aspect of studying metabolites is to carry out reaction phenotyping studies [39] and to identify the enzymes responsible for the metabolism of an NME [40,41]. In later stages of discovery, the radiolabeled form of

the NME is used to carry out bile-duct-cannulated (BDC) studies in rodents or other toxicological species. These studies provide a semiquantitative estimation of the NME absorbed and metabolites formed under *in vivo* conditions, including information about conjugated and reactive metabolites [42].

In recent years, advances in *in silico* modeling have made it possible to screen compounds for undesirable ADMET properties, preferably before synthesis, which saves time and is also more economical for drug discovery. *In silico* methods such as Admet Predictor, Pre-ADME, ChemSilico, KnowItAll, and Pharma Algorithms help in predicting properties such as permeability, elimination, half-life, plasma protein binding, and oral bioavailability [43–46]. However, these techniques are fairly new and require extensive development and validation investments before they can be used as a predictive tool for optimization in drug discovery and drug design. The *in silico* models for metabolite predictions can be classified as being local or global. The global methods are empirically based expert systems that follow expert rules for predictions and include META, MetabolExpert, TIMES, MetaDrug, KnowItAll, and Meteor [47–49]. Local methods such as Compact and Camitro are based on the quantitative structure–metabolism relationships (QSMRs) and are derived from physiochemical, structural, or quantum mechanical properties [50,51].

7.3.2 Development-Stage Studies

In a developmental setting, drug metabolism studies are carried out with greater rigor, and cross-species comparison of *in vitro* (liver microsomes, S9 fraction, hepatocytes, and/or tissue slices) metabolites, nonclinical ADME studies, and human ADME studies are performed using the radiolabeled (^{14}C or ^3H) form of the NME [31]. Unlike the discovery stage studies, the *in vitro* and nonclinical ADME studies are carried out at more pharmacologically relevant doses. Among the nonclinical ADME studies, mass balance studies provide information about elimination routes, biotransformation pathways, and possibly gender-related differences in metabolism. Traditionally, mass balance studies are conducted around the same time as first-in-human (FIH) studies, but the timing of these studies can be delayed until after the proof-of-concept or clinical phase II studies.

In order to circumvent the late-stage attrition of NMEs, due to lack of efficacy or safety of the drug molecule, microdosing (1/100 of the pharmacologically effective dose) of radioactive drugs in clinical testing is supported by the US FDA or the EMEA to precede phase I studies and are called *phase 0* or *Exploratory IND* studies. Highly sensitive instruments such as accelerator mass spectrometry (AMS) and positron emission tomography (PET) are useful for the analysis of PK samples from microdosing studies. Even though the AMS technique can quantify ^{14}C -labeled compounds with attomole (10^{-18}M) sensitivity, additional chromatographic fractionation and separation needs to be carried out for the metabolites and NMEs before analysis is carried out by AMS. To overcome this limitation, the fast-scanning QTRAP 5500 hybrid mass spectrometer has been utilized to analyze samples from microdosing studies [52–54]. The combination of SRM-IDA (selected reaction monitoring–information-dependent acquisition) and list of possible metabolites were used to quantify the NME and to identify potential metabolites [55].

The efficacious human dose is predicted by scaling factors (e.g. allometric scaling) using PK and pharmacodynamic (PD) data derived from *in vitro* and *in vivo* studies

[56,57] for IND-enabling toxicological studies and FIH studies. Among the FIH studies single ascending dose (SAD) studies are carried out with small groups of patients to monitor for adverse side effects. Dose levels are escalated based on the early SAD studies to reach maximum tolerated dose (MTD) [58]. The next step is to carry out multiple ascending dose (MAD) studies to better understand the PK and PD of the NME. In addition to PK parameters and parent exposure, regulatory authorities (FDA/EMA) recommend determining circulating metabolites in humans and exposure to the same metabolites in preclinical species undergoing toxicological evaluations [59,60]. However, differences in LC-MS responses for a structurally related NME and its metabolites limit direct quantitation of metabolites during metabolite detection and characterization experiments. Ideally, the synthesis of an authentic standard is required for quantitative estimation of a metabolite by LC-MS. As cost- and resource-effective alternatives, several groups have evaluated techniques based on UV [61,62], collision-activated dissociation (CAD), LC-captive spray ionization (CSI) [60,63], NMR [64], radioactivity [65,66], and nano/micro-ESI [67–70] to analyze samples from SAD and MAD studies. These alternative techniques have been demonstrated to provide semiquantitative estimates of the metabolites in the absence of reference standards. A human ADME study is carried out before phase III to determine the total disposition of the NME [71,72], including mass balance, routes of metabolite/NME elimination, and biotransformation pathways. Metabolite identification and profiling from the ^{14}C -RAD profiles also help in correlating the results obtained from the SAD/MAD studies and nonclinical species undergoing toxicological studies [72].

7.4 MASS ANALYZERS IN SUPPORT OF DRUG METABOLISM STUDIES

Some of the common goals of drug discovery and development metabolism studies include detection, characterization, and quantitation of drug-derived materials in biological samples. The five commonly used mass analyzers at various stages of drug discovery and development can be classified into two categories: beam-type analyzers (TOF, magnetic sector, and QMF) and ion trapping analyzers (quadrupole and ion cyclotron resonance) [73]. Each of these analyzers has distinct capabilities of mass resolving power, mass accuracy, mass range, linear dynamic range, speed, and ionization compatibility [5]. The need to develop more efficient and high performance mass spectrometers has led to the advent of hybrid mass spectrometers, which coalesce the potential (or qualities) of multiple analyzers and ion manipulation devices into one instrument.

An important aspect of drug metabolism studies is the correct structural elucidation of metabolites, which can be achieved by utilizing tandem mass spectrometry (MS/MS). Hybrid instruments can also be classified as tandem-in-space instruments, in which both mass analyzers are physically separated, and tandem-in-time instruments, in which ion trapping, manipulations, and detection are achieved in sequentially timed events. Tandem-in-space instruments include sector instruments in forward or reverse geometry, Q-TOF, and Qq-LIT (QTRAP); tandem-in-time instruments include Fourier transform ion cyclotron resonance (FTICR) and ITs. The newest generation of hybrid mass spectrometers, for example, LIT-FTICR, LIT-OT, and QMF-OT combine aspects of tandem-in-space and tandem-in-time MS/MS experiments.

7.4.1 Nonhybrid Mass Analyzers

7.4.1.1 Quadrupole Mass Filter (QMF). The QMF or analyzer was developed in parallel with the QIT. A quadrupole mass analyzer consists of four parallel rods that have fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focused on and passed along the middle of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular m/z will have a stable trajectory and thus pass through to the detector. The RF and DC potentials are varied to allow ions of different m/z to pass through to the detector and thus build up a mass spectrum. Stand-alone single quadrupole mass spectrometers are often not used in drug metabolism studies because these types of mass analyzers lack the ability to perform MS/MS (MS^n) experiments to provide structural information about drug metabolites. On the other hand, a triple quadrupole mass analyzer, constructed using two QMFs and a multipole collision cell, can be utilized for acquiring full scan as well as MS/MS spectra. Because triple quadrupoles are tandem-in-space mass spectrometers, they are not ideally suited for performing sequential MS^n experiments. As shown in Table 7.2, SRM, product ion scans, constant neutral loss scans (CNLSs), and precursor ion scans (PISs) allow triple quadrupole mass spectrometers to be ideally suited for the quantification of parent NMEs (PK) as well as for identification of unknown metabolites.

Triple quadrupole mass spectrometers are the choice of instruments for majority of the preclinical and clinical PK studies, as well as metabolic stability studies, due to their high sensitivity and specificity for quantitation in the SRM mode. The mass range of the instrument is from 30 to 1800/2000 Da, and fragmentation patterns using collision-induced dissociation (CID) results in direct bond cleavage and formation of metastable ions, resulting in rich spectra. Triple quadrupole mass spectrometers are the choice of instruments for metabolic stability studies due to their high sensitivity and specificity for quantitation experiments using SRM mode [74–77]. Despite the high sensitivity in the SRM mode, triple quadrupoles are not ideally suited for detection and identification of metabolites due to low resolution, poor duty cycle, and low full scan sensitivity. If an NME series or an NME exhibits undesired PK properties in triple-quadrupole-based metabolic stability screening assays, metabolite profiling studies are initiated as a second-tier assay using samples obtained from higher drug concentrations. Incubations with higher NME concentrations are required to detect molecular ions of metabolites using low sensitivity Q1 or Q3 scan (full scan) of triple quadrupole mass spectrometers. During the early 2000, ThermoFisher redesigned the TSQ700 and TSQ7000 series triple quadrupole mass spectrometers to keep the RF as high as possible at the maximum RF voltage to achieve higher mass resolution. Although the new TSQ Quantum series triple quadrupole mass spectrometers provided a mass resolution >5000 , the higher resolution mode of operation still required slower scan speeds and scarification of sensitivity [78]. To improve sensitivity by transmitting higher number of ions from an ion source into the Q1 region and to provide some options to scan faster, the electrodynamic ion funnel technology, introduced by Smith and coworkers [79,80], has been incorporated with triple quadrupole mass spectrometers. However, the inability to perform MS^n experiments and to provide HRMS with liquid chromatographically suitable scan speeds limits the utility of triple quadrupole mass spectrometers in the mainstream metabolite identification laboratories.

TABLE 7.2 Summary of Triple Quadrupole MS/MS Scanning Modes for LOR (m/z 383.2) and its Major Fragment Ion of m/z 259.1

Scan Type	MS1 (Q1) Function	Collision Cell (Q2) Function	MS2 (Q3) Function	Purpose
Product ion	Parent or precursor isolation (e.g., for LOR, m/z 382.7–383.7) FIXED	Fragmentation of a narrow selected range of ions (e.g., for LOR, m/z 382.7–383.7)	Separation of fragments (e.g., for LOR, m/z 50–393) SCAN	Structural elucidation
Precursor ion	Passes a wide window of ions (e.g., 280–900 Da for LOR) SCAN	Fragments a wide window of ions (e.g., for LOR, 280–900 Da)	Set for a particular fragment (m/z 275 generated by oxidation of the m/z 259 fragment ion of LOR) FIXED	Structurally related unknowns/metabolites from complex biological matrix
Neutral loss	Passes a wide window of ions (e.g., 250–950 Da for LOR) SCAN	Fragments a wide window of ions (e.g., for LOR, 250–950 Da)	Separates a wide window of ions (Q1 and Q3 are offset by the neutral; 176 for all glucuronide metabolites of LOR or its metabolites) OFFSET SCAN	Structurally related unknowns/metabolites from complex biological matrix
SRM/MRM	Parent or precursor isolation (e.g., for DL, m/z 310.6–311.6) FIXED	Fragmentation of a narrow selected range of ions (e.g., for DL, m/z 310.6–311.6)	Set for a particular fragment (e.g., m/z 258.6–259.6 for DL) FIXED	Quantification

Abbreviations: LOR, loratadine; DL, desloratadine; MRM, multiple reaction monitoring.

7.4.1.2 QIT (3D Trap). The QIT analyzer utilizes a cylindrical ring and two endcap electrodes to make a 3D quadrupolar field capable of selectively trapping or ejecting ions. Although the fundamentals and concept of QIT were realized in the late 1950s by Wolfgang Paul and coworkers, difficulty in machining the hyperbolic electrodes and slower scanning mass-selective stability mode of operation limited the use of QIT to only physics research laboratories. In 1983, George Stafford and coworkers at the Finnigan Corp contributed to the advancement of QIT technology by incorporating (i) mass-selective instability mode of operation and (ii) bath or damping gas (i.e., helium) [81]. The new incorporations improved resolution, peak shape, trapping efficiency, and MS/MS time efficiency of QIT mass spectrometers. During the 1990s, because of the advances in machining technology combined with mass-selective instability mode of operation in the presence of damping gas and the ease of coupling to liquid chromatography, compatible ionization sources allowed QIT technology to develop into an analytical tool of choice for structural elucidation. A decade ago, drug discovery stage systematic approach to metabolite characterization and identification involved triple quadrupole and QIT mass spectrometers as frontline instruments [2]. Some of the advantages of using QIT for full scan MS and MS/MS experiments are listed in Table 7.3. A similar approach involving triple quadrupole and QIT mass spectrometers was also used in development-stage metabolite identification studies in support regulatory filings of several drugs, including desloratadine [82], lonafarnib [83],

TABLE 7.3 Comparison of Scan Modes and Associated Sensitivity When Using a Quadrupole Ion Trap and Quadrupole Mass Filter Mass Analyzers

Scan Type	QIT	QMF	QIT vs QMF
Full scan (Q1 or Q3, or 100–1100 Da) Total scan time = 1 s	A group of ions (m/z 100–1100) will be trapped for 750 ms and then emptied within next 250 ms. Trap will repeat the same process 20 times over a 20-s wide peak.	In any given time, ions of one m/z are present and allowed to pass through a QMF to the detector and all other ions are rejected. So, each m/z will be allowed to pass for 1 ms.	750 ms/1 ms = 750 QIT is 750 times more sensitive
Full scan product ion (MS/MS, 100–500) Total scan time = 1 s	Same as above, a group of ions are trapped for 750 ms; isolated, fragmented, and detected within a second. So, each m/z is collected for 750 ms.	Each m/z is sequentially scanned to the detector for 2.5 ms (1 s/400 Da = 2.5 ms/Da)	750 ms/2.5 ms = 300 QIT is 300 times more sensitive
SRM	Ions will be collected for 750 ms and then isolated and fragmented. Duty cycle is <75%.	Q1 and Q3 are set to pass a single m/z . Duty cycle is 100% because no scanning is required (10–250 ms per SRM).	A QMF provides an order of magnitude better LOQ for quantification experiments than a trap

Abbreviation: LOQ, limit of quantification.

saxagliptin [84,85], sitagliptin [86,87], prasugrel [88], clenbuterol [89], and dasatinib [90]. Although QITs are ideally suited for qualitative analysis, a number of groups have demonstrated the utility of QIT for simultaneous qualitative and quantitative analyses of metabolites and drug-related species [91]. To provide additional options for simultaneous qualitative and quantitative analyses, Bruker has introduced an ultrasensitive IT (amaZon™) with dual ion funnel technology, which has a scan speed of 52,000 Da/s at better than a unit resolution, for fast UPLC applications and maximum duty cycle in data-dependent MS/MS and MSⁿ. It can achieve mass resolving power up to 20,000 in full scan mode across the 50–3000 *m/z* range. Zero Delay Alternating™ acquisition allows high speed ion polarity switching without compromising sensitivity and speed. Up to 20 Hz MS data acquisition in both polarities is ideal in combination with fast ultrahigh pressure liquid chromatography (UHPLC) separations. It is also supported by spectral MSⁿ libraries for MS/MS-based multicomponent screening. Over the 10-year span from 1995 to 2005, QIT mass spectrometers were preferred for metabolite profiling workflow due to their excellent sensitivity, high speed (duty cycle), compact footprint, ease of use, ease of coupling to HPLC, reasonable cost, and ability to carry out MSⁿ experiments [2,92]. Poor trapping efficiencies, limited dynamic range, and space-charge effects resulting from small trap volume limited the sustainability of QIT as a frontline instrument for metabolite identification studies. Additionally, low mass cutoff or the 1/3 rule for MS/MS as compared to triple quadrupole often limits the utility of QIT for unknown structural elucidation. However, if sufficient ion signal from a sample is available, one has the option to circumvent the low mass cutoff by performing MS3 or MS4 experiments for gathering information about low mass fragment ions.

7.4.1.3 LIT (2D Trap). LIT, which is also referred to as *2D trap*, was introduced in 2002 [93] as a stand-alone mass spectrometer, known as *LTQ*, or as a part of triple quadrupole (QTRAP) [94] and in 2005 as a part LTQ-OT or LTQ-FTICR [95]. This section mainly focuses on the stand-alone LIT, and the hybrid analyzers are discussed in later sections. LIT is a variation on the transmission quadrupole mass analyzer and is constructed such that the ions can be either analyzed immediately in full scan mode or used for trapping to carry out MS/MS experiments. This instrument has significant improvements with respect to trapping efficiency, ion capacity, scan speed, and sensitivity for MS/MS compared to traditional 3D ITs. Table 7.4 compares some of the DMPK analytical performance characteristics of a QIT and LIT with those from a triple quadrupole mass spectrometer [96]. Since 2002, several LIT models,

TABLE 7.4 Comparison of Performance characteristics of QIT, LIT, and Triple Quadrupole Mass Spectrometers

Mass Spectrometer	Scan Time	Points/Peak	RSD (%)	LOD (pg/μL)	LOQ (pg/μL)
LCQ Deca XP Max (QIT)	MS: 575 ms MS ⁿ : 1000 ms	3–5	10	1.0	2.5
LTQ (LIT)	MS: 80 ms MS ⁿ : 150 ms	15–18	<2	<1.0	<1.0
TSQ Quantum Ultra (QMF)	SRM: 35 ms	>50	3	1.0	2.5

Abbreviations: LOD, limit of detection; RSD, relative standard deviation.

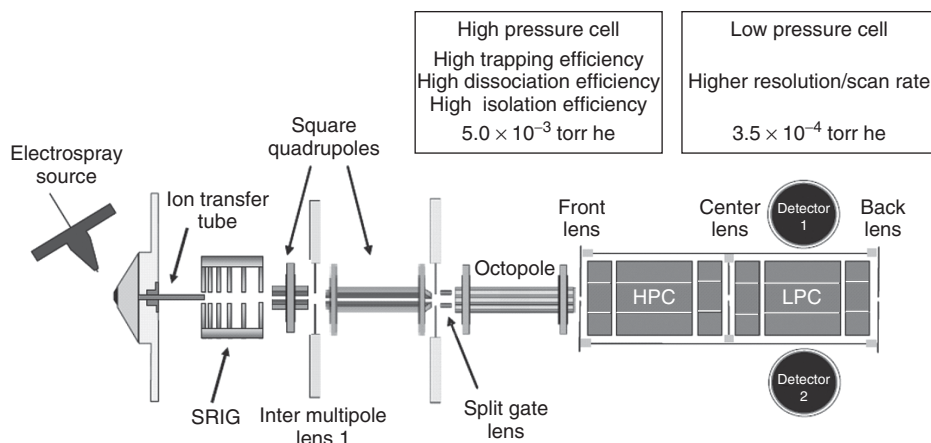


Figure 7.1 Schematic showing the ThermoFisher dual-cell linear ion trap (LTQ-Velos) mass spectrometer and the optics used for transferring ions from the ESI source to the mass analyzer.

each with improved performance characteristics, have been introduced including LTQ classic, LTQ-XL, and LTQ-Velos. Overall, separating the LTQ cell into high pressure and low pressure regions for ion accumulation/manipulation and detection, respectively, allows LTQ-Velos to gain 2–5X in sensitivity and scan speed over LTQ-XL (Fig. 7.1). Improved scan speed (10 Hz) allows the LTQ-Velos to accommodate analytes separated using UHPLC as well as on-the-fly positive/negative switching for detecting phase I and phase II metabolites by either polarity mode. One of the limitations of LIT for structural elucidation is MS/MS spectra with limited fragmentation information and low mass cutoff. Comparison of imatinib MS/MS spectra obtained using triple quadrupole and LIT mass spectrometers is shown in Fig. 7.2 [97]. While the MS/MS spectrum of imatinib (m/z 494) from a triple quadrupole is information rich, that from LIT provides only a single major fragment (m/z 394). Subsequent MS/MS of m/z 394 fragment provides additional fragmentation information. The fast-scanning capabilities of LIT allows to circumvent the low mass cutoff limitation by performing a modified form of CID involving pulsing of the q -values (PQD). Under PQD conditions, resonant activation of a precursor ion is achieved (100 μ s vs 1–2 ms) with high RF amplitude. This, in turn, allows the RF amplitude to be lowered very quickly to values which allow trapping of the low mass fragment ions [98]. Overall, PQD is not as sensitive as CID but provides the option to obtain low mass fragmentation information [99,100]. Another advantage of LIT stems from its fast-scanning capabilities, which is the ability to collect simultaneous qualitative and quantitative information of metabolites and drug-related species [96].

7.4.1.4 TOF. TOF mass analyzers have ions gated from the source region by an electrical pulse, which are accelerated down a TOF tube and have the same energy, where the low mass ions travel at a higher velocity, reaching the detector quicker than the high mass ions. Owing to their narrow dynamic range, TOF instruments have limited application in quantitation experiments. TOFs have an unlimited mass range as the ions are not trapped (similar to the quadrupole, IT, or FTICR) nor are their flight paths curved (magnetic sectors), and their detection efficiencies induce practical limits of a

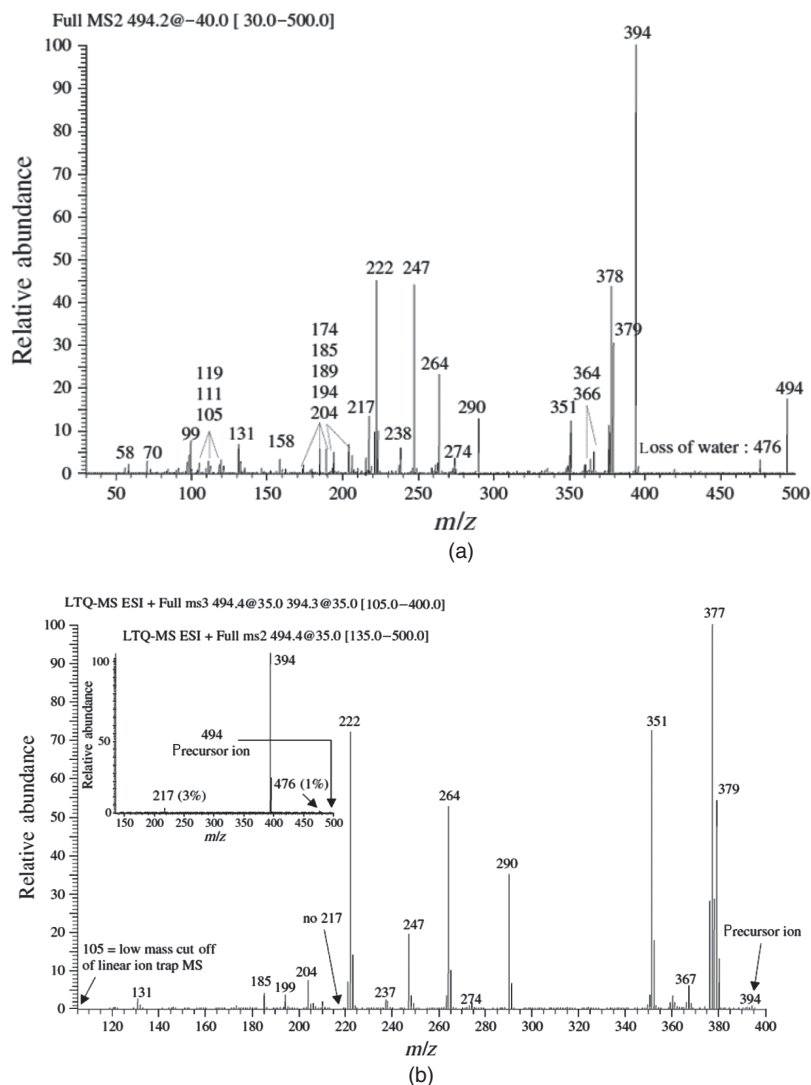


Figure 7.2 Comparison of imatinib MS/MS and MS³ information obtained using (a) triple quadrupole and (b) linear ion trap mass spectrometers. *Source:* With permission from Ref. 97.

few hundred kilodaltons. Several groups have evaluated stand-alone TOF analyzers for performing integrated qualitative and quantitative bioanalyses. One of the earlier evaluations using a micromass liquid chromatography–TOF (LCT) mass analyzer involved simultaneous quantification of drugs in rat plasma and confirmation of their metabolites based on accurate mass and elemental compositions [101]. Most recently, Nagele and Fandino [102,103] used LC-ESI-TOF with multiple column and fast liquid chromatography to study the metabolic stability of drugs and identify their metabolites (Fig. 7.3). Overall, stand-alone TOFs are seldom used in the mainstream metabolite identification

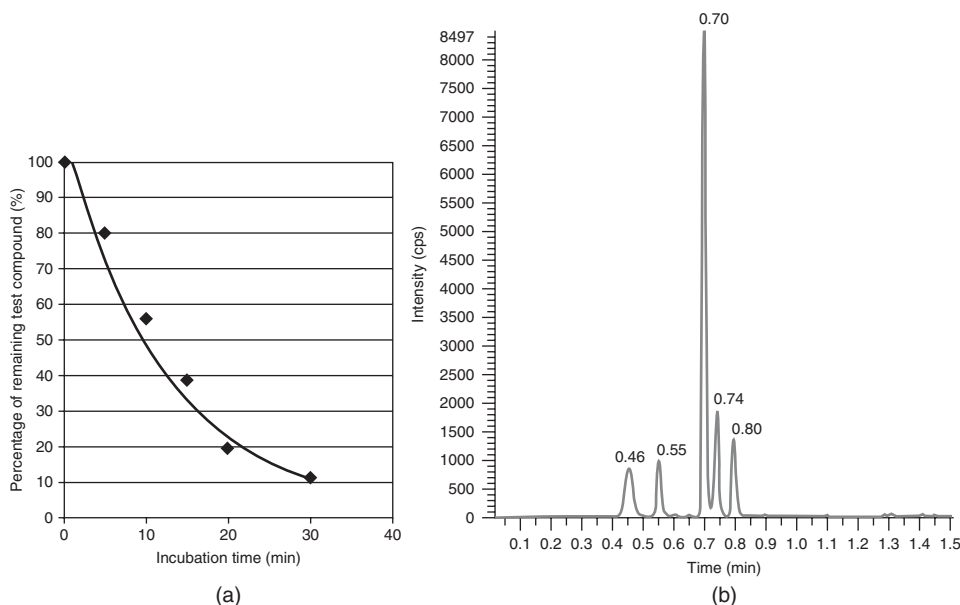


Figure 7.3 (a) Determination of metabolic stability of buspirone and (b) identification of five monooxy metabolites of buspirone by fast LC-TOF using an integrated qualitative/quantitative bioanalytical approach. After 20 min of incubation, about 80% of the buspirone was metabolized. (a) The metabolic stability plot was generated by plotting incubation time with respect to extracted ion chromatogram peak area for buspirone $[M + H]^+$ ($C_{21}H_{32}N_5O_2$) = m/z 386.2556. (b) Monooxy metabolites of buspirone $[M + H]^+$ ($C_{21}H_{32}N_5O_3$) = m/z 402.2505 were extracted from the full scan TOF-MS spectrum. Five monooxy buspirone metabolites were identified by accurate mass measurement and elemental formulae confirmation. *Source*: With permission from Ref. 102.

studies due to their inability to perform MS/MS experiments. However, TOF couples extremely well with pulsed ion sources such as matrix-assisted laser desorption ionization (MALDI), and its excellent characteristics of high mass resolution and duty cycle allows it to be coupled to another TOF, QMF, or ITs to generate tandem instruments, such as TOF-TOF, Q-TOF, or IT-TOF, respectively. Among these tandem mass spectrometers, TOF-TOF is ideally suited for MALDI imaging experiments and to study metabolites distributed across various tissue compartments [104–107]. Q-TOF and IT-TOF (see earlier discussion) mass spectrometers are predominantly used for metabolite identification studies or integrated qualitative and quantitative bioanalyses.

7.4.1.5 FTICR. The FTICR mass analyzer is capable of providing the highest resolving power among all available mass analyzers. The technique of ICR-MS was first published in the mid-1950s when it was demonstrated as the instrument for measurement of very small mass differences at very high precision [108]. The technique remained largely an academic tool until the application of FT methods by Marshall and Comisarow in the early 1970s [109,110]. It is now one of the most sensitive methods with unlimited resolution. Since ions are measured using frequencies characteristic of the m/z value within the ICR trap using a combination of magnetic field

and voltages, ions are not destroyed by ejection for detection. Therefore, the same ions can be measured over and over or subjected to multiple experiments, ideally MS/MS or MS^n for structural elucidation, as well as other gas-phase ion–molecule reactions. Because frequencies can be measured most accurately than ion current or TOF, mass measurements with sub-ppm mass accuracies and $>100,000$ mass resolving power are routine with FTICR-MS. However, for routine metabolism studies, stand-alone FTICR mass spectrometers are not considered as a mainstream mass analyzer due to practical limitations resulting from operational pressure requirements in the range of 10^{-8} to 10^{-10} torr. Conversely, the operational pressure requirements for stand-alone LITs and QMFs are in the range of 10^{-5} to 10^{-7} torr and make these types of mass spectrometers readily amenable to coupling with HPLC- and/or UHPLC-based high throughput assays.

7.4.2 Hybrid Mass Analyzers

The need to develop more efficient and high performance mass spectrometers has led to the advent of hybrid mass spectrometers, which coalesces the potential (or qualities) of multiple analyzers and ion manipulation devices into one instrument. For example, the ability of LITs and QMFs to operate in the vacuum pressure range of 10^{-5} to 10^{-7} torr makes these types of mass spectrometers ideally suited for coupling with TOF- and FTMS-based mass analyzers for capitalizing on their high resolution capabilities. Among the various hybrid mass analyzers, QTRAP, Q-TOF, and LTQ-OT have evolved as frontline instruments for drug metabolism studies involving metabolite profiling.

7.4.2.1 QTRAP. QTRAP is a hybrid instrument that combines the attributes of ITs and triple quadrupoles into a single mass spectrometer. The salient features of the QTRAP include the selective scans (CNLS, PIS, SRM) [111] of a triple quadrupole with high sensitivity, high speed (duty cycle), and full scan features of the IT, allowing both qualitative and quantitative analyses of metabolites [112–115]. Similar to stand-alone LIT (2D IT) mass spectrometers, which were developed to improve detection limits over traditional QIT mass spectrometers, QTRAPs also have greater ion storage capabilities, and trapping and storage can be carried out without space-charge effects, which leads to increased sensitivity. A QTRAP's ion path consists of three quadrupoles, where Q3 can be operated in a standard mass-resolving triple quadrupole mode or used as an LIT (Figure 7.4). The novelty of a QTRAP instrument is that it has retained the mass filtering capability and provided the functionality of the LIT. When the instrument is operated in the LIT mode, an entrance potential barrier and exit potential barrier is created axially to contain the ions. In addition, a radial RF field is applied to contain the ions. Fundamental instrumentation details are described in a paper by Hager [113]. A QTRAP is capable of scanning at 250–4000 Da/s and its resolution is dependent on scan speed. In contrast to 3D ITs, helium is replaced by nitrogen to cool the ions and carry out CAD for ion trap fragmentation. The instrument can be switched rapidly (in about 5 ms) between triple quadrupole and LIT scans, which allows the acquisition of both LIT and quadrupole scans in the same cycle. MS/MS experiments are carried out by the selection of the precursor ion in Q1 using quadrupole DC isolation. Fragmentation is then carried out in Q2 (high pressure linear acceleration collision cell), and the resulting fragment ions are collected in Q3, which is operated

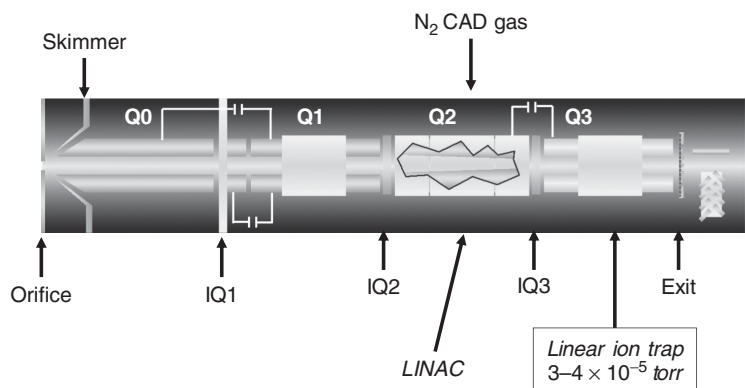


Figure 7.4 Schematic of AB Sciex QTRAP mass spectrometer. IQ, interquadrupole lens; LINAC, linear acceleration high pressure collision cell. (See color insert.)

in the LIT mode. Since ion selection is carried out in Q1, the trapping efficiency is greatly increased for the analyte because Q2 is filled with the ions of interest only. The increased trap capacity of the LIT (nearly 70 $\times$) as compared to the QIT plays an important role in the increased sensitivity of the LIT. In addition, the collision cell in the LITs allows higher energy fragmentation compared to traditional traps, leading to a rich fragmentation spectra containing primary and secondary fragmentation pathways. Owing to the absence of the low mass cutoff, more fragments at lower mass range can be observed.

The LIT has a variety of scan functions, which are described in the following.

1. *MS*. Enhanced MS is carried out to obtain a full scan MS spectra or survey scan in a defined scan range. During the EMS scan function, Q1 and Q2 are operated in RF-only mode with no collision energy and ions are trapped in Q3 (LIT), which are then detected. EMS provides the most sensitive survey scan for subsequent MS/MS scans.
2. *EPI*. During an enhanced product ion, the precursor ion is selected in Q1, fragmentation is carried out in Q2, and ions are trapped in Q3 and then scanned out for generating product ion spectra. What makes the EPI scan one of the most sensitive MS/MS scan function is the concurrent trapping of ions in the Q0 region while the isolation, fragmentation, and detection events are occurring.
3. *ER*. Enhanced-resolution scan is used to obtain mass spectra at higher resolution, especially on the order of <0.25 Da. Mass resolving power in the range of 5000–6000 at m/z 700 is achievable with scan speeds of 250 Da/s.
4. *MS³*. This type of scan function involves precursor ion selection in Q1, fragmentation in Q2, and trapping precursor/product ions in Q3 before switching to the LIT mode of operation for RF/DC isolation of a product ion followed by single frequency excitation in Q3 (LIT mode) before generation of product ion spectra. The MS³ mode spectra exhibit low mass cutoff.
5. *EMC*. The enhanced multiply charged scan removes singly charged ions and enriches multiply charged ions. This function helps in removing signals due to background/matrix and improves S/N ratio.

6. *TDF*. Time-delayed fragmentation helps to diminish the amount of secondary fragment ions, which simplifies complex MS/MS spectra. Under TDF conditions, ions are activated en route from Q2 to Q3. In Q3, activated ions are allowed to thermally cool before fragment ions are collected. Early fragments are rejected using the LIT mode stability parameters, and the fragments are collected after a delay time. The user has time-delayed control over the mass range.

It is also important to note that QTRAPs provide the option to utilize a collision energy spread (CES) function that changes the collision energy during a trap fill and EPI scan. For example, if CE is set at 30 with a CES of 15, the MS/MS spectra will show all the ions from the fragmentation at 15, 30, and 45. This feature is useful while acquiring qualitative data to identify metabolites, where it can be difficult to predetermine the optimum collision energy to be used for acquiring the data.

The various scan modes available in the QTRAPs have made it possible for the identification and characterization of metabolites. The triple quadrupole and LIT scan modes can be utilized for a variety of experiments: EMS (LIT single MS mode), PIS based on parent fragmentation pattern or phase II conjugate loss (phosphate, sulfate charged loss), CNLS based on phase II neutral conjugate loss (glucuronide, glutathione), and theoretical SRM based on biotransformation of parent molecule [116]. These modes can be coupled with automatic product ion experiments (IDA) in the QTRAP, where acquisition of both triple quadrupole survey scans is followed by MS/MS spectra being acquired in the IT in an IDA manner.

Qualitative metabolite identification was first reported for gemfibrozil with an API 2000 QTRAP, and quantification was reported for propranolol in plasma samples [116]. Hager and Le Blanc [115] demonstrated that metabolite identification could be improved using selective triple quadrupole MS survey scans to trigger the product ion spectra using automated IDA. Triple quadrupole and LIT scan modes can be used in several ways: LIT single MS mode, PIS based on parent compound fragmentation or for phase II conjugates (glucuronide or glutathione), and theoretical SRM to monitor biotransformation reactions of the parent compound. Very detailed work on metabolite identification and quantitation has been presented by Hopfgartner *et al.* [117] for several compounds including trocade, tolcapone, and remikiren. The ability to utilize selective MS/MS scans helped identify metabolites in picogram levels in very complex matrices [117,118].

In more recent years, quantitative and qualitative profiling of endocannabinoids (ECs) in human plasma was performed by an IDA experiment with SRM as the survey scan and EPI as the dependent scan. This method was rapid, highly sensitive, and selective to assess quantitation and identify ECs [119]. Quantitative and qualitative workflows using QTRAP technology have been extended by Qiao *et al.*, [120], King *et al.*, [121], and Li *et al.*, [122]. The essence of these experiments is based on the selective triple quadrupole scans, sensitive product ion scans, and the ability to perform SRM for targeted and untargeted metabolites. Good agreement was observed between SRM only and combined full scan and SRM data. In summary, QTRAP is suitable for laboratories where quantitative PK/TK (toxicokinetic) bioanalysis is the main focus and occasionally selected samples or studies involving qualitative bioanalysis need to be handled. Overall, QTRAP QQQ mode of operation allows one to use the triple quadrupole mode SRM methods for targeted quantification while the scan

speeds achievable with the LIT mode of operation provide options for collection of nontargeted information about metabolites, degradants, impurities, and biomarkers.

7.4.2.2 Q-TOF. A schematic of a Q-TOF mass spectrometer is shown in Fig. 7.5. Q-TOF is a hybrid mass spectrometer where a quadrupole mass analyzer (excellent sensitivity for SRM experiments) is coupled with a TOF mass analyzer (high resolution and efficient duty cycle) and involves replacement of the Q3 of the triple quadrupole mass spectrometer with the TOF. In the recent years, there has been much improvement in scan speeds, dynamic range, signal-to-noise (S/N) ratio, resolution, and the coupling with UHPLC, making the Q-TOF an instrument where integrated qualitative and quantitative bioanalytical studies can be carried out with ease [123–127].

Ions generated from atmospheric pressure ionization (API) or MALDI are “gated” from the source region by an electrical field pulse and accelerated down the TOF tube. The ions with lower m/z values travel with a higher velocity than those with higher m/z values. The calibration of accelerating fields and resulting flight times provides mass analysis. Similar to any analytical instrument, the analyte’s mass accuracy is dependent on the mass axis calibration. This can be performed by external or internal calibration procedures. External calibration can be easily performed with all mass analyzers and is the most preferred option. Frequent calibrations are not required with nominal mass or low resolution mass spectrometers (triple quadrupoles and ITs) because the degree of mass accuracy is limited to >500 ppm. Similarly, mass measurements with <5 ppm mass accuracies are routine with most FTMS with periodic (monthly or semiannually) external calibration [128]. However, with TOF-based mass analyzers, mass measurements are impacted by temperature changes and related changes in the ions’ TOF within the field-free region. Therefore, frequent mass calibration is a critical component for achieving <5 ppm mass accuracy and is an essential daily routine to be performed before running batches of samples. Most modern Q-TOF systems can

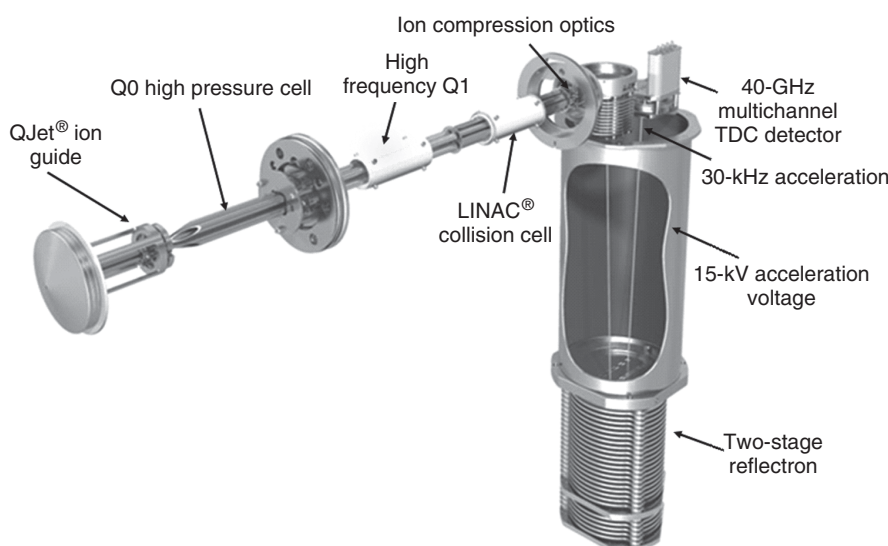


Figure 7.5 Ion path for AB Sciex TripleTOF 5600 Q-TOF mass spectrometer.

be automatically calibrated using reference compounds that are stored in vials with software-controlled automated valves [129–131]. In the Waters Synapt Q-TOF, mass accuracy can further be improved by a reference mass (known as a *lock mass*) that is introduced via a second ESI probe to the ion source for analysis within each run [36,128]. Whenever resolution and mass range settings are changed, the mass calibration procedure should be repeated. Similarly, the TripleTOF 5600 (AB Sciex) has a unique calibrant delivery system that is programmed to automatically inject a calibration mixture, via an atmospheric pressure chemical ionization (APCI) probe, at regular intervals during the analysis of sample batches. This process is completely under software control, provided the user configures the instrument before the submission of sample batches. The instrument software automatically checks the resolution setting and instrumental parameters (MS and MS/MS) for the analytical run and calibrates the system if needed. This eliminates the need for user intervention when the instrument parameters and experiments are changed for multiple batch submissions especially during unattended overnight analysis.

As shown in Figs. 7.6–7.8, a collision cell is located between the quadrupole and the TOF analyzer to induce fragmentation in MS/MS experiments. The quadrupole can be used either in narrow-band pass mode for acquisition of MS/MS data on selected ions or in wide-band pass mode for all ions to enter the collision cell. When the ions are not selected, and only RF voltage is applied to the quadrupole, the ions will pass the collision cell without undergoing any fragmentation and will be analyzed by the TOF in full scan MS mode. Subsequent analysis of ions and fragments is carried out in the TOF analyzer, which uses either an analog-to-digital converter (ADC) or a time-to-digital converter (TDC) [132,133]. The Q-TOF systems from Agilent Technologies, Bruker Daltonics, and Waters are equipped with ADC-based detection techniques, whereas the TripleTOF 5600 (AB Sciex) is equipped with a TDC detector [126]. The linearity and mass accuracy of highly abundant ion signals are impacted when using TDC detectors because of the signal saturation and inherent dead time [129]. However, in newer TOF instruments, detector saturation is minimized by incorporation of dynamic ion transmission controls with unique gating technologies to modulate the flux of the ions entering the flight tube. An added advantage of the TDC detection system is a lower background noise compared to the ADC, which in turn provides better sensitivity and/or S/N ratio particularly for lower mass ions. The TripleTOF 5600 resolved most of the dynamic range limitations of TDC by incorporating a four-channel 40 GHz TDC detector and a 25 keV accelerating voltage combined with unique gating controls and fast electronics to produce 25-ps time resolution and mass accuracy across the full mass range [134].

Until the introduction of time-lag focusing in the mid-1980s and the reflectron in the mid-1970s, TOFs were considered a low resolution mass analyzer with a very limited dynamic range. Time-lag focusing improved mass resolution by simultaneously correcting for the initial spatial and kinetic energy distributions of the ions. The reflectron design, a common feature in today's TOF-based mass analyzers, corrects for the kinetic energy distribution of the ions. Collectively, implementation of time-lag focusing and the reflectron improved the resolution of TOFs from several hundreds to thousands [136,137]. Furthermore, improvements in mass resolving power and mass accuracy resulted from flight tube temperature stabilization, novel detectors, and fast

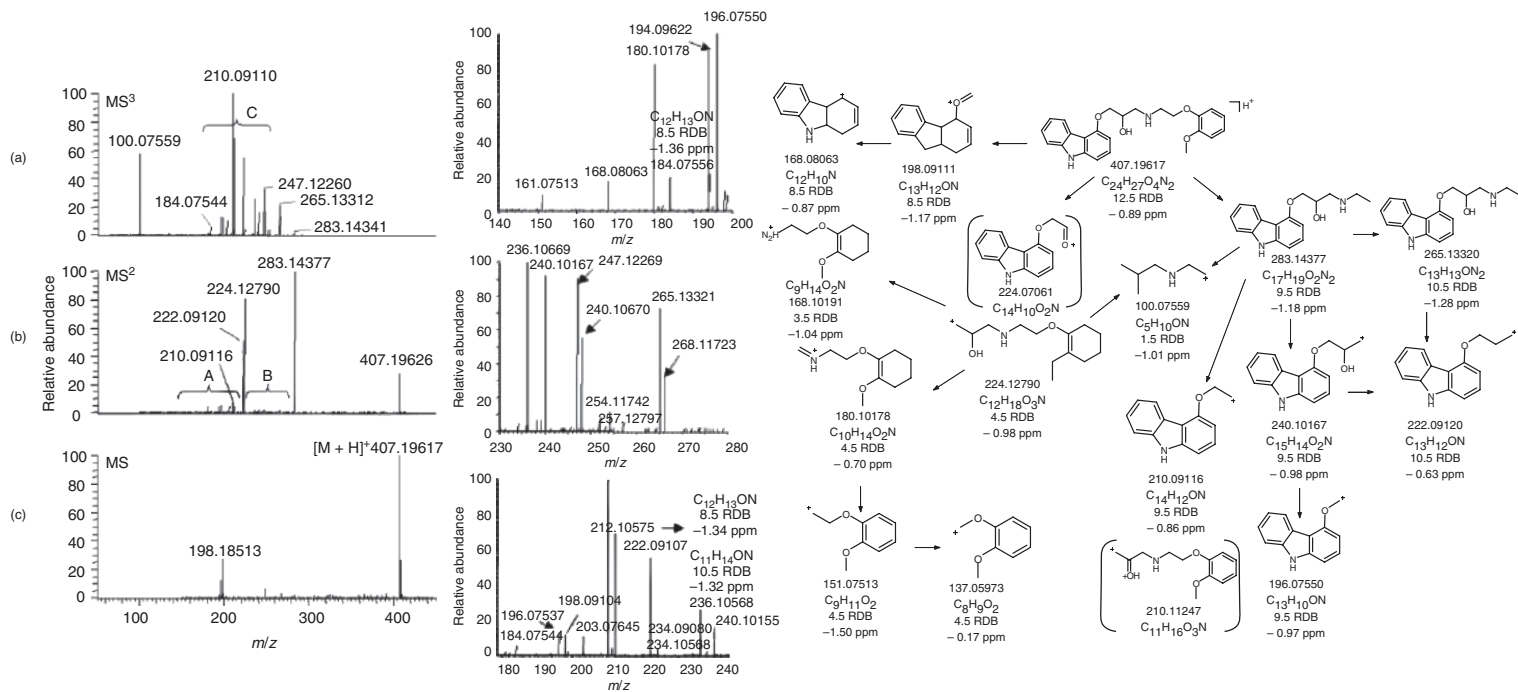


Figure 7.6 (a) MS³, (b) MS/MS, and (c) MS spectra of carevidol obtained using LTQ-Orbitrap Classic and the proposed fragmentation pathway. *Source:* With permission from Ref. 135.

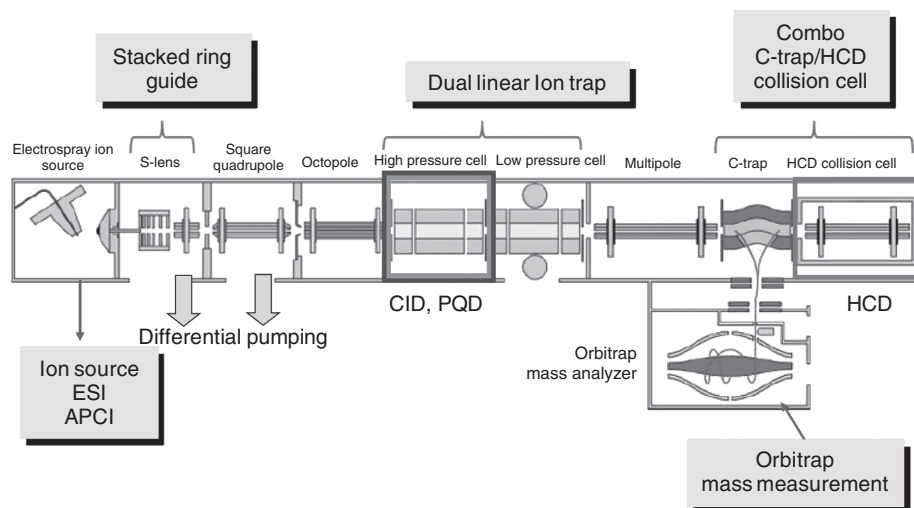


Figure 7.7 Ion path for ThermoFisher LTQ-Orbitrap Velos™ mass spectrometer, and the various fragmentation options available for structural elucidation of drug metabolites. CID, collision-induced dissociation; PQD, pulsed Q collision-induced dissociation; HCD, higher energy collisional dissociation. (See color insert.)

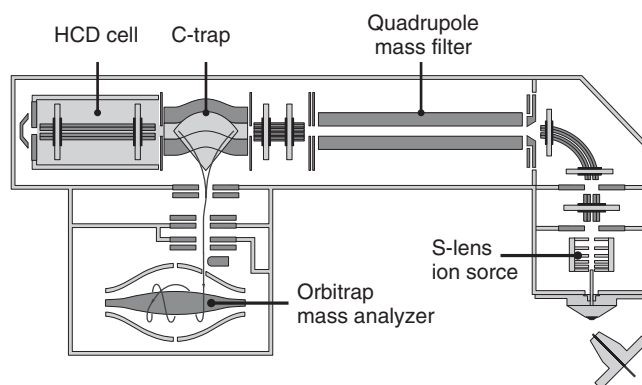


Figure 7.8 Ion path for ThermoFisher Q-Exactive mass spectrometer.

electronics [36,138]. Overall, the current Q-TOF mass spectrometer is an inherently fast technique and provides the user the option to collect full scan data as well as targeted MS/MS or information-dependent MS/MS data (Section 7.4.2.4). Availability of the full scan MS or survey scan data provides the flexibility to examine the data set using a wide array of postacquisition software for probing unexpected metabolites as well as biomarkers or degradants [139–142].

7.4.2.3 Orbitrap (OT). Various OT-based hybrid mass analyzers were introduced over the years, starting in 2005. OT utilizes electrical fields between sections of a roughly egg-shaped outer electrode and an inner electrode or a spindle, where the ions' orbit and their oscillation are recorded as image current on the spindle. Similar to FTICR, in OT, ions are measured using frequencies characteristic of the m/z values.

In contrast to FTICR, where a combination of magnetic field and electrostatic potentials is used to trap and manipulate the ions within an ICR cell, ions in OT are manipulated using a combination of electrostatic potentials. Since OTs measure motions of ions, similar to FTICR, it is important to maintain ultrahigh vacuum in the range of 10^{-7} to 10^{-8} torr. Ultrahigh vacuum requirements prevent OT from functioning as a stand-alone mass analyzer. However, ions from an API source can be first introduced into either an LIT or a QMF, which are capable of functioning at 10^{-5} to 10^{-7} torr before transferring into OT for analysis. Similar to any trap-based mass analyzers, the performance of an OT mass analyzer is also affected by space-charge effects, which occurs when too many ions reside in the IT, leading to a distortion of the electric field. This in turn can impact mass resolution, detection, and mass accuracy. The automatic gain control (AGC) procedure, implemented for minimizing space-charge effects, uses a short prescan to determine the ion current within the mass range of interest and enables the storage of a fixed number of ions ("AGC target value") in the following analytical scan [143,144]. This function is carried out by alternate prescan and analytical scans. If the prescan identifies an excessively high total ion current (TIC), the injection time for the consecutive analytical scan will be subsequently reduced. Typically, a user can select among three AGC target values: (i) 3×10^6 for a high dynamic range or to maximize quantitative performance, (ii) 1×10^6 for a balanced scan, and (iii) 5×10^5 for best mass accuracy in untargeted analysis.

Metabolic profiling of pharmaceutical entities requires comprehensive structure elucidation of the parent compound and its metabolites. The fragmentation of the parent drug is used as a reference, and the site of biotransformation or the "soft spot" can be determined after comparing the product ion spectra of the parent and the metabolite. Often, multiple MS/MS experiments such as MS^3 or MS^n experiments are useful in determining the metabolite structure. The availability of accurate, fast, and sensitive mass measurement and several CID fragmentation options allowed Lim *et al.* [135] to study the metabolism of carvedilol. In the workflow followed by Lim *et al.* [135], an LTQ-OT classic mass spectrometer operated at a resolving power of 60,000 was used to perform parent mass list triggered data-dependent MS^n accurate mass analysis. As shown in Fig. 7.6, the initial step to understand the fragmentation pathway of carvedilol and lineage of its product ions involved MS, MS/MS, and MS^3 experiments. Fragment ion structures proposed by Mass Frontier (fragmentation prediction software) were identified and confirmed using elemental compositions proposed by HRMS. At present, OT-based mass analyzers are standard platforms for detection, characterization, and identification of metabolites from *in vitro* and *in vivo* samples. Steady improvements have been made to OT platforms in two fronts: (i) increase scan speeds to accommodate separations based on UHPLC and (ii) provide richer fragmentation information for structural elucidation using alternate fragmentation techniques such as higher energy collision dissociation (HCD) and pulsed Q collision-induced dissociation (PQD). Improvements in scan speed and fragmentation techniques were achieved in the LTQ-OT VelosTM mass spectrometer (Figure 7.7) [145–147] and are discussed in Section 7.2.6.2.

As shown in Fig. 7.8, in the newly released hybrid OT mass spectrometer, Q-Exactive, the LTQ component of an LTQ-OT is replaced with a QMF [148]. Another way of looking at the new line of hybrid OT instruments is the mass selection capability provided in a stand-alone benchtop OT mass spectrometer "Exactive." The Exactive mass spectrometer has been explored for small-molecule quantitation [149]

and integrated qualitative and quantitative [141] workflows, but the lack of mass selection for fragmentation has thus far limited its application to selected areas in the DMPK arena. Unlike the LTQ-OT series of mass spectrometers, where switching between positive and negative HRMS modes of data collection can take hours, the Q-Exactive is capable of switching between positive and negative modes within minutes. Recent evaluations clearly show that Q-Exactive is ideally suited for high throughput screening with integrated qualitative and quantitative analyses. The Q-Exactive is capable of providing a resolution of 100,000 and 140,000 at m/z 400 and 200, respectively, and is equipped with an HCD cell for performing mass-selective CID experiments. Ions generated in the API source are transmitted using a QMF into a curved linear trap (c-Trap) or HCD cell before being transmitted into the OT for FT mass analysis. MS/MS data are collected using the QMF to select precursor ion of interest and HCD cell to perform CID experiments. Similar to any FT-based mass analyzers, mass resolution is dependent on scan speeds and for an ion at m/z 200, the resolution could vary between 10,000 and 100,000 with scan speeds between 10 and 1 Hz, respectively.

7.4.2.4 Metabolite Fragmentation Information. To eliminate the need to perform multiple injections of the same sample and utilize an array of mass spectrometric and chromatographic methods to decipher metabolism information and arrive at structural information about metabolites, Wrona *et al.* [150] and Bateman *et al.* [151] introduced the “All-in-One” or MS^E (where E represents the collision energy) experimental approach. In the MS^E approach, data is collected using two scan functions as shown in Figure 7.9. In the first function, Q1 is set to pass a mass range, for example, m/z 100–800, and Q2 is then set at a “low” collision energy (5 eV) that provides

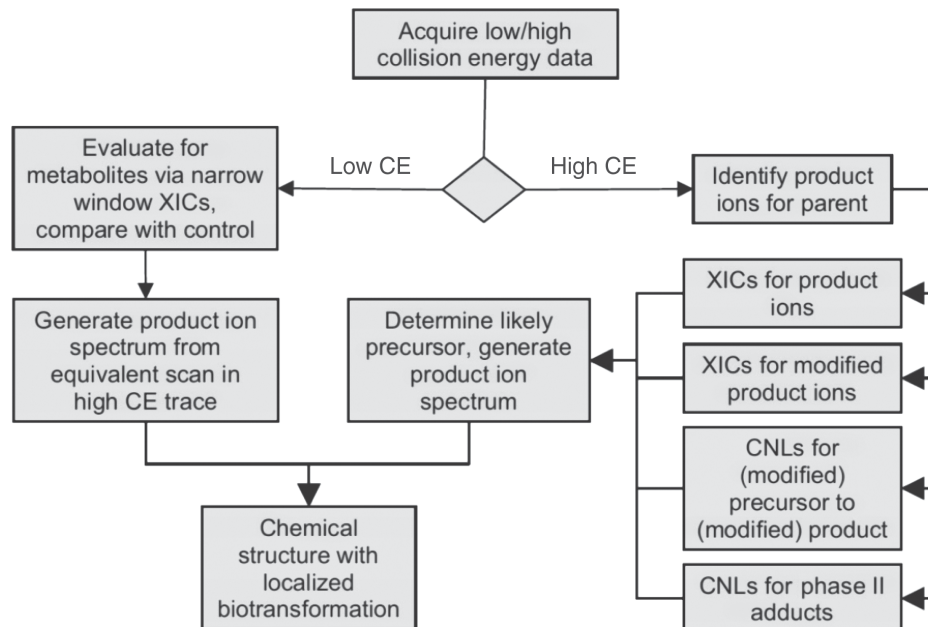


Figure 7.9 All-in-One or MS^E (where E represents the collision energy) experimental approach. CNL, constant neutral loss; XIC, extracted ion chromatogram. Source: With permission from Ref. 150.

transmission of intact ions through the collision cell. These ions are pushed into the TOF analyzer and detected with high resolution [8000 full-width-at-half-maximum (FWHM)] and mass accuracy (<10 ppm). The second scan function also scans in the same mass range, but Q2 is set at a “high” (15–35 eV) collision energy that fragments all ions that are transmitted through Q1. Again, the product ions are detected by the TOF analyzer. Hence, two mass spectra, one with intact molecular ion information (“low energy” function) and other with fragment ion information (“high energy” function) are generated alternating low/high energy data acquisition (MS^E) in the wide-band mode. The advantage of obtaining full scan spectra is that comprehensive data for all metabolites and adducts can be generated, including doubly and singly charged adducts. Key diagnostic ions for accurate neutral losses and precursor ions can be selected by comparing the low energy and high energy fragmentation traces, especially for the detection of GSH adducts [152]. The mass window is set for ± 10 mDa. This narrow range allows high selectivity and excludes false positives from the matrix. Since all data is collected in one run, postacquisition processing of fragment ions is possible by a variety of data processing algorithms [153,154]. Other workflows with a similar theme to MS^E [150,151,155] include the recently demonstrated FragAll or MS^{ALL} [156] or SWATH [157,158]. Even though MS^E experiments can provide detailed information of the product ion spectra, it is sometimes difficult to determine the exact soft spot of a metabolite and it may be necessary to resort to a combination of MS-based techniques such as stable isotope labeling [5], H/D exchange [159], or even LC-NMR [26].

Several Q-TOF-based options are also available to acquire a combination of full scan and MS/MS data under UHPLC timescale (analyte peak widths 3–7 s) for adapting the combined qualitative and quantitative (qual/quant) workflow in the DMPK arena. The combined Q-TOF-based qual/quant workflow for DMPK applications was recently described by Ramanathan *et al.* [160], Ranasinghe *et al.* [134], Campbell and Le Blanc [161], and Fung *et al.* [162]. The qual/quant workflow can be implemented as shown in Figure 7.10. In the approaches shown in Figure 7.10, once a full scan HRMS spectrum is acquired, an MS/MS experiment can be triggered based on the criteria provided by the operator. The threshold-based MS/MS data collection method is referred to as *IDA* or *data-dependent acquisition*.

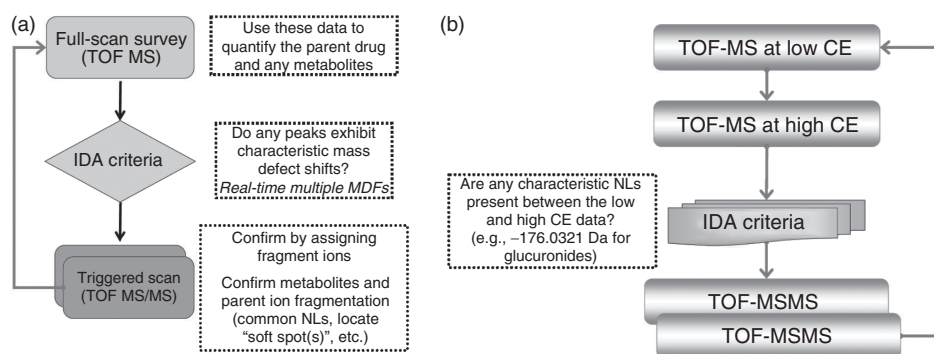


Figure 7.10 Two Q-TOF MS-based methods applicable for integrated qualitative/quantitative workflows. (A) Combination of TOF-MS survey scan and the on-the-fly mass defect filter (MDF)-triggered MS/MS or other information-dependent triggered MS/MS. (B) Coupled low- and high-collision energy (CE) experiments that can trigger a confirmatory MS/MS scan based on on-the-fly constant neutral loss (NL) detection. *Source:* With permission from Ref. 161.

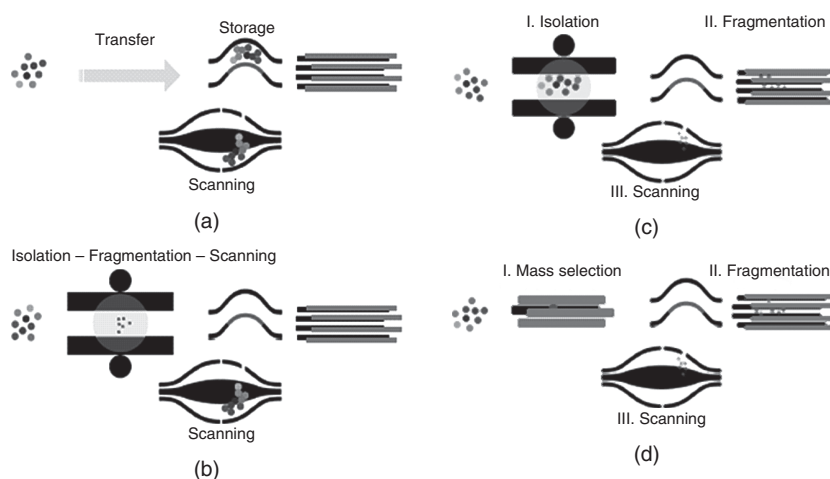


Figure 7.11 Fragmentation options available in orbitrap (OT)-based mass spectrometers. (a) Exactive: no mass selection is available for CID experiments but non-mass-selective all-ion fragmentation is possible. (b) LTQ-OT Velos (CID): selection in time (trap-trap); mass selection and CID are possible in the LTQ (ion-trap-like fragmentation). (c) LTQ-OT Velos (HCD: selection in time (trap-trap); mass selection in the LTQ and CID in the HCD cell (triple-quadrupole-like fragmentation). (d) Q-Exactive: selection in space (filter-trap); mass selection using QMF and CID in the HCD cell. *Source:* With permission from Ref. 148.

Fragmentation options available in OT-based mass spectrometers are summarized in Figure 7.11. No precursor ion selection is available for CID experiments in a stand-alone OT or the Exactive but non-mass-selective all-ion fragmentation (AIF) is an option [163]. When AIF is used, the mass spectrometer collects alternating full scan MS and MS/MS scan spectra. Although the AIF feature is similar to the MS^E available on the Q-TOF mass spectrometers, deciphering the fragmentation pathways and linking the origin of each fragment is difficult without sufficient liquid chromatographic separation or precursor ion selected fragmentation [163]. As shown in Figure 7.12 and Table 7.5, three precursor ion selectable MS/MS options are available in the LTQ-OT Velos mass spectrometer: (i) CID, precursor ion selection and fragmentation in the LTQ and detection using either the LTQ (low resolution) or the OT (high resolution); (ii) PQD, time events are identical to CID but the Q-value is pulsed to enhance trapping of the low mass fragment ions; and (iii) HCD, precursor ion selection in the LTQ, fragmentation in the HCD cell, and detection using the OT. Recently, work from our laboratory demonstrated that small molecules (MW <600 Da) and their metabolites can be efficiently fragmented with high accuracy using both HCD and PQD [99]. In the example shown in Figure 7.12, the CID, HCD, and PQD spectra of hydroxywarfarin obtained using a LTQ-OT Velos mass spectrometer are compared. While some of the low mass fragment ions were absent in the CID spectrum, the fragmentation process under the CID conditions was efficient and most sensitive. The PQD spectrum showed a unique fragment ion at m/z 189.0181 that helped to narrow the site of hydroxylation, but one of the major fragment ions at m/z 179.0339 was absent. The MS/MS techniques available on the LTQ-OT Velos mass spectrometer can be rank-ordered based on the sensitivity results as CID > HCD > PQD. Overall, the HCD and PQD spectra were rich in fragmentation information and helped to narrow the site of hydroxylation.

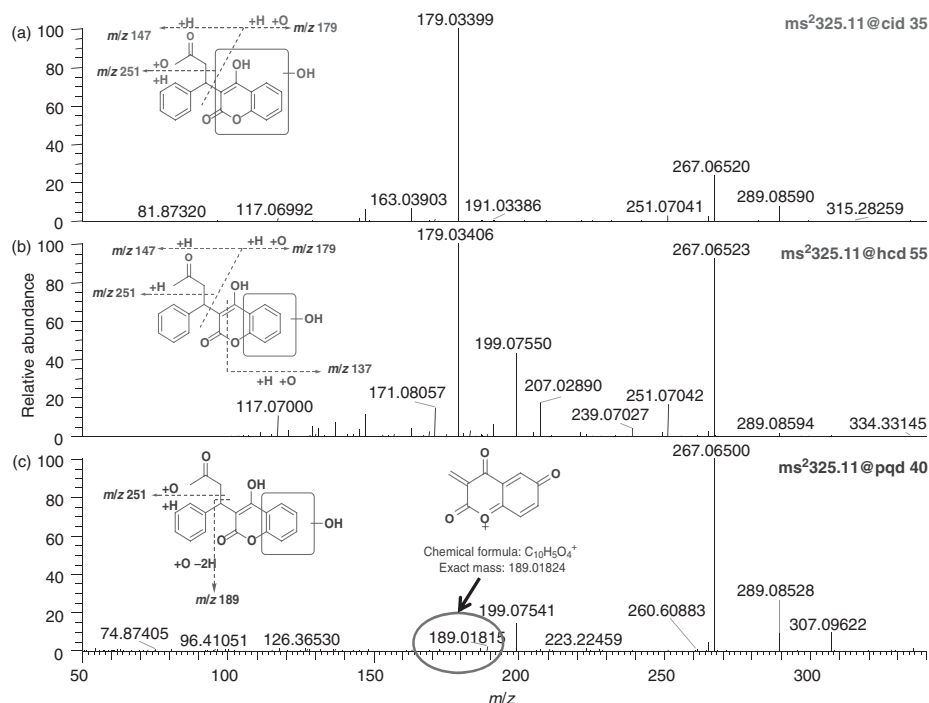


Figure 7.12 Comparison of (a) CID, (b) HCD, and (c) PQD spectra for hydroxywarfarin obtained using an LTQ-OT Velos mass spectrometer.

TABLE 7.5 Fragmentation Modes in LTQ-OT Velos Mass Spectrometer

Activation Type	CID	HCD	PQD
Precursor selection	LIT	LIT	LIT
Collision cell	LIT	HCD	LIT
Collision gas	Helium	Nitrogen	Helium
Collision energy ^a	~35	40–75	35–60
Detection of ions	IT/Orbitrap	Orbitrap	IT/Orbitrap
Activation, Q-value	0.25(constant)	N/A	>0.6
Dissociation	single	single	3-step
Mass range	Low mass cutoff (“1/3 rule”)	No low mass cutoff	No low mass cutoff
Fragmentation	MS ⁿ	MS ²	MS ⁿ

^aNormalized collision energy.

7.4.2.5 Mass Defect Filtering. According to currently accepted mass measurement convention, the mass of carbon $^{12}\text{C} = 12.000000$ Da and the exact masses of all the other elements are either slightly higher or lower than their integral value. For example, the exact mass of $^1\text{H} = 1.007825$ Da and $^{16}\text{O} = 15.994910$ Da. The ^1H is therefore $+0.007825$ Da and ^{16}O is -0.00509 Da. Therefore, the term *mass defect* refers to the difference between the exact mass of an element or compound and its closest integer value [164]. Although the utility of mass defect for identifying isotopes

of elements was realized during the 1920s [165], the application of mass defect to filter drugs and their metabolites present in biological samples was only demonstrated very recently [35,151,166,167]. At present, mass defect filter (MDF) can be applied post acquisition on every HRMS platform and during real-time acquisition on selected platforms [161]. One of the prerequisites to use MDF is that data must be collected using high mass accuracy, high mass resolution, and full scan MS mode. A combination of high resolution measurement and the mass defect of the parent drug can be used to readily discern drug-derived ions from the background ions. Postacquisition application of MDF is demonstrated in Fig. 7.13. Comparison of unfiltered data (a) with those obtained using extraction window of m/z 400–600 (b) and MDF with a filter of 10 mDa above and 30 mDa below the fractional exact mass of the parent drug (c) shows that MDF clearly filters out all the background signal and sheds light on the drug-related components.

Another option is to apply MDF during real-time acquisition or “on-the-fly.” In the example shown in Figure 7.14, buspirone product ion at m/z 122.0713 was used to screen for metabolites or drug-derived components (precursors) forming a product ion with the exact mass of 122.0713 [134]. Detection of the product ion at m/z 122.0713 suggests that the pyrimidine moiety of buspirone is not modified. One of the advantages of this approach is the fact that the number of product ion spectra or the number of hits was low, and primarily related to the metabolites, due to the high specificity of the on-the-fly MDF [166]. On-the-fly MDF-triggered MS/MS is a bonus to postacquisition probing of the full scan data where MS/MS of the *in vivo* metabolites are not available unless the samples are reinjected and analyzed by targeted MS/MS experiments [134]. The mass defects and accurate masses of both product ions and the precursor ions confirm the site of oxidation, and the tentative structures of the *in vivo* metabolites are assigned. Although multiple MDF templates can be used to ensure metabolites outside the 100 mDa MDF window are not missed, in the approach shown in Fig. 7.14, single MDF/analyte was used for on-the-fly MDF experiments [168]. As a fail-safe approach, all full scan survey raw data files can be subjected to postacquisition multiple MDF.

7.4.2.6 Isotope Pattern Filtering. In addition to the option to apply MDF post acquisition, other advantages of acquiring full scan HRMS data include preservation of information about additional analytes from an analysis and preservation of isotopic patterns of analytes of interest and background ions. Pharmaceutical drugs that contain chlorine (Cl) or bromine (Br) or strategically introduced stable-labeled isotopes such as ^{13}C or ^{15}N at a known percentage generate a unique MS isotope patterns. Frequently, metabolites or degradants maintain a similar isotope pattern. Therefore, the isotope pattern can be used as a filter to discern drug-related components in complex biological matrices. In an IPF workflow, if an analyte contains chlorine (Cl), the relative abundances of the peak [A] or $[\text{M} + \text{H}]^+$ generated by the monoisotopic ions including the ^{35}Cl and isotopic peaks $[\text{A} + 1]$ and $[\text{A} + 2]$ generated mainly due to ^{13}C and ^{37}Cl isotopic contributions are examined, if the $[\text{A}]/[\text{A} + 1]$ and $[\text{A}]/[\text{A} + 2]$ peak ratios. If the isotopic pair ratios match the user-defined ratio then the components will be considered as drug-derived materials and extracted into the ion chromatograms or additional MS/MS experiments can be triggered for structural elucidation. Today, almost all HRMS platforms offer some form of postacquisition isotope pattern filtering (IPF) algorithm and selected vendors even offer on-the-fly IPF-triggered MS/MS options.

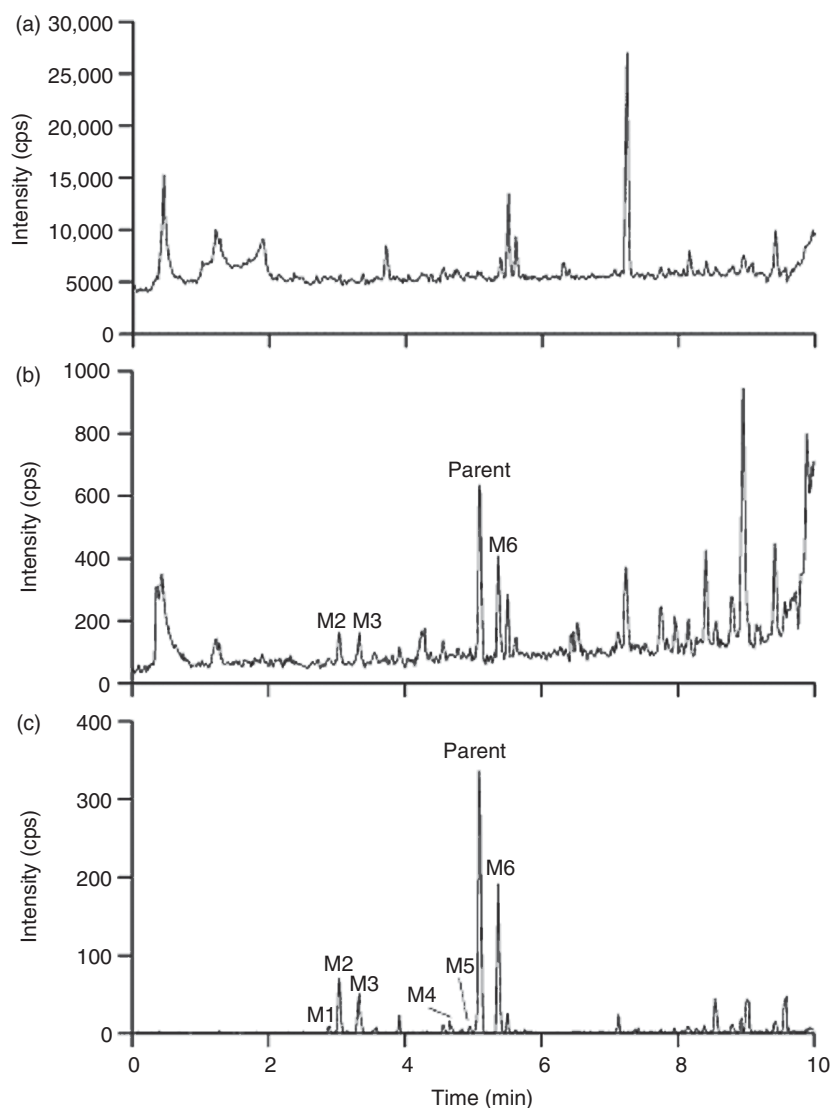


Figure 7.13 Application of “postacquisition” MDF as part of metabolite detection, characterization, and identification strategy for an extract of rat plasma following administration of a proprietary drug (L-006235): (a) unfiltered data, (b) extracted ion chromatogram (m/z 400–600), and (c) MDF using the fractional mass of the proprietary drug (L-006235) (XIC window $+10/-30$ mDa). *Source:* With permission from Ref. 151.

The IPF option has been explored to screen for reactive metabolites as well as to scout for drug-derived components. Ma *et al.* [169] evaluated the reliability and effectiveness of the IPF technique using an LTQ mass spectrometer. In this evaluation, human liver microsome incubations enriched with a 1:0.8 mixture of nonlabeled GSH/stable-isotope-labeled GSH were used to study GSH adducts of carbamazepine, diclofenac, 4-ethylphenol, acetaminophen, *p*-cresol, and omeprazole. Without utilizing HRMS techniques, Ma *et al.* [169] demonstrated that when employed as

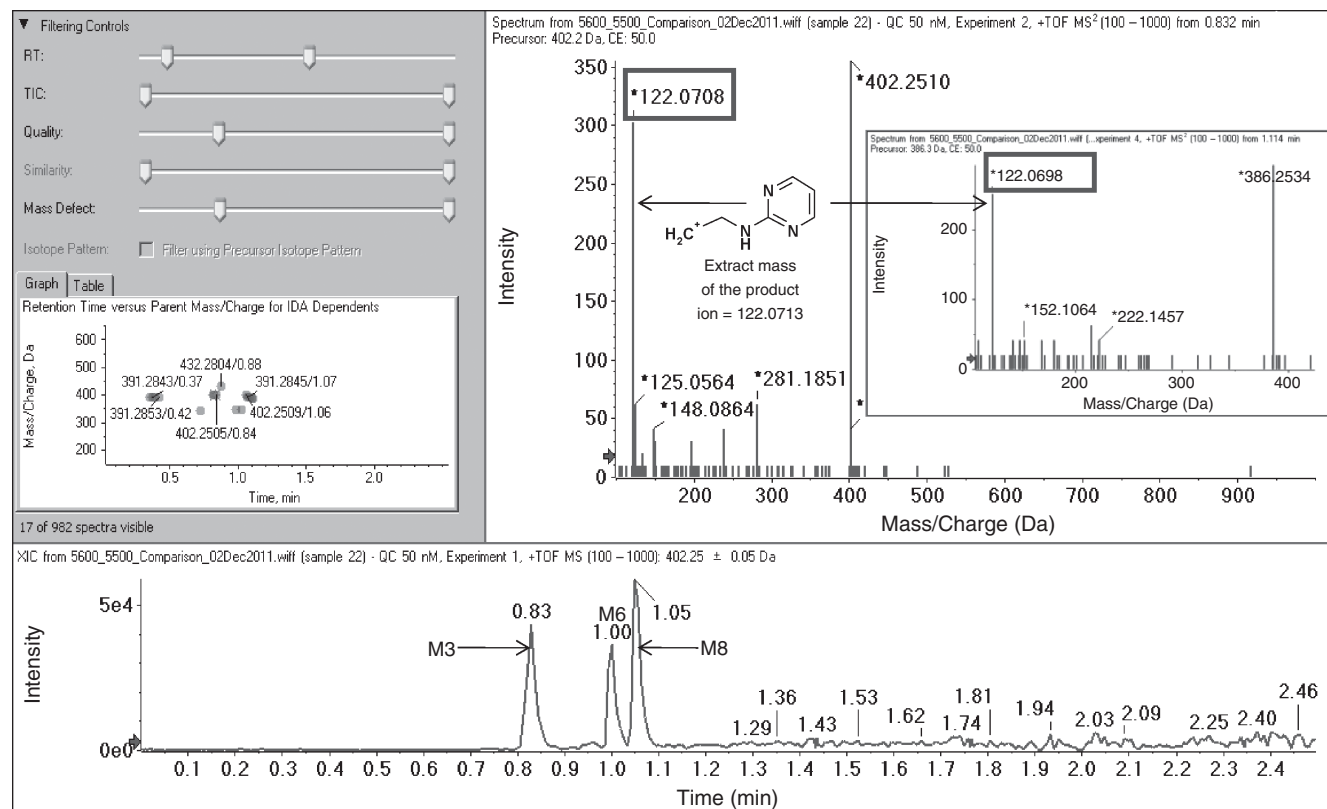


Figure 7.14 Application of “on-the-fly” MDF as part of the integrated qualitative/quantitative bioanalysis to scout for metabolites in *in vivo* samples.
Source: With permission from Ref. 134.

a postacquisition data mining tool, the IPF technique was very powerful and effective in detecting low levels of GSH adducts. A similar workflow using a TOF-based mass spectrometer and brominated analog of glutathione, *N*-(2-bromocarbobenzyloxy)-GSH (GSH-Br), was evaluated by Le Blanc *et al.* [170]. In this study, GSH-Br was used to trap reactive metabolites formed following the incubation of acetaminophen, fipexide, trimethoprim, and clozapine in rat liver microsomes.

Lim *et al.* [171] used a LTQ-OT mass spectrometer and exploited HRMS in combination with ISP-triggered data-dependent product ion scan to successfully detect and characterize GSH conjugates of acetaminophen, tienilic acid, clozapine, ticlopidine, and mifepristone. To take advantage of the IPF workflow, human liver microsome incubations were carried out using a 2:1 mixture of nonlabeled GSH/ $^{13}\text{C}_2^{15}\text{N}$ -GSH. The IPF workflow used by Lim *et al.* [171] allowed not only the detection of GSH adducts but also the detection and characterization of cyano-*N*-methylene iminium ion conjugates of verapamil and its O-desmethyl metabolites. Recently, a similar accurate mass-based IPF method was demonstrated by MsMetrix [172], a company engaged in developing software tools for metabolite identification. In this approach, a 1:1 mixture of nonlabeled GSH/ $^{13}\text{C}_2^{15}\text{N}$ -GSH was used as a trapping agent to generate GSH conjugates with a unique (*A*) and (*A* + 2) doublet with an exact mass difference of 3.0037 Da. The MsMetrix approach was successfully used to detect GSH conjugates of clozapine, diclofenac, imipramine, and ticlopidine following incubation with human live microsomes. Most recently, Zhang *et al.* [173] evaluated the MsXelerator software, a software approach developed by the MsMetrix group, to detect and quantify GSH adducts. In this evaluation, various nonlabeled GSH/labeled GSH ratios were evaluated using LTQ-OT Velos and TripleTOF 5600 mass spectrometers. A ratio of 3:1 of nonlabeled GSH/labeled GSH was found to be sufficient to detect most GSH adducts. An attractive outcome of this study was that MsXelerator software was found to be capable of processing raw data files from multiple vendors and detecting GSH adducts without difficulty.

7.4.2.7 Software Tools for Metabolite Detection. The availability of various HRMS technologies discussed previously clearly shows that the acquisition of analytical data is no longer the bottleneck of metabolite detection, identification, and characterization. This limitation now falls in processing and interpretation of data sets to make meaningful conclusions, independent of instrument model, and beyond just the hybrid mass analyzers. The Waters MetaboLynx application manager runs the samples scheduled for analysis by LC-MS, detects expected/unexpected metabolites, and reports within a data browser mass spectrometric and chromatographic evidence for the automated metabolite assignment [125]. Software packages with similar capabilities include MetWorks (ThermoFisher) and MetabolitePilot (AB Sciex). These software packages provide opportunities for most scientists to decipher metabolism information from a sample rather than limiting the task to mass spectrometry or metabolism specialists. Most of these programs work by comparing two LC-HRMS raw data files, one is from the sample of interest (dosed or +NADPH) and another from a control (predose or placebo-dosed or -NADPH) sample. Drug-derived components are detected using user-defined MDF, IPF, or MS/MS criteria. Despite the limitations of the software packages, including the inability to produce a comprehensive list of all theoretically possible biotransformation processes of a drug and the inability to detect metabolites present at low levels, many software tools are being adopted widely and

consequently as their benefits are being realized. The current limitation of these software tools is the inability to process raw data files, both qualitative and quantitative, from multiple platforms or vendors. However, several software vendors including the MsMetrix and Mass-MetaSite groups are actively engaged with pharmaceutical scientists to develop instrument vendor neutral software tools [172–174]. Such software tools would be paramount for seamless functioning of a globalized scientific community involving multiple scientists from multiple functional areas accessing the same data file.

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8 Imaging Mass Spectrometry

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8.1 Introduction	1
8.2 Method Overview	4
8.3 Applications	19
8.4 Perspectives	36
References	37

8.1 INTRODUCTION

Molecular imaging is one of the cornerstones of the experimental methods available for understanding the spatial complexity of biological tissues and cells. Ideally, imaging methods should provide detailed information of a number of tissue components and provide a resolution that surpasses that of individual cells. Most current methodologies often require *a priori* knowledge of the molecules of interest. Microscopy has long been one of the most powerful techniques to study the distribution of biologically relevant compounds in tissues. Immunohistochemical methods were first studied in 1942 in the work of Coons *et al.* [1]. They presented a method employing the specificity of an antibody labeled with fluorescein for the localization of antigens under a fluorescence microscope. It was a generic method for the histological localization of any antigen of interest. The antibody could be conjugated with simple chemical compounds without destroying its capacity to react with the antigen. One of the limitations of immunohistochemistry is that simultaneous visualization of different antigens can be very challenging and posttranslational modifications of proteins can be difficult to detect because of the lack of an available antibody specific for these modifications.

The ability of modern proteomic techniques to identify and quantify the levels of up to thousands of proteins from a tissue or biofluid sample has transformed medical and biological research. In addition to global protein profiling [2], methods have been developed that target protein isoforms [3,4] or their interactions [5], specific pathological entities [6], or subcellular components [7]. It has been increasingly recognized how the rich molecular information provided by this array of approaches offers new possibilities in the clinical field. This ranges from new insights into the molecular changes associated with pathogenesis, the identification of new therapeutic targets, to improved diagnosis

and prognosis through the determination of biomarker proteins and protein profiles associated with a disease and its progression [8]. Many clinical institutions have initiated research programs for the identification of disease biomarker proteins and protein profiles, mostly in easily accessible body fluids such as plasma, serum, and urine.

Difficulties associated with sample volume and complexity, dilution of the biomarkers, and the intrinsic variability of the protein content of body fluids have led to the alternative strategy of searching for biomarkers in the affected tissue. Recent notable examples include a combination of tissue microdissection, protein extraction, and extensive peptide/protein separation to quantitatively investigate the changes in protein content associated with the different stages of diseases [6,8].

Several groups have demonstrated how mass spectrometry (MS)-based methods can be directly applied to tissue. Rapid progress in the field now allows the parallel analysis of 100–200 proteins, high sensitivity and selectivity, and the ability to distinguish between close variants [9–11]. Histology-directed tissue profiling, in which localized MS is used to generate peptide and protein signatures of specific regions of a tissue previously annotated by a pathologist, has demonstrated how the signatures of the different regions can be used to classify the tissue [12,13]. These classifications, based on the nontargeted analysis of the peptides and proteins present in specific regions of the tissue, demonstrate the ability of this technique to acquire clinically relevant data. The candidate markers determined by these classifications are based on the levels of many peptides and proteins and provide an example of a general observation in biomarker research, namely, that a single protein biomarker may not be sufficient to annotate the complexity of real biological systems [14,15]. Protein profiling by imaging MS has been performed on human brain tumors [11,16,17], rat pituitary [18], mouse brain [19], mouse prostate [10], mouse epididymis [9], and in animal models for neurological disorders including Parkinson's disease [20] and Alzheimer's disease (AD) [21].

Imaging MS can be considered a spatially resolved direct tissue analysis and uses the same tools to simultaneously analyze the distribution of 100–200 proteins in tissue and to associate the differential protein images with histopathological analysis. Several studies have already shown how imaging MS can be used to chart the variation in protein content in pathological tissues and revealed that changes in distribution and biochemical content are widespread [22]. The spatiochemical information obtained directly from the tissue (thus not diluted by body fluids) and without prior knowledge of the sample's biomolecular content has great potential as a discovery tool to identify those changes associated with a specific diagnosis/prognosis. In many regards, it is the aim of imaging MS to become a peptide/protein screening that complements histopathological analysis.

Imaging MS comprises the collection of a mass spectrum at each of a regular series of points across the tissue section. Using these spectra, it is possible to plot the relative intensity of the individual m/z peaks in the mass spectrum across the tissue section, visualizing the distribution of the individual molecular ions. A two-dimensional map can be generated for each mass peak of interest in the mass spectrum and depicted as an intensity or pseudocolor heat map. Reproducible sample preparation, mass spectral acquisition, and data processing allow these maps to be compared between related samples to assess which biomolecular changes are associated with disease (and its progression). In current experimental workflows, the imaging MS analysis is aligned with an optical image of the tissue section (H&E stained after the imaging MS experiment) [23,24]. This allows the direct comparison of the specific biomolecules with the tissue's

morphology or the determination of the molecular content of histological regions of interest. Advanced data-analysis tools enable the data sets to be interrogated to reveal which peptides and proteins have similar profiles [25–27], which are correlated with specific histological features [27] and can be used to classify the tumor type of the tissue [27,28].

One main advantage is its lack of *a priori* knowledge since any compound of interest at the surface of the sample can potentially be detected. Imaging MS is very useful as a discovery tool where a tissue section can be investigated without knowing in advance what specific proteins have changed in a comparative study. Furthermore, imaging MS also allows the detection of posttranslational modifications, given that these generally involve mass changes. Unfortunately, many of the fixation approaches developed for optical and electron microscopy are not suitable for imaging MS, and so new MS-specific protocols have been designed, this is described in the following sections. Imaging MS has a significant potential to be used in a targeted approach as well, such as for monitoring drug delivery and metabolism. Imaging MS can visualize and distinguish the parent drug and its metabolites in contrast to autoradiography as no labeling is required.

While profiling MS of a tissue section can be of interest to analyze discrete regions of a tissue, imaging MS becomes necessary when the focus is the protein distribution information. The analysis of various cell types can be correlated to the tissue architecture, thereby enabling peptides and proteins specific to the tumor and/or adjacent stroma to be identified. High resolution images are desirable when a defined substructure is present; however, they may also be utilized as a discovery tool for a suspected yet otherwise not morphologically detectable transformation such as has been observed for tumor margins [29].

Immunohistochemical methods allow localization of antigens, antibodies, and numerous other cell and tissue components and have facilitated the development of specific diagnostic tests. Imaging MS offers the potential for the simultaneous analysis of many molecular species present in a single biopsy regardless of the availability of specific antibodies. Furthermore molecules spanning multiple molecular classes, which may be involved in a disease state, can be investigated. For clinicians, this will permit molecular assessment of tumor biopsies with the potential to identify subpopulations that are not evident based on the cellular phenotype determined microscopically [30]. In addition, this technology also permits imaging the distribution of low molecular weight compounds such as lipids, drugs, and metabolites, opening new possibilities for the measurement of concomitant protein changes in specific tissues after systemic drug administration.

Imaging MS has been used in a wide variety of applications since its development by Caprioli *et al.* in 1997 [18]. Improvements have been made in sample handling, spatial resolution, sensitivity, robustness, speed, and instrumentation, and molecular images up to a resolution of 5 μm can now be acquired. Today, imaging MS is applied in different research fields, from fundamental research to clinical applications (cancer research, neuroscience, and pharmaceutical research).

Imaging MS is increasingly used in clinical proteomics as a tool for candidate biomarker discovery. A large range of biomolecules, spanning multiple molecular classes, can be simultaneously analyzed without predefining the molecules of interest (e.g., labeling). This multiplex, nontargeted analysis enables a comparative analysis of the biomolecular content of diseased samples and, as such, may be used to complement

established histopathological practice. However, before imaging MS can be routinely used in a clinical setting, a large number of patient samples need to be compared to provide statistical foundation necessary for a new diagnostic tool. Good collaborations with pathologists and surgeons are crucial for ensuring the quality of the samples and the interpretation of the subsequent experimental results.

Designing an imaging MS experiment includes several important aspects that will optimize signal quality. Sample preparation for imaging MS plays a crucial role in generating reproducible quality data, and this is described below.

8.2 METHOD OVERVIEW

The workflow of a typical MALDI (matrix-assisted laser desorption/ionization) imaging MS experiment is given in Fig. 8.1, and all different steps are described in detail in this section. Sample handling, from the point of collection and isolation to the final data acquisition, is one among the most crucial steps in imaging MS for achieving qualitative and reproducible results. Goodwin *et al.* [31] have given a schematic representation of the MALDI imaging MS workflow, with an overview of which factors need to be considered to minimize variation and which factors can be used to optimize performance based on four different key stages (tissue collection, tissue cutting, tissue pretreatment, and matrix application), which we have discussed in the following paragraphs (Fig. 8.2). It is essential to maintain the integrity and spatial localization of the molecules in the original tissue. Since the introduction of imaging MS, many reviews have been published that address sample handling and sample preparation [30,32–37].

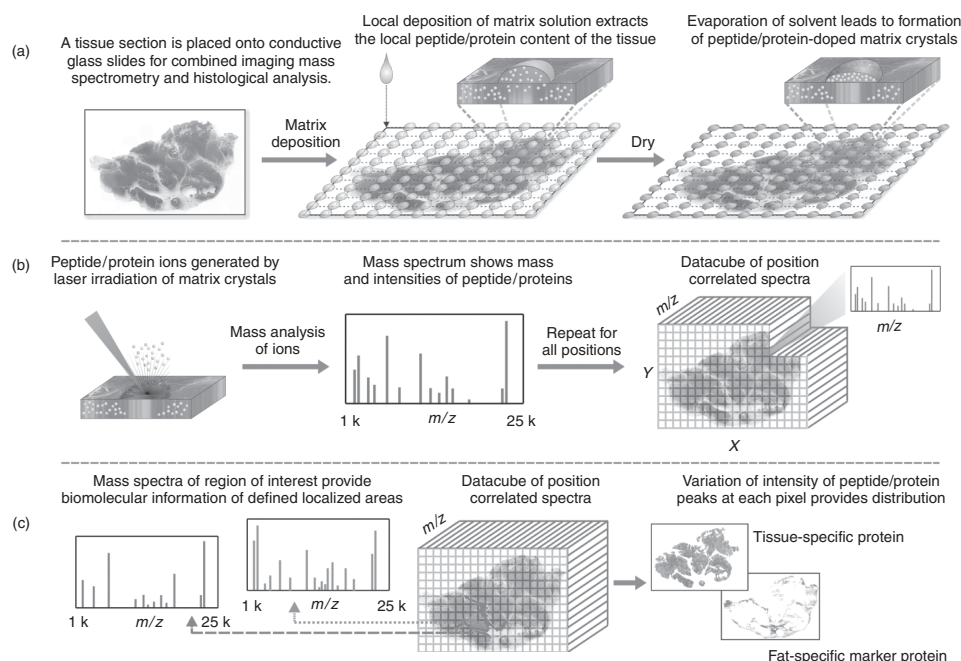


Figure 8.1 Schematic of the MALDI imaging MS workflow.

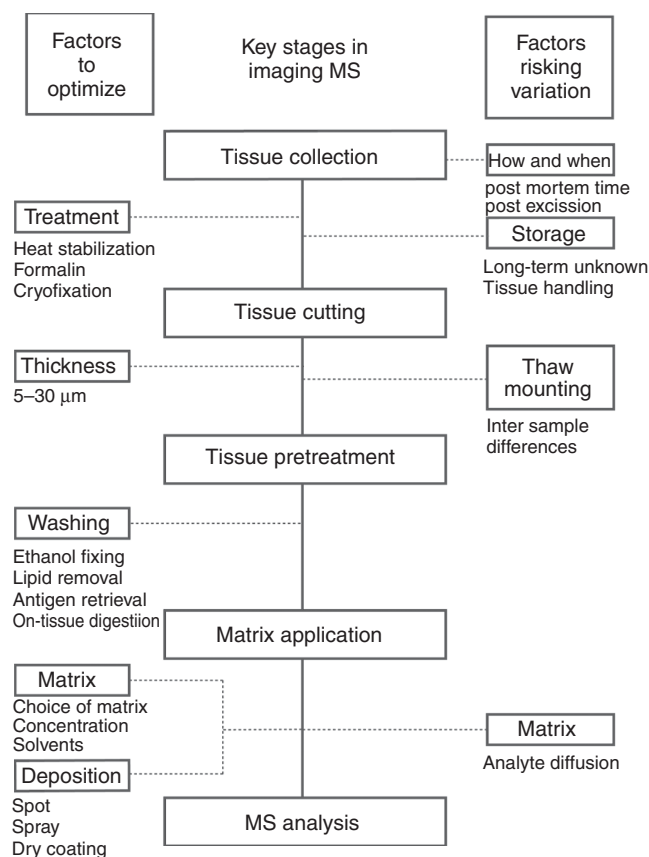


Figure 8.2 MALDI imaging MS workflow and the experimental factors that must be considered to minimize variation and optimize performance. The manual nature of these steps can make them highly prone to user artifacts unless stringent protocols are followed [38].

8.2.1 The Tissue Sample

The integrity of a tissue sample is key to any analysis of its biomolecular content. An imaging MS procedure starts with tissue collection, and this is the first important step toward high quality and reproducible results. Tissue collection must be performed in accordance with the appropriate ethical guidelines. It has been shown that postmortem changes can occur within minutes [31]. Techniques to limit the impact of postmortem degradation suppress enzymatic activity by denaturation of proteins, chemical fixation, or cryofixation. In animal studies, a common condition to preserve tissue morphology as well as the native distribution of biomolecules is that tissues must be rapidly dissected following animal sacrifice and flash-frozen in liquid isopentane or nitrogen. For the collection of tissue specimens derived from humans, standardized protocols and well-documented procedures should be agreed on/consulted with the surgeons and the pathologists. After freezing, tissues should be stored at -80°C .

The majority of the imaging MS studies have been performed on flash-frozen tissue sections, although recent developments have enabled imaging of formalin-fixed paraffin-embedded (FFPE) tissues [39,40]. Formalin fixation stabilizes proteins by

chemical cross-linking, thus preventing postmortem enzymatic proteolysis while maintaining the cellular histology [41]. FFPE tissue blocks are stored at room temperature. The cross-linked proteins are difficult to extract from the tissue with standard matrix application procedures. To circumvent the cross-linking induced by formalin fixation, a protocol based on ethanol fixation [42] or on RCL2/CS100 fixation (a non-cross-linking organic fixative [43]) in combination with paraffin embedding has been developed and proved to be suitable for proteomic analysis and tissue imaging. The advantage of using FFPE tissues for imaging MS is the large existing collections of FFPE tissues in hospital tissue banks: The ability to search for biomolecular signatures associated with specific pathological features and known clinical outcomes would represent a powerful tool for clinical biomarker research. However, in order to achieve this goal, it will be necessary to identify which tissues are still suitable for analysis, as the retrospective nature of the tissue banks means that many of the tissues may have been collected and processed using methods less suited to modern bioanalytical techniques.

After freezing, the tissue can be fixed to the cryostat stage using a droplet of water or 10% gelatin. The use of optimal cutting temperature (OCT) embedding media should be avoided as much as possible because this compound includes many water-soluble polyethylene glycol compounds that significantly interfere with the imaging MS experiment [32]. The potential interference from these compounds is such that if one is using a shared cryostat, the cryostat should be comprehensively cleaned before use. When an embedding medium is required for sectioning, 10% gelatin or agarose can be used [44]. The frozen tissues are then sectioned and transferred onto cold indium-tin oxide (ITO)-coated glass slides [45] or other holders suitable for imaging MS experiments. It is important to deposit the section uniformly without scratches [46]. The tissues are normally mounted onto the target plate or glass slide by gently heating the underside of the slide with a finger. Immediately after sectioning, the tissue section should be dried for at least 10 min in a vacuum desiccator to remove residual water from the tissue.

To date, the majority of imaging experiments has been focused on the analysis of thin frozen tissue sections. The sections, 5–50 μm thick, are collected at -5 to -25°C using a cryomicrotome. For each tissue, the cutting temperature may need to be optimized. Thicker sections are easier to collect; however, thinner sections have been shown to provide higher quality mass spectra [47]. When a study is set up, wherein multiple sections are required, it is recommended to collect these sections at the same time to prevent repeated handling of the tissue, although side effects such as oxidation may increase [38].

Goodwin *et al.* [31] showed postmortem degradation in brain tissue section. The intensity of a range of markers was seen to vary when tissue sections were kept on room temperature and on timescales of 30 s to 3 h; while some peptides/proteins were stable, the intensities of several peptides were found either to decrease (degradation) or increase (degradation product) (Fig. 8.3). Furthermore, it was shown that within each of these classes, there is clearly a range of rates at which the markers are observed to change, which could be location dependent. These results indicate that warming a sample for a short period, for example, during sectioning and mounting, may perturb the markers observed, in particular, when human tissue samples retrieved from the hospital are used. To guard against misinterpreting artifacts as real markers, a rigorous sample handling procedure should be employed. Approaches to prevent post-mortem degradation have been developed, for example, Denator AB has released the

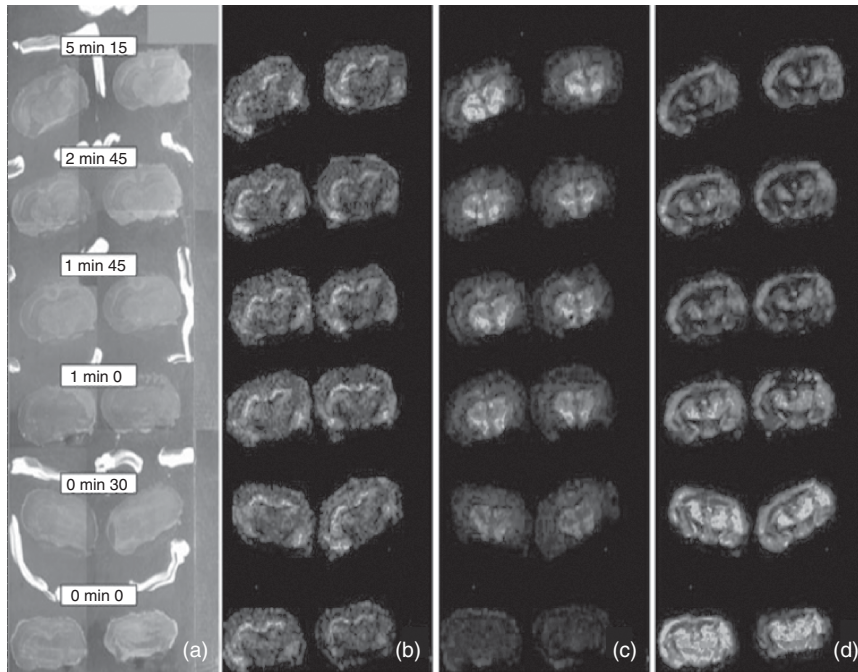


Figure 8.3 Investigation of postmortem degradation in MALDI imaging MS. Optical and MALDI imaging MS analyses of 14- μm -thick mouse brain sections thaw-mounted onto ITO-coated slides. (a) Optical image of the mounted transcoronal sections. Strong white areas are Tipp-ExTM spots applied to the slide to coregister images. MALDI imaging MS analyses after 0, 0.5, 1, 1.75, 2.75, and 5.25 min for peptide peaks at m/z (b) 2608, (c) 2479, and (d) 6724 [31]. (See color insert.)

tissue stabilization system, Stabilizer T1 (Denator AB, Sweden), that rapidly inactivates enzymes by heat-induced denaturation and reduces postmortem degradation to improve the analysis of fresh and frozen tissue samples [48,49]. In an initial study, it was shown that posttranslational phosphorylations of analyzed proteins were stable and peptide extracts were devoid of abundant protein degradation fragments [48]. However, it should be noted that after treatment with the Stabilizer T1, the tissues could be more fragile and therefore difficult to handle. Goodwin *et al.* [49] have reported that changes to the tissue structure have also been found to have a negative effect on the quality of the imaging MS data, although they improved the stability. Given this information, it is imperative in clinical investigations that tissue collection protocols should be standardized to reduce the likelihood of sample-processing artifacts.

8.2.2 Sample Preparation

8.2.2.1 Wash. Washing the tissue sections before matrix deposition has proven to be useful to improve spectra quality by increasing the ion yields and the number of signals observed [50]. One of the main purposes of washing tissue sections is to remove salts, lipids, and excessive amounts of hemoglobin to enhance MALDI signals. The most common washing procedure consists of a 30-s wash in ice-cold 70% ethanol

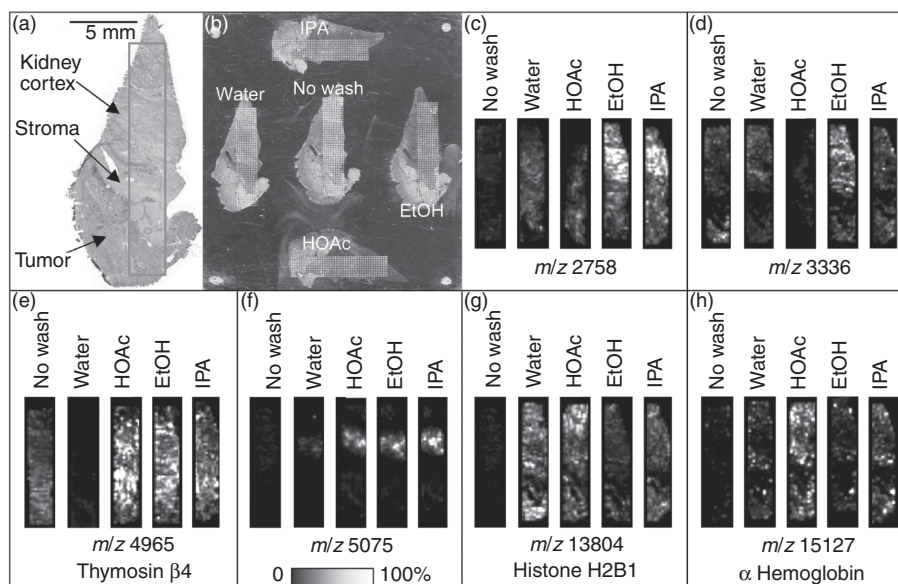


Figure 8.4 Imaging MS analysis of serial sections cut from a clear cell renal cell carcinoma resection not washed or after washing with isopropanol, ethanol, acetic acid, or water. (a) Photomicrograph of a serial H&E stained section detailing the histology of the biopsy. Areas of normal kidney cortex, stroma, and tumor are visible. The area outlined was analyzed. (b) Photomicrograph of the sections used for IMS (ion mass spectrometry) after washing and automated matrix deposition. (c–h) Ion density maps for selected protein signals from the unwashed and washed sections [50]. HOAc, acetic acid; EtOH, ethanol; IPA, isopropanol. (See color insert.)

to remove salts and cell debris, followed by a 100% ethanol wash to dehydrate and fixate the tissue. For the removal of lipids, tissue sections can be washed in organic solvents, for example, chloroform or xylene [47]. In general, the washing conditions must be optimized for specific imaging MS experiments since different tissues may require different washes.

The washing strategy also influences which biomolecules can be analyzed. Seeley *et al.* [50] systematically explored the effects of several solvent combinations for washing tissue sections. Different molecules were detected after different washes, so combining different washes can create complementary data sets describing different molecules (Fig. 8.4) [50].

It is important to be aware of the fact that there is always a potential risk of molecular diffusion during any wash procedure. Tissue sections that are fixed can be kept for several days (up to seven) in a desiccator without noticeable degradation of MALDI spectra [45]. For small-molecule analysis, such as drugs and metabolites, the wash procedures are usually omitted as they may easily delocalize the analyte of interest.

Several methods have been reported that attempt to reverse formalin fixation to “retrieve” antigens. Antigen retrieval typically involves the application of a high temperature treatment along with the use of a buffer solution, in an effort to reverse protein cross-linking and return the tissue to its native state [40]. A schematic representation is given in Fig. 8.5. First, the paraffin is removed from the FFPE sections

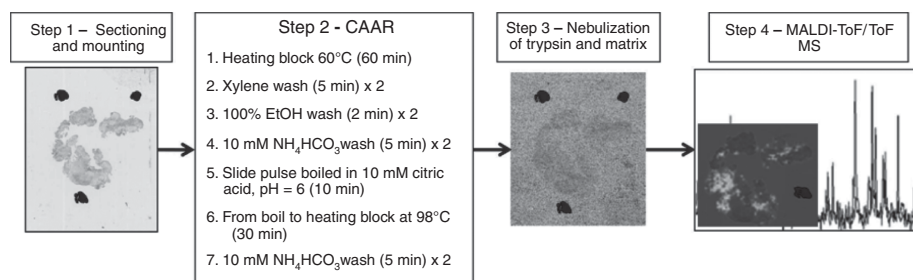


Figure 8.5 Citric acid antigen retrieval (CAAR) method applied to imaging MS of FFPE tissue. In step 1, sections cut from the FFPE block are mounted onto an ITO-coated slide. Step 2 describes the antigen retrieval process. In step 3, the proteins are cleaved into proteolytic peptides and then extracted by the deposition of the MALDI matrix solution. Step 4 shows an example of the mass spectra and images that can be obtained [53].

using xylene (100%, twice for 20 min), followed by graded ethanol washes (100%, twice for 5 min, and successive washes of 95%, 80%, and 70% ethanol for 5 min). Then the tissue can be treated either by heating the section in a tris-EDTA buffer pH 9 at 95°C for 20 min [51,52] or by using a high temperature citric acid buffer [53].

The methylene bonds are difficult to break without destroying the peptide backbone. To solve this problem, endopeptidases such as trypsin are used directly on tissue sections to break down the protein network and retrieve the digested peptides that can additionally be used for protein identification [28].

Detection of intact proteins of a molecular mass >25 kDa has been much less successful than that of peptides and small proteins. One strategy to analyze those proteins is based on proteolytic digestion of the tissue's proteins followed by MALDI imaging MS of their tryptic peptides. In principle, this “bottom-up” strategy could enable proteins of any mass to be detected. The disadvantage is that all information regarding protein isoforms is lost. For example, in an on-tissue digestion investigation, the glucose-regulated protein 78 kDa (Grp78) was identified as being associated with human pancreatic adenocarcinoma (often referred to as *pancreatic cancer*). However, in a subsequent article, Scholz *et al.* [54] reported that several Grp78 isoforms in murine pancreas displayed significant changes solely due to postmortem protein degradation (F-test $p < 0.05$). It is much more difficult to control protein degradation in human tissue samples, and unless the tryptic peptides contain the specific posttranslational modifications of a protein's isoforms, information regarding protein isoforms is omitted.

Several strategies based on tissue washes have been developed to detect proteins of higher molecular mass. For example, a 28-kDa integral crystalline lens membrane protein could be detected after extensive washing with water to deplete highly abundant soluble proteins, followed by application of a matrix solution containing a high percentage of organic solvent [55]. This procedure would be difficult to apply as a general strategy as its success is based solely on extensive on-tissue protein purification of membrane proteins (which remain anchored to the insoluble membrane during the repeated water washes).

Another strategy has used a layered matrix preparation that enabled proteins in the 25- to 50-kDa range to be detected. It was found that Triton X-100, compatible with MS up to a concentration of 1%, aided in the detection of higher m/z proteins from tissue [56]. In short, a tissue section is placed on a drop of sinapinic acid (SA,

3,5-dimethoxy-4-hydroxycinnamic acid) matrix dissolved in 90% EtOH and 0.5% Triton X-100. Once dry, a drop of SA/xylene suspension was placed on top of the tissue section, followed by a layer of SA/90% EtOH and a layer of SA/50% acetonitrile (ACN). Franck *et al.* [57] developed a sample preparation strategy using the solvents 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2,2,2-trifluoroethanol (TFE) for detection of high mass proteins with MALDI MS. Unfortunately, the very low viscosity and harsh chemical abrasiveness of HFIP and TFE make it difficult to apply this procedure with the automated sample preparation stations commonly used for MALDI imaging MS.

8.2.2.2 Matrix. The correct choice of matrix is crucial for a successful MALDI imaging MS experiment. The matrix, usually an aromatic acid, is used to absorb energy from the irradiating light source (usually a UV laser). Ablation of the matrix and gas-phase chemistry leads to the generation of gas-phase pseudomolecular ions [58]. Depending on the mass range analyzed and chemical properties of the molecules of interest, the matrix and solvent conditions should be chosen accordingly [36,45, 59–61].

The matrix α -cyano-4-hydroxycinnamic acid (CHCA) is commonly used for lower molecular weight masses (peptides <3000 Da), while SA is used for higher molecular masses (peptides >3000 Da and proteins). Phospholipid analysis typically involves either 2,5-dihydroxy benzoic acid (DHB) or 2,6-dihydroxyacetophenone (DHAP), and finally, CHCA and DHB are also often used for pharmaceuticals [62,63]. Ionic matrices, composed of a binary mixture of the matrix and a base (e.g., CHCA with aniline or *N*, *N*-dimethylaniline), have been reported to provide increased sensitivity and higher resistance to vacuum sublation [47]. The matrix 9-aminoacridine (9-AA) is used for the analysis of acidic metabolites and glycerophospholipids [64,65]. A summary of different matrices is given in Table 8.1.

The goal of the matrix solution is to effectively dissolve the analyte of interest and thus extract it from the tissue. The matrix solution typically consists of an organic solvent such as ACN or methanol, water, the matrix, and trifluoroacetic acid (TFA). The solvent composition, the matrix concentration, and the TFA concentration have all been shown to affect the protein profile obtained from a tissue [32]. It is for this reason that the matrix solution needs to be optimized for each application and then carefully and reproducibly applied to the tissue in order to obtain images that accurately represent the distribution of the molecules of interest within the tissue [32].

8.2.2.3 Matrix Application. MALDI MS requires the presence of matrix crystals. Imaging MS has been used to investigate the distribution of analytes within matrix crystals and has indicated a partial segregation within and between the crystals [66].

TABLE 8.1 A Summary of Matrices used in MALDI Imaging MS Experiments

Abbreviation	Chemical Name	Application
DHB	2,5-Dihydroxybenzoic acid	Lipids, peptides, carbohydrates
CHCA	α -Cyano-4-hydroxycinnamic acid	Peptides
SA	Sinapinic acid	Proteins
DHAP	2,6-Dihydroxyacetophenone	Lipids
PA	Picolinic acid	Oligonucleotides
9-AA	9-Aminoacridine	Metabolites

To avoid such artifacts, the matrix crystals need to be significantly smaller than the pixel size of the image. For imaging MS of peptides and proteins, the most common application, the analyte molecules need to be incorporated into the growing matrix crystals for high sensitivity analyses. In general, larger crystals have extracted more analyte from the tissue, leading to higher signals, but generate lower spatial resolution images. Smaller crystals maintain high spatial fidelity, enabling higher spatial resolution images to be obtained, but contain less extracted analyte and are thus associated with lower sensitivity.

Initial imaging MS experiments used a manual TLC (thin-layer chromatography) sprayer to apply a uniform matrix layer onto the tissue, and this remains one of the first methods used by new groups entering the field because of its low cost. However, it is generally associated with low reproducibility and the use of one of the commercially available automated systems is strongly recommended. These devices are based on either microspotting or spray deposition. The microspotters sequentially deposit 100- to 200-pL droplets of matrix solution according to a predefined array, whereas the sprayers deposit sequential layers of small droplets over the sample.

The important factors to consider are how does the deposition method affect extraction of the tissues' peptides and proteins? and does the deposition method influence matrix crystallization? Sequential deposition of tens of 100- to 200-pL-sized droplets, such that each position of the array is sampled using nanoliter-sized droplets of matrix solution, has been found to be highly effective at dissolving peptides and proteins in the tissue and incorporating them into the matrix crystals. For efficient extraction, the spray devices need to apply a sufficiently "wet" layer of matrix onto the tissue, but care must be taken to avoid depositing too many droplets onto the tissue, as flooding causes lateral migration. Several instruments have been developed to apply the matrix, for example, the ImagePrep device (Bruker, Germany) and the TM Sprayer (Leap Technologies, USA). The ImagePrep device attempts to prevent flooding and control matrix deposition by using an optical sensor to monitor the sample's wetness as well as the thickness of the matrix layer. Alternatively, the TM Sprayer uses a heated spray to ensure more rapid solvent evaporation. Using these commercial spray devices or a laboratory-built system to deposit multiple layers of matrix solution (incorporating multiple steps of peptide and protein extraction, redissolution, and recrystallization) a uniform layer of peptide/protein-doped matrix crystals can be obtained. Following matrix coating, samples maybe coated with a thin layer of metal such as gold to avoid sample charging and thereby increase the detected signals [39,40].

Several solvent-free matrix dry-coating methods have been developed for small-molecule analysis. Matrix sublimation can produce an even layer of small crystals across the tissue section [67]. The deposition can be readily controlled with time, temperature, and pressure settings and is highly reproducible from one sample to the next. This technique eliminates the potential for analyte migration during solution-based matrix application and produces very small crystals that are necessary for high spatial resolution measurements. However, the dry-coating approach is not suitable for peptide or protein analysis because these compounds need to be incorporated into the matrix crystals.

For the detection of lipids on tissue sections, a 20- μm sieve has also been used to deposit finely ground matrix onto tissue. The lipid signals obtained were comparable to those from spray-coated sections, producing identical localization patterns even with this simpler and faster sample preparation [68]. This approach was found to yield highly

reproducible results, eliminating much of the variance caused by operator differences and making it an attractive alternative to solvent-based methods. A similar method uses a ball-mill device to press the matrix through a metal mesh. The mesh (20 μm) produces 1- to 12- μm crystals (DHB, CHCA matrices) in 1 min, as determined by optical microscopy, permitting fast uniform matrix coverage. Even 1- to 5- μm sized crystals could be obtained using a 3- μm mesh [69].

8.2.3 The Experiment

8.2.3.1 The Spatial Resolution. The spatial resolution of an imaging MS experiment determines the size of the spatial features that can be resolved. The resolution is dependent on the sample preparation, signal intensity per pixel, the capabilities of the instrument (laser spot on target), and the distance between raster points. The laser spot size is dependent on the ion source, the focusing optics, and the geometry of the source. The spatial resolution desired is an important factor in determining the method of matrix application.

Imaging by using an array of small discrete droplets, deposited using an automated spotting device, limits sample migration to within the diameter of the matrix droplet, typically $\approx 150\ \mu\text{m}$. An array of such spots, with a 200- μm pitch, would be referred to as having a spatial resolution of 200 μm .

Recently, McDonnell *et al.* [70] indicated the protein concentration range accessible for MALDI imaging MS (Table 8.2). The table shows the amount of a specific protein in each pixel as a function of spatial resolution, tissue thickness, and protein concentration. Assuming a detection limit of 1 attomol (10^{-18} mol), a 10- μm pixel is the highest spatial resolution achievable for a 1- μM peptide present in a 10- μm -thick tissue section. At smaller pixel sizes, there is simply insufficient peptide/protein available to detect it using current MALDI strategies. In practice, the achievable resolution will be less because the efficiency of protein extraction, ionization, and detection is lower than 100%. Without significant improvements in sensitivity, high spatial resolution analysis will be limited to more abundant species that ionize well, such as lipids or peptides in endocrine tissues [70]. For example Rompp *et al.* [71] have reported high mass resolution and high spatial resolution (around 5 μm) of lipids and peptides in pituitary tissue.

The spatial resolution limitations highlighted here refer to the amount of protein present in a voxel and thus would also apply to a profiling workflow utilizing laser-capture microdissection, protein extraction, trypsin digestion, and LC-MS/MS. MALDI analysis of tissues can generate mass spectra containing hundreds of distinct peptide and protein peaks from a single $150 \times 150\text{-}\mu\text{m}$ pixel. Assuming an average cell size of 20 μm , this corresponds to < 60 cells. This is much smaller than the samples typically analyzed in a proteomics workflow using protein extraction and LC-MS/MS analysis and is a testament to the successful on-tissue fractionation of the ion, lipid, peptide, and protein components. Cornett *et al.* [12] have shown how the specificity and reproducibility of direct tissue profiling can be similar to those obtained using laser-capture microdissection but used far fewer cells.

8.2.3.2 Identification. In an imaging MS experiment, different proteins are distinguished and characterized by their intrinsic molecular mass; however, additional experiments are necessary to assign identities to these masses. In-source decay can be used to obtain N-terminal sequence information to identify proteins in a “top-down” strategy [72]. However, the lack of a precursor ion selection step can make

TABLE 8.2 Total Amount of a Specific Protein as a Function of Pixel Size, Tissue Thickness, and Protein Concentration^a. Combinations highlighted in green may be considered accessible in a modern MALDI ToF mass spectrometer

Concentration [M]	Tissue Thickness 10 μm					Tissue Thickness 2 μm	
	100 μm	50 μm	20 μm	10 μm	5 μm	5 μm	2 μm
1.00E−03	100 fmol	25 fmol	4 fmol	1 fmol	250 amol	50 amol	8 amol
1.00E−04	10 fmol	2.50 fmol	400 amol	100 amol	25 amol	5 amol	0.8 amol
1.00E−05	1 fmol	250 amol	40 amol	10 amol	2.5 amol	0.5 amol	0.08 amol
1.00E−06	100 amol	25 amol	4 amol	1 amol	0.25 amol	0.05 amol	0.008 amol
1.00E−07	10 amol	2.5 amol	0.4 amol	0.1 amol	0.025 amol	0.005 amol	
1.00E−08	1 amol	0.25 amol	0.04 amol	0.01 amol	0.0025 amol		
1.00E−09	0.1 amol	0.025 amol	0.004 mol	0.001 amol			
1.00E−10	0.01 amol	0.0025 amol					
1.00E−11	0.001 amol						
1.00E−12							

^aRef. 70.

in-source decay more difficult to apply to the analysis of complex samples such as a tissue section. Furthermore, the low charge state (the majority of MALDI-generated ions have unit charge) and large size of proteins make ion activation methods such as collision-activated dissociation inefficient. For these reasons, protein identification is normally performed using LC-MS/MS of tissue extracts or via on-tissue digestion protocols [39]. On-tissue digestion protocols deposit a trypsin solution in the same way as used for matrix application, thus forming proteolytic peptides of each protein while maintaining their original spatial distribution. Several groups have now demonstrated that on-tissue digestion combined with MALDI imaging MS can be used to identify and localize proteins through their tryptic peptides; among others, it has been applied to lung cancer [28], breast cancer [51], prostate cancer [73], and pancreatic adenocarcinoma [74]. Recently, N-terminal peptide derivatization strategies were shown to provide improved peptide identification by directing the fragmentation to provide a complete y -fragment series [75]. The increased complexity of the composition of the tissues following on-tissue digestion (every protein will form many different proteolytic fragments) means that on-tissue digestion and MALDI imaging MS analyses reporting new protein biomarkers will still need to be independently verified using additional means. Cazares *et al.* [73] recently used a combination of intact peptide and protein MALDI imaging MS, on-tissue digestion, and immunohistochemical staining (IHC) to demonstrate the localization of a new candidate protein biomarker in prostate cancer tumors. To identify lipid species, preliminary peak assignments can be obtained from the LIPID MAPS database (<http://lipidmaps.org>) and then further confirmed by performing MS/MS analysis. Neutral losses can provide headgroup information as well as identify the fatty acid groups of the lipids [76].

8.2.3.3 Mass Analyzers. Imaging MS has benefitted from recent technological innovations in the MS field. The most commonly used mass analyzers for imaging MS are ToF (time of flight) (linear, reflectron, and orthogonal), quadrupoles, hybrid instruments such as ToF/ToF or Q-ToF (quadrupole time of flight), ion-mobility ToF, and ion trap (IT) (QIT, Orbitrap, and FT-ICR (Fourier transform ion cyclotron resonance)). An overview of the commercial instruments that are used in the MALDI imaging MS field and their performance capabilities can be found on www.maldi-msi.org.

The majority of MALDI imaging MS is performed using ToF mass analyzers because they offer the combination of parallel detection of a broad mass range, high sensitivity, and high speed. The linear geometry is commonly used to image proteins because of the need for a high mass range, while the reflectron geometry is used to resolve and identify endogenous peptides and tryptic peptides [this geometry is more limited in mass range (<6 kDa)]. An orthogonal ToF enables the performance of the mass analyzer to be independent of the ionization step, thereby maintaining optimum performance of the mass analyzer throughout the tissue section [77]. The addition of ion mobility to a mass analyzer adds an additional, partially independent, separation dimension that distinguishes between ions on the basis of their gas-phase cross section and thereby further improves the separation of drugs, metabolites, lipids, and peptides [78]. The Fourier transform techniques, FT-ICR and Orbitrap, provide very high mass resolution capabilities, which can distinguish between isobaric species and thus is very useful for imaging lipids, drugs, and metabolites [79].

The detection sensitivity of the typical microchannel plate detector used in a ToF mass analyzer decreases at higher m/z because of the lower intrinsic ion-to-electron

conversion efficiency of slower moving ions and detection saturation [80]. When a ToF MS is equipped with a high mass detection system (CovalX, Switzerland), the detection of macromolecules, up to 1.2 MDa, with nanomolar sensitivity can be achieved. Recently, we showed that this detector with the sample preparations described above allowed the detection of large ions (>50 kDa) direct from tissue with higher sensitivity [81].

Hopfgartner *et al.* [82] have reported the use of a multiple reaction monitoring (MRM) instrument for MALDI imaging of pharmaceuticals and their metabolites in whole-body sections. MRM instruments can provide higher sensitivity than other mass analyzers, as well as very high speed, but require the mass and tandem mass spectra of each analyte of interest to be known before the experiment. MALDI MRM imaging is limited to the analysis of a small number of species owing to the finite number of laser shots that can be applied to a specific region of tissue and still yields biomolecular ions; nevertheless, the high sensitivity and high speed of the technique could make it a powerful tool for validation of candidate biomarkers in clinical tissue cohorts [83].

These examples demonstrate that high quality MALDI imaging MS experiments can be performed with most modern MALDI mass spectrometers, provided the samples have been prepared well. Sample preparation and the analysis of the resulting data are frequently the limiting factors in the workflow. Newly released MALDI mass spectrometers continue to offer improvements in speed, higher mass resolution, higher spatial resolution, and additional separation dimensions; all of which combine to generate larger data sets that need to be efficiently analyzed to take full advantage of the improved data quality and quantity.

8.2.3.4 Quantitation. Tissue-specific response curves have been used to quantify the amount of drug compounds in animal tissue. A dilution series of drug standards is applied on a set of tissues and the average response curve is determined [63]. For whole-body imaging MS experiments, it has been reported that the determination of relative response factors, for each organ in the whole-body section, could provide quantitative results in agreement with whole-body autoradiography [84]. This was performed by applying a uniform coating of the drug over an adjacent section and using the MALDI-generated ion signals to determine a relative response. Robust sample preparation methods of identical MALDI imaging MS experiments are crucial for quantifying MS images [63,85].

8.2.3.5 Integrating Histology and Imaging MS. A very important aspect of imaging MS analysis of tissue is to correlate the MS images with the morphological structures shown in histological images. Since the introduction of ITO glass slides, which enabled staining and imaging MS of the same tissue section [86], it is now a more common procedure to H&E stain the tissues after imaging MS analysis. After the measurement, the matrix is removed by washing the sections in 70% methanol twice for 30 s, followed by a standard H&E staining protocol. The optical image of the stained sections can then be coregistered with the MS image. The pathologist can annotate areas of particular interest, enabling a direct comparative analysis of the biomolecular signatures obtained from the specific histology features. Such histology-defined analyses have already led to the identification of several novel biomarkers, for example, in human brain tumors [11,23,27,28,73,87].

There are stains available, such as cresyl violet, Terry's polychrome, methylene blue, nuclear fast red, and toluidine blue, that are MS compatible with a minimal risk of protein delocalization [86]. These nuclear stainings can be of great help in providing higher accuracy for the imaging MS by focusing on specific morphological regions of interest. This technique can also be used in laser-capture microdissection to allow capture and direct analysis of specific cells of interest. Cornett *et al.* [12] have used histology-directed profiling to record the biomolecular profiles of different cell types.

8.2.3.6 Data Analysis. The analysis of imaging MS data can be separated into three distinct steps, namely, data preprocessing, statistical analysis, and visualization. Figure 8.6 shows a schematic representation of the data acquisition, data processing, and correlation strategies, which can be used [88]. First, the mass spectra are processed to reduce random noise (mass spectral smoothing), remove the nonspecific background signal prevalent in MALDI-MS spectra from tissue (background subtraction), correct for inhomogeneities in the matrix coverage (total-ion-count (TIC) normalization), and finally, align every pixel's mass spectrum onto a common m/z scale (mass spectral alignment). It has been shown how each of these steps can significantly improve the qualitative and quantitative information obtained from imaging MS experiments [88].

The statistical analysis performed on imaging MS data sets is largely determined by the goal of the experiment. MALDI imaging MS generates large and highly dimensional data sets that can describe the distributions of hundreds of peptide and protein signals. A comparative analysis of the different cell types in a tumor, as defined by histology, can be used to identify biomarker peptides and proteins specific to each cell type or to find peptide and protein profiles (incorporating multiple peptides and proteins)

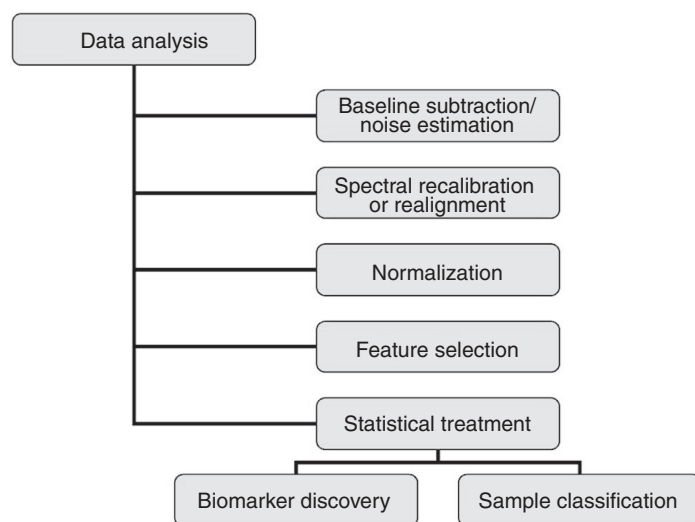


Figure 8.6 Data processing and analysis workflow. The mass spectra are processed by removing the background signal, alignment of each pixel's m/z scale, normalization to each pixel's TIC, and finally peak selection. The reduced data from peak selection can then be subject to statistical analysis in order to identify specific features that discriminate between two (or more) specified classes, biomarker discovery, or to classify tissue based on their biomolecular content [88].

that discriminate between the different cell types. Such supervised, histology-defined classification of tissues has been performed using several different tools, including linear discriminant analysis [26], genetic algorithms [23], and support vector machines [23,28]. Groseclose *et al.* [28] used MALDI imaging MS to analyze a tissue microarray containing 112 needle-core biopsies from lung tumors diagnosed as adenocarcinoma and squamous cell carcinoma. A support vector machine classifier based on 73 tryptic peptides enabled regions marked as adenocarcinoma by a pathologist to be classified with an accuracy of 97.9% (140/143 spectra) and squamous cell carcinoma with an accuracy of 98.6% (141/143 spectra).

These classification studies are based on the prior histological annotation of the tissue. One of the potential advantages of MALDI imaging MS is that it can define a tissue based on its peptide and protein signature and thereby identify regions that display a molecular signature associated with a tumor but which have not yet undergone morphological transformation. For example, MALDI imaging MS analysis (supported by LC-MS) found colorectal-carcinoma-related proteins in histologically benign polyps, from which the authors inferred that these apparently benign polyps could have malignant potential [89]; recently, Willems *et al.* [90] demonstrated that imaging MS could reveal the intratumor heterogeneity of intermediate-grade myxoid fibrosarcoma tumors (which showed the same nodal structure in multiple patient samples).

A variety of data-analysis tools have been applied to examine the innate correlations within MALDI imaging MS data sets [26]. The most common, principal component analysis (PCA), is an unsupervised multivariate technique that maximizes the variance (spread) in the data to identify the peptides or proteins that are responsible for the largest variation in the data set [91]. The result is a series of orthogonal principal components (PCs) that describe, in descending order, the maximum variance in the data set, the next maximum variance (orthogonal to PC1), and so on. PCA is adept at finding the major variances within the data sets but will only reveal the molecular distributions of interest if the peptides and proteins have sufficient signal to noise (for their distributions to be described in earlier PC's than the noise). One of the tools beginning to establish itself in MALDI imaging MS is hierarchical cluster analysis [27]. This technique groups the pixels according to a hierarchical ordering of the similarity of their spectra. The resulting dendrogram displays the hierarchy and provides a simple tool to investigate the relationships between the pixels and thus to navigate the spatial variation of the tissue's peptide and protein profiles.

An imaging MS analysis creates a mass spectrum for every pixel of the image, each of which contains a multitude of peaks that describe the mass and intensity of specific biomolecules. These rich multiplex analyses generate very large data sets, which has limited most analysis of the innate variation in the biomolecular content of the tissue to a small numbers of tissues. Recently, McDonnell *et al.* [92] developed automated feature detection and extraction algorithms for imaging MS data sets that can effectively distill each pixel's mass spectral data into the features of interest and thereby reduce the data load by $\times 100$ –1000.

Finally, the visualization of 2D and 3D imaging MS data, whether the distribution of a single species or the results of a statistical analysis, is typically achieved using false color images. It is recommended that the user limits the number of channels (masses or results of data analysis) to a maximum of three. Three channels can be effectively communicated using RGB scales and clearly display overlapping regions in another color. A number of software packages are available to generate images from data

generated in an imaging MS experiment, for example, BioMap, Fleximaging, Axima2 Analyze, and Image Quest.

8.2.4 Novel Approaches—Targeted Analysis, Tissue Stretch, and Automation

Several methods have been developed that provide new variations to the imaging mass technique.

8.2.4.1 Targeted Imaging MS. This technique has been developed to enhance the specificity of the analysis. Functionalized antibodies carry a photocleavable reporter group that, can be detected by MALDI MS. The antibodies interact with an antigen of interest, and the localization of this antigen can be determined by analyzing the photocleavable probe. Two different methods have been developed: TagMass [71] and Targeted multiplex MS IMaging (TAMSIM) [93,94]. The TagMass procedure is based on specific MALDI imaging of proteins using a probe labeled with a photocleavable linker attached to a small peptide. The small peptide acts as the reporter molecule detected in the mass spectrometer and thus is used to image the distribution of the antigen. TAMSIM uses small, intrinsically ionic molecules as the reporters, for enhanced sensitivity. Different reporter molecules can be designed, allowing multiple markers to be tagged (Fig. 8.7) [93,94].

The tissue is incubated with the functionalized antibodies, up to three different tags have been reported in a single assay, without the risk of mutual quenching that can

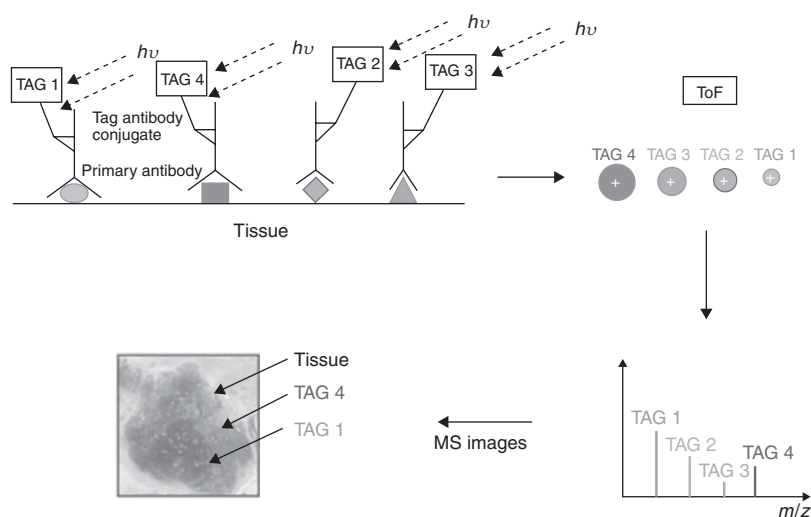


Figure 8.7 TAMSIM: mass spectrometry analysis of mass-tag conjugated antibodies. Primary antibodies are incubated with the tissue section to bind with the antigen in the tissue. Secondary antibodies that each carries a specific mass tag are specifically attached to each primary antibody. The slide containing the tissue is then introduced into the mass spectrometer, where the pulsed UV laser of the MALDI ion sources cleaves the mass tags from their antibodies and releases them into the gas phase. The m/z values of the mass tags, provided by the mass spectrometer, differentiate between the different mass tags and thus provide antigen specificity. Spatially correlated analysis enables the distribution of each antigen in the tissue [93]. (See color insert.)

occur in fluorescence imaging. After irradiation of the tissue with laser, the reporter molecules are released from the antibodies and detected in the mass analyzer. Using labeled oligonucleotide and antibody probes, Lemaire *et al.* [95] have demonstrated how TagMass can be used to record the distribution of proenkephalin mRNA and a 180-kDa membrane protein. Recently, Thierry *et al.* [93,94] used TAMSIM to localize synaptophysin, chromogranin, insulin, calcitonin, and somatostatin in pancreatic neuroendocrine cells and the cancer markers PS100 and HMB45 in human lymph node and liver invaded by metastatic melanoma. Some of these proteins are below the detection threshold in a standard imaging MS experiment. Moreover targeted imaging MS can be applied to both frozen and FFPE tissue sections.

8.2.4.2 Tissue Stretch. In this method, a tissue section is placed onto an array of glass beads embedded in a parafilm membrane [96]. By stretching the membrane, the tissue section is fragmented into small individual pieces ($\sim 40\mu\text{m}$ in size) that can be individually prepared for MALDI-MS analysis. The physical separation between beads prevents analyte redistribution during matrix application and thus allows larger matrix droplets to be applied, thus enabling longer analyte extraction periods and increased sensitivity. This sample preparation has also been found to reduce the presence of alkali metal adducts [96]. This technology has been made more practical by the development of automated image reconstruction algorithms [97].

8.2.4.3 Automation. The low throughput of imaging MS has been one of the bottlenecks in investigating the clinical potential of using the multiplex molecular images as a novel molecular histological technique. Recently, an automated setup, consisting of a controlled environment sample storage chamber, a sample loading robot, and a MALDI-ToF/ToF MS, all controlled by a single user interface, was developed [98]. This approach in combination with the new high speed ToF/ToF instruments such as the AB Sciex ToF/ToF 5800, the ultrafleXtreme ToF/ToF from Bruker Daltonics, and the AB Sciex FlashQuant Triple Quad will provide the robustness and increased throughput for high throughput analyses.

8.3 APPLICATIONS

8.3.1 Oncology

Imaging MS can contribute significantly to cancer research as a tool to help diagnose tumors and provide indicators for the susceptibility to recurrence and progression. It can also be used as a discovery tool to detect biomarkers that are specific for early-stage tumor formation. MALDI imaging MS has already demonstrated its ability to distinguish cancerous tissue, differentiate between tumor types and tumor grades, and to locate protein biomarkers. Recently, an extensive review on imaging MS in cancer research was published by McDonnell *et al.* [70].

8.3.1.1 Brain Tumors. Meningiomas can be classified into three major grades: benign (grade I), atypical (grade II), and malignant (grade III). MALDI-MS profiling and imaging experiments of brain tumor tissues have shown how direct tissue analysis can locate gliomas as well as distinguish between the different tumor types,

grades, and subclasses [9,13,16,86,99,100]. For example, the protein thymosin β 4 was found to be primarily located in the proliferating head of the tumor, whereas the protein S100A4 was found localized in the tumor core [16].

Chaurand *et al.* [101] have shown how the levels of the calcium-binding protein S100B (and independently verified using IHC) can be used to distinguish high grade and low grade glioma. MALDI profiling MS has even revealed protein profiles that are correlated with tumor histology and patient survival [17]. Statistical analysis of the MALDI-MS profiles from 108 glioma patients, or the subset corresponding to 57 patients diagnosed with grade IV malignant gliomas, could identify protein profiles associated with shorter-term or longer-term survival.

To further develop imaging MS for clinical applications and to develop a reference database for predictive classification of brain tumors, Agar *et al.* [102] set up a study to evaluate predictive classification of brain tumors using imaging MS. A preliminary classifier based on a support vector machine model was able to distinguish between meningioma image spectra from the nontumor brain and gliomas and thus to enable class imaging of surgical tissue. It was demonstrated that the development of the classifier was very sensitive to data preparation parameters such as recalibration and the peak picking algorithm. Similar conclusions have been previously reported for clinical profiling experiments [103]. The performance of a classification algorithm is sensitive to systematic but nonconstant variations in the data due to differential sample processing, mass analysis methods, or data-processing routines. Consequently, it is crucial for clinical applications that standardized protocols are followed.

Three-dimensional MALDI imaging [19,104] has also been applied to brain tumors, specifically 3D imaging coregistered with *in vivo* magnetic resonance imaging (MRI) [105,106]. The integration of MALDI imaging MS can help establish the molecular phenotypes associated with the anatomical features detected with MRI and may help to assess if these changes in molecular phenotype occur before anatomical transformation [89]. The distribution of the astrocytic phosphoprotein Pea15, a protein previously determined to be elevated in grade III gliomas [17], is shown in Fig. 8.8, volume rendered against the corresponding *in vivo* MRI data. Good agreement could be obtained between the areas highlighted by MRI and areas with high Pea15 content. Figure 8.8b shows a comparison of 2D overlays of the MALDI imaging MS data and different MRI coefficients, as well as the results of a region-of-interest (ROI) analysis comparing regions inside and outside the tumor (in the cortex). It can be seen that both MRI and MALDI imaging MS can distinguish the tumor. The enhanced T_1 (longitudinal relaxation time weighted), diminished T_2 (transverse relaxation time weighted), and diminished ADC (apparent diffusion coefficient) signals observed in the MRI scans may be explained by differences in cell density, water, and protein content.

8.3.1.2 Breast Cancer. Direct MALDI analysis of mouse mammary tumor virus/HER2 transgenic mouse has revealed protein profiles that appear to predict the antitumor action of molecular therapies [13]. When treated with the erbB receptor inhibitors OSI-774 and Herceptin, inhibition of proliferation and induction of apoptosis and tumor reduction were predicted by a >80% reduction in thymosin β 4 and ubiquitin. The effects were time and dose dependent and confirmed by a MALDI imaging MS analysis, in which the spatial distributions of the proteins were inversely correlated with that of the small-molecule inhibitor.

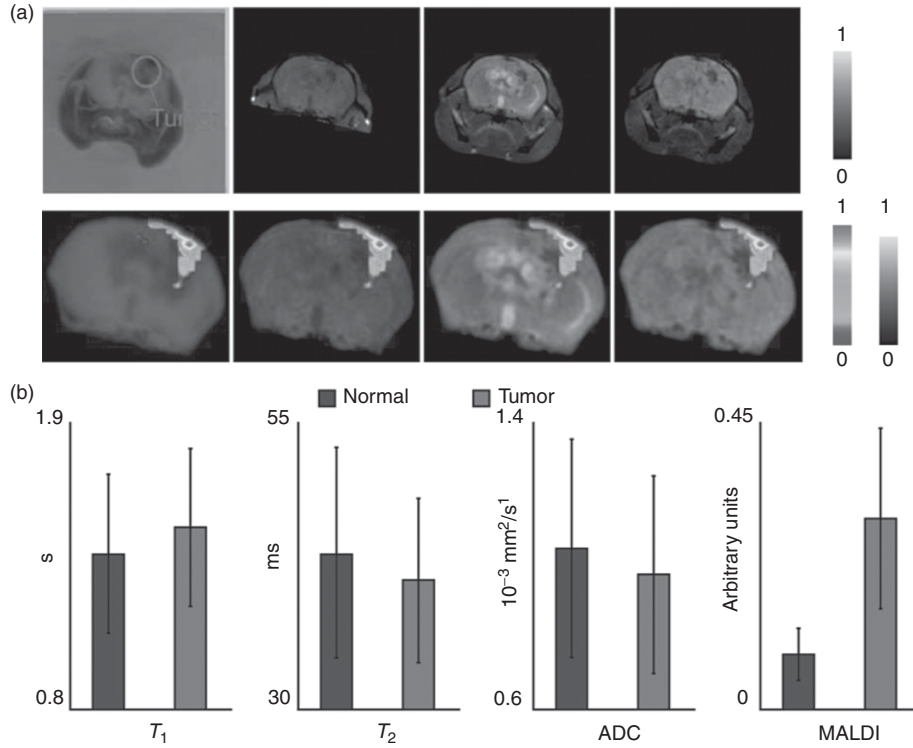


Figure 8.8 Intermodal imaging. (a) Coregistered MALDI imaging MS and magnetic resonance imaging data from a whole mouse head. (Top) Corresponding slices from coregistered blockface, T_{1w} , T_{2w} , and diffusion weighted volumes, rendered in normalized grayscale (0 is black, 1 is white). (Bottom) The same slice with the MALDI imaging MS distribution of the protein Pea 15, a biomarker for grade III glioma, overlaid. (b) Results of a ROI analysis for manually selected normal and tumor tissue [106]. (See color insert.)

Knowledge of estrogen receptor (ER), progesterone receptor (PR), and, more recently, growth factor receptor HER2/neu (ERBB2) status has been critical in the evolution of modern targeted therapy and remains essential for making informed therapeutic decisions. Recently, it was demonstrated that a multiplex assay of the mRNA of these three proteins provided significantly improved diagnostic capabilities and could provide the personal “theranostic test” to guide therapy selection [107]. Rauser *et al.* [108] have demonstrated that MALDI imaging MS may determine HER2 status directly from patient tissues. MALDI imaging MS analysis of 48 breast cancer tissue samples revealed specific peptide and protein expression changes associated with HER2 status, which could classify tissues with 83% sensitivity, 92% specificity, and an overall accuracy of 89%. The cysteine-rich intestinal protein 1 was identified as one of the proteins strongly correlated with HER2 overexpression and demonstrates how MALDI imaging MS may help develop new tissue diagnostic procedures.

MALDI MS has been used to investigate the heterogeneous nature of protein expression in breast cancer tissues [50,105]. The profiling and MALDI imaging results shown in Fig. 8.9a revealed how the different regions of a breast cancer tissue section can

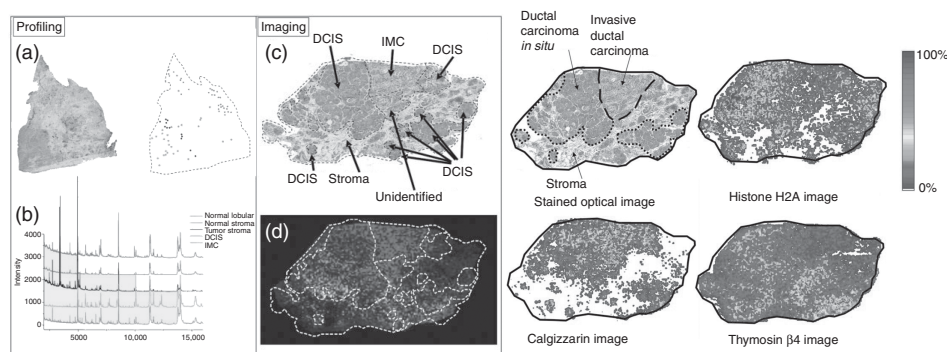


Figure 8.9 MALDI profiling versus imaging MS of human breast cancer tissue. (a) H&E stained tissue, and localized areas selected according to cell type. (b) MALDI-MS profiles obtained from cell types indicated in (a). (c) Histologically annotated H&E stained tissue section. (d) MALDI imaging MS analysis reveals distributions of specific proteins throughout the heterogeneous tissue section [105]. DCIS, ductal carcinoma *in situ*; IMC, invasive mammary cancer. (See color insert.)

generate different protein profiles. The images indicate that the individual proteins have quite heterogeneous spatial distributions but do not appear to follow the histologically annotated regions. Figure 8.9b shows the distributions of three proteins that are clearly associated with the different regions of the breast carcinoma tissue: histone H2A displays its highest abundance in the regions of the tissue histologically annotated as ductal carcinoma *in situ*, calgizzarin in invasive carcinoma, and thymosin $\beta 4$ in stroma [105].

MALDI imaging MS analysis has also been performed on FFPE breast cancer tissues using in-tissue digestion protocols [51,109] or targeted imaging of a known candidate biomarker protein [110]. Instead of endeavoring to find new biomarker profiles, Seuma *et al.* [110] used Au/Ag-tagged antibodies and the quantitative elemental imaging technique, laser ablation inductively coupled plasma MS, to quantitatively image known candidate biomarkers. The technique displayed good reproducibility and quantification, with limits of detection of ≈ 50 fmol for both Au- and Ag-tagged antibodies. The advantage of such targeted approaches is that they can exploit the findings of the large number of serum/plasma-profiling studies that have been undertaken in recent years.

A recent systematic review on the reproducibility of MS-based serum profiling for diagnosis of breast cancer revealed that despite substantial differences in experimental design, experimental conditions, data analysis, and even analytical reproducibility, a common set of discriminating and reproducible peaks could be found, indicating the presence of breast cancer [111]. The targeted imaging of these corroborated proteins in tissue microarrays could be performed using tagged antibody strategies.

8.3.1.3 Lung Cancer. Direct tissue analysis using MALDI profiling MS has been shown to be effective for accurately classifying and predicting histological groups as well as survival in resected non-small-cell lung cancer [112]. From a data set consisting of more than 1600 distinct protein peaks, differentially expressed peaks were found that could classify lung cancer histologies, distinguish primary tumors from metastases to the lung from other sites, and classify nodal involvement. A subset of the proteins, consisting of just 15 distinct protein peaks, was found that could distinguish between

patients with resected non-small-cell lung cancer and had poor prognosis (median survival 6 months, $n = 25$) and those who had good prognosis (median survival 33 months, $n = 41$, $p < 0.0001$). The above profiling study was performed by comparing the protein signatures from 1-mm regions of tissues from 79 lung tumors and 14 normal lung tissues; consequently, each profile effectively averages the signals from the highly heterogeneous tissue in each sample area (tumor regions chosen for analysis contained a tumor cellularity $>70\%$ [112]). The same group recently used MALDI imaging MS to analyze a small lung cancer tissue microarray containing 112 needle-core biopsies. An H&E stained section of the tissue microarray was analyzed by a pathologist using light microscopy and the cancer, noncancer, and normal regions were demarcated in each needle biopsy. This annotated tissue section was then aligned with an adjacent tissue section, which was then used for in-tissue digestion MALDI imaging MS. A subset of the needle biopsies was then used as a training set, in which the origin of each mass spectrum was annotated with the histologist's diagnosis (Fig. 8.10a). Even though the data sets included tryptic peptides that were found to be located in just a subset of each specific class [28], a support vector machine based classifier using 73 tryptic peptide peaks could classify the regions in the test samples (thus not included in the training set) marked as adenocarcinoma by a pathologist, with an accuracy of 97.9% (140/143 spectra), and squamous cell carcinoma, with an accuracy of 98.6% (141/143 spectra) (Fig. 8.10b).

8.3.1.4 Ovarian Cancer. Fournier and coworkers have reported an in-depth investigation for ovarian cancer candidate protein biomarkers using MALDI profiling of tissue extracts and MALDI imaging MS, which showed that the Reg-alpha fragment of the 11S proteasome activator complex is a new candidate protein biomarker. The candidate biomarker was identified by LC fractionation of the proteins from tissue extracts and determination of the appropriate fraction using MALDI profiling, followed by tryptic digestion and LC-MS/MS of the proteins in the fraction of interest. The results of the MALDI imaging MS investigation were then compared with IHC analysis using the anti-Reg-alpha C-terminal antibody and targeted MALDI imaging by using a primary antibody directed against the C-terminal part of human Reg-alpha and a secondary antibody modified with a photocleavable linker and a reporter peptide for MALDI-MS detection. Further analysis of the MALDI imaging data sets has demonstrated that hierarchical cluster analysis is highly effective in distinguishing the different regions of the tissue [113]. Liu *et al.* [114] used imaging MS to investigate the elevation of sulfatides in ovarian cancer in combination with transcriptomic and lipidomic approaches. In Fig. 8.11, pseudocolor ion images of sulfatides in ovarian carcinoma tissue are shown; sulfatides were only detected in the ovarian epithelial carcinoma and not in normal ovarian tissue (data not shown) [114]. Previous studies have shown that sulfatides are elevated in many types of cancer, including ovarian cancers, on the basis of colorimetric assay, which might have cross-reacted with other lipids (such as cardiolipin, phosphatidylserine, and phosphatidylinositol (PI)) [115]. Microarray mRNA data using a sphingolipid pathway map indicated that epithelial ovarian cancer cells might have elevated sulfatides, which was confirmed by LC ESI-MS/MS.

8.3.1.5 Prostate Cancer. Schwamborn *et al.* [23] have analyzed prostate cancer tissues using MALDI imaging MS to search for new and reliable candidate protein biomarkers. After the MALDI imaging MS experiment, the remaining matrix was

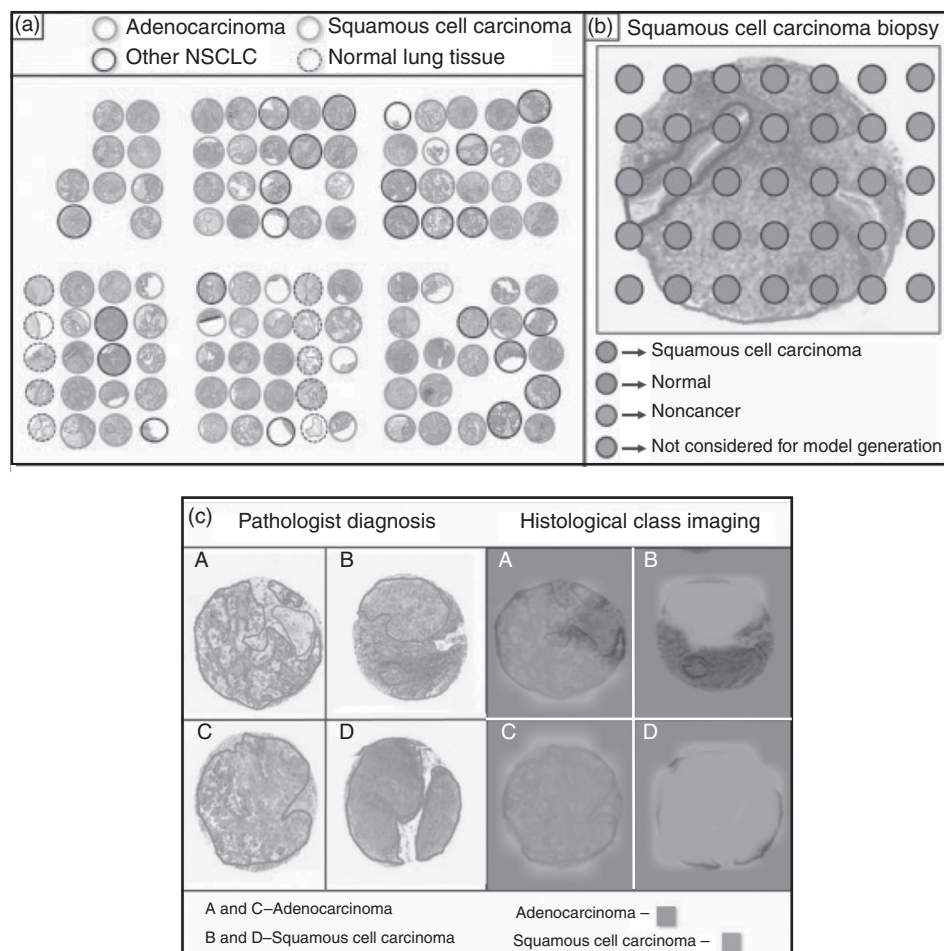


Figure 8.10 Classification of a lung cancer tissue microarray using MALDI imaging MS. (a) Optical image of an H&E stained section of the microarray and pathological diagnosis. (b) For training the diagnostic classifier, each spectrum is grouped based on the histological region from which it was acquired. (c) MALDI-imaging-MS-based classification of four biopsies compared to the diagnosis based on histology [28]. NSCLC, non-small-cell lung cancer. (See color insert.)

removed by incubating the slides twice in methanol (100%, at room temperature for 5 min) and once in acetone (100%, at room temperature for 5 min). The tissues were then H&E stained and a histological analysis performed. A support vector machine model was then built using histologically defined regions of interest (normal and cancerous areas). A false color image was created indicating those areas determined to be cancerous and compared with the results of a pathohistological analysis of the H&E stained section [23]. It was found that both regions could be distinguished with an overall cross-validation of 88% and a sensitivity and specificity of 85.21% and 90.74%, respectively, using 22 peptide and protein peaks [23]. The same data set, analyzed using just five peaks and a genetic algorithm, provided an overall cross-validation, sensitivity, and specificity of 77%, 70%, and 84%, respectively [23].

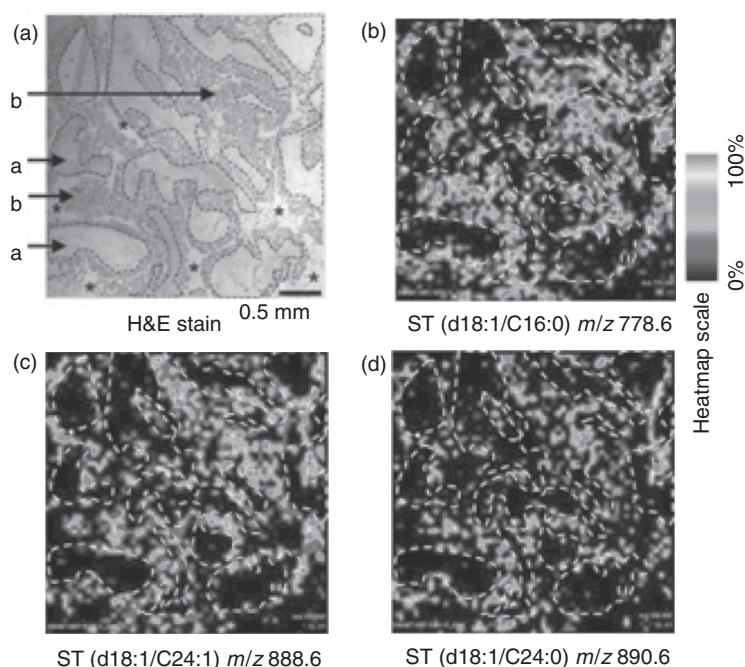


Figure 8.11 Visualization of the localization of sulfatides in ovarian carcinoma using MALDI imaging MS. (a) H&E stained thin section of an ovarian carcinoma and normal tissue. Distinctive morphological features have been outlined and superimposed on the (b–d) mass spectrometry images to aid in comparison. (b–d) Pseudocolor ion images of the ions at m/z (b) 778.6, (c) 888.6, and (d) 890.6 [114]. (See color insert.)

Cazares *et al.* recently reported a larger-scale MALDI imaging MS investigation of prostate cancer that led to the detection of a candidate biomarker. A discovery set of 11 cancer-containing tissues and 10 benign tissues was examined and a single peptide ion at m/z 4355 was found that could discriminate prostate cancer from normal tissue. This finding was then verified on a separate validation set of 54 tissue sections (23 tumor and 31 benign sections). This peptide was then identified as a fragment of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 2 (MEKK2) by tandem MS analysis of tissue lysates. On-tissue digestion MALDI imaging MS detected multiple tryptic peptides of the candidate biomarker, which were colocalized in prostate cancer tumors but not in benign or normal tissue, and immunohistochemical staining of MEKK2 showed increased expression in the tumor. This clinical study highlights the potential of MALDI imaging MS to significantly contribute to patient diagnosis and to identify new candidate biomarkers: in this case, MALDI imaging MS provided the first demonstration of MEKK2 overexpression in prostate tumors and its potential correlation with cancer stage/grade [73,116].

Chaurand *et al.* [10] compared normal mouse prostates with prostate tumors derived from genetically engineered mice (LPB-Tag mouse model for prostate cancer) by MALDI profiling and imaging MS to identify proteins common to normal prostatic morphogenesis and tumorigenesis. They showed that some secreted proteins were absent in tumors, indicating that expression of these differentiated proteins is decreased

or lost during tumor cell proliferation. Cyclophilin A (CypA), a protein found in other cancers, was detected with differential α N-terminal acetylation. Immunohistochemistry and Western blot analysis demonstrated the presence of CypA both in mouse prostate tumors and in human benign prostatic hyperplasia and prostate adenocarcinoma tissue sections, although the posttranslational modifications could not be discriminated by those techniques (in contrast to the MALDI imaging MS).

8.3.1.6 Tumors of the Gastrointestinal Tract. Following the prostate cancer analysis and the lung cancer tissue microarray analysis shown in Fig. 8.10, Dijidja *et al.* [117] recently demonstrated that MALDI imaging MS could be used to classify a pancreatic cancer tissue microarray and identified a number of proteins that appear to discriminate between different tumor classes. Figure 8.12 shows the distribution of some of the observed peptide signals within the tissue microarray sections. These peptides were identified as actin, periostin, hemoglobin α -chain, and histone 2A. Figure 8.13 displays the distribution of ion signals at m/z 944, 1032, and 1477 identified as histone 2A, histone H3, and type I collagen, respectively, which were found mainly in the tumor region. Figure 8.13d–f shows the H&E staining images of the section, which correspond with the MS images. All these analyses are based on tissue sections previously annotated by a trained pathologist. Although it is good practice to first test a new imaging method against established pathological analysis, such an

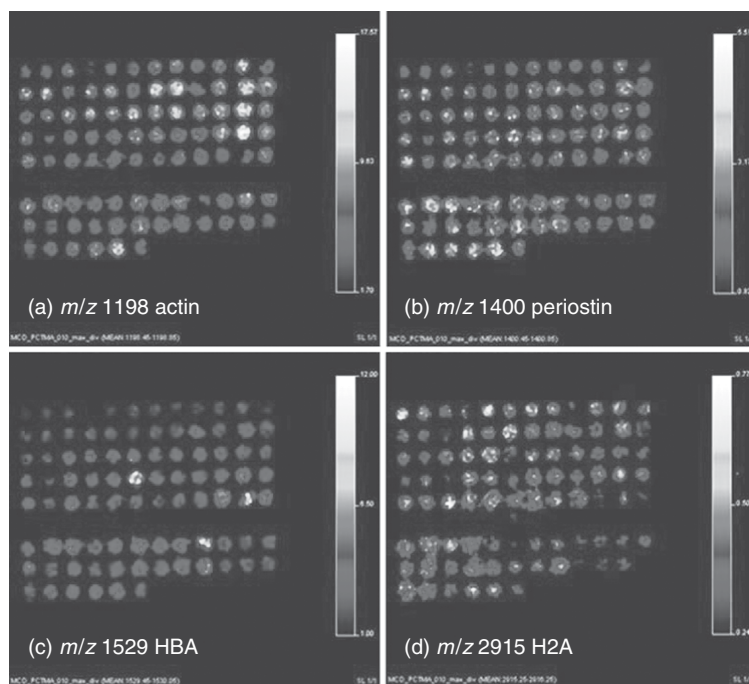


Figure 8.12 MALDI imaging MS images of the localization of proteolytic peptides within a tissue microarray. The distributions of proteolytic peptides from (a) actin, (b) periostin, (c) hemoglobin α -chain, and (d) histone H2A are displayed [117]. (See color insert.)

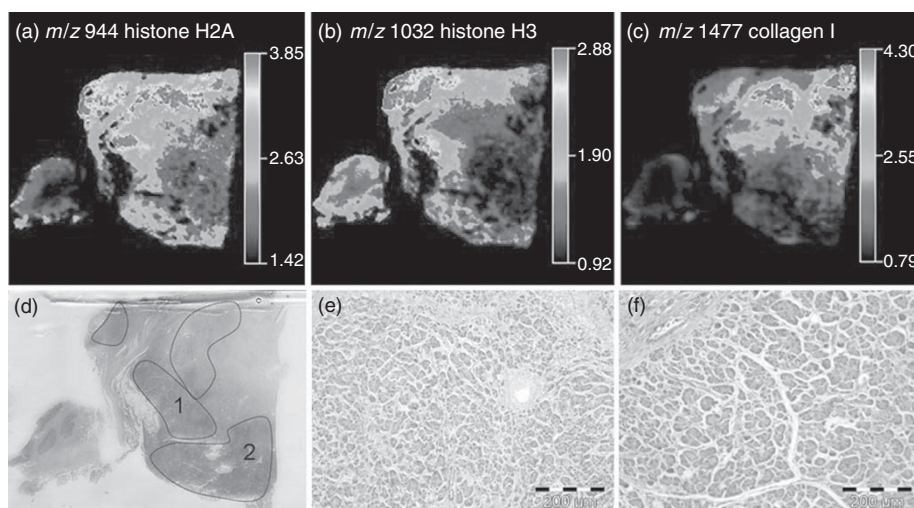


Figure 8.13 MALDI imaging MS images of the distribution of (a) histone H2A, (b) histone H3, and (c) type I collagen tryptic peptides. Images were normalized against the matrix adduct at m/z 877. (d) Optical image of the H&E stained FFPE human pancreatic cancer with the localization of tumor regions. (e,f) The $\times 10$ magnification images acquired from tumor regions 1 and 2, showing tumor cells [117]. (See color insert.)

analysis will not highlight molecular differences that occur before the emergence of pathological entities.

A recent study combining MALDI imaging MS and LC-MS identified colorectal-carcinoma-related proteins in *histologically benign* polyps [89]. Examination of multiple benign polyps with MALDI imaging MS revealed colorectal-carcinoma-related proteins in the transition zone between the polyp and normal peripheral tissue, the area of greatest neovascularity and growth. This raised the concern that although a pathologist reporting no tumor in the normal tissue is correct, histology alone could underestimate the extent of preneoplastic changes (already reflected in different peptide/protein expression profiles detected by MALDI imaging MS) and progression of the lesion toward malignancy [89].

The ability to detect changes in disease-associated protein expression before their histological transformation is one of the main advantages of the nontargeted nature of the technique and arguably crucial to identify potential early-stage biomarkers. A new diagnostic method for separating benign from malignant colon polyps, based on histopathology in combination with MALDI imaging MS, may provide physicians with a more accurate diagnosis. The MALDI imaging MS measurements could also include candidate biomarkers spanning multiple molecular classes; for example, Shimma *et al.* [118] have reported that the lipids from a human colon cancer liver metastasis tissue sample clearly differentiate the cancerous regions of the tissue from the normal regions.

Gradual changes in expression levels of candidate biomarker proteins, changes in the relative levels of different proteins [15], and candidate biomarker proteins that are represented by weaker peaks in the mass spectrum require advanced data-analysis

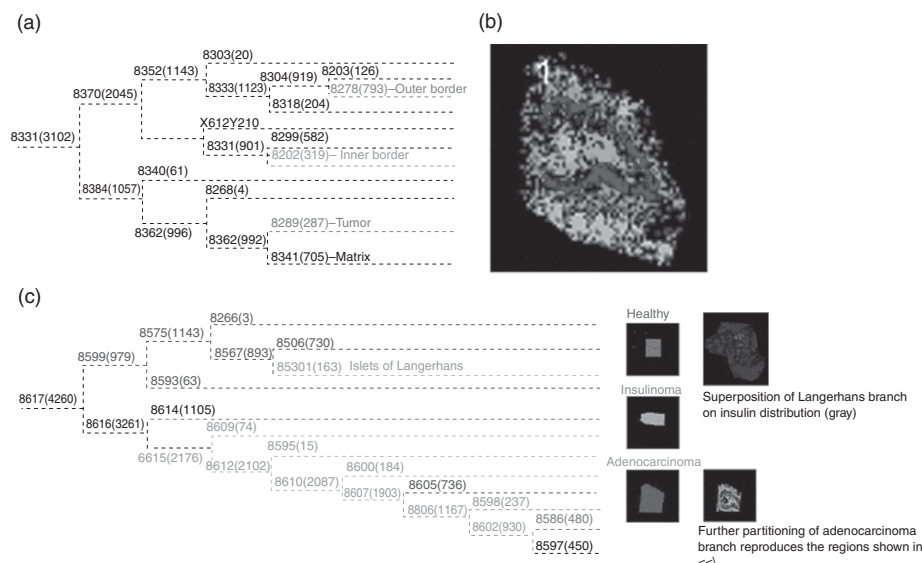


Figure 8.14 MALDI imaging MS analysis of pancreatic adenocarcinoma, insulinoma, and healthy tissue. Hierarchical cluster analysis of (a) the pancreatic adenocarcinoma data set reveals (b) the substructure in the tissue. (c) Hierarchical cluster analysis of the combined data sets distinguishes the adenocarcinoma, insulinoma, and healthy tissues as well as the substructure in the subsequent branches [70]. (See color insert.)

tools to identify or confirm the different molecular signatures associated with the different regions of the tissue. This is especially true if the goal of an experiment is to identify different regions of tissue, based on their molecular content, independent of histological analysis (e.g., to identify molecular changes that occur before histological transformation).

The hierarchical cluster analysis of pancreatic ductal adenocarcinoma shown in Fig. 8.14 clearly reveals the spatiochemical substructure of the tissue, including an area bordering the tumor core that was verified in the original MALDI imaging MS data [70]. Furthermore, it was shown that the same analysis, independent of prior histological annotation, could differentiate between healthy pancreas, ductal adenocarcinoma, and insulinoma tissue *and* indicate the different regions of each tissue.

Deininger *et al.* [27] have used hierarchical cluster analysis to highlight the different features in nonneoplastic stomach mucosa and gastric cancer tissues. After demonstrating that each histological region could be distinguished solely on the basis of their different peptide and protein profiles (thereby demonstrating the technique's capability to reproduce histological features), it was postulated that MALDI imaging MS may be able to detect phenotypic differences invisible to classical histological techniques (Section 8.3.1.7). This study was performed on a small number of surgical gastric cancer samples. Kim *et al.* [119] performed a prospective clinical study to evaluate the feasibility of using MALDI imaging MS on frozen endoscopic biopsy samples for gastric cancer from 63 gastric cancer patients and 43 healthy volunteers. Half of the samples were used to develop a classification model and were validated using the other half. It was found that both regions could be distinguished with high predictive values

(positive and negative predictive values for cancer, 96.8% and 91.3%, respectively; sensitivity 93.8%; and specificity, 95.5%) using 73 peptide and protein peaks. Signals that were overexpressed in tumors were identified as α -defensin, α -defensin/2, calgranulin A, and calgranulin/B. Furthermore, they could distinguish early stage from more advanced-stage tumors.

8.3.1.7 Intratumor Heterogeneity. One of the foremost concerns in clinical oncology and pathology is ensuring complete tumor removal [120]. Local recurrence of cancer occurs quite often, despite microscopically confirmed negative surgical resection margins, and is likely related to the presence of abnormal, fully neoplastic or preneoplastic cells adjacent to the tumor that cannot be recognized by conventional light microscopy [121]. Tumor margins can be studied by antibody-based approaches or by nuclear staining procedures, which are limited to visualization of cellular morphology [122]. Identification of proteins that define the molecular microenvironment of the neoplastic process may become an important part of the evaluation of cancer tissue, potentially helping guide surgical resection procedures. MALDI imaging MS can significantly improve the assessment of microenvironment at the molecular level and thereby not only facilitate a better understanding of tumor invasion but also provide potential markers to aid in histological assessments that help ensure complete tumor removal. Caldwell and coworkers [121,123] used this technique to reveal that changes in protein content associated with a fibrous histiocytoma can extend far into adjacent histologically normal tissue. Kang *et al.* [124] reported how MALDI imaging MS could be used to identify proteins upregulated in tumor interface zones, the region between tumors and normal tissues where epithelial–mesenchymal transition occurs. It was proposed that these proteins maybe more effective prognostic biomarkers (than proteins that are upregulated within the tumor core). Plastin 2 and peroxiredoxin 1 were identified, and independent validation of their upregulation in the interface zones of ovarian cancer was performed using fluorescence microscopy.

More recently, Oppenheimer *et al.* [29] performed an in-depth investigation of tumor interface zones of renal cell carcinoma (RCC) and revealed that normal tissue adjacent to the tumor expresses many of the molecular characteristics of the tumor. They studied the correlation of the molecular changes in the margin-normal tissue with tumor aggressiveness. Mass spectra were acquired from the tissue containing tumor and the attached normal tissue, which allowed the mapping of proteins in tumor and adjacent normal tissue. The optical image of an H&E stained section was used to define the tumor border (Fig. 8.15a). A total of 40 significant features (m/z values) were plotted for 25 patients. Most samples expressed protein patterns exemplified by line “D” in Fig. 8.15b, where features are underexpressed in the tumor and the margin as compared to the nontumor tissue. The characteristics monitored were the point at which the maximum rate of change occurred and the point at which the feature amplitude began to plateau. There was not a clear correlation between the pathological diagnosis and the distance of compromised tissue beyond the histological tumor border. For the m/z features noted, expression patterns in most samples followed the aforementioned pattern illustrated in Fig. 8.15b. There was a small group of samples in which margin trends could not be assessed [29].

The imaging MS study of gastric cancer referred to above also provided the first indication that imaging MS may help reveal heterogeneity within tumors. Hierarchical

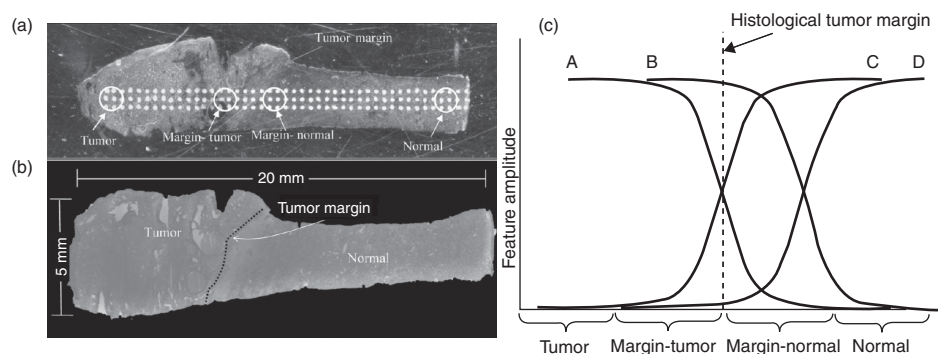


Figure 8.15 MALDI imaging MS analysis of tumor margins. (a) Optical image of tumor and adjacent normal tissue on a MALDI plate with regions of interest marked. (b) Optical image of a corresponding H&E stained section marked by a pathologist. (c) The observed patterns of the molecular features traversing the histological tumor margin. Lines A and C represent features that begin changing at the histological margin, while lines B and D represent features that change after the histological margin [29]. (See color insert.)

cluster analysis of an imaging data set revealed detailed clustering within the tumor that did not correspond to histologically distinct regions. The same data-analysis tool was recently used by Willems *et al.* [90] to demonstrate that intermediate-grade myxofibrosarcoma, a soft tissue tumor, possessed distinct areas with high-grade-like and low-grade-like character. To test if the regions could be further subdivided, on the basis of their biochemical signatures, and if the same intratumor heterogeneity is present in multiple tissues, six different localized regions of interest were selected in a single tissue sample and a support vector machine classification model was created. Application of this model to multiple intermediate-grade myxofibrosarcoma patient samples revealed that many intermediate-grade tissues exhibited significant biochemical intratumor heterogeneity. Furthermore, the results indicate that the areas previously highlighted as low-grade-like and high-grade-like consist of multiple nodules with different protein signatures. Low grade myxofibrosarcoma is microscopically characterized by a multinodular growth pattern [125]. The biomolecular heterogeneity might be due to metabolic and epigenetic differences in the tumor, though, strong evidence indicates that intratumor heterogeneity also arises from genetically distinct clones within the tumor [126,127]. Myxofibrosarcoma is characterized by nonspecific cytogenetic aberrations that increase with grade. It is thought that the tumors progress through multiple nodules, one of which becomes more dominant and progresses toward high grade myxofibrosarcoma (as has been shown in other neoplasms) [128–130]. Most human malignancies originate from a single cell but display intratumor heterogeneity by the time of diagnosis.

8.3.2 Degeneration—Neurological, Muscular, and Ocular

Molecular images of different neuropeptides have been reported, some of which were validated using immunohistochemistry [131]. Besides molecular images of peptides and proteins, different classes of lipids have also been imaged [132–138].

Wisztoski *et al.* [60] have reviewed the application of imaging MS in nervous tissues of both vertebrates and invertebrates with a focus on recent studies of neurodegenerative diseases. Langstrom *et al.* [139] have reviewed *in vitro* imaging techniques as a complement to *in vivo* positron-emission tomography (PET) and single photon emission computed tomography (SPECT) imaging in neurodegenerative diseases.

In rat brain frozen tissue sections treated with 6-hydroxydopamine, a neurotoxin commonly used to lesion dopaminergic pathways and generate experimental models for Parkinson disease, it was shown that transelongation factor 1, hexokinase, and neurofilament-M were downregulated as previously shown [140], whereas peroxidoredoxin 6, F1 ATPase, and α -enolase were upregulated [20,40]. Stauber *et al.* [40] identified three new putative biomarkers for Parkinson disease using FFPE 6-hydroxydopamine-treated rat brain tissues. Protein identification was obtained using MALDI MS direct profiling with nanoLC-nanoESI-IT MS. They confirmed that hexokinase and neurofilament-M proteins were downregulated, whereas the collapsing proteins response mediator 1 and 2, peroxidoredoxin 6, F1 ATPase, ubiquitin, and α -enolase were upregulated.

AD is a progressive neurodegenerative disorder characterized by the gradual onset of dementia. The pathological hallmarks of the disease are amyloid β plaques, neurofibrillary tangles, synaptic loss, and reactive gliosis. The localization, characterization, and quantification of amyloid β peptides have been studied in APP23 mouse brains [21,141]. Amyloid β peptides 1–40 and 1–42 were the most abundant amyloid peptides in those brain areas, which were identified by MS/MS analysis.

Scraper, a protein which is localized at the synapses in neurons, is an ubiquitin E3 ligase that is involved in the decomposition of Rab3-interacting molecule 1, an important regulator of synaptic plasticity, and thus regulates synaptic transmission. The *Scraper* knockout mouse brain showed two types of neurodegenerative pathologies, spongiform neurodegeneration and shrinkage of neuronal cells. In a comparison of a WT and SCR-KO mouse brains, both imaging MS and 2DE analysis detected altered molecules. There were several differences in information obtained from each procedure [142]; however, the additional value of imaging MS was that it showed the localization of several altered molecular species in the SCR-KO mouse brain spanning several molecular classes, including lipids, peptides, and small molecular weight proteins (<10 kDa), which are difficult to detect with 2DE analysis.

The *mdx* mouse is a model of Duchenne muscular dystrophy (DMD). Duchenne is a neuromuscular inherited, autosomal recessive disease. In DMD patients and *mdx* mice, modifications of the lipid composition of dystrophin-deficient muscle analyzed by radiolabeling, gas chromatography, or thin-layer chromatography [143] as well as oxidative damage [144] have been demonstrated. The most important difficulty remains the fact that in muscle biopsies, the muscle fibers are mixed with adipose and conjunctive tissues, which can be difficult to separate. The degeneration/regeneration process in muscles of *mdx* mouse was studied using MALDI-ToF profiling and cluster ToF secondary ion mass spectrometry (SIMS) imaging. Three regions in the *mdx* mouse legs could be distinguished: a severely damaged region, a second degenerating region (oxidative stress and deregulation of the PI cycle), and a third stable region [145,146]. ToF-SIMS imaging has the major advantage, over alternative methods, of allowing direct and simultaneous collection of mass spectra and ion images.

The determination of molecular composition and individual compound localization on a tissue section, at micrometer scale and without the need of any prior sample

treatment, makes the analysis easier, more straightforward, and the closest possible to physiological conditions.

Mass spectra revealed differences in the distribution of fatty acids, phospholipids, diglycerides (DGs), and triglycerides (TGs). Vitamin E and PIs were found to concentrate within the cells, whereas intact phosphocholines accumulated over the most damaged areas of the dystrophic muscles, together with cholesterol and sphingomyelin species. Fatty acyl chain composition varied depending on the region, allowing estimation of the local damage extent [147]. Recently, Benabdellah *et al.* showed inversion of the PC34:2/PC34:1 intensity ratio between destructed areas (from dystrophic muscle) and control areas (healthy muscle and structured areas from dystrophic muscle), which was interpreted as evidence of restoration of membrane lipid composition after treatment of *mdx* mice with molsidomine [64,65].

MALDI tissue profiling and imaging of proteins in ocular lenses have been developed to investigate the distribution of lens proteins [55,148–151]. Because of the lack of protein turnover, lens proteins undergo various age-related posttranslational modifications that include truncation, deamidation, glycation, and phosphorylation. The modifications accumulate over the lifetime of mammalian species and possibly contribute to lens opacification and cataractogenesis by inducing aggregation of lens proteins. While immunolabeling can be used to investigate several protein distribution patterns, its ability to simultaneously detect more than four specific antibody probes is limited principally due to the small number of discrete recording channels available. Furthermore standard immunohistochemical approaches can be challenged by the lack of specific antibodies to phosphorylated forms, the inability to detect specific truncation products, and the large number of individual experiments required to obtain this information. Molecular images showed the spatial distribution of the integral lens protein aquaporin, and its truncated products, namely, MP20, opsin, and α -crystallin, and their modified forms in one single experiment [55,148–151]. α A-crystallin was extensively degraded in the lens core; α B-crystalline degradation was limited and phosphorylation was most abundant in the middle cortex [55,148–150]. The results were validated by identification of the proteins and the modification by additional reverse-phase liquid chromatography of soluble extracts from specific tissue regions followed by tandem MS [150]. In addition, the regional distribution of lipids in porcine lens were mapped. The intraocular lens contains high levels of both cholesterol and sphingolipids, which are believed to be functionally important for normal lens physiology. Seven lens sphingomyelins and two ceramide-1-phosphates were imaged and further assigned by imaging FT-ICR MS. Glycosylated sphingolipids or sphingolipid breakdown products were not detected. Surprisingly, neither the cholesterol ion nor its dehydrated fragment ion could be detected by imaging MS, although the cholesterol molecules could be detected by SIMS and desorption electrospray ionization (DESI). This is probably due to the strong integration of cholesterol molecules in the lens [152].

8.3.3 Drugs, Tracers, and Metabolites

8.3.3.1 Drugs and Tracers. In 2003, Reyzer *et al.* [153] demonstrated that imaging MS could be used to trace the distribution of pharmaceuticals in various organs. It has rapidly developed into highly effective technique for imaging the distribution of small organic molecules, both exogenous drugs and endogenous metabolic intermediates

[154]. The advantage of this technique for metabolite imaging is that it does not require any labels or specific probes; it is a nontargeted method, and thereby many types of molecules can be analyzed simultaneously. Moreover, the parent drug and its metabolites can be distinguished [155,156], which is not possible with autoradiography or fluorescence imaging. Rohner *et al.* [157] reviewed MALDI imaging MS and described imaging work being carried out at Novartis. From the same group, there was also a report describing some of the practical aspects of obtaining imaging MS data for drug and metabolite distributions [85].

The mass range containing most drugs and metabolites can be densely populated with many ions of similar mass. Consequently, many experiments have used MS/MS to increase specificity. The increased resolving power of ion-mobility MS or FT-ICR MS enables drugs and metabolites to be distinguished using MS [132,136,155,156, 158–160]. MALDI imaging MS has attracted great interest from drug development companies. In recent years, numerous studies have been reported, where imaging MS has been used to monitor the distribution of an administered drug and its metabolites, for example, antitumor drugs (vinblastine, imatinib, banoxantrone, oxaliplatin, and diazepam) [155,156, 160–162], antipsychotics (olanzapine and clozapine) [48,119,121], antianxiety drugs and hypnotics (temazepam), treatment of chronic obstructive pulmonary disease (tiotropium) [63,163], HIV protease inhibitors [164], and an antifungal drug (ketoconazole)[62].

As described earlier, it is very important to avoid delocalization of the analyte and care must be taken when using MS to ensure there are no interfering peaks from the matrix or from endogenous compounds present in the tissue. Sample preparation procedures including the choice of matrix extraction solvents and detection method may need to be developed because of the range of structures, solubility, and physicochemical properties of drug compounds [165]. Several recent papers describe sample preparation protocols for drug imaging in which elimination of matrix-derived ions are developed such as the use of nanoparticle-based ionization [136,166], matrix-enhanced surface-assisted desorption/ionization [167], desorption/ionization on silicon [168], and nanostructure-initiator MS [169].

Imaging MS for low molecular weight compounds has been analyzed on ToF, IT, Q-ToF, IT-ToF, FT-ICR, and QqQLIT (a triple quadrupole in which the final quadrupole is a quadrupolar linear ion trap) mass analyzers [155,156]. Recent developments using MRM showed that images of a complete rat section could be accomplished with a 1000 Hz laser frequency and a rastering speed of about 18 mm/s, so a whole image of a drug compound distribution could be measured within 15 min [82].

Stoeckli *et al.* [85] and Hiesh *et al.* [84] showed that imaging MS provided detailed drug distribution images that are comparable to traditional whole-body autoradiography using radiolabeled compounds. Both methods showed high levels of a compound in several organs, while low levels in the blood were also detected. Autoradiography requires laborious and expensive synthesis and labeling of a tag with the risk of altering the pharmacological properties of the drug. Unlike autoradiography, imaging MS can distinguish the medicinally intact drugs from their metabolites. Khatib-Shahidi *et al.* [156] showed that in complete rat sections, the intact drug reached the target organ (the brain), whereas its metabolites were localized in the bladder (Fig. 8.16a).

Furthermore, the time course of the metabolism of parent drugs into demethylated and hydroxymethylated metabolites was visualized over the whole-body section (Fig. 8.16b). In this study, notable decreases of olanzapine were observed everywhere

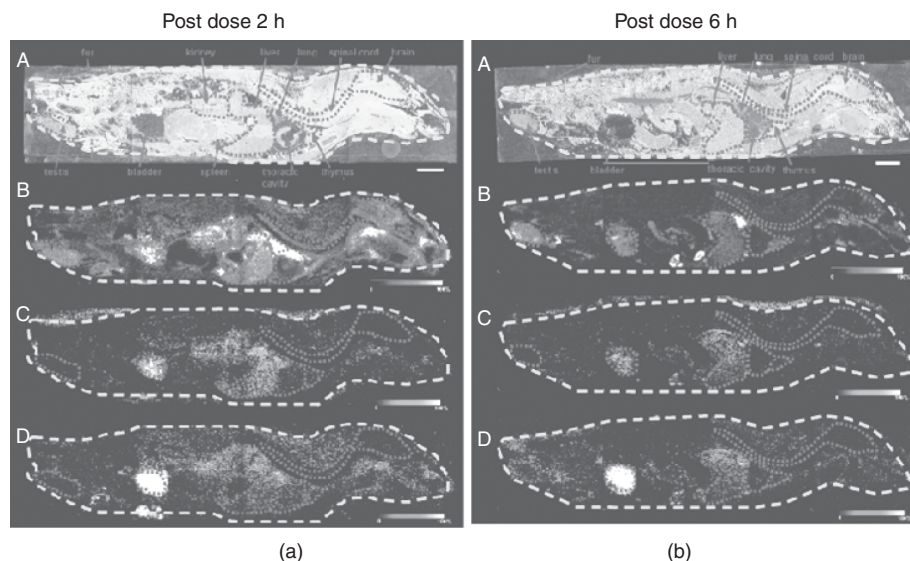


Figure 8.16 MALDI imaging MS of the drug olanzapine and its metabolites in a whole rat sagittal tissue section (a) 2 h post dose and (b) 6 h post dose. (A) Optical images of tissue sections, in which the organs have been outlined with a dark gray dotted line. (B) MS/MS images of olanzapine (m/z 256). (C) MS/MS ion image of *N*-desmethyl metabolite (m/z 256). (D) MS/MS ion image of 2-hydroxymethyl metabolite (m/z 272). Scale bar = 1 cm [156]. (See color insert.)

in the body except the testis and bladder, while the metabolized compounds accumulated in the bladder. The differentiation of clozapine concentrations within the rat brain was confirmed using a fast HPLC/MS/MS method. Close examination of the distribution of drugs within specific organs can also be used to assess drug penetration. Bouslimani *et al.* [161] showed that the platinum antitumor drug oxaliplatin was localized exclusively in the kidney cortex, suggesting that it did not penetrate deep into the organ.

The spatiochemical information provided by imaging MS has also been used to investigate the distribution of pharmaceutical compounds in tablet formulations [170]. Tablets are compacted pharmaceutical dosage forms that contain active drug compounds and excipients. These results indicate that imaging MS can assist in the development of drug-delivery systems as well as in lead development. MALDI imaging MS was recently exploited to independently measure the distribution of MRI contrast-enhancement agents in both frozen and paraformaldehyde-fixed mouse livers, which were independently validated by both an *in vivo* MRI and an *ex vivo* inductively coupled plasma atomic emission spectroscopy [171]. The development of targeted MRI contrast-enhancement agents is one of the key tools being developed for early, non-invasive detection of disease biomarkers, and imaging MS could provide an independent measurement of its distribution in tissue to ensure its colocalization with the target biomarker.

8.3.3.2 Endogenous Metabolites. Endogenous metabolites can give insight into metabolic changes linked to drug administration or disease. Among the endogenous

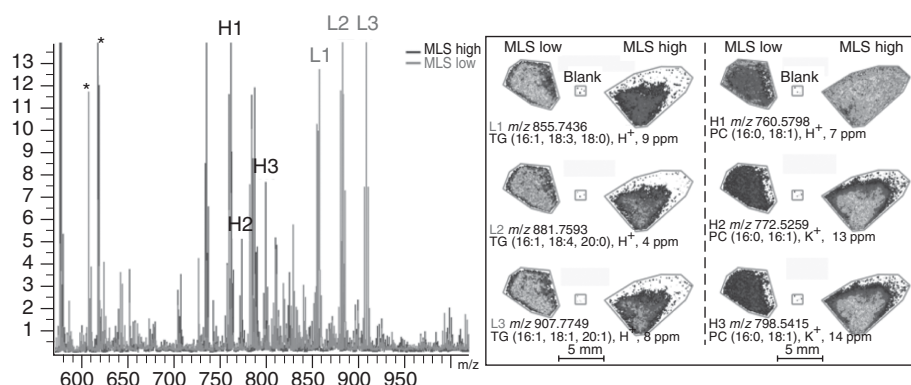


Figure 8.17 Lipid imaging mass spectrometry of high and low grade myxoid liposarcoma (MLS) reveals differential lipid profiles, and the spatial distributions show the localization of specific lipids in one tumor grade. The lipids were identified using tandem mass spectrometry. The experimental mass, the assignment, and the mass accuracy of the assignments are provided alongside the image [90]. *Matrix clusters. (See color insert.)

metabolites, simple lipids (e.g., cholesterol, acylglycerides, and fatty acids) and complex lipids (e.g., glycerophospholipids and glycosphingolipids), as well as aminoacids, flavonoids, and oligosaccharides, have been investigated and reviewed by Sugiura and Setou [154].

Lipid analysis of myxoid liposarcomas by imaging MS revealed differences between low and high grade tumors [90]. Low and high grade tumors each contain specifically expressed lipids whose images clearly reveal their localization to one grade (Fig. 8.17). These lipids were then identified using MS/MS and the LIPID MAPS database. The spectra from high grade tumors revealed higher levels of phosphocholines. Phosphocholines are commonly detected by MALDI and are thought to be derived from (cell) membranes. The higher levels of these lipids in high grade tumors are consistent with the higher cellularity of higher grade tumors [172]. Low grade myxoid liposarcoma contained additional peaks because of triacylglycerols. Active PPAR γ signaling promotes adipocytic differentiation in preadipocytes and is active in myxoid liposarcoma cells [173]. The data suggest decreased PPAR γ -regulated fatty acid synthesis in myxoid liposarcoma upon increase in grade, histologically reflected in a diminished adipocytic phenotype, and consistent with recent work that established that PPAR γ inactivation by the FUS/DDIT3 liposarcoma-specific fusion protein is required for liposarcoma development [174].

The molecular ion distribution of intact lipids directly from single neuroblastoma cells was studied using matrix-enhanced (ME) SIMS. The ME-SIMS spectrum of these cell surfaces showed several lipid species in the mass range between m/z 700 and 900, ceramide, diacylglycerol species between m/z 500 and 700, and organic fragments in the lower mass range. High cholesterol signal intensity was found at the border of the cells, the plasma membrane, compared to the center of the cells, corresponding to the nuclei [175].

Background matrix peaks can be abundant in the low m/z range and can severely complicate metabolite imaging MS experiments. In order to improve the analysis of metabolites using imaging MS, different matrices have been investigated; 9-AA has

been reported as a highly promising matrix for metabolite analysis. No matrix signal was detected in the m/z range between 300 and 1000, the mass range where metabolite ion peaks are expected to be detected [64]. Furthermore, 13 primary metabolites at m/z values 323 (uridine monophosphate), 328 (cyclic adenosine monophosphate), 339 (fructose-1,6-biphosphate), 346 (adenosine monophosphate), 362 (guanosine monophosphate), 403 (uridine diphosphate), 426 (adenosine diphosphate), 442 (guanosine diphosphate), 506 (adenosine triphosphate), 522 (guanosine triphosphate), 558 (ADP-ribose), 565 (UDP-glucose), and 606 (UDP-GlcNAc) were observed, whereas none of them was detected using other matrices [64]. Identifications were made by tandem MS/MS comparing fragment fingerprints obtained from the tissue section and from standard samples. The distributions of those primary metabolites with a sensitivity of attomoles per pixel on rat brain sections were shown. As primary metabolites are known to be biomarkers of several brain disorders, imaging MS can be useful to study those metabolic changes [64].

8.4 PERSPECTIVES

Since its introduction in the late 1990s, MALDI imaging MS technology has made tremendous advances. Imaging MS is now used to investigate many different molecular classes, including peptides, proteins, lipids, metabolites, and drugs. The potential application of MALDI imaging MS in neurodegenerative diseases, cancer, viral infections, and drug metabolism lies in its ability to generate profiles in a nontargeted manner. These abilities enable the technique to detect unexpected and *a priori* unknown changes in the protein content of the tissue, for example, the presence of colorectal carcinoma biomarkers in histologically benign tissue [89], and to describe the tissue by a spectrum of peptides and proteins. MALDI imaging MS can also be adapted to span multiple molecular classes using essentially the same technique but different sample preparation strategies (peptides, proteins, lipids [118], and metabolites [64]), and can be integrated with established *in vitro* and *in vivo* analyses, thus the scope for complementary analyses is open. To investigate this potential in the clinic, the application largely driving the field, the biomolecular signatures obtained from the supervised and unsupervised analysis of the tissues are beginning to be tested across the large number of samples typically included in clinical cohorts [73].

To date, the classification of tissues using MALDI imaging MS has been based on established histological analysis [23,28,73]. The mass spectra obtained from a set of histologically annotated tissue sections are used to create a classifier, which is then applied to other tissue sections to test its performance. The ability of MALDI imaging MS to reproduce established histological classification demonstrates its ability to acquire clinically relevant data and can be considered the necessary first step of any new diagnostic/prognostic tool. The identification of new candidate protein profile biomarkers based on MALDI imaging MS, independent of histological analysis and thus able to detect biochemical changes before the appearance of pathological entities, has been performed for a small number of tissue sections [27,87]. Arguably, the next step in the development of MALDI-imaging-MS-based biomarker discovery is the development of classifiers based on the simultaneous analysis of tissue arrays. Such high throughput analysis will exploit the recently announced high speed MALDI mass spectrometers and imaging MS automation [98].

MALDI imaging MS is a rapidly developing tool that has great potential for improved diagnostic/prognostic capabilities. Nevertheless, it will be most effective when implemented as part of a concerted effort utilizing a range of complementary techniques and expertise.

MALDI imaging MS on small molecules has opened a new frontier both in pharmaceutical and toxicological research and in the developmental phase of new drugs. The potential of this technique lies in its ability to visualize and distinguish the parent drug and its metabolites and is an attractive alternative for monitoring drug distribution within animal models, with much faster results and lower cost than the traditional whole-body autoradiography method [85,176]. The possibility to monitor in parallel multiple analytes, with high specificity and rapid acquisition, makes it a very promising tool for drug development. Furthermore, this technique can study in one analysis the ability of the compound of interest to reach the target organ, the drug penetration into the target organ, and the diffusion to undesirable organs [161]. It offers the possibility of not only examining and tracking drug metabolism in whole animal sections but also following polymer substrate degradation and drug release.

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9 Gas-Phase Ion Mobility-Mass Spectrometry (IM-MS) and Tandem IM-MS/MS Strategies for Metabolism Studies and Metabolomics

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9.1 Introduction	1
9.2 An overview of ion mobility techniques	2
9.3 Integrated ion mobility–mass spectrometry	11
9.4 Tandem methods for ion mobility–mass spectrometry/mass spectrometry	18
9.5 Summary	24
Acknowledgments	25
References	26

9.1 INTRODUCTION

Metabolite analysis is a demanding initiative for analytical technologies. It is estimated that between 4000 and 20,000 distinct metabolites are expressed in eukaryotic organisms, spanning between seven and nine orders of magnitude in concentration range [1,2], which necessitates highly sensitive and broad dynamic range analyses. This is particularly true when considering xenobiotic metabolites, in which all feasible biotransformations that an administered drug may undergo must also be considered. These may occur over a large dynamic range of possible concentrations, and depending on the dose administered, may require highly sensitive analytical techniques [3]. Metabolism is a dynamic process, requiring measurement strategies that provide high sample throughput and instrument duty cycle. A further challenge is that metabolites represent a diverse set of chemical classes possessing disparate polarities, such that there is no single extraction or separation technique that can handle most, much less all, metabolites present. Because of the shortcoming of current technologies, contemporary profiling strategies are necessarily focused on a relatively small number of known metabolites and thus are noncomprehensive. Nevertheless, targeted analysis and

low complexity metabolite profiling approaches have been highly successful in drug discovery and development [4,5].

Established bioanalytical methods for studying metabolites are predominately based on nuclear magnetic resonance (NMR) and mass spectrometry (MS). MS methods are widely used and are routinely combined with additional separations [e.g., gas chromatography (GC) and liquid chromatography (LC) before MS identification] to improve instrument sensitivity and dynamic range to detect lower abundance analytes. In parallel with advances in MS has been the development of postionization gas-phase separations based on ion mobility (IM) [6–8]. IM is a compartmentalized technology that offers a combined separation and analysis that is ca. four to six orders of magnitude faster than GC and LC, respectively [9]. Since gas-phase ionization is a prerequisite for both IM and MS, the two methods are commonly coupled into a single analysis technique referred to as *IM-MS*, which allows separations on the basis of two physical properties: IM (size) and ion mass (weight). IM-MS is still an emerging technology, and one major challenge associated with using IM-MS for metabolite profiling is the limited amount of validation that can be drawn from the literature. Another challenge is the limited orthogonality between IM and MS separation dimensions, since analyte size and mass are two closely correlated physical properties [10]. Nevertheless, the extremely high speeds of the separation (micro- to milliseconds) and the potential for enhancing existing MS methodologies offer great promise for IM-MS to become a complementary bioanalytical technique for dynamic metabolic and metabolomics analyses.

This chapter provides an overview of the IM technique and a review of the various analytical approaches that utilize IM for metabolomics. The coupling of IM and MS is discussed in light of the analytical advantages and the additional information gained in these two-dimensional analyses. In recent years, high resolution mass spectrometers have allowed for the determination of metabolite identity based on high mass accuracy measurements, which allow for the determination of metabolic transformations based on mass defect filtering, which is further enhanced in combination with IM. Tandem MS experiments (MS/MS) are important methods for elucidating analyte identity, and the common methods of operation are outlined. The addition of IM capabilities to MS instrumentation permits several useful combinations of tandem experiments (e.g., IM-MS/MS, MS/IM-MS, and MS-IM-MS/MS), which enhance the current commercial MS/MS capabilities; these are discussed in detail at the end of this chapter.

9.2 AN OVERVIEW OF ION MOBILITY TECHNIQUES

IM can be broadly defined as the *kinetic behavior* (velocity) of ions in a neutral gas. Differences in the velocity of two ions, even those isomeric in mass, can be used to differentiate the two ions experimentally. The analytical technique that utilizes the property of IM to separate ions is often called *IM spectrometry* to draw parallels with MS. Other names such as gas-phase electrophoresis [11] and plasma chromatography [12] have also been used. In terms of the technology, IM spectrometry closely resembles MS; however, the separation mechanism is very similar to chromatography, which can cause confusion if the fundamentals of the IM method are described from either (a spectrometric or chromatographic) perspective [13]. In this chapter, we refer to the analytical technique simply as IM and make important distinctions where necessary.

From a pedagogical perspective, all IM techniques can be conceptualized as a series of opposing vector forces: (i) the electric field force that directs ions across a defined region and (ii) the opposing force of ion–gas collisions that impedes this forward motion. In the simplest IM experiment, ions are pulled by an electric field through a chamber filled with a constant pressure of gas and disperse in time based on differences in their mobility. The number of ion–gas collisions is related to the size of the ion. Thus, for two ions of equal mass but different sizes, the smaller of the two will have a higher ionic mobility (velocity) and traverse the region the fastest, arriving on the other side of the region first. This kind of IM experiment is known as the *drift tube IM technique* and is the most commonly encountered IM method in the literature.

The term *ion mobility* does not refer to any single experimental method but it encompasses a family of different analytical techniques, all of which utilize the same basic concept of an ion's gas-phase mobility to generate an analytically useful separation. As with MS, IM techniques can be broadly divided into two categories: (i) temporally dispersive methods, which separate all ions in time but along the same spatial path, and (ii) spatially dispersive methods, which separate ions along different trajectories. The conceptual differences between temporal and spatial dispersion are depicted in Fig. 9.1. Temporally dispersive IM methods can obtain a complete IM spectrum in a single measurement cycle, while spatially dispersive IM techniques while spatially dispersive IM techniques transmit only a narrow IM range transmit only a narrow IM range of ions at a time. Members of the spatially dispersive IM category are considered scanning methods, which filter ions based on their mobilities and require that a parameter (typically a voltage) be scanned in order to generate the broadband spectrum. Table 9.1 contains a listing of several different IM methods along with their defining attributes. The distinction between any two IM techniques can be made by comparing the relative direction of the various field forces (electric field and gas flow) with respect to the motion of the ion. Note here that the ion motion will preferentially follow one of the forces such that the ion velocity vector is aligned with one of the force vectors. A listing of the analogous MS method for each IM technique is also provided in Table 9.1.

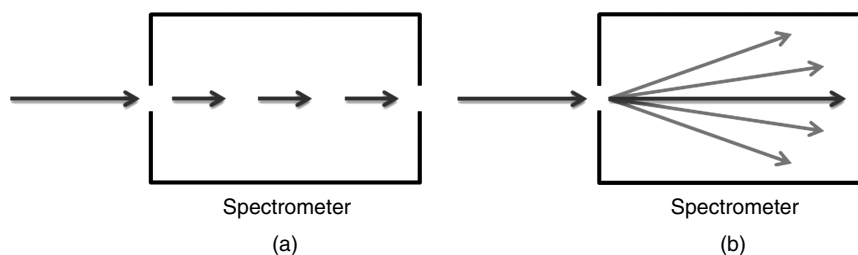


Figure 9.1 Conceptual schematic of the two types of separation schemes for ion-mobility techniques. (a) Temporally dispersive separation methods partition a pulse of ions into different mobility values along the same spatial trajectory. Ions of different mobilities arrive at different times during each measurement pulse. (b) Spatially dispersive separation methods partition a continuous beam of ions into different mobility values along different spatial trajectories, with each trajectory representing ions possessing common gas-phase mobilities.

TABLE 9.1 The Different Ion Mobility Techniques, Their Commercial Availability, Defining Features, and Currently Achieved Resolving Powers Reported in the Literature, Including the Analogous Mass Spectrometry Methods for Each Ion Mobility Technique

	Ion Mobility Technique	Commercial IM-MS Instruments (Available as of 2010)	Vectors			Resolving Power ^a Achieved	MS Analog	References
			Ion Motion	Electric Field	Gas Flow			
Temporally dispersive	Drift tube ion mobility spectrometry Plasma chromatography Ion chromatography	ESI-IM-Q (Excellims); www.excellims.com MALDI-IM-TOF (Ionwerks); www.ionwerks.com ESI-IM-TOF (Tofwerk AG); www.tofwerk.com	→	→	0	240 ^b	Time of flight	7,9
	Traveling wave IM	Synapt HDMS (Waters); www.waters.com	→	→→→→	0	40 ^c	“Solitron” (mass selective traveling wave ^d)	14
Spatially dispersive (scanning)	Differential high/low field IM High field asymmetric waveform IMS (FAIMS) Differential mobility spectrometry (DMS)	FAIMS (Thermo Scientific) ^e ; www.thermo.com DMS interface (Sionex) ^e ; www.sionex.com	→	→	↑	200 ^f	Quadrupole	15,16

Differential mobility analyzer (DMA)	DMA interface (SEADM) ^e ;	→	↑	→	50 ^g to 80 ^h	Magnetic sector	17,18
Gas-phase electrophoretic molecular mass analyzer (GEMMA)	www.seadm.com Aerosol TOFMS (TSI); www.tsi.com						

References are provided directing the reader to comprehensive reviews on the separation described.

^aIt should be noted that resolving power does not adequately represent the separation efficiency of certain ion mobility methods, particularly the spatially dispersive IM techniques. For these methods, peak capacity is a more representative metric of separation ability (Section 9.3.2).

^bA resolving power of >300 has been observed for a special circular path drift tube that operates in a narrow bandpass mode 19,20.

^cExperimentally observed on the Synapt Generation 2 (G2) for data obtained from the authors' laboratory.

^dRef. 21.

^eOffered as a front-end IM interface for a variety of commercial MS instruments.

^fRef. 22.

^gRef. 23.

^hPerformance specifications for the SEADM DMA interface.

The following sections describe each of the specific IM techniques in more detail. We start with a qualitative description of the drift tube IM method and use concepts discussed there as the foundation for understanding other IM techniques. These methods are initially discussed in the absence of MS; however, the IM techniques are framed as modular rather than as stand-alone or end detection technologies. Governing equations, although important for each technique, are avoided in this chapter in order to convey the qualitative operational principles clearly. The reader is directed toward specific references given in Table 9.1 if a more thorough understanding of each technique is sought.

9.2.1 Drift Tube Ion Mobility Spectrometry

The drift tube IM technique is the most familiar IM method and has been a commercial technology since the 1970s, although it has primarily been used as a stand-alone technology for chemical screening in security applications [24]. Drift tube instruments can operate at either ambient pressure (ca. 760 Torr) or reduced pressure (ca. 1–10 Torr), the latter more amenable to coupling with vacuum MS techniques. Ambient pressure drift tubes do not need bulky vacuum systems, and owing to the high gas number densities, the IM separation occurs in a relatively short distance, so these instruments are often preferred as low cost, portable chemical detectors. Reduced-pressure drift tubes are more costly and technically complex due to the added vacuum hardware, but they offer very high measurement precision and accuracy and are readily coupled to mass spectrometers. Complicating ion chemistry is often avoided at reduced pressures, where ions experience fewer interactions with trace gas impurities inherently present in the drift gases used. Reduced-pressure drift tubes are generally preferred in research applications where high quality ion measurements are desired, but one major disadvantage has traditionally been that these instruments were not readily available commercially. This situation has changed in the last five years or so as several commercial vendors have begun offering high performance drift tube IM-MS equipment.

The basic components and operational principle of the drift tube IM technique are depicted in Fig. 9.2a. A uniform electric field is defined along the length of a tube by a series of stacked ring electrodes. The tube is filled with an inert gas, usually helium or nitrogen, which is maintained at a constant pressure and low flow conditions. A pulse of ions is introduced at one end and allowed to drift down the length of the tube by the influence of the electric field via electrophoretic migration. During the ion's drift, it collides many times with the drift gas ($>10^4$ collisions at 1 Torr), which slows the ion's forward motion. The amount of time any given ion spends in the drift tube is directly related to the number of collisions it experiences, which in turn depends on its collision cross section or nominal gas-phase size and shape. This is analogous in principle to GC, where the less volatile (lower vapor pressure) analyte will spend more time in the column. The measured drift times in the IM experiment are essentially the same in principle as retention times measured in chromatography, but it is important to note that IM is not chromatography in the strictest sense, as there is no stationary phase (and thus no phase partitioning) but a constantly moving density of gas.

Because the electric field, gas pressure (number density), temperature, and tube length are (ideally) all kept constant in the drift tube IM experiment, the relationship between the measured ion drift time and the collision cross section is direct and can

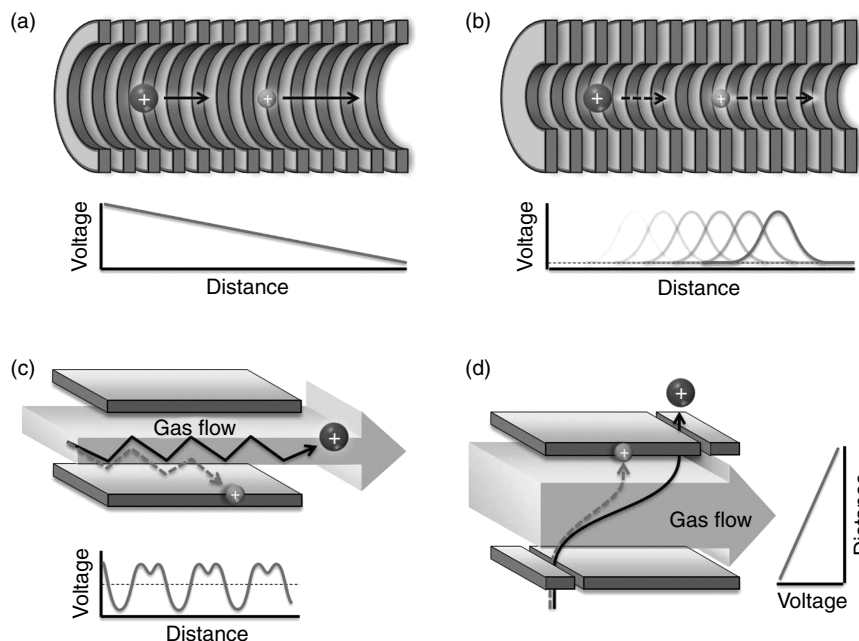


Figure 9.2 The operation principles governing the four ion mobility techniques. (a) The drift tube ion-mobility method disperses ions of differing mobilities temporally along the same trajectory by utilizing a continuous declining potential across a gas-filled region. (b) The traveling wave ion-mobility method achieves temporally dispersive mobility separations using a traveling wave potential across a gas-filled region. (c) The high field asymmetric waveform ion-mobility method disperses ions spatially along different trajectories using an orthogonally applied asymmetric waveform as ions are entrained in a gas flow. (d) The differential mobility analyzer disperses ions spatially by mobility using a flow of gas and an orthogonally applied uniform electric field.

be determined from first principle kinetic equations [25]. The measurement of absolute gas-phase collision cross sections of ions is an important feature of the drift tube experiment. The interpretation of the collision cross section value is, however, not straightforward since effects such as neutral gas polarization can contribute to nonideal (inelastic) ion–gas interactions [26]. This is particularly significant for small molecules such as metabolites, where the analyte polarity can contribute to a more attractive ion–gas interaction (a “sticky” collision), which will make the collision cross section appear larger than the physical size of the gas-phase analyte ion. Thus, it is difficult to assign any physical meaning to the collision cross sections measured for small molecules in the IM experiment. For larger analyte ions, greater than about m/z 500, the size effects become the significant contributor to the measured drift times, and in this case, collision cross section is a close representation of the physical size and shape of the gas-phase ion. The use of nonpolar drift gases also improves the accuracy of the collision cross section measurement, which is why helium is often preferred in research drift tube instruments. One prevailing advantage of drift tube instruments over the other IM techniques described in this chapter is the very high resolving powers that

can be achieved (>200) [19], which makes drift tubes important IM instrumentation for core research and development.

9.2.2 Traveling Wave Ion Mobility Spectrometry

Traveling wave is a relatively new IM technique but has already shown great promise to become a routine method for chemical analysis. This is largely due to the rapid commercialization of the technology, which is now widely available integrated into a high end mass spectrometer platform [14]. The first commercial traveling wave instrument was released in 2006 (Synapt HDMS, Waters) and has since seen a major revision (Synapt G2, 2009), which has improved the IM resolving power by about a factor of 4 (currently ca. 40–50) and offers mass resolving powers of up to ca. 50,000. The traveling wave concept itself can be traced back to early accelerator physics applications [27] and early work with using radio frequency (RF) fields to determine ionic mobilities [28,29], but until recently, there have been no attempts made at translating this technology to separating ions by mobility on an analytical scale.

The basic components of the traveling wave IM device is shown in Fig. 9.2b and are essentially the same as what can be found in a drift tube IM instrument. The drift region consists of a stacked ring ion guide and is filled with a low static pressure (1–5 Torr) of nitrogen. The rings serve two purposes: (i) a sinusoidal RF potential is applied to confine ions to the center axis of the device to improve instrument sensitivity, while (ii) a pulsed voltage is applied on sets of rings and rapidly switched from one end of the device to the other to generate the traveling wave potential for IM separation. Ions that are introduced to the device in narrow pulses will be picked up by the wave and pushed down the length of the drift region where they experience many collisions with the nitrogen gas. Some ions experience more collisions with the drift gas and are pushed against the wave until they fall behind it and wait for the next wave to carry them forward again. The number of times any given ion will tumble behind the wave is directly related to its ionic mobility—larger ions are inherently more difficult to push forward than small ions. Thus, the device disperses ions based on their mobilities in much the same way as the drift tube experiment, although fundamentally this is achieved in a very different manner. Because the wave travels at a fixed velocity, only ions that fall behind the wave will separate—those ions that are pushed along by a single wave will arrive at the same drift time and result in no useful mobility separation. Factors such as the wave velocity and voltage amplitude must be tuned in order to obtain a good mobility separation. The current second-generation traveling wave instrument (Synapt G2) can achieve IM resolving powers of ca. 45 (authors' laboratory).

Because of the complex potentials utilized in the traveling wave IM instrument, there is no direct first principle correlation between measured drift times and collision cross sections. For those interested in obtaining structural information from the IM experiment, this is seen as a disadvantage when using the traveling wave IM technique, though, methods have been proposed. By preparing a calibration curve with standards of known collision cross section, structural values have been extracted using traveling wave IM. This method is limited to the range of the calibration curve, which typically limits this method to the analysis of a particular biomolecular class. Additionally, methods of dynamically changing the traveling wave velocity for the determination

of mobility have been presented as well, though, this method is not commercially available and not particularly feasible for complex sample analysis. However, there are two distinct advantages that traveling wave IM offer over traditional drift tube IM. First, the axially confining electric fields result in very little ion loss and thus very good analyte sensitivity. Second, the traveling wave device is much easier to couple to other instrumentation due to the fact that the potential at the start and exit of the device remains the same. In contrast, coupling instrumentation such as MS to a drift tube IM device requires either the front or back component be biased to a nonzero electric potential, increasing technical complexity. A third advantage might be that traveling wave instruments are becoming much more accessible to mainstream analytical research and will no doubt see some exciting applications in the near future, particularly in the area of drug research and discovery.

Both the drift tube and traveling wave IM techniques disperse ions temporally along the same space, which permits an entire broadband mobility spectrum to be generated in each experimental measurement cycle. This has the obvious advantage of allowing one to obtain data on several analyte peaks at the same time. One limitation of temporally dispersive approaches is that ions are introduced in pulses, requiring that each measurement be complete before another pulse is admitted (a duty cycle), which reduces the overall sample throughput of the technique. In some cases, it is desirable to only monitor a few select analyte signals continuously, and so the next two IM techniques discussed act as narrow bandpass mobility filters and are particularly well suited for analyte monitoring applications.

9.2.3 High Field Asymmetric Waveform Ion Mobility and Differential Mobility Spectrometry

Consider now a continuous stream of ions entrained in a gas flow that passes between two parallel plates (Fig. 9.2c). One plate is kept at a constant potential, usually ground, and on the other plate is applied an alternating (AC) voltage. This AC voltage is kept at the same average potential as the opposite plate but alternates between a short duration, high amplitude and a longer duration, low amplitude (an asymmetric waveform) (Fig. 9.2c). The total magnitude of each push/pull potential is the same, such that the ions experience the same average electric field for each cycle, but in opposite directions. If the mobility of the ion during the push cycle is the same as during the pull cycle, the ion's average displacement will be zero. However, the gas-phase mobility of an ion has an electric field dependence such that it changes between going from high field (ca. 20,000 V/cm) to low field (ca. 1000 V/cm) [30]. This results in a different net displacement of the ion when subjected to the short duration, high field wave cycle than when it experiences the longer duration, lower field wave cycle. Ultimately, this results in ions that are not balanced by the asymmetric waveform to be neutralized onto one of the plates. This device will transmit a narrow bandpass of ions whose high field and low field IM is exactly matched by the waveform. An additional compensation voltage is applied to either plate to allow the device to be scanned, thus filtering ions possessing different changes in high/low field ion mobilities.

Devices that utilize this principle of IM separation have been described by several names, the two most popular being high field asymmetric waveform ion-mobility spectrometry (FAIMS) and differential mobility spectrometry (DMS). In Table 9.1, we refer to both techniques as *differential high/low field IM*. Both terms describe the same

general technique with defining differences being in the geometry of the electrodes used and how ions are detected in stand-alone applications. FAIMS and DMS devices do not separate purely by IM but by the difference in high/low field IM (the first derivative), so the observed separations are often subtly different from those derived from the other IM methods. In many cases, these devices can resolve ion species that are difficult to separate by traditional IM methods, such as leucine and isoleucine [31]. Additionally, one can couple FAIMS or DMS with a temporally dispersive IM method (i.e., drift tube or traveling wave IM spectrometry) and achieve excellent orthogonality between the two separations [32], which is analogous in principle to two-dimensional GC using polar and nonpolar columns. FAIMS and DMS have been successfully used to separate a variety of small molecules and both technologies are commercially available as front-end separators for mass spectrometers (FAIMS, Thermo Scientific, and DMS, Sionex). Because these devices operate at or near ambient pressure, they are readily compatible with atmospheric pressure ionization sources.

The high/low field IM behavior and transition between differential behaviors are still poorly understood. Because there is no first principle correlation between the change in IM and the low field collision cross section, analyte size information is difficult to obtain with any accuracy using the differential high–low field IM technique. Gas-phase collision cross sections can be determined in differential high–low field IM through special energy loss experiments using triple quadrupole MS [33].

9.2.4 Differential Mobility Analyzer

Differential mobility analyzers (DMAs) are a special class of IM instruments used primarily for measuring the size of aerosol particles >5 nm in diameter. These instruments are not commonly used for small molecules due to high diffusive ion losses; however, the method is included here to complete the discussion of IM technologies. The reader may skip to the next section if more relevant discussion regarding IM techniques for metabolite analysis is preferred.

Building on the differential high/low field IM technique described in the previous section, consider now a similar experimental setup, whereby ions are entrained in a flowing gas and allowed to pass between two parallel plates as shown in Fig. 9.2d. Instead of an alternating voltage, a direct voltage is applied across the two plates creating a uniform electric potential gradient perpendicular to the ion transit. This will cause ions to migrate from one plate to the other as they are being pulled along by the gas flow. All ions of the same charge will experience the same force of the electric field, but because of differences in their ion mobilities, smaller ions will be displaced at a shorter distance than larger ions. This results in a spatial dispersion of ions based on their ionic mobilities. Practical designs of this type of transverse field IM instrument utilize small apertures or slits on the parallel plates, which are offset at some defined distance from one another, as depicted in Fig. 9.2d. Ions are introduced to the gas stream through one slit, and the voltage is tuned to allow ions with different mobilities to pass through the second slit, in much the same way as tuning the m/z of a magnetic sector MS instrument. With the exit slit design, the instrument operates in a scanning mode. The exit slit can also be replaced by an array detector, which allows for broadband IM spectrum to be measured [34]; however, this design cannot be coupled as a front end to MS (e.g., IM-MS).

These instruments are sometimes called *aspirator mobility analyzers* due to the aspiration effect of introducing ions into the gas stream. More commonly they are referred to as *differential mobility analyzer (DMA)* and by the trade name gas-phase electrophoretic molecular mobility analyzer (GEMMA), the latter now referred to as *macroIMS* (TSI Incorporated). The DMA nomenclature is oftentimes confused with the DMS technique described in the previous section, but it should be noted that these two IM techniques separate ions by two very distinct mechanisms, specifically the DMA differentiates ions based on aerodynamic ionic mobility (IM in the presence of a gas flow), whereas DMS exploits IM differences between high and low electric field conditions. DMA and GEMMA have been highly successful in measuring particle sizes down to the nanometer range [17]. Smaller ions are more difficult to transmit due to diffusive losses and the instrument performance is inherently tied to the size of the slits used—larger slits improve sensitivity at a cost of resolution and vice versa. Recent technological improvements show promise for extending the usable size range down to small molecular ions with high sensitivity and IM resolution [23].

As with drift tube IM instruments, DMA measurements can be used to determine the ion's effective size since there is a direct relationship between the experimental conditions (transmission voltage and gas flow rate) and the experimentally measured gas-phase IM. A hard sphere model is used to relate the measured IM value to an effective size. As mentioned previously, DMA instruments are currently limited to the analysis of relatively large ions (tens of nanometers or greater in diameter), requiring that effects such as surface roughness be accounted for in the size calculation [35]. Nonetheless, because the DMA experimental separation parameters (voltage, drift length, and gas flow) are well characterized, the resulting measurement precision of the DMA technique is high.

9.3 INTEGRATED ION MOBILITY–MASS SPECTROMETRY

All the IM methods described previously can operate as a stand-alone chemical analysis technology (i.e., an IM spectrometer). One shortcoming of IM analysis alone is that the measured ionic mobility is a relative property of the analyte and will change with respect to different experimental conditions (e.g., drift gas pressure, temperature, and composition). Consequently, confident analyte identification using IM spectrometry information alone (via the reduced mobility) relies on rigorous method validation and even then is subject to specific constraints on the system being studied [36–38]. In contrast, MS measures an inherent physical property of the analyte (mass or more specifically, the mass-to-charge ratio), which can be used for analyte identification purposes, as the property of ion mass is readily comparable across instrument platforms. MS and tandem MS methods are the cornerstone technology of many analyte characterization initiatives, a fact that is reflected in the numerous contributions to this volume. Owing to the requirement that the analyte be ionized, IM is particularly suited for coupling with MS. The first IM-MS instruments appeared in the 1960s in response to the need to better characterize the ion chemistry observed in drift tubes [39,40]. Bioanalytical drift tube mass spectrometers emerged in the 1990s owing to the development of soft ionization methods (MALDI and ESI (electrospray ionization), which are discussed in other chapters) [6]. IM-MS is now widely utilized and regarded as a technique in and of itself [41], much like the integration of LC to MS (LC-MS). Most

of the technical challenges associated with coupling the elevated pressure IM stage to a vacuum MS have been solved, largely due to parallel initiatives in improving the transfer efficiency of ions from atmospheric ionization sources to MS [42]. As mentioned previously, commercially available IM-MS instruments are now emerging and offer little to no compromise in performance as compared with the familiar MS platforms in which they are coupled. Thus, researchers familiar with existing commercial MS technologies will find that IM capabilities enhance existing methodologies built upon MS analysis.

9.3.1 Ion Mobility–Mass Spectrometry Instrumentation

Figure 9.3a illustrates the basic components of an IM-MS instrument. Sample introduction can include direct infusion, GC, or LC. Virtually every ionization source that has been used in MS has been coupled to an IM-MS instrument [41], including all the ionization techniques covered in this volume. Likewise, virtually every combination of IM and MS has been attempted. Temporally dispersive IM (drift tube and traveling wave) combined with temporally dispersive MS (time of flight, TOF) is a particularly useful combination since both techniques offer broadband analysis for each experimental cycle [43]. Another useful combination is coupling a scanning IM (e.g., FAIMS or DMS) to a scanning MS (an ion trap), which allows selective monitoring and analysis experiments [44]. Instruments based on bracketing an IM between two mass spectrometer stages (MS-IM-MS) have also been developed [45], allowing several useful stages of tandem ion experiment to be conducted [46]. We discuss various combinations of tandem IM/MS in the later sections. Commonality between all these instrument configurations is the back-end mass analysis, which provides a comprehensive characterization of the sample by mass or, specifically, the mass-to-charge ratio. For the purposes of this chapter, we focus primarily on IM-MS instruments that incorporate the first stage of mass selection and tandem capabilities with a high resolution mass spectrometer (the so-called high performance IM-MS, Fig. 9.3b). Although the literature defines high resolution mass spectrometers with a degree of disparity, we consider high

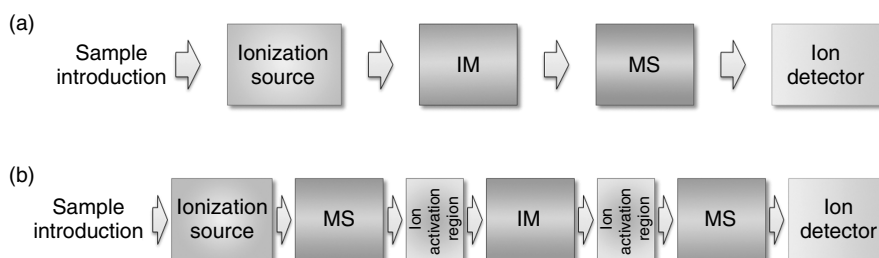


Figure 9.3 (a) A schematic of the basic components of an ion mobility–mass spectrometer. From left to right: sample is introduced, ionized, then separated first by ion mobility and then by mass, and finally, detected. (b) A “high performance” ion mobility–mass spectrometer can include a mass spectrometer before the ion-mobility spectrometer and several stages of ion activation located between each spectrometer component. Additionally, the spectrometers operate with high precision (resolving power) and sensitivity, enabling high quality measurements of mobility and mass to be made.

resolution as the ability to obtain part-per-million mass precision (e.g., 1000 ± 0.015 Da, or 20,000 resolving power) within the mass-to-charge range of interest.

9.3.2 Orthogonality and Peak Capacity in Ion Mobility–Mass Spectrometry Analyses

The observed mobility–mass correlation in IM-MS data limits the orthogonality of the two separations. Completely orthogonal measurements would distribute data throughout two-dimensional separation space with no correlation between the data sets, as each separation is contingent upon chemical or physical properties that are independent of one another [47]. In other words, data projected on a two-dimensional separation plot would occupy all the separations space, as illustrated conceptually in Fig. 9.4a, where analyte signals distribute themselves across the full two-dimensional area. This high orthogonality is often observed, for example, in 2D gel electrophoresis [48,49], where the two dimensions of separation are analyte mass and isoelectric point—two unrelated analyte parameters. The degree of orthogonality of IM-MS separations is low due to the correlation of mass and size [50]. This is apparent when considering an ion of increasing mass, which will inherently possess a larger volume and therefore occupy an established region of separations space, a mobility–mass correlation termed *conformational space* [51]. Low orthogonality inherently limits the peak capacity (ϕ) of the IM separation, which is the maximum number of resolvable peaks within the separation window, as illustrated in Fig. 9.4b for a hypothetical IM-MS spectrum [52]. Here, the

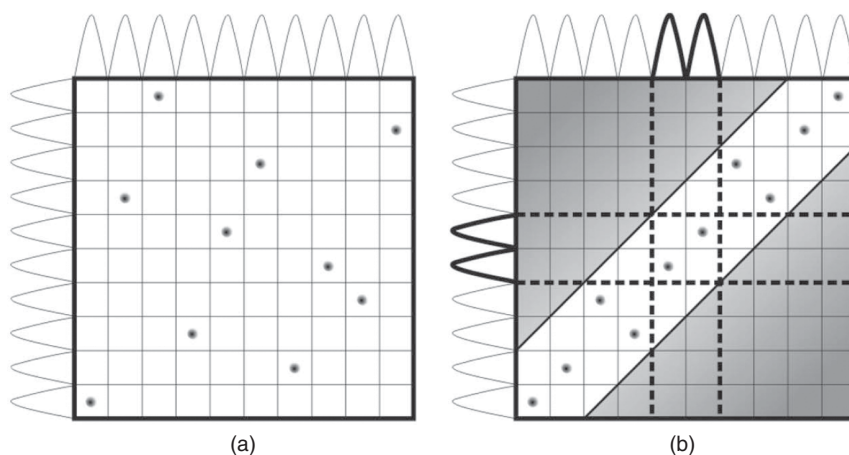


Figure 9.4 (a) A theoretical distribution of peaks (represented in black in grid space) in two-dimensional space for completely orthogonal separations. Each axis corresponds to a theoretical separation parameter. The high orthogonality allows for the occupancy of all two-dimensional space, thus having a peak capacity equivalent to the product of the two peak capacities. (b) Two separations with low orthogonality. The region of two-dimensional separations space that peaks occupy is limited to a fraction of all separations space. The region of analysis space is narrowed to that shown in the overlapping regions, for example. This limits peak capacity, as the total peak capacity is no longer the product of the two peak capacities, but a corrected value that is a fraction of the maximum. The gray areas are regions of separation space, in which no signal appears.

narrow distribution of data requires that the analysis window be narrowed accordingly, depicted with the dotted lines in Fig. 9.4b. Previous IM studies have reported a peak capacity of ~ 5 for the IM dimension alone, which is low when compared with other separation methods [10]. However, the strength of IM is that when combined with MS, the overall peak capacity of the MS dimension is increased multiplicatively (specifically, fivefold, for this example). As there is virtually no compromise in analytical performance when IM is added to MS, this represents a significant improvement in analytical performance. For example, IM-MS has recently demonstrated a peak capacity of 10^3 for metabolites in a single IM-MS spectrum, which is comparable to that of highly orthogonal methods, such as reverse phase LC-MS ($\phi = 1000\text{--}1500$, *Shewanella oneidensis* lysate) [53], two-dimensional GC-MS (GC $\times$ GC-MS, $\phi = 1200$, lean C57BL/6 mice spleen extract, $\phi = 1800$ human serum) [54,55], and capillary electrophoresis-MS (CE-MS, $\phi = 1692$, *Bacillus subtilis* extract) [56]. These examples represent general separation benchmarks for each analytical platform, and while useful for qualitative comparative purposes, it should be noted that the samples, conditions, and mass spectrometers are not consistent across each study.

9.3.3 Ion Mobility–Mass Spectrometry Analysis Speed

The main strength IM holds over conventional separations techniques is that it occurs post ionization, which means separations are performed entirely in the gas phase. This results in a separation that occurs on the order of hundreds of microseconds to milliseconds. As a result, thousands of IM spectra can be acquired each second. When compared with previously presented separation methods that are utilized before ionization (e.g., LC, CE, and GC), the time required for a mobility separation to occur is several orders of magnitude faster. An analysis speed comparison of several separation techniques is provided in Fig. 9.5. For a typical metabolomics study, high performance LC, two-dimensional GC, and CE separations occur on the order of minutes to hours. Additionally, GC separations often require analyte derivatization to promote volatility,

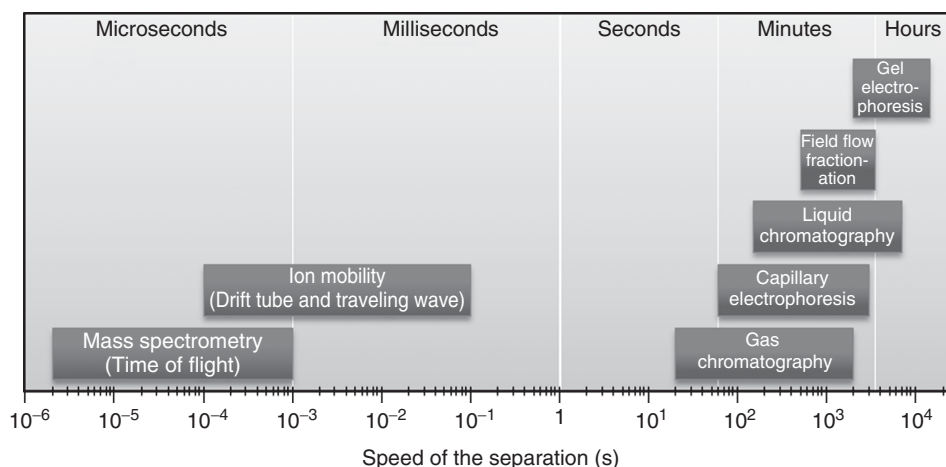


Figure 9.5 Speeds for separation techniques commonly applied to metabolite analysis. Note the logarithmic scaling on the abscissa.

TABLE 9.2 Several Single-Dimensional Separation Methods and the Parameters for Separation, Peak Capacity (ϕ), and Peak Production Rate (ϕ/s)

Separation Method (Single Dimension)	Separation Parameter	Peak Capacity (ϕ)	Signal Peak Production Rate (ϕ/s)	References
Capillary electrophoresis (CE)	Electrophoretic mobility	1000	0.5	62
Gas chromatography (GC)	Vapor pressure	75	6	63
Liquid chromatography (LC)	Hydrophobicity	60	0.06	64,65
Ultrahigh pressure LC		300	0.2	
1D slab gel electrophoresis	Isoelectric point, mass	100	0.03	66
Field-flow fractionation (FFF)	Hydrodynamic radius	10	0.005	66,67
Ion mobility (IM)	Gas-phase cross section	5	500	10

which adds multiple hours in addition to the separation [55,57–61]. While the orthogonality and peak capacity of IM-MS remains comparable to other two-dimensional methods, it is the high speed of IM-MS that affords a great analytical advantage in terms of the peak production rate, as illustrated in Table 9.2 [9].

In addition to the high data production rate of IM-MS, another key advantage of coupling time dispersive IM and MS is the complimentary nature of the timescales for each dimension of analysis. Because time dispersive IM separations (millisecond) occur two to three orders of magnitude slower than time dispersive MS (microsecond), hundreds of mass spectra can be acquired for each IM spectrum, which benefits the sampling resolution across the IM dimension [68]. This temporal sampling benefit is commonly exploited in LC-MS where each LC trace is sampled numerous times by the MS. One criticism of LC-MS has been that the immensely disparate timescales (six to eight orders of magnitude) ineffectively utilize the throughput of the mass spectrometer. IM readily bridges this time disparity between LC and MS, which has been demonstrated for the three-dimensional analysis of complex biological samples [69]. CE-IM-MS has also been demonstrated to improve the sensitivity of MS for low abundance analytes in complex biological samples [70]. Combining front-end separation techniques with IM-MS is still an emerging area that holds great promise for improving the chemical analysis for both contemporary and future applications.

9.3.4 Information Content of Ion Mobility–Mass Spectrometry Analyses

One favorable consequence of the data correlation between IM and mass is the potential for extracting additional information regarding specific analytes within the sample.

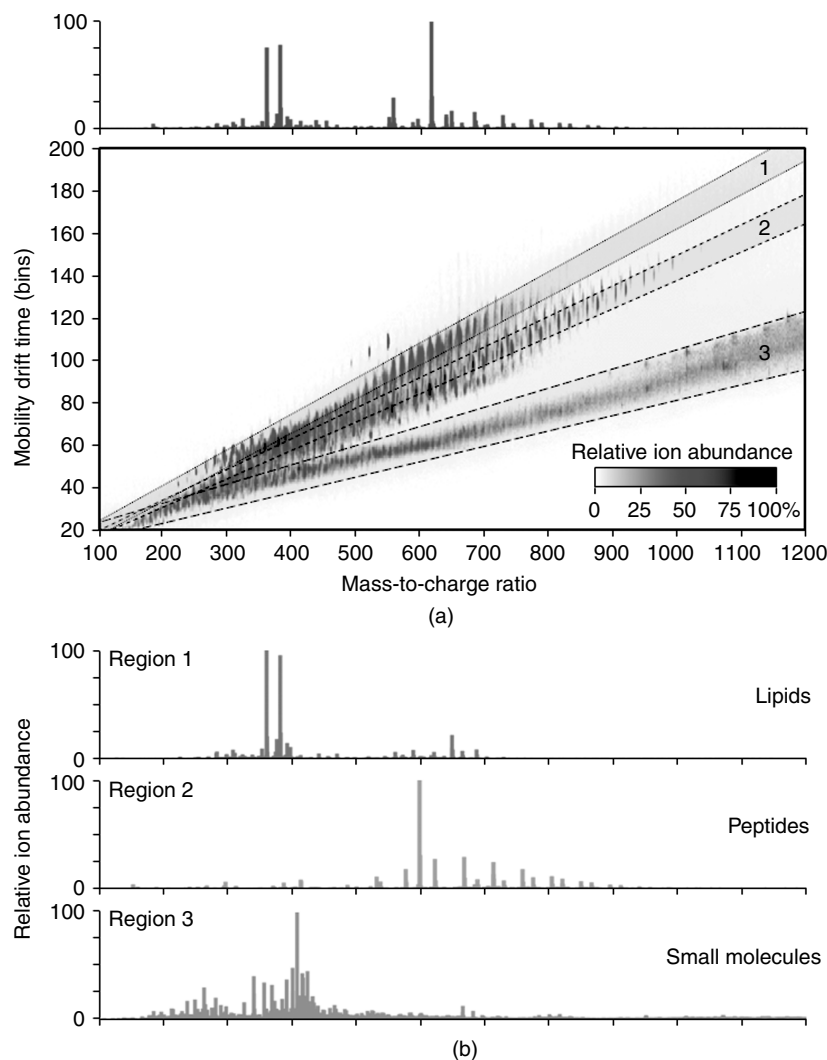


Figure 9.6 (a) Shown is a nanoelectrospray ionization–ion mobility–mass spectrum of a methanol extract of rat blood. Mass-to-charge ratio is depicted on the abscissa, with mobility drift time represented on the ordinate (ms). Peaks are false colored to illustrate relative abundance, with the scale displayed in the inset. Shown above the IM-MS plot is the corresponding mass spectrum for the entire sample. Regions corresponding to various mobility–mass correlation lines are highlighted and labeled, with the extracted mass spectra displayed in (b). Preliminary identifications are assigned based on conformational space occupation. (See color insert.)

This complimentary information can be used in a number of ways for more confident identification and characterization of analyte signals within the sample. Figure 9.6a contains a two-dimensional projection of IM-mass data for a nanoelectrosprayed sample of a methanol extract of rat blood. This data was obtained in <1 min in the authors' laboratory using a traveling wave IM-MS instrument. The spectrum displays m/z (Da) on the abscissa and drift time (ms) on the ordinate. The spectrum is false colored

(heat mapped) to represent relative signal abundances and is composed of almost exclusively singly charged ions due to the nano-ESI conditions utilized. Immediately obvious is the close correlation between the mobility and mass, which manifests as a clustering of data along a mobility–mass correlation line [71]. This results from the intuitive scaling of the analyte size with its weight. Figure 9.6b illustrates the advantage of selective interrogation of data corresponding to the mobility–mass correlation for lipids, peptides, and small molecules. The individual mass spectrum is extracted directly from the combined mobility–mass spectra (Fig. 9.6a). The result is the extraction of relevant analyte signals, based on the distillation of chemical noise, which greatly improves the amount of information that can be gleaned from the data. This strategy has recently been shown to be applicable to metabolite identification for the IM-MS analysis of human blood [72], rat lymph fluid [73], and *Escherichia coli* extracts [74].

One of the challenges of metabolite studies is the diverse representation of chemical species classified as metabolites. These include both large (e.g., peptides, lipids, and glycosides) and small (e.g., alkaloids, terpenes, and phenols) biomolecules, but traditionally focused on the latter because of the available technologies for small-molecule analysis (e.g., GC-MS). As described previously, the IM-MS analysis provides an advantageous dimension of information in the form of molecular class-specific mobility–mass correlation lines, which can be used for identification purposes and also for isolation and extraction of certain chemical signals. From a global analysis perspective, this has the advantage of interrogating a large amount of chemical data simultaneously, which is particularly advantageous for whole systems approaches [75]. As an example of the utility of IM-MS for whole systems studies, Fig. 9.7a depicts a mapping of ion collision cross section (IM) versus ion mass for a large sample

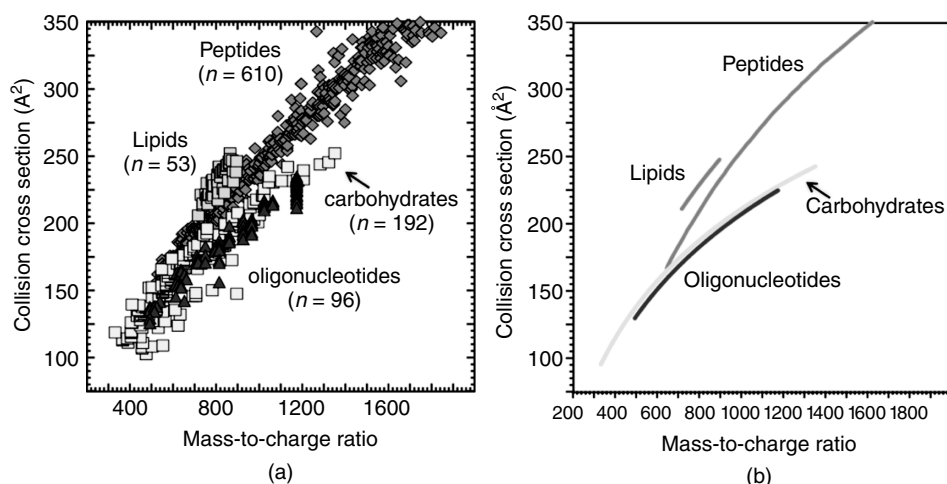


Figure 9.7 (a) The mass-to-charge values and calculated collision cross sections (in Å²) obtained using a drift tube ion mobility–mass spectrometer for 53 lipids, 610 peptides, 192 carbohydrates, and 96 oligonucleotides, illustrating the separation of biomolecular classes in two-dimensional space using IM-MS. (b) Logarithmic regression fits applied to the biomolecular class distributions in (a). *Source:* Adapted from Fenn LS, Kliman M, Mahsutt A, Zhao SR, McLean JA. *Anal Bioanal Chem* 2009;394:235. Fig. 9.1(a,b), with kind permission from Springer Science+Business Media. (See color insert.)

number ($n = 951$) representing four biomolecular chemical classes: lipids, carbohydrates, peptides, and nucleotides. Figure 9.7b contains logarithmic regression fits of the data sets in Fig. 9.7a and illustrates the average mobility–mass correlation for each biomolecular class. Note that the IM data normally projected as drift time has been converted to collision cross section (in $\text{\AA}^2$). What is immediately obvious is that the average mobility–mass correlation is different for each class, with notable disparity between peptides and carbohydrates. The data projections in Fig. 9.6 represent an initial attempt at mapping mobility–mass data space for a large data set representative of several molecular classes. The ultimate goal of such a project is to generate a reliable “atlas” of conformational space of biologically relevant molecules and apply this *a priori* knowledge toward an integrated “omics” approach [76].

9.4 TANDEM METHODS FOR ION MOBILITY–MASS SPECTROMETRY/MASS SPECTROMETRY

IM has so far been discussed with regard to the analytical merit of performing fast separation of mixtures and using the resulting separation to locate relevant chemical signals that may otherwise be disregarded in a single dimension of mass analysis, such as the case of structural isomers. While the identification of significant analyte signals (markers) in the spectrometric analysis is certainly important, it is more challenging to then assign these signals a chemical identity [77]. Tandem MS experiments (MS/MS) are a critical aspect of MS approaches to metabolite identification. MS/MS involves two mass separation steps with ion fragmentation occurring in between. The first MS is used to isolate one mass to charge, which is then subjected to a fragmentation step (most commonly through energetic gas collisions in a pressurized chamber), causing decomposition of the selected precursor ion into fragments. The fragment ions are then analyzed in a second MS stage to generate a fragment ion mass spectrum. Fragmentation data is used to determine the precursor ion’s identity based on predicted fragmentation pathways [78]. Additionally, there are several useful “scans” that can be utilized with MS/MS in order to monitor, for example, specific product ions that are known metabolic markers for a particular drug. These tandem strategies are briefly outlined later in this chapter. Tandem MS experiments are performed using either serial stages of MS instrumentation (tandem in space) or an ion trap instrument (tandem in time). When we discuss tandem experiments in IM-MS, we have dealt only with tandem in space arrangements (primarily as a result of the lack of development of ion-trapping-based IM technologies, although it is noted that such a device has been suggested theoretically [79]).

9.4.1 Scanning Modes for Tandem Mass Spectrometry

There are several “scan modes” that are useful in multistage tandem MS for metabolite screening applications. These modes differ only in whether each MS stage is operated in narrow (selected MS) or broadband (full MS) mode. With two MS stages (including a fragmentation stage in between), there are four possible combinations of scan modes that can be utilized. Figure 9.8 illustrates these four scan modes, namely, (a) precursor ion scan (full–selected), (b) neutral loss scan (full–full), (c) product ion scan (selected–full), and (d) selected reaction monitoring (selected–selected). Traditionally, these modes were applied to triple quadrupole instruments, but the general methodology

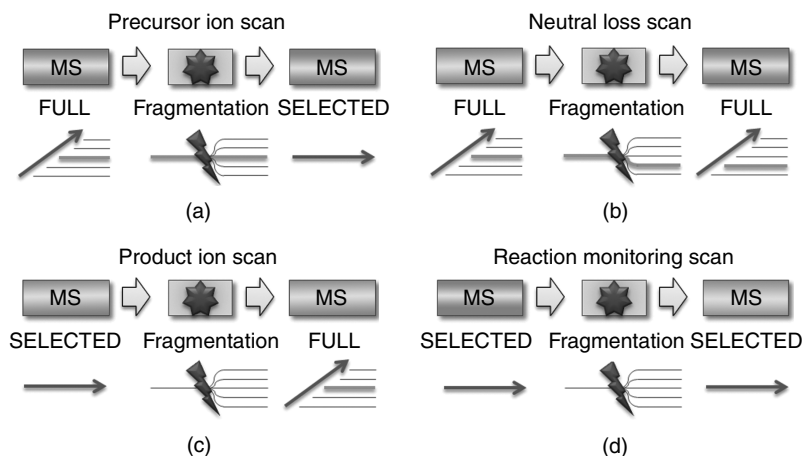


Figure 9.8 Illustrations of the various MS/MS scanning methods for two tandem MS stages. Fragmentation occurs between the two mass analyzers, as depicted. (a) A precursor ion scan, where the first mass analyzer performs a full scan, while the second mass analyzer is fixed to a given m/z that corresponds to an expected product ion. (b) A neutral loss scan, where both mass analyzers perform a full scan, with the second mass analyzer set to a fixed mass-to-charge difference from the first. Commonly, the analyzer offset corresponds to a known biotransformation. (c) A product ion scan in which the first mass analyzer isolates a particular mass-to-charge value and the second mass analyzer performs a full scan to obtain all resulting fragments. (d) A reaction monitoring scan in which the first mass analyzer selects a specific mass-to-charge value and the second mass analyzer is set to selectively monitor for the appearance of a particular product ion.

has since carried over to other MS/MS instrumentation, such as Q-TOF (quadrupole time of flight), TOF-TOF, and, to some extent, various ion trap platforms. The underlying difference in applying these scan modes to different instrument platforms is in the resulting performance: TOF instruments generally sacrifice sensitivity and broad-range linear response (e.g., quantitative ability) for high speed and high resolution/mass accuracy. Ion traps trade some degree of performance (speed and mass resolution) for experimental versatility; most notably, the capability to do multiple stages of tandem experiments (MS^n , where $n > 2$). Because TOF instruments obtain a broadband mass spectrum, the precursor and selective reaction monitoring experiments are performed post data acquisition. The following is a brief description of each scan mode as the modes are applied in tandem MS/MS experimentation.

The precursor ion scan (Fig. 9.8a) and neutral loss scan (Fig. 9.8b) modes are the first tandem MS experiments that are utilized in a metabolite study. These MS/MS experiments are considered as reasonable preliminary methods since the information gained is broadly encompassing and requires very little *a priori* knowledge regarding the system under investigation. In a precursor ion scan, an expected product ion is selected with the second MS stage, and the first MS stage is scanned through a range of m/z values until the expected product ion signal appears. The product ions of interest represent common metabolic alterations, such as glucuronidation ($M + 176$). Additionally, the second MS stage can be set to monitor the m/z of the parent compound itself in order to discover metabolites that consist an unaltered portion of the

parent compound, such as oxidation ($M + 16$). Because the fragmentation step in an MS/MS experiment results in both fragment ions and fragment neutrals, the mass spectrometer is blind to the latter. To overcome this fundamental limitation, the two MS stages can be set to transmit a fixed m/z difference from one another, representing a characteristic neutral loss. For example, to locate an oxidation product metabolite, the first MS stage is scanned through a range of m/z values and the fragment ion, $M - 16$, is monitored by the second MS stage. This will find all precursor ions that have been oxidized.

The product ion scan (Fig. 9.8c) is the conventional mode for MS/MS experiments. In a product ion scan, a precursor ion m/z is isolated and transmitted by the first MS stage, fragmented, and the entire fragment ion spectrum is obtained in the second MS stage. The product ion scan is useful for characterizing a precursor ion once it has been identified as being of interest. Finally, the reaction monitoring experiment (Fig. 9.8d) is a powerful and specific mode of MS/MS operation used primarily when quantitative information is sought. In this mode, an m/z of interest is selected by the first MS, fragmented, and a specific m/z is monitored by the second MS. Prior knowledge regarding the expected product ions is necessary. A single or multiple set of fragment ion m/z values can be monitored for each precursor ion transmitted by the first MS.

As mentioned previously, these scanning modes were originally developed for triple quadrupole instrumentation, but recent advances in hybrid MS instrumentation has expanded their usage to other platforms. The Q-TOF configuration is a particularly important instrument platform which replaces the final MS stage of a triple quadrupole with a broadband TOF MS. This has the significant advantage that TOF MS performs with greater analyte sensitivity in its conventional broadband MS mode than a quadrupole operating in a full-scan MS mode. The other important analytical advantage that Q-TOF instruments hold over a triple quadrupole platform is higher speed and, consequently, higher sample throughput. Finally, the high mass resolving power capabilities of TOF greatly improve the mass measurement accuracy. Contemporary Q-TOF instruments are capable of resolving powers in excess of 40,000, with a theoretically unlimited mass range. Macromolecules in the MDa (10^6 mass units) range have recently been studied using Q-TOF instruments [80].

9.4.2 High Performance Tandem Ion Mobility–Mass Spectrometry Modes

The addition of IM to multistage MS instruments offers greater experimental flexibility with regard to tandem experiments. For example, the use of an FAIMS device at the front end of an MS instrument introduces an added dimension of chemical noise discrimination that serves to enhance important ion signals appearing at low abundance [81]. A more versatile example is the addition of an IM dispersive stage into a hybrid Q-TOF instrument, which creates the useful MS-IM-MS configuration (specifically, a Q-IM-TOF). Because of the necessary transition between the two vacuum MS stages into the pressurized IM, elevated pressure ion guides are utilized between each stage to efficiently transfer ions into and out of the IM device, which creates an advantageous addition of two collision cells in the MS-IM-MS instrument configuration. Thus, collisional activation of ions can be accomplished before or after the IM separation, which creates a number of useful tandem arrangements. The next section deals with the various tandem configurations afforded by the MS-IM-MS configuration,

and we consider some of the added information that can be gleaned from each stage of tandem analysis. Regarding nomenclature, the hyphen designates two coupled analyzers (e.g., IM-MS) while the forward slash designation (e.g., MS/MS) denotes tandem capabilities (i.e., a collision cell between the two components).

When considering an analytical configuration that brackets a mobility separation between two mass analyzers (MS-IM-MS), the first possibility is to select an m/z window with the first MS stage and immediately induce fragmentation before the IM-MS analysis (i.e., MS/IM-MS). This will generate a comprehensive 2D IM-MS spectrum of resulting fragment ions (Fig. 9.9a). This strategy is identical to performing a product ion scan, but with the added dimensionality of the IM separation, resulting in separation of product peaks based on both mobility and mass-to-charge ratio. The practical benefit is the separation of product ions in chemical space, allowing for the distillation of the fragmentation spectrum. However, isobaric species will not be separated before fragmentation, and the resulting product ion spectrum may reflect multiple isobaric species not easily differentiated.

An alternative mode of operation is to first select an m/z of interest, mobility-separate, and then fragment the ions following the IM region (i.e., MS-IM/MS). As a result, a particular mass of interest is dispersed based on mobility before fragmentation and secondary mass analysis. An example spectrum is contained in Fig. 9.9b. Because ion fragmentation follows the IM separation, the resulting fragment ions will have mobility drift times correlated to their precursor ion signal. Thus, this method allows for the initial separation of isobaric ions based on their different mobilities, while simultaneously correlating the product ions to their precursors. Thus, both chemical noise and isobaric signal overlap are attenuated through this fragmentation method. The MS-IM/MS method can greatly simplify analyte identification based on the removal of contaminating product peaks contributed by isobaric ions.

A third mode of operation that can be utilized in the MS-IM-MS configuration is to isolate an m/z in the first MS stage, induce dissociation, mobility-separate the resulting fragments, and finally, fragment once again before the secondary mass analysis (e.g., MS/IM/MS). This mode of operation (Fig. 9.9c) results in mobility-correlated secondary product ions. Figure 9.10 contains tandem data for the IM-MS analysis of the small carbohydrate, lacto-*N*-fucopentaose 1 and illustrates two modes of tandem IM-MS. In Fig. 9.10a, the ion fragmentation occurs before the IM-MS analysis, which results in a two-dimensional spectrum of fragment ions separated in both mobility and mass space. In Fig. 9.10b, ions are induced to fragment both before and immediately following the IM analysis, resulting in a second stage of fragmentation (a pseudo-MS³ experiment). In the latter example, the secondary fragments align themselves to the precursor ion signals in which they originated since the IM separation occurs before their formation. The novel aspect of this mode of tandem experimentation (MS/IM/MS) is that simultaneous ion activation can be achieved, generating a complete precursor and fragment ion spectrum during each experimental measurement cycle.

9.4.3 Advantages of High Mass Accuracy and High Resolution in Metabolite Identification

In drug metabolism studies, the molecular formula of a metabolite of interest is a key piece of information. This information is most easily obtained by measuring the exact mass of the ion to high accuracy. High resolution MS can obtain a mass measurement

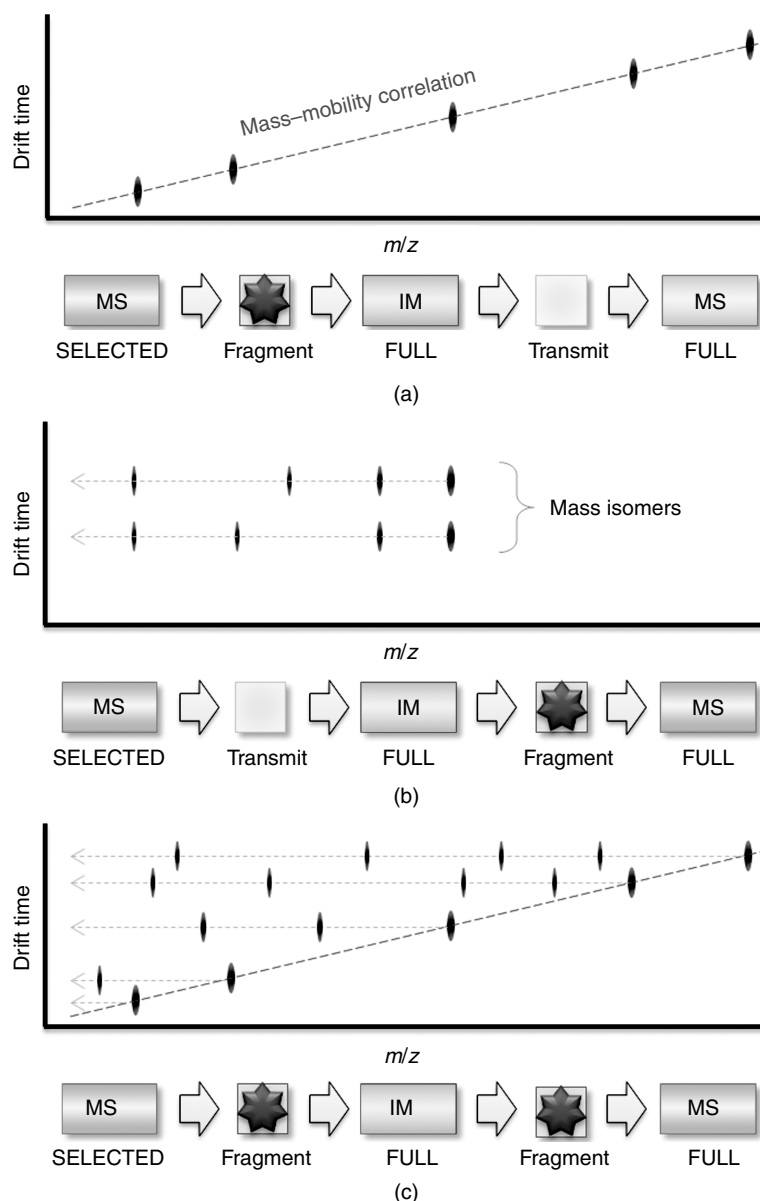


Figure 9.9 Three different tandem modes for MS-IM-MS instrumentation. Each two-dimensional spectrum corresponds to a single m/z value isolated by the first MS stage. (a) MS/IM-MS mode in which fragmentation precedes the IM-MS analysis. (b) MS-IM/MS mode in which the mobility spectrum is generated before ion fragmentation, resulting in alignment of the fragment ions to their precursor ion's measured mobility value. As illustrated, this tandem mode is particularly useful for isobaric ions that cannot be differentiated by MS alone. (c) MS/IM/MS mode in which an isolated m/z value is fragmented both before and after the ion-mobility analysis, resulting in parallel fragment ion alignment, correlating to their respective precursor ion signals. In these examples, only singly charged ions are considered.

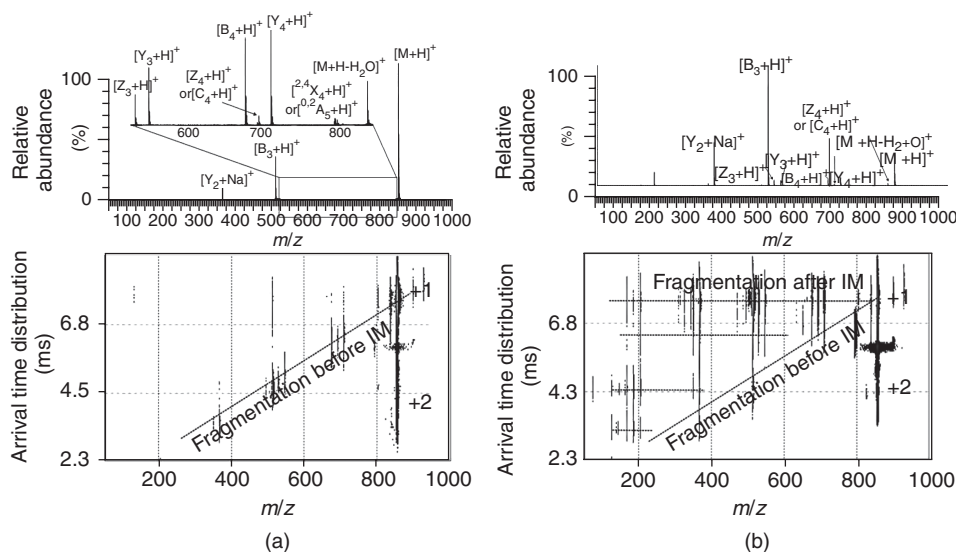


Figure 9.10 Ion mobility–mass spectra of the small carbohydrate, lacto-*N*-fucopentaose 1, illustrating two tandem IM-MS modes. (a) Spectra from fragmentation before IM-MS analysis, which results in dispersion of fragment ion signal across both dimensions of data analysis. (b) Spectra resulting from two stages of fragmentation, one before the IM analysis and one immediately following the IM. In the latter case, fragment ion signal is dispersed across the mobility dimension and secondary fragment ion signals align isobaric in mobility, resulting in simultaneous fragmentation of all ions resulting from the first stage of activation. *Source:* For the mass spectra (in both (a) and (b)), the carbohydrate nomenclature used is from Domon and Costello [82]. Adapted from McLean JA, Fenn LS, Enders JR, Mass Spectrometry Imaging, Methods in Molecular Biology 656, Rubakhin, SS, Sweedler, JV (eds.) 2010. Fig. 21.7, with kind permission from Springer Science+Business Media.

with high precision, which factors directly into the accuracy of the mass measurement. By obtaining an accurate mass measurement with high confidence, one can narrow down the number of possible molecular formulas pertaining to the measured mass.

When utilizing high resolution MS, an approach has been applied to metabolite identification that relies primarily on high mass accuracy to query the entire sample post separation [83]. This method is termed MS^E and functions by initially acquiring data over a large mass range (ca. 100–1000 Da). Subsequently, collision energy is imparted to the same range of ions in a ramped method from low to high energy. This results in data that combines the full scan with corresponding fragments. Using the high mass accuracy and drift time separations of the instrument, product ions can be correlated back to their respective precursor ions based on mass defect and mobility. This resolves the issue of discarding ions on the basis of selective fragmentation, as this method allows for the fragmentation of all ions within a given time range. High mass accuracy is maintained through the use of an internal standard for proper calibration. What results is the ability to perform broadband fragmentation and monitor product ion transitions, as commonly done in scanning experiments, over an entire mass range. A fundamental limitation lies in the fragmentation of isobaric species that are inherently present in complex samples, as product ions for both isobars will be present in the fragmentation spectrum, resulting in convoluted data sets. This is commonly addressed

by performing a preionization separation. Fortunately, the IM separation can distinguish isobars in the mobility dimension. As a result, an additional degree of discrimination is present in compiling a list of product ions for a given precursor.

Tandem MS inherently has limitations. Most notably, this method is insensitive to low intensity precursor ions, as these are often overlooked by automated systems if a particular peak is transient in nature. Selected precursor ions must be well above the limits of detection for a given tandem platform in order to generate meaningful tandem spectra. Isobaric species, which would commonly convolute tandem spectra, may be separated using IM post ionization separations to resolve isobars. While IM-MS enhances tandem experiments, currently, IM separations are insufficient for the routine resolution of structurally similar isobars, for example, compounds that differ by a single chiral center. This is where high resolution structural measurements, such as NMR, are required. However, these methods allow for the relatively rapid interrogation of complex samples, allowing distillation of robust data sets to significant analytes.

We began this chapter by stating the technological challenges of metabolite analysis are substantial. While this is accurate, the number of metabolites expressed by eukaryotes is small in comparison to the number of proteins, which is estimated somewhere at ca. 200,000 for humans. If IM-MS and related technologies are to be commissioned into the global “omics” initiatives, then there is perhaps no greater potential for impact than in metabolite analysis. Future work should seek to implement the real-time acquisition of metabolic information to better represent the dynamic nature of the metabolome. The temporal resolution necessary to understand transient biological responses will likely require the ability to acquire spectra at a rate that exceeds our current analytical capacity using preionization separations. This conundrum lends itself strongly to the case for IM-MS as a means of rapid analysis of drug metabolism. By coupling IM-MS with microfluidics that allow for the trapping and maintenance of cell colonies, a global profile of a microenvironment can be acquired with high temporal resolution. This permits a more complete understanding of the underlying metabolic processes that preclude particular metabolites, while potentially circumventing the dilution issue commonly associated with bulk cultures. Although many technical issues exist with coupling microfluidic environments directly to IM-MSs (e.g., ion suppression by non-informative, high abundance metabolites, and salts), these are surmountable and have been addressed in a modular fashion.

9.5 SUMMARY

IM spectrometry is the separation of gas-phase ions on the basis of differential velocities through an inert buffer gas under the influence of an electric field, this rate being termed *mobility*. Four main IM separation methods exist (drift tube IM spectrometry, traveling wave IM spectrometry, high field asymmetric waveform IM or DMS, and differential mobility analyzer), each utilizing a different approach for separations of gas-phase ions. These methods, although present in various geometries and grounded in disparate physical principles, have the common thread of performing a postionization separation based on mobility. Drift tube and traveling wave IM temporally separate ions along the same space, which has the advantage of generating a broadband spectrum for each measurement cycle, while limited to separating discontinuous

ion pulses. High field asymmetric waveform IM and differential mobility analyzers separate ions along different spatial trajectories, allowing for a continuous beam of ions to be transmitted, which possess a narrow band of mobilities. The latter techniques must be scanned across an instrument parameter (a voltage) to generate a broadband IM spectrum.

Each IM separation method has been integrated with MS for two-dimensional separations based on both mobility and mass-to-charge ratio. These two analytical methods are readily coupled (IM-MS), as both separations occur post ionization, and result in rapid separations occurring on the order of milliseconds and microseconds, respectively. This temporal disparity allows for hundreds of mass spectra to be obtained for each mobility spectrum. For high performance MS applications, broadband dispersion mobility separations are commonly implemented, which allow for global metabolome profiles to be obtained. Because the ionic mobility is a function of gas-phase size and a function of mass, a two-dimensional IM-MS analysis results in a clustering of data along a narrow region of mobility and mass separation space. Thus, the degree of orthogonality between IM and MS is low; however, the rate at which information is generated is very high, with signal peak production rates ca. two orders of magnitude greater than other analytical separations. The correlation between mobility and mass data exhibits a dependence on intramolecular forces, monomer subunit composition, and degree of branching, which allows for the preliminary determination of biomolecular and metabolite class based on mobility. IM-MS allows spectra acquisition temporal resolution on the order of milliseconds, dispersion of chemical noise in two dimensions, separation of isobaric species based on structural differences, and preliminary determination of molecular class based on mobility. IM-MS is also readily coupled to any liquid-phase preionization separation through ESI.

The ability to perform fragmentation between separations in an IM-MS instrument allows for identification studies (tandem experiments) that enhance precursor ion identification confidence. Fragmentation is commonly prefaced with a mass selection step using a scanning mass analyzer (e.g., a quadrupole MS) in order to isolate a single mass-to-charge window. Fragmentation can occur either before (MS/IM-MS) or after (MS-IM/MS) the IM separation, resulting in product ions or isobaric precursor ions separated in mobility space, respectively. Fragmentation before mobility separation results in mobility-resolved product ions, and these product ions can be further fragmented after mobility separation (MS/IM/MS), resulting in secondary product ions that can be correlated to primary product ions on the basis of the congruent mobilities. Broadband fragmentation can also be performed on relatively simple samples using a large mass window (ca. 100–1000 Da), a ramped fragmentation energy profile, and a high performance mass spectrometer. In the latter example, product ions are correlated to precursor ions based on mass defect. Increased confidence is obtained through the use of mobility correlation to this broadband fragmentation method, as isobaric ions will convolute data otherwise.

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10 Inductively Coupled Plasma Mass Spectrometry for Analysis of Metal-Containing Pharmaceuticals

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10.1	Introduction	1
10.2	ICP-MS	2
10.3	Quantitative determinations	3
10.4	LC-ICP-MS	4
10.5	CE-ICP-MS	7
10.6	LA-ICP-MS	11
10.7	Applications	11
10.8	Summary	22
	Abbreviations	22
	References	23

10.1 INTRODUCTION

The inductively coupled plasma mass spectrometry (ICP-MS) technique was developed in the 1970s based on the need for a mass spectrometer capable of accepting solid mineral samples and providing multielement analyses at levels down to 10 ng/g in an analysis time of few minutes [1]. Thus, the technique was the successor of atomic absorption spectrometry and intensively used for analysis of geological samples.

In the last decades, the technique has evolved as hyphenation to different separation systems and has become the state of the art in the research field often referred to as *metallomics*. This research field has been described as “the comprehensive analysis of the entirety of metal and metalloid species within a cell or a tissue type” [2]. This field includes speciation analysis of essential and toxic elements as well as interaction of metal-based drugs (MBDs) with cell constituents and macromolecules.

The major advantages of the ICP-MS technique are the capability of multielement analysis, a very large sensitivity, and on-line sample introduction. The most

important feature, however, is that the detector response of the ICP-MS, in contrary to UV- and ES-MS (electrospray-mass spectrometry) detection, is independent of the analyte structure. Thus, unknown metabolites can be detected and quantified based on the ICP-MS detectable element in the metabolites. This makes ICP-MS an obvious detector in analysis of MBDs.

10.2 ICP-MS

10.2.1 The Instrument

ICP-MS is a hard ionization mass spectrometric technique that produces positive ions of single atoms in a high temperature argon plasma. Most often, the sample is introduced in solution, although solid material can be introduced using laser ablation. For analysis of pharmaceuticals, however, liquid sample introduction is predominant. The sample is pumped into a nebulizer, where an aerosol is produced by a flow of argon and transferred to a spray chamber. In the spray chamber, large droplets are discarded allowing only a fine mist of liquid to enter the argon plasma where the aerosol is desolvated, molecules are vaporized and decomposed into atoms, and finally the atoms are excited and ionized.

The argon plasma is an electric conducting gaseous mixture of argon, argon ions, and electrons sustained by a fluctuating magnetic field produced by a radio frequency induction coil. The plasma temperature of 6–10,000 K gives rise to complete sample combustion and a very efficient ionization. Positively charged ions are extracted from atmospheric pressure into the vacuum of the mass spectrometer through a set of cones. In the mass spectrometer, the ions are separated based on their mass to charge ratio (m/z). In routine instruments, the mass spectrometer is most often quadrupole based, operating at mass ranges of 3–300 with unit resolution, although high resolution instruments with a resolution of up to 10,000 are also commercially available [3].

The ionization efficiency of the plasma is dependent on the first ionization energies of the measured elements (Table 10.1). Metals are, in general, efficiently ionized and ICP-MS is therefore intensively used in inorganic analysis as the detection limits for most elements are at the subparts per billion level.

10.2.2 Interferences

One major challenge in ICP-MS analysis is the presence or formation of interferences. These are often divided into spectroscopic and nonspectroscopic interferences. Spectroscopic interferences appear at the same m/z value as that of the analyte, for example, the overlap of ^{196}Hg on ^{196}Pt . Such an overlap can most often be overcome by the choice of another isotope of the analyte. Another group of spectroscopic interferences is the polyatomic ions derived from the plasma gas (e.g., $^{40}\text{Ar}_2^+$), atmospheric gasses, sample solvent, or sample matrix constituents (e.g., $^{12}\text{C}^{40}\text{Ar}^+$ on $^{52}\text{Cr}^+$). The number of possible interferences decreases with increasing atomic mass, hence few interferences compromise the measurement of metals such as platinum and gold compared to the lighter metals such as iron and chromium. A comprehensive list of possible interferences on most elements has been given by May and Wiedmeyer [4]. To avoid polyatomic interferences, instruments equipped with a collision or reaction cell can

TABLE 10.1 First Ionization Energy and Degree of Ionization for Selected Elements

Atomic Number	Element	Most Abundant Isotope	Percentage	First Ionization Energy (kJ mol)	Degree of Ionization (%)
12	Carbon	¹² C	98.9	1086	5
15	Phosphorus	³¹ P	100	1012	33
16	Sulfur	³² S	95.0	1000	14
18	Argon	⁴⁰ Ar	99.6	1520	0.04
22	Titanium	⁴⁸ Ti	73.8	658	99
23	Vanadium	⁵¹ V	99.8	650	99
26	Iron	⁵⁶ Fe	91.7	759	96
33	Arsenic	⁷⁵ As	100	947	52
44	Ruthenium	¹⁰² Ru	31.6	711	96
51	Antimony	¹²¹ Sb	57.4	834	78
64	Gadolinium	¹⁵⁸ Gd	24.9	593	93
74	Tungsten	¹⁸⁴ W	30.7	770	94
78	Platinum	¹⁹⁵ Pt	33.8	870	62
79	Gold	¹⁹⁷ Au	100	890	51
83	Bismuth	²⁰⁹ Bi	100	703	92

be used. In a collision cell, the polyatomic ions are disintegrated by collision with an inert gas such as He, while in the reaction cell, the interference is removed from the m/z value of the analyte by reaction with a gas resulting in reaction products with m/z values different from the analyte [5]. These second-generation instruments are most commonly used today. Use of high mass resolution sector field instruments is an alternative, but a much more expensive solution to the interference problems. These instruments are capable of resolving minute differences in the masses of the analyte ion and the interfering ions [6].

The nonspectroscopic interferences are observed as changed sensitivity of the analyte signal induced by matrix constituents, for example, high salt concentrations. These interferences may be caused by changes in nebulization and ionization efficiency, in ion transport efficiency, in ion extraction, or combinations of these [7]. Elements with low mass or high ionization energies are most severely affected [8]. The nonspectroscopic interferences are often referred to as *matrix interferences*.

10.3 QUANTITATIVE DETERMINATIONS

The multielement capabilities of ICP-MS are routinely applied in the related area of determination of inorganic impurities in drugs and active pharmaceutical ingredients, an area not covered in this chapter, but that has been reviewed recently [9]. Drugs containing metals are often analysed by ICP-MS due to the low limits of detections, high sensitivity and few interferences for most drug related metal-ions (Pt, Ru and Au), few interferences are present. The multielemental capabilities of ICP-MS are rarely used, although an internal standard or a few other elements are sometimes measured along with the selected metal in order to correct for changes in instrument sensitivity over time or to monitor potential polyatomic interferences. The determination of total metal-containing drugs using ICP-MS is often applied in metabolism, distribution, and

excretion studies or in uptake experiments using cancer cell line models. This requires analysis of biological samples such as serum, urine, or cell samples. These are all difficult to analyze due to the small sample volume available or matrix effects from salts, proteins, and other biomolecules. Thus, sample preparation and quantification are critical in order to achieve accurate and precise results.

Two different approaches can be chosen for the sample preparation of biological samples. Most simple is the application of a dilution with an appropriate diluent [10–12], thereby keeping the drug in solution in its existing form. For dilutions, there is a risk of coprecipitation of the metal, as proteins and other such molecules might precipitate over time. Alternatively, acid digestion with concentrated acids (plus hydrogen peroxide) can be applied, reducing organic molecules to CO₂ and H₂O and bringing the metal ions in their ionic form [13–18]. In general, acid digestions should be preferred, resulting in more stable solutions of the metal ion of interest. Another advantage of acid digestions is that quantification using external calibration is possible, while the more laborious standard addition calibration or careful preparation of matrix-matched standard is needed, when samples are simply diluted before analysis. Acid digestions are, however, more time consuming and require more sample handling steps. Most accurate and precise quantification is achieved applying isotope dilution using an enriched isotope of the analyte as a standard (e.g., ¹⁹⁴Pt for the quantification of platinum). Isotope dilution quantification can be either unspecific applying a solution of the enriched isotope as standard or specific for which the enriched isotope is incorporated into a specific compound, for example, [¹⁹⁴Pt(NH₃)₂Cl₂]. These approaches were used by Sar *et al.* [19] for the accurate and precise determination of platinum in DNA samples.

Many of the drugs are lipophilic, and adsorption to the surfaces of sample preparation vials and sample wells can be a problem. This problem was addressed by Egger *et al.* [17], who developed a protocol for determination of the cellular uptake of metal compounds in adherent cancer cell lines, a protocol that takes into account adsorption and blank correction. Utilizing this protocol for determination of the cellular uptake of Pt and Ru drugs, they were able to measure the cellular uptake experiments with good precision and accuracy.

Certified reference materials (CRMs) are widely used for validation of ICP-MS methods for determination of total metal concentrations, but for Pt and Ru, only a few biological CRMs with certified concentrations exist, and there is a clear need for new CRMs in the metallo-drug area. Thus, accuracy and precision are most often evaluated using in-house quality control samples. Bettinelli [11] carefully evaluated the analytical uncertainty associated with the determination of total Pt in plasma, plasma ultrafiltrate, and urine using ICP-MS. The instrumental LOD was at submicrogram per liter level and the limits of quantification (LOQs) (10σ) were at low microgram per liter level for plasma, ultrafiltrate, and urine [11,12]. Similar detection limits led to an LOQ of 0.5 μg/kg in DNA samples [12]. Figures of merit in the same range have been found by others [13,15,16,18], while even better LODs can be achieved with high resolution ICP-MS instrumentation [14].

10.4 LC-ICP-MS

Coupling of LC (liquid chromatography) systems with flow rates of 0.1–1 mL/min to ICP-MS is quite simple, as these flow rates are readily compatible with the sample

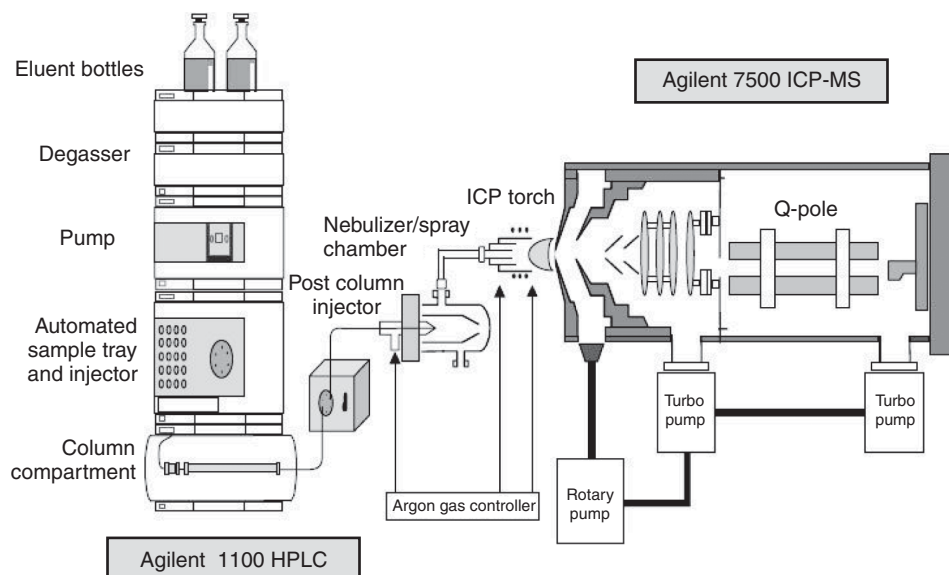


Figure 10.1 Schematic overview of LC-ICP-MS. *Source:* ©Agilent Technologies, Inc. 2010. Courtesy of Agilent Technologies, Inc., with permission.

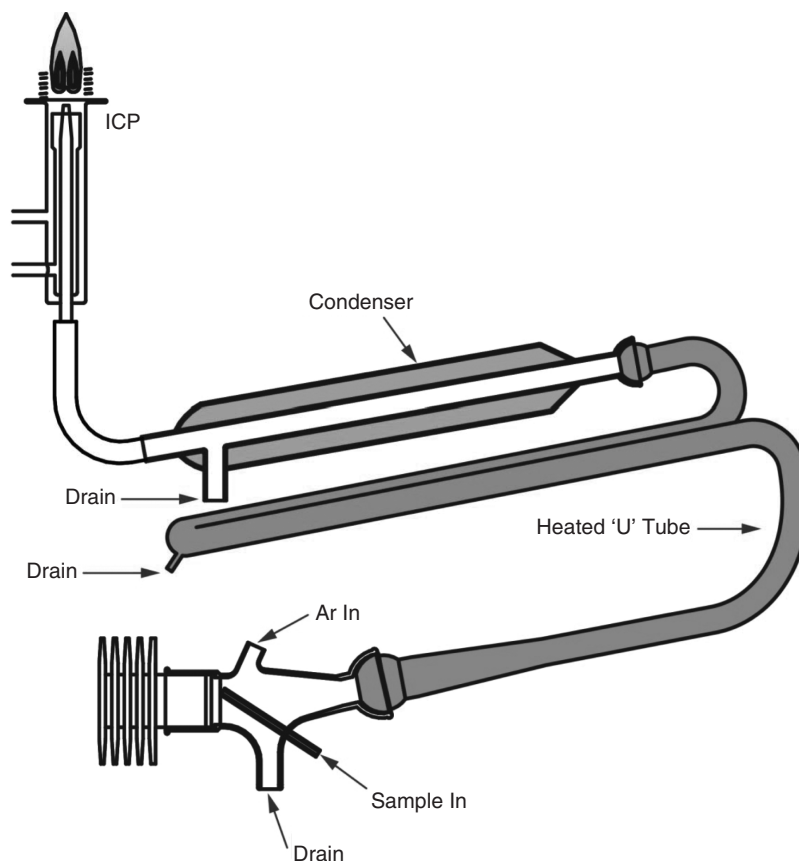
uptake around 1 mL/min for the ICP-MS instrument. The coupling of an LC system to an ICP-MS instrument is illustrated in Fig. 10.1. Several reviews on this coupling have been published [20–22]. Most of the early work on LC-ICP-MS coupling was performed using ion-exchange chromatography, as the eluents in such systems are, most often, aqueous buffers and elution is regulated by the salt concentration. As the ICP-MS, in contrast to organic MS, is quite tolerant toward moderately high salt concentrations, ion-exchange columns were, for a long period, the most popular columns for the hyphenation.

10.4.1 Organic Solvents

When more lipophilic substances, such as pharmaceuticals and their metabolites, have to be separated, reversed phase (RP) chromatography involving eluents containing large amounts of organic solvents is the most common separation principle used. In most drug metabolism studies, linear gradient elution from 5% to 95% organic solvents is often applied as a standard procedure. This introduces several analytical challenges, as the plasma only tolerates small amounts of organic solvents. Severe suppression of the signal followed by plasma instability is observed by increasing the organic solvent load and eventually the plasma is extinguished. The influence of organic solvents on the plasma is complex and involves interaction between solvent and plasma as well as change of aerosol characteristics in the spray chamber [23,24]. Introduction of organic solvent cools the plasma and changes ionization characteristics, leading to decreased sensitivity. In contrast, the addition of organic solvent to an eluent decreases surface tension and improves the aerosol by changing the droplet distribution toward smaller droplets, which results in improved transport efficiency and increased sensitivity. However, the interaction between plasma and solvent most often dominates, and increasing the radio frequency (RF) power with increasing organic solvents is necessary.

As the sensitivity of the ICP-MS signal is highly dependent on the organic solvent content of the solution, and the sensitivity in general decreases when the amount of organic solvent is increased, gradient elution would compromise quantifications, as sensitivity would change with the elution gradient.

Several approaches for minimizing the influence of organic solvent on the sensitivity have been reported. Solvent combustion could be enhanced by adding oxygen to the nebulizer gas. A combination of 5% oxygen in the nebulizer gas and spray chamber cooling has been a common setup reported in many studies. The plasma load can be reduced by reducing the eluent flow rate or by flow splitting, cooling the spray chamber, or complete removal of the solvent before introducing to the ICP-MS. Complete removal of the solvent is used in membrane desolvation units, where the eluent is heated and removed. These systems are often used in combination with an ultrasonic nebulizer (Fig. 10.2). In principle, these systems should allow for using gradient elution. However, great care should be taken that the analyte itself does not escape with the solvent, and several studies have shown that different species show different sensitivities in such systems. A review of different approaches to circumvent the problems



Schematic of CETAC U6000AT+ Ultrasonic Nebulizer / Membrane Desolvator

Figure 10.2 Schematic overview of membrane desolvation unit. *Source:* CETAC Technologies, with permission.

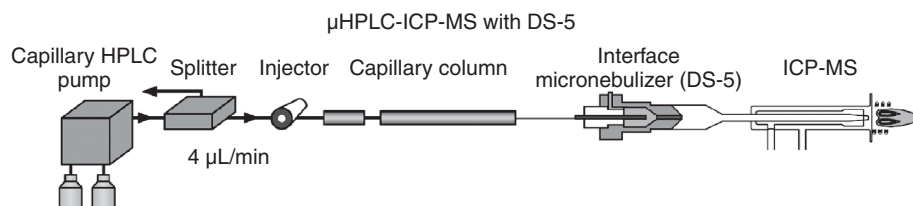


Figure 10.3 Schematic overview of capillary LC interfaced with ICP-MS via a micronebulizer system. *Source:* From CETAC, with permission.

introduced by organic solvents in drug metabolism studies of metal-containing and nonmetal drugs has recently been published [25].

10.4.2 Small Bore Columns and Low Flow Nebulizers

The common chromatographic column inner diameter was 4.6 mm just a decade ago, increasing interest in “green chemistry,” and subsequent interest in diminishing the organic solvent consumption has led to decreasing column diameters to 2 mm, 1 mm, and even down to capillary sizes of <100 μm . The increasing interest in analyzing very small sample sizes has fortified this tendency. Thus, the use of microbore columns in combination with low flow nebulizers has become increasingly popular for hyphenation with ICP-MS.

The lower flow of microbore columns demands the use of low flow nebulizers capable of delivering stable nebulization of flow rates in the order of 1–100 $\mu\text{L}/\text{min}$. These are known as the *direct injection high efficiency nebulizers* (DIHENs). Although the sample flow is lowered with these nebulizers, the sensitivity is still good, as the nebulizers introduce the whole sample flow into the plasma in contrast to the use of spray chambers where only a few percent of the sample reaches the plasma. The first of these nebulizers was very fragile and difficult to operate, while newer systems have become more robust. An example of a microflow sample introduction system is shown in Fig. 10.3. The handling of such systems, however, is still not a matter of routine [26].

Although the use of narrow-bore columns and low flow rates diminish the problem with plasma instability, stable signals are only obtained with isocratic elution schemes. A new approach to circumvent the influence of changing sensitivity is to oppose the gradient by introducing a counterflow, resulting in a constant load of organic solvent to the plasma[27].

Examples of LC-ICP-MS applications are listed under the individual elements in the following sections. The structures of the most commonly analyzed MBDs are given in Fig. 10.4.

10.5 CE-ICP-MS

Capillary electrophoresis (CE) is based on the migration of ions in an electric field. Thus, this separation principle is different from LC and could be a useful alternative technique. The main idea behind coupling of CE to ICP-MS is to exploit the sensitive and element-specific detection of ICP-MS with the high resolution of CE. Many MBDs have no or limited UV absorption limiting the use of CE-UV. Although hyphenation of CE and ICP-MS is not simple, it offers several advantages: a very low injection

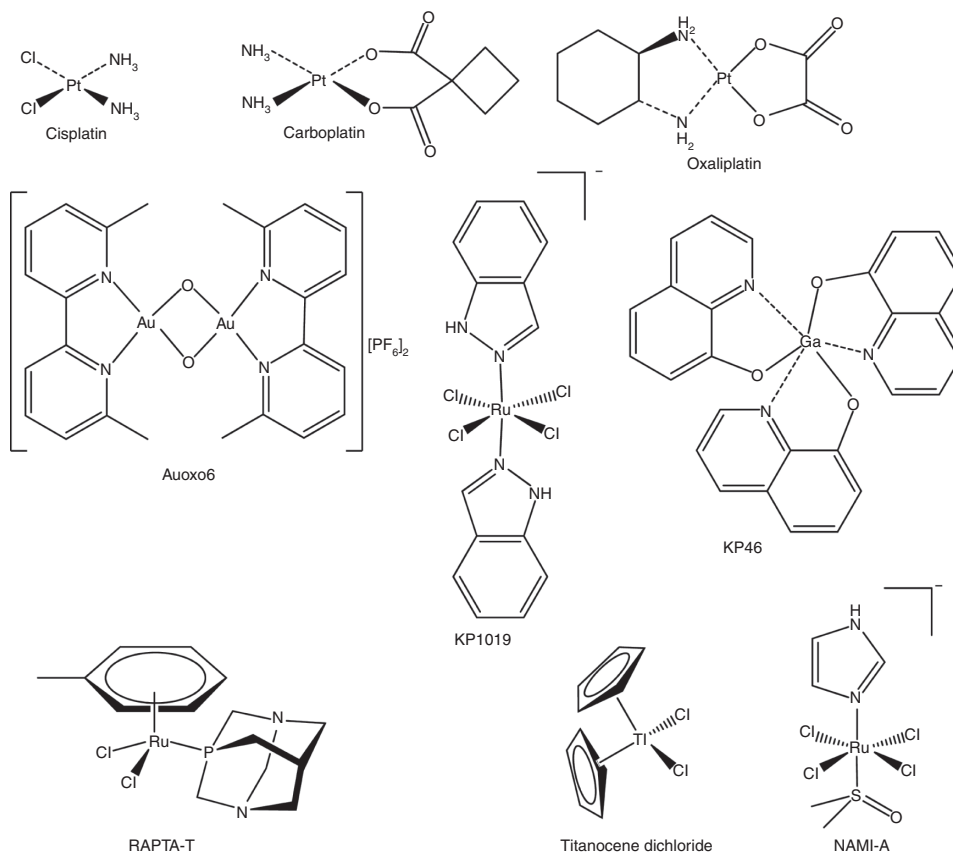


Figure 10.4 Structures of selected metal-based drugs.

volume in nanoliters, an alternative separation method to LC, low buffer consumption, and high number of theoretical plates.

10.5.1 Interfaces

The first paper on hyphenation of CE and ICP-MS was published by Olesik in 1995 [28], and since then, several papers have presented different ways of hyphenation. Today, two CE-ICP-MS interfaces are commercially available. In contrast to LC-ICP-MS, hyphenation is not straightforward as the low flow from the CE must be adapted to the flow acquired by the nebulizer, the electric connection must be maintained at the outlet of the CE capillary, and any laminar flow in the CE capillary must be avoided due to suction from the nebulizer. Figure 10.5 shows the hyphenation of CE and ICP-MS through a sheath flow interface. Here, a sheath flow is used to obtain a total flow of 5–10 $\mu\text{L}/\text{min}$, the electric connection is maintained through a grounded Pt electrode placed in a sheath liquid, a low consuming nebulizer is used, and liquid levels in the CE and sheath liquid are adjusted to avoid siphoning. A detailed description and technical aspects of hyphenation of CE and ICP-MS can be found in a recent review [29].

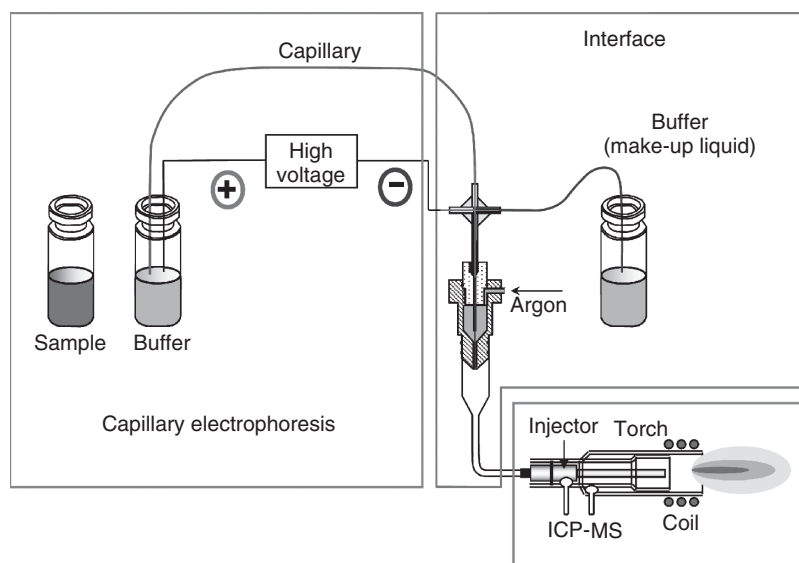


Figure 10.5 Schematic overview of CE-ICP-MS with the CEI-100 interface. *Source:* With permission from CETAC Technologies.

10.5.2 Quantitative Aspects

CE-ICP-MS has primarily been used for qualitative and semiquantitative analyses. A major drawback of the technique is changes in sensitivity during the day. Two ways of circumventing this problem has been proposed: addition of a trace element (external standard) to the sheath liquid [30] and the use of internal standard in the samples [31]. This leads to improved precision. Figure 10.6 shows an electropherogram of

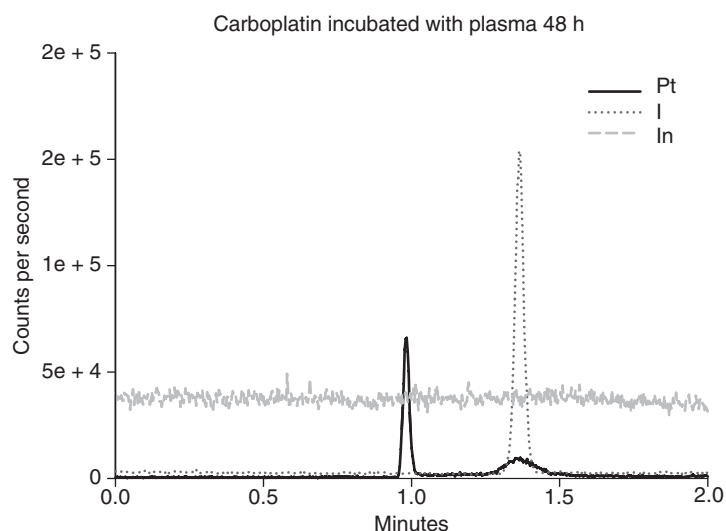


Figure 10.6 CE-ICP-MS analysis of incubation of plasma and carboplatin for 48 h at 37°C. Sodium diatrizoate is added as an internal standard before analysis, and In is used as an external standard. (See color insert.)

TABLE 10.2 CE-ICP-MS Application

Isotopes	Analyte	Subject	Interface	Buffer	Year	References
¹⁹⁴ Pt ¹⁹⁵ Pt ¹⁹⁵ Pt ⁷² Ge	Cisplatin and cisplatin analogs	Interaction with HSA	CEI-100	15 mM phosphate pH 7.4	2004	[30]
³⁴ S ⁵⁶ Fe ⁶⁹ Ga ⁷¹ Ga ⁷² Ge	KP46Ga(NO ₃)	Interaction with HSA and Tf	CEI-100	Ammonium carbonate pH 7.4	2009	[34]
¹¹⁵ In ¹²⁷ I ¹⁹⁴ Pt ¹⁹⁵ Pt ¹⁹⁶ Pt	Carboplatin	Interaction with plasma	CEI-100	10 mM phosphate pH 7.4	2009	[31]
¹⁰⁰ Ru ¹⁰² Ru ⁷² Ge	KP1019	Interaction with HSA and Tf and stability	CEI-100	10 mM phosphate buffer	2007	[35]
⁷² Ge ¹⁰¹ Ru ¹⁰² Ru ¹⁰⁴ Ru	KP1019, RAPTA-C, KP418	Stability and interaction with 5'-dGMP	CEI-100	50 mM formic acid	2008	[36]
³⁴ S ⁷² Ge ⁶⁴ Zn ⁶⁶ Zn	Zn(II) complexes	Interaction with HSA and apo-Tf and serum	CEI-100	20 mM Tris or 10 mM ammonium carbonate	2009	[37]
³⁴ S ⁷² Ge ¹⁰¹ Ru ¹⁰² Ru ¹⁰⁴ Ru	KP1019	Human plasma interaction with HSA and apo-Tf and serum/plasma	CEI-100	Polybrene-coated capillaries, 50 mM formic acid (pH 2.7).	2008	[33]
¹⁹⁷ Au	HAuCl ₄	Interaction with L-histidine	Laboratory made	50 mM Tris pH 7.5	2000	[38]
⁶⁹ Ga ⁵⁷ Fe ⁷² Ge	Ga drug	Stability and interaction with HSA, Tf, and serum	CEI-100	10 mM phosphate buffer, containing 10 mM NaCl	2009	[39]

carboplatin incubated in plasma. Indium was added to the sheath liquid, and sodium diatrizoate (iodine) was used as an internal standard to correct for changes in sensitivity.

ICP-MS is a mass sensitive detector, meaning the signal is dependent on the total amount of analyte in the plasma. This explains why the detection limit for CE-ICP-MS is higher than that for LC-ICP-MS, as the injection volume is in nanoliters and

microliters, respectively. Furthermore, the sample is diluted due to the use of a sheath flow [32].

CE-ICP-MS has been applied to the analysis of various MBDs, with primary focus on stability and interactions with biomolecules, particularly proteins. Most work is based on model systems using human serum albumin (HSA), transferrin (Tf), or plasma/serum. However, the method has been applied to the analysis of a plasma sample from a patient treated with the experiment Ru drug KP1019 [33]. Table 10.2 gives an overview of some of the recent research on MBDs using CE-ICP-MS.

The main challenge of analyzing proteins is to minimize adsorption due to hydrophobic or ionic interactions between the protein and the capillary surface, which leads to broad or tailing peaks. Using coated CE capillaries or adding a modifier to the buffer minimizes the problem. Furthermore, the background electrolyte should resemble the incubation buffer and physiological conditions should be used during sample preparation to avoid alternation of weak interactions during sample preparation and analysis [34]. A high background electrolyte concentration also increases the likelihood of clogging the nebulizer [32].

CE-ICP-MS analysis is still not a matter of routine. The technique has been compared to capillary LC-ICP-MS for analysis of metalloproteins. Using the same injection volume, similar performance of the two systems was observed. Capillary LC-ICP-MS was more robust and offered the opportunity to inject a larger sample volume and use on-line preconcentration of the sample before analysis. This is important when analyzing real samples with low concentration [40]. Thus, over time, CE-ICP-MS systems may be outperformed by capillary LC-ICP-MS in most applications.

10.6 LA-ICP-MS

In contrast to LC-ICP-MS, for which samples must be dissolved before analysis, laser ablation (LA)-ICP-MS offers the opportunity to measure solid samples by laser ablation of a small sample quantity, which is transferred to the ICP-MS. LA-ICP-MS has not gained great prevalence for the analysis of drugs. A general problem with LA-ICP-MS quantification is that it is difficult to obtain suited standards. The first application of LA-ICP-MS in the analysis of MBDs was to identify the platinum-containing proteins in *Escherichia coli* cells treated with cisplatin before separation with gel electrophoresis [41]. Examples of recent applications are quantification of Pt along a single hair strand from a patient treated with cisplatin [42] and scanning of kidney tissue from a mouse treated with cisplatin to create images of the distribution of ^{63}Cu , ^{64}Zn , and ^{196}Pt [43].

10.7 APPLICATIONS

The first application of LC-ICP-MS for MBD analysis is analysis of the gold-containing drug, auranofin [44]. The hydrolysis products of cisplatin and reaction products with methionine, cysteine, and glutathione were investigated using ion-pair LC-ICP-MS [45]. In addition to monitoring interactions with small biomolecules and metabolites, the interaction with proteins has been of general interest. In early investigations, the fraction of free and protein-bound metal was analyzed after ultrafiltration, after

which on-line-SEC-ICP-MS monitoring the interaction between proteins and Pt- or Ru-based drugs was introduced [46]. Following is an overview based on the individual elements.

10.7.1 Platinum

Since Rosenberg, in 1965 by accident, discovered cisplatin [47], various platinum compounds have been synthesized and tested for their cytotoxicity. Today, three Pt-based drugs are approved worldwide as anticancer drugs. The structures of cisplatin, carboplatin, and oxaliplatin are given in Fig. 10.4. Cisplatin is very efficient in the treatment of certain cancer types, particularly testicular cancer and some solid tumors. The limited application; severe side effects such as kidney damage, loss of hearing, damage to the nervous tissue; and intrinsic or acquired resistance to the treatment have led to the synthesis of many new Pt-based drug candidates. At first, most new drug candidates were cisplatin analogs, based on a square planar Pt(II) center with two monodentate or one bidentate leaving groups, but the oral Pt(IV) drug satraplatin (JM216) has also entered clinical trials [48]. In total, around 40 Pt-based drugs have been investigated in clinical trials and more than 1000 have been synthesized [49].

10.7.1.1 ICP-MS and Platinum. Platinum has six stable isotopes ^{190}Pt , ^{192}Pt , ^{194}Pt , ^{195}Pt , ^{196}Pt , and ^{198}Pt . The three most abundant isotopes ^{194}Pt , ^{195}Pt , and ^{196}Pt have a relative abundance of 32.9%, 33.8%, and 25.2%, respectively. They are all subject to isobaric interference of ^{178}HfO , ^{179}HfO , and $^{180}\text{HfO}/^{180}\text{WO}$, respectively, but this is normally not a problem, as Hf and W are not present in biological samples and the formation of metal oxides can be controlled through plasma conditions. Furthermore, ^{196}Pt is subject to isobaric interference of ^{196}Hg . Instrumental detection limits for determination of total Pt in subnanogram per liter range have been reported for quadrupole-based ICP-MS [18], while detection limits for LC-ICP-MS have been reported to be around or below 1 $\mu\text{g/L}$ using a quadrupole and the detection limit improved by a factor of 10 using sector field ICP-MS [50,51].

10.7.1.2 DNA-Binding Mechanism. The general mechanism of Pt-based drugs involves binding to DNA in the cell nucleus. When cisplatin enters the cell, it undergoes hydrolysis because of low chloride concentration inside the cell, $\sim 5\text{ mM}$. The major binding site for cisplatin on DNA is on the guanine base, and it forms an intrastrand cross-link [52]. DNA is bent leading to inhibition of transcription and in the end to apoptosis or necrosis [53]. As formation of DNA adducts is believed to be crucial for cell death, studies of the nature of the binding have been conducted. Recently, an LC-ICP-MS method was developed to quantify cisplatin–DNA adducts formed after incubation of cisplatin cell carcinoma cultures. Three methods all applying isotope dilution analysis (IDA) and species-specific or unspecific spiking were used for quantification. A method for quantifying the amount of Pt bound to DNA, extracted from peripheral blood mononuclear cells and tumor tissue from patients treated with cisplatin, has been developed. The results were compared to the alternative ^{32}P -postlabelling method and found to be more precise, sensitive, and less laborious [54].

10.7.1.3 Interaction Studies. Pt(II) is a soft metal with a high affinity toward soft ligands such as sulfur. Therefore, interactions between biomolecules and Pt-based drugs are common. Table 10.3 gives an overview of some of the recent research obtained using LC-ICP-MS. Cisplatin, carboplatin, and oxaliplatin are all administered intravenously. Therefore, interactions with serum protein have gained particular interest.

One of the features of ICP-MS is multielement detection. This has been utilized in several papers on interaction with proteins and biomolecules. All serum proteins contain sulfur as a result of the presence of the sulfur-containing amino acids, methionine and cysteine. This gives the opportunity to simultaneously monitor Pt and S in interaction studies. In addition, Tf contains iron, and in serum, around 30% of the Tf is loaded with iron. This has been used to demonstrate that the incubation of transferrin with cisplatin did not displace iron from transferrin [19,65]. Recent studies, however, contradicted this observation [65]. Interaction between metallothionein (MT) and cisplatin has also been monitored with Zn, Cd, and Pt [56,66]. Sensitivity of S is much lower than that of Pt. This is due to the high ionization potential of S and the fact that the most abundant isotope ^{32}S (95%) is subject to interference from $^{16}\text{O}_2$. Instead, ^{34}S (4.2%) can be monitored or a dynamic reaction cell can be used to move $^{32}\text{S}^+$ to $^{32}\text{S}^{16}\text{O}^+$. Figure 10.7 shows the simultaneous monitoring of Pt, S, and Fe in iron-saturated apotransferrin incubated with cisplatin [19].

10.7.1.4 Stability of Pt-Based Drugs During Sample Preparation and Analysis.

Whenever performing speciation analysis, the integrity of the sample is of primary concern. However, only few papers have dealt with this problem. Tran *et al.* [67] analyzed cell lysates from T289 malignant melanoma cells exposed to cisplatin using hydrophilic interaction chromatography (HILIC)-ICP-MS. Chemical lysis, osmosis, and freeze thaw were compared. Chromatograms of the lysates showed different results, stressing the need for critical evaluation of the lysis method. In addition, they also found a difference in the total amount of Pt extracted from the cells using the aforementioned lysis methods. Esteban-Fernandez *et al.* [60] evaluated the interaction strength between cisplatin biomolecules under denaturing conditions using LC-ICP-MS and found that the interaction was strong, as no changes were observed. As previously mentioned, conditions applied during separation may affect the binding of metals to proteins [34]. The choice of mobile phase may also lead to artifacts. Heudi *et al.* [68] found that phosphoric acid, in contrast to formic acid, interacted with monoaquacisplatin. The above-mentioned papers all demonstrated the need to carefully evaluate the sample preparation method and separation method in order to avoid altering the sample and creating artifacts.

10.7.1.5 Uptake in Cancer Cells—Total Determination of Pt in Various Fractions.

In the search for new Pt drugs, there has been an interest in establishing a relationship between the cytotoxicity and the uptake of Pt in the cells and the amount of Pt bound to DNA. Also, the distribution of Pt between different cellular compartments has been investigated. After incubation of the drug, the cells were divided into nucleus, DNA, mitochondria, cytosol, and medium. The concentration was determined relative to the amount of protein, which was used as a measure of the amount of cells. In addition to the determination of distribution in the cellular compartments, ICP-MS has been used to determine the pharmacokinetic parameters. A recent review summarizes different investigations on the pharmacokinetic parameters for oxaliplatin [69]. An overview of selected papers on total determination of Pt can be found in Table 10.4.

TABLE 10.3 Application of LC-ICP-MS in the Analysis of Platinum-Based Drugs

Isotopes	Analyte	Subject	Separation	Year	References
¹⁹⁵ Pt ³⁴ S ⁵⁴ Fe	Cisplatin	Interaction with HSA, Tf, IgG, and serum	AEC	2008	[19]
¹⁹⁴ Pt ¹⁹⁵ Pt ²⁰⁵ Tl	Cisplatin	Quantification of cisplatin and monoaquacisplatin in plasma UF and cell culture medium UF	RP	2006	[55]
⁶⁶ Zn ¹¹¹ Cd ¹⁹⁴ Pt ¹⁹⁵ Pt	Cisplatin	<i>In vitro</i> interaction with MT and GSH Analysis of rat kidney, liver, and inner ear cytosol	SEC	2007	[56]
¹⁹⁴ Pt ¹⁹⁵ Pt	Cisplatin	Quantification of isolated DNA adducts from cell carcinoma cultures and a larvae using different IDA strategies	RP narrow bore	2009	[19]
¹⁹⁵ Pt ⁵⁷ Fe	Cisplatin, carboplatin, and oxaliplatin	<i>In vitro</i> interaction with Hb and <i>in vitro</i> incubation with whole blood and analysis of red blood cells	SEC	2004	[57]
¹⁹⁴ Pt ¹⁹⁵ Pt ¹⁹⁶ Pt	Cisplatin, carboplatin, and oxaliplatin	Incubation with whole blood Analysis of plasma fraction	HILIC	2009	[13]
¹⁹⁵ Pt	Carboplatin	Incubation with HSA and IgG, analysis of human plasma, <i>in vitro</i> incubation with plasma	SEC	2007	[58]
¹⁹⁴ Pt ¹⁹⁵ Pt ¹⁹⁶ Pt	Carboplatin	Human urine analyzed using isotope dilution	RP	2008	[59]
¹⁹⁴ Pt ¹⁹⁵ Pt ¹⁹¹ Ir ¹⁹¹ Ir	Cisplatin, (carboplatin, oxaliplatin)	Analysis of cytosol from rat ear and kidney after monodosage of cisplatin. Total determination in mitochondria, nucleus and ear, kidney, and liver cytosol	SEC	2008	[60]

TABLE 10.3 (continued)

Isotopes	Analyte	Subject	Separation	Year	References
^{195}Pt ^{56}Fe	Oxaliplatin	Quantification of profiling oxaliplatin–protein complexes in blood samples	SEC	2006	[61]
^{194}Pt ^{195}Pt ^{205}Tl	Satraplatin, metabolite of satraplatin, cisplatin	Incubation with human plasma, determination of reversible or irreversible binding and stability/degradation	RP	2008	[62]
^{194}Pt ^{195}Pt ^{205}Tl	Oxaliplatin	Human plasma, quantification of intact oxaliplatin	RP	2008	[63]
$^{32}\text{S}^{16}\text{O}$ ^{102}Ru ^{195}Pt	Cisplatin	Incubation with serum	SEC-AEX and CIM	2010	[64]

Abbreviations: CIM, convective interaction media; GSH, glutathione; AEC, anion-exchange chromatography.

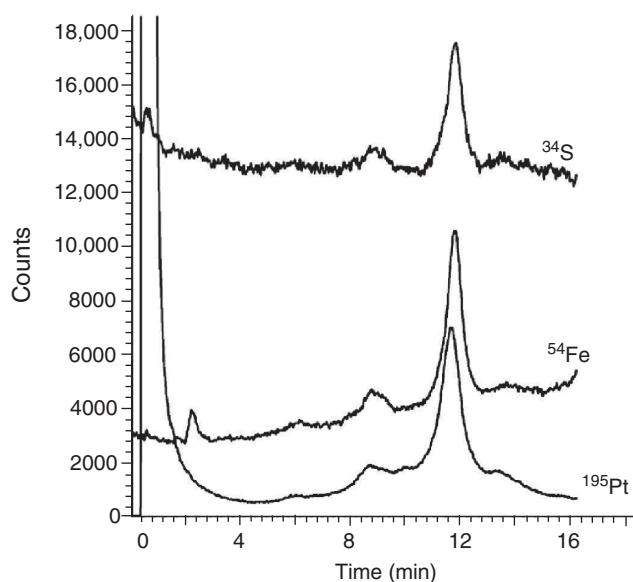


Figure 10.7 HPLC separation of iron-loaded transferrin incubated with cisplatin (1:10) with simultaneous detection of S, Fe, and Pt. *Source:* Reproduced from D. Esteban-Fernandez, M. Montes-Bayon, E. B. Gonzalez, M. M. G. Gomez, M. A. Palacios, A. Sanz-Medel. *J Anal At Spectrom* 2008;23:378–384. *Source:* By permission of the Royal Society of Chemistry. Ref. 19.

TABLE 10.4 Examples of Determination of Total Platinum

Isotopes	Analyte	Subject	Sample Preparation	Year	References
¹⁹⁴ Pt ¹⁹⁵ Pt	Cisplatin	Comparison of different cell lysis methods	Chemical lysis, osmosis and freeze thaw	2009	[67]
⁶⁹ Ga ¹⁰² Ru ¹¹⁵ In ¹⁹⁵ Pt	Cisplatin and KP1019	Development of protocol to determine uptake in adherent tumor cells	Acid digestion	2009	[17]
¹⁹⁵ Pt ¹¹⁵ In	Cisplatin, carboplatin and oxaliplatin and experimental Pt drugs	Uptake/accumulation after prolonged incubation of cancer cells with various Pt compounds	Acid digestion	2003	[16]
¹⁹⁵ Pt ¹⁹³ Ir	Oxaliplatin	Validation of microwave-assisted mineralization method for Pt in plasma and PUF	Acid digestion	2008	[18]
¹⁹⁵ Pt ¹⁹⁴ Pt	Cisplatin	Determination of Pt DNA ratio	Buran lysis buffer	2007	[12]
¹⁹⁴ Pt ¹⁹⁵ Pt	Cisplatin, carboplatin, oxaliplatin	Determination of Pt DNA ratio at 37°C and 43°C	Acid digestion	2008	[70]
¹⁹⁴ Pt ¹⁹¹ Ir	Cisplatin	Total Pt in DNA extracts from human blood mononuclear cells and tissue	Acid digestion	2008	[54]
¹⁹⁵ Pt ¹⁹³ Ir	Oxaliplatin	Pharmacokinetics, Pt in PUF, plasma, and whole blood	Acid digestion	2000	[71]
¹⁹⁴ Pt ¹⁹¹ Ir	Cisplatin	The effects of sulfur-containing compounds and gemcitabine on the binding of cisplatin to plasma proteins and DNA	Acid digestion	2008	[72]

Abbreviations: PUF, plasma ultrafiltrate; UF, ultrafiltrate.

10.7.1.6 Drug-Delivery Systems. Owing to the side effects from treatment with Pt drugs, drug-delivery systems have gained attention. A targeted delivery of the drug to the tumor could enhance efficacy and minimize side effects. ICP-MS has been used to monitor the amount of encapsulated drug and the uptake of drug in cancer cells. Passive targeting takes advantage of the enhanced permeability and retention (EPR)

of macromolecules, nanoparticles, and liposomes. EPR arises because of both vascular permeability stimulated by extravasation and increased retention from the inefficient drainage of macromolecules by the lymphatic system [73]. Several ways have been explored: encapsulation of the drug in nanoparticles [74,75] or microspheres [76] and binding of the drug to macromolecules [77,78].

10.7.2 Gold

Gold-containing drugs were originally developed for the treatment of arthritis [79]. In recent years, gold complexes have also attracted attention as possible anticancer drugs. Numerous gold(I) complexes have been evaluated for cytotoxicity and antitumor activity [80]. Also, gold(III) complexes demonstrate antitumor properties, an example is Auoxo6, a binuclear gold(III) complex (Fig. 10.4) [80,81]. Gold(III) complexes typically display the same electronic configuration and similar structure and reactivity as platinum(II) complexes. However, apoptosis induced by gold(III) complexes seems to be triggered by mitochondrial damage and not by prevention of DNA replication, as in the case of cisplatin [80]. Despite the efforts in discovering and developing new gold complexes for cancer treatment, no drugs have yet been approved. Hence, gold-containing drugs are still limited to arthritis treatment. These comprise the polymers of sodium thiopropanolsulfonate-*S*-gold(I), sodium bis(thiosulfate), sodium aurothiomalate (myocrisin), and aurothioglucose (solganol) and the monomer triethylphosphine-goldtetraacetylthioglucose (auranofin and Ridaura). Recently, a new promising field applying gold nanoparticles as drug-delivery vehicles has emerged. Gold nanoparticles can be functionalized with a thiolated poly(ethyleneglycol) monolayer onto which a drug can be linked. Gold nanoparticles have been loaded with doxorubicin [82] and oxaliplatin [75]. In both cases, a high cellular uptake was observed along with improved or at least similar cytotoxicity to that of the free drug.

10.7.2.1 ICP-MS and Gold. Gold is monoisotopic (^{197}Au), and because of its high first ionization potential, the degree of ionization in the argon plasma is low (51%). But since polyatomic interferences of m/z 197 are virtually nonexistent when analyzing biological samples and the complete Au signal is seen on one isotope, instrument detection limits in the 1–3 ng/L range can still be achieved. However, one important prerequisite is to ensure that Au is in a soluble form during the sample preparation processes (e.g., as the $[\text{AuCl}_6]^{3-}$ ion) to prevent reduction and precipitation of solid Au particles. Furthermore, Au drugs are often lipophilic and are easily reduced to metallic Au, making sample preparation and ICP-MS analysis a challenge. As a consequence, very few studies on the application of ICP-MS for investigation of Au-containing drugs have been published. One exception is the study by Zhao *et al.* [83], who used LC-ICP-MS to determine Au-containing metabolites in urine sample from patients treated with myocrisin and auranofin, the main metabolite found was $[\text{Au}(\text{CN})_2]^-$. Surprisingly, new studies on the application of ICP-MS technology in the investigation of Au-containing drugs have not been published in the last 10 years, but the new interest in the application of Au nanoparticles as drug-delivery vehicles may prompt a renewed interest in the use of ICP-MS for analysis of gold-containing drugs (Table 10.5).

TABLE 10.5 Application of LC-ICP-MS in the Analysis of Gold-Based Drugs

Isotopes	Analyte	Subject	Separation	Year	References
^{197}Au	Auranofin and myochrysine and their metabolites	Human urine	Ion-pair RP	1992	[83]
^{197}Au	Myochrysine	<i>In vitro</i> incubation with RBCs and analysis of human RBCs	SEC, FIA, ion-pair RP	1995	[84]
Cu, Cd, Zn, Au	Auranofin	Human blood	SEC, WAX	1989	[44]
^{197}Au	Auranofin, myochrysine, and solganol	Human urine and blood	Ion-pair RP	1993	[85]

Abbreviations: FIA, flow injection analysis; RBCs, red blood cells; WAX, weak anion exchange; SEC, size exclusion chromatography.

10.7.3 Ruthenium

In recent years, there has been a growing interest in ruthenium complexes as anticancer drugs. Two Ru(III) drugs with octahedral coordination chemistry, NAMI-A (imidazole Ru(III) dimethyl sulfoxide complex) and KP1019 (indazolium[transtetrachlorobis(1H-indazole)ruthenate(III)]), have successfully completed phase I clinical trials. NAMI-A showed efficacy as an antimetastatic agent, whereas KP1019 exhibited activity against colorectal carcinomas and primary human tumors. Other ruthenium complexes that exhibit favorable pharmacological profiles are under investigation, for example, RAPTA complexes that are ruthenium(II)-arene complexes bearing the 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane ligand, the structures are shown in Fig. 10.4. Ruthenium binds strongly to DNA by mechanisms similar to the ones proposed for Pt(II) complexes. Protein binding is supposed to play a role in the mechanism of action, as Ru(III) uptake by cells is mediated by transferrin transport. This transport mechanism offers opportunities to deliver not only Fe but also Ru complexes inside the cancer cells improving tumor selectivity [86]. Ruthenium(II)-chloroquine complexes have recently been shown to be potential antimalaria drugs [87].

10.7.3.1 ICP-MS and Ruthenium. Ruthenium has seven stable isotopes ^{96}Ru , ^{98}Ru , ^{99}Ru , ^{100}Ru , ^{101}Ru , ^{102}Ru , and ^{104}Ru , the latter three being the most abundant with abundances of 17.1%, 31.6%, and 18.6%, respectively. Ruthenium is almost fully ionized (96%) in the argon plasma. Potential interferences are few, but since most originate from matrix elements (e.g., $^{40}\text{Ar}^{61}\text{Ni}^+$, $^{64}\text{Ni}^{37}\text{Cl}^+$, and $^{85}\text{Rb}^{16}\text{O}^+$ on $^{101}\text{Ru}^+$ and $^{62}\text{Ni}^{40}\text{Ar}^+$, $^{66}\text{Zn}^{36}\text{Ar}^+$, and $^{65}\text{Cu}^{37}\text{Cl}^+$ on $^{102}\text{Ru}^+$), interferences can be a problem when analyzing biological samples, as transition metals such as Ni, Cu, and Zn often occur in relatively high concentrations. Still, these interferences can often be avoided by the use of multiple Ru isotopes and careful monitoring of transition metal levels during analysis. Detection limits for total determination of Ru in biological samples below 0.1 $\mu\text{g/L}$ can be obtained on a routine basis [10], while detection limits for LC-ICP-MS are in 2–10 $\mu\text{g/L}$ range (Table 10.6) [88].

TABLE 10.6 Example of Application of LC-ICP-MS in the Analysis of Ruthenium

Isotopes	Analyte	Subject	Separation	Year	References
⁵⁴ Fe ⁵⁶ Fe ¹⁹⁵ Pt ¹⁹⁶ Pt ¹⁹⁸ Pt ¹⁰⁰ Ru ¹⁰¹ Ru ¹⁰² Ru	RAPTA and cisplatin	Interaction with HSA and transferrin (Tf) at various ratio between drug and protein and different concentrations	SEC	2010	[65]
¹⁹⁴ Pt ¹⁹⁵ Pt ¹⁹⁶ Pt ¹⁰⁰ Ru ¹⁰¹ Ru ¹⁰² Ru ⁶³ Cu ¹¹⁴ Cd ³¹ P	Three experimental Ru drugs and cisplatin	Interaction with serum proteins, release of protein-bound Ru after treatment with EDTA	SEC	1999	[46]
³² S ¹⁶ O ⁵⁶ Fe ¹⁰² Ru	KP1019	Human plasma and interaction with plasma <i>in vitro</i>	SEC + AEX	2005	[88]
³² S ¹⁶ O ¹¹⁵ In ¹⁰² Ru	KP1019	Pioneer study of the use of immunoaffinity LC	Immunoaffinity SEC	2010	[89]

Abbreviations: AEX, anion exchange; SEC, size exclusion chromatography.

10.7.3.2 Interaction Studies. The first study on the interaction of a ruthenium-based drug with serum proteins by ICP-MS detection was performed by Szpunar *et al.* [46]. They observed that the ruthenium drug was bound to proteins with a molecular mass of 50–70 kDa. No free drug was observed, as neither the drug nor its hydrolyzed products could be eluted from the column. The same approach was taken by Sulyok *et al.* [88]. They analyzed the drug KP1019 by SEC-ICP-MS and also observed that the ruthenium signal appeared in the 50- to 70-kDa band. By LC-ES-MS, they identified two proteins in the band, HSA and Tf. The stoichiometry of the KP1019 protein binding was determined by the Ru/S ratio. Less than 2% of KP1019, in an incubated mixture of HSA and Tf, was found to bind to Tf. This example illustrates how the multielemental detection feature of ICP-MS provides qualitative as well as quantitative information on the drug–protein binding [88]. Recently, Groessl *et al.* [65] used SEC-ICP-MS to study the interaction of RAPTA complexes with transferrin and found that the affinity was higher for holotransferrin (iron saturated) than for apotransferrin (iron free), suggesting an iron-mediated Ru binding mechanism. Similar results were obtained by examination of the interaction of KP1019 with HSA and Tf using CE-ICP-MS. Hartinger *et al.* [90] developed a two-dimensional chromatographic scheme for studying interactions of KP1019 with serum proteins in plasma samples from a patient by monitoring Ru, ³²S¹⁶O⁺, and ⁵⁶Fe⁺. This study highlights the strength of ICP-MSs multielemental capabilities.

10.7.3.3 Determination of Total Ru in Biological Samples. Brouwers *et al.* [10] successfully developed an ICP-MS method for the determination of Ru in human plasma filtrate, plasma, and urine samples from an NAMI-A phase I clinical trial. Sample preparation was a simple dilution with either 1% HNO₃ or 0.01% EDTA-triton before analysis. Potential polyatomic interferences were avoided by monitoring the ¹⁰²Ru isotope, which was the isotope least affected by interferences. Carefully correcting for the adsorption, an LOD of 0.44 fg Ru per cell was achieved along with good accuracy and precision on repeated cellular uptake experiments. These two studies clearly demonstrate that ICP-MS is a very powerful technique for the determination of Ru in biological samples and that controlled sample preparation is of utmost importance.

The availability of RAPTA complexes linked to recombinant HSA has been investigated with the purpose of exploiting the EPR effect [91].

10.7.4 Other Metal-Containing Drugs

Several other metal complexes have been investigated in search of new drugs. Some of these, such as Gd(III)-, Ga(III)-, and Ti(IV)-complexes, have entered clinical trials. Some of the most prominent examples of potential metal-containing drugs are outlined below and summarized in Table 10.7.

10.7.4.1 Gadolinium. Motexafine gadolinium is a member of the texaphyrins or “expanded porphyrins” class of macromolecules capable of coordinating large metal ions [96]. It has been subject to several clinical trials for its anticancer activity and is also under investigation as a site-directing molecule improving delivery of carboplatin [94,100]. Gadolinium complexes, for example, gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) are widely used as magnetic resonance imaging (MRI) contrast agents due to gadolinium’s ability to enhance the intracellular ¹H MRI signal. Contrast agents should be nontoxic and excreted rapidly, as they are often given in gram quantities. Gadolinium has seven isotopes, and ICP-MS sensitivity and accuracy are good, provided that potential interferences from various lanthanide oxides are resolved or minimized. ICP-MS has been used to study the pharmacokinetics and biodistribution of motexafin in plasma and tissue [94] and to follow the excretion and stability of Gd-DTPA in urine from a patient, using SEC-ICP-MS. No Gd-DTPA metabolites were found, and the complete dose (> 99%) was excreted within 24 h [95].

10.7.4.2 Gallium. A small group of Ga(III) complexes has entered clinical trials as oral anticancer drugs. One of these was designed to be similar to the iron(III)–maltol complex, which is known to provide iron in a bioavailable, readily absorbable form. These gallium complexes are interesting, as Ga³⁺ is more bioavailable than other metal ions (e.g., Ru³⁺) and allows administration as an oral drug. In the cell, Ga(III) impairs DNA replication leading to apoptosis through the mitochondrial pathway [101].

Gallium has two isotopes, ⁶⁹Ga and ⁷¹Ga. The ICP-MS analysis of gallium is hampered by many polyatomic interferences (various ClO₂⁺ and SO₂⁺ ions) plus interference from doubly-charged ¹³⁸Ba²⁺ ions, which makes accurate determination of total gallium at low levels in biological levels difficult. CE-ICP-MS has been used to investigate the binding of KP46 to plasma proteins. By simultaneous monitoring of ⁶⁹Ga⁺, ³⁴S⁺, and ⁵⁶Fe⁺, it was shown that KP46 preferably binds to transferrin [34].

TABLE 10.7 Examples of Application of LC-ICP-MS in the Analysis of Other Metals

Isotope	Analyte	Subject	Separation	Year	References
²¹ Sb ¹²³ Sb ¹¹⁵ In	Glucantime (<i>N</i> -methyl meglumine antimoniate)	Human whole blood, plasma, urine, and hair	AEX	2002	[92,93]
¹⁶⁰ Gd ¹⁵⁹ Tb	Motexafin gadolinium	Stability of the drug in plasma	RP	2006	[94]
¹⁵⁸ Gd	Gd-DTPA (DTPA: diethy- lenetriamino- pentaacetic)	Human urine	SEC	2004	[95]
¹⁸⁴ W ¹⁸⁶ W ¹⁹¹ Ir	Sodium tungstate	Interaction with proteins, incubation with serum	SEC	2008	[96]
⁵¹ V	V(III) and V(V)	Interactions with human serum	AEX	2005	[97]
³⁴ S ⁴⁴ Ca ⁴⁶ Ti ⁴⁷ Ti ⁴⁸ Ti ⁴⁹ Ti	Ti citrate	Interaction with Tf and human serum, species-specific isotope dilution analysis	SEC and AEC	2008	[98]
²⁰⁷ Tl ²⁰⁹ Bi	Bismuth citrate (ranitidine)	Interaction with transferrin and albumin, competition	Off-line LC	2003	[99]

Abbreviations: AEX, anion exchange; SEC, size exclusion chromatography.

10.7.4.3 Titanium. The organometallic agent titanocene dichloride [Cp₂TiCl₂] (Fig. 10.4) has entered clinical trials as an anticancer drug [102–104]. To our knowledge, no ICP-MS analysis has been carried out on titanocenes. Titanium analysis using ICP-MS in biological samples is, in general, difficult due to heavy interference from polyatomic ions and isobaric overlap in the mass range of Ti isotopes (⁴⁶Ti, ⁴⁷Ti, ⁴⁸Ti, ⁴⁹Ti, and ⁵⁰Ti); furthermore, careful sample preparation is critical as titanium can be difficult to maintain in solution. As a consequence, there are few papers describing the ICP-MS analysis of Ti in biological samples, one exception is the paper by Sarmiento-Gonzalez *et al.* [98], who studied the binding of Ti(IV) to transferrin in blood serum using LC-ICP-MS.

10.7.4.4 Vanadium, Zinc, Rhodium, and Osmium. Vanadium and zinc complexes have been suggested to be involved in the regulation of glucose level in the body and thereby mimic the biological effects of insulin. Studies on binding of vanadium and zinc to transferrin and other human serum proteins utilizing various hyphenated ICP-MS techniques have been reported [37,97,105]. Like for Ti and Ga, ICP-MS analysis of V and Zn is hampered by a large number of polyatomic interferences and can

be difficult. By applying hyphenated techniques and by careful monitoring of blank levels/contamination, ICP-MS analysis of low levels of V and Zn in biological samples is possible.

Recently, rhodium and osmium analogs to the ruthenium RAPTA drugs have been synthesized, these complexes show similar cytotoxicity in cancer cell lines as that of the Ru RAPTA complexes [106]. However, no ICP-MS studies on these compounds have been reported.

10.8 SUMMARY

ICP-MS is a hard ionization MS technique characterized by a high and structure-independent sensitivity as all MBDs and their metabolites are converted into the M^+ ions in the plasma. When interferences have been overcome by use of collision or reaction cell instruments, instrumental detection limits at the nanogram per liter level are obtained for most metals.

A distinctive feature of MBDs is their interactions with proteins and other biomacromolecules. LC-ICP-MS is the method of choice for analysis of these interactions. The system tolerates well high salt concentrations used in ion-exchange chromatography, while the high organic solvent loads used in RP chromatography is not readily compatible with plasma. This problem is most easily solved by diminishing column dimensions, resulting in lower flow rates of the organic solvent introduced. Microbore and capillary column separations will probably become the method of choice before long.

CE coupling to ICP-MS is not as straightforward as the LC coupling and has not gained a broad use presumably because of the difficulties of hyphenating and the higher LODs than those of LC-ICP-MS. However, as CE offers a complementary separation technique to LC, it still finds applications in certain research areas.

Hyphenated ICP-MS systems have been applied to numerous experiments on elucidation of metal–drug reactions with plasma proteins and DNA, which is a target for several of these drugs. These comprise primarily platinum-containing drug compounds, as cisplatin has been used for decades, and ruthenium and gold are gaining increasing attention as alternative metals in new anticancer agents. The multielement capability of the technique is exploited by simultaneous monitoring of the metal and sulfur to examine protein binding, in general, and simultaneous monitoring of iron or phosphorus to examine binding to the iron-containing transferrin and DNA, respectively. As the interest in MBDs involving a variety of possible metals for different purposes grows rapidly, the use of ICP-MS will increase correspondingly.

ABBREVIATIONS

AEX	Anion Exchange
CE	Capillary Electrophoresis
CIM	Convective Interaction Media
DNA	Deoxyribonucleic Acid
GSH	Glutathione
FIA	Flow Injection Analysis
Hb	Hemoglobin

HILIC	Hydrophilic Interaction Chromatography
HSA	Human Serum Albumin
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IDA	Isotope Dilution Analysis
IgG	Immunoglobulin G
IP	Ion Pair
LA	Laser Ablation
LC	Liquid Chromatography
MRI	Magnetic Resonance Imaging
MT	Metallothionein
PUF	Plasma Ultrafiltrate
RP	Reversed Phase
RBCs	Red Blood Cells
SEC	Size Exclusion Chromatography
UF	Ultrafiltrate
WAX	Weak Anion Exchange

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11 On-Line Electrochemical/LC-MS Techniques for Profiling and Characterizing Metabolites

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11.1 Summary	1
11.2 Overview of electrochemical (EC) flow cells	2
11.3 Electrochemistry in series with LC-MS	3
11.4 EC-Array techniques	9
11.5 Conclusion	14
References	14

11.1 SUMMARY

Over the last decade, there has been a steady increase in the number of publications that describe on-line combination of electrochemical (EC) techniques with liquid chromatography-mass spectrometry (EC-LC-MS) [1,2]. These techniques have been shown to possess unique capabilities to both utilize and study oxidative and reductive (redox) reactions. This can provide not only analytical utility (e.g., LC detection) but also an insight that can be beneficial to drug discovery and development processes. This latter capability is largely based on the relevance of redox processes to many chemical and biochemical aspects of drug development, including chemical oxidative degradation [3], oxidative metabolism and formation of reactive species [4,5], and the relevance of oxidative stress in disease and toxicity [6]. The incorporation of rugged commercially available EC cells into the existing LC-MS systems and protocols is typically inexpensive and readily implemented. These and other factors have led to an increased use of these techniques at strategic times within the drug development process.

This chapter is an overview of several EC/LC-MS approaches, specifically those that employ three-electrode EC flow cells for controlled potential electrolysis. We describe some basic theories and a number of applications including those that use EC as reaction devices in series with MS and those involving parallel EC and MS for HPLC detection. Serial EC-MS applications include generating and characterizing products that often correspond to metabolites, degradants, or short-lived species of interest. The

quantitative and qualitative aspects of parallel EC-Array-MS are also described for studying chemical oxidative stability, for profiling metabolites and degradants and for metabolomics. To facilitate expanded adoption of EC/LC-MS techniques, our emphasis is on basic methodology and practical considerations.

11.2 OVERVIEW OF ELECTROCHEMICAL (EC) FLOW CELLS

The common uses of three-electrode EC flow cells include HPLC detection (LCEC) [7–10] and hydrodynamic voltammetry [11]. There are many excellent sources of EC information that can be consulted for detailed description of theory and applications [12–15]. Briefly, the cell functions by establishing a specific applied potential between a reference electrode (RE) and a working electrode (WE). The auxiliary electrode (AE), through electrical feedback via a potentiostat, provides the energy necessary to maintain the potential and relies on solution conductivity (typically mobile phase with ≥ 20 mM buffer) to complete the circuit. This energy drives electrolysis of solution-phase species (i.e., analyte and reactant) at the WE surface. The majority of applications described below involve recording EC cell current and associated mass spectra as a function of, and as a result of, the applied potential.

EC cells with carbon-based WE are the most widely used for LCEC and, as such, are the primary focus of this discussion. Other WE materials (e.g., Au, Ag, and Pt), while more advantageous for certain applications [16,17], are typically more prone to surface passivation and are generally less practical for many solvent and pH conditions [16]. There are several possible EC flow cell designs, but only three basic geometries are in widespread use: thin-layer, wall-jet, and porous flow through. Thin-layer and wall-jet amperometric cells have small surface area WE, and when using typical HPLC flow rates (i.e., 0.2–2.0 mL/min), only a small percentage (typically $<5\%$) of analyte comes into close-enough proximity to the WE to be oxidized or reduced. Response is therefore affected by changes in flow rate, and the overall yield of reaction products is typically low. Also, electrode maintenance is frequently required because of WE passivation, particularly, when analyzing complex matrices or a large number of samples [18]. Thin-layer and wall-jet cells are widely used in the analysis of limited volume samples, such as microdialysis perfusates. In these instances, the combination of low volume EC cells with microbore chromatography can provide limits of detection in the femtogram range. A third class of EC flow cell utilizes high surface area microporous WE to achieve higher electrolysis efficiencies (typically $>95\%$ at 2.0 mL/min). This design is termed *coulometric* since the integrated response (peak area) represents the charge realized (coulombs) from nearly complete electrolysis of analyte as described by Faraday's law. A disadvantage to the coulometric cell design is that the WE is not accessible to mechanical resurfacing. However, in comparison to thin-layer and wall-jet cells, coulometric cells provide higher yield and more reproducible reactions (i.e., response) and require much less maintenance [8–10]. These advantages have led to its more widespread use in the analysis of complex matrices and in higher throughput approaches including those that utilize gradient elution HPLC. Further discussion is focused on two basic implementations of coulometric EC: (i) a single cell upstream and in series with MS and (ii) multiple cells (EC-Array) parallel with MS.

11.3 ELECTROCHEMISTRY IN SERIES WITH LC-MS

11.3.1 General Considerations

Several laboratories have used single EC cells as a simple “add-on” to a typical LC-MS setup. The common experimental configurations (Fig. 11.1) include flow injection, direct infusion, and both pre- and postcolumn EC/LC-MS. Several design aspects must be considered when adding an EC cell to an LC-MS system, such as solution conductivity, electrical grounding, and cell volume. Supporting electrolyte is required to provide solution conductivity. Common LC-MS mobile phases containing, for example, 0.1% formic acid or 5–10 mM ammonium acetate may have insufficient conductivity, and the resulting *IR* drop must be compensated for by using higher applied potentials. While this may be acceptable for some experiments, the use of more conductive solutions is generally preferred. This is particularly important for LCEC detection and for generating hydrodynamic voltammograms (HDVs) where low solution conductivity may lead to poor response, peak tailing, and loss of voltammetric resolution. Volatile buffers (e.g., ammonium acetate or ammonium formate) of at least 20 mM concentration typically provide sufficient conductivity and pH buffering. While higher buffering capacity may provide improved control of chromatography and detection, it is important to consider potential adverse effects on MS detection (e.g., ionization suppression, adduct formation, background noise). A further consideration is related to the use of conductive fluids and high voltage ion sources. The liquid inlet to the ion source (e.g., electrospray) of most LC-MS instruments is electrically grounded. However, in some instruments, there exists the possibility that current flow may occur from the high voltage ion source through a conductive fluid. Since many LC eluents are somewhat conductive, grounding the fluid line is typically recommended, irrespective of the use of EC. Current flow through the fluid line may compromise analyte integrity since it can affect the interfacial potential of distal, upstream, wetted components (e.g., injector and analytical column), which can lead to unwanted redox reactions [19]. A simple way to ground the fluid line is to use a stainless steel fluidic union connected to the ground of the MS high voltage power supply, as previously described [20].

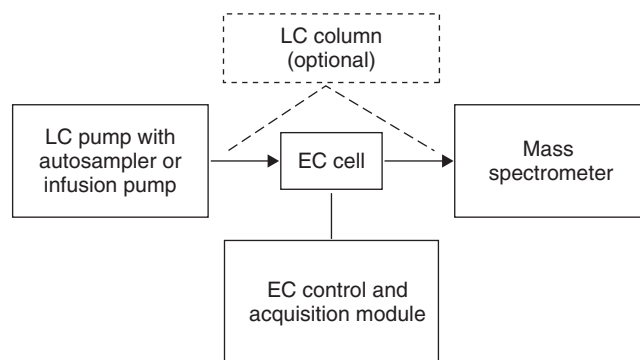


Figure 11.1 Representative serial EC/LC-MS configurations using either an ESA Model 5021A or 5030 coulometric EC cell controlled with Coulochem III for MS Detector (ESA Biosciences, Inc., Chelmsford, MA, USA).

11.3.2 Applications Using Flow Injection Analysis

Serial EC/LC-MS configurations are used in drug discovery and development to study the susceptibility of a compound to oxidation (or reduction), the nature of products and short-lived intermediates, and the associated reaction pathways. These data are used to provide insight to chemical stability, metabolism, and toxicity in a variety of contexts from rapid early-stage screening to more in-depth studies. A common approach is to use flow injection analysis (FIA) for medium throughput analysis of a series of compounds [21]. Each compound is typically analyzed at several EC potentials (e.g., 0, 400, 800, and 1200 mV vs Pd) to quickly (e.g., <30 s per injection) generate voltammetric data, which are indicative of relative ease of EC oxidation. The corresponding MS data are then used to provide information on the likely chemical sites of oxidation and the nature of products. These data are typically generated for analogous series to provide input to lead optimization strategies or to provide structural alerts for oxidatively unstable compounds. It should be noted that instantaneous voltammetric data may also be generated from a single injection by using several EC cells arranged in series, each successive cell maintained at a higher potential than the preceding cell. This technique, typically conducted in early-stage discovery for studying oxidative stability, is discussed in Section 12.4.

11.3.3 Pre- and Postcolumn EC with LC-MS

A logical extension of FIA with serial EC/LC-MS is to incorporate a separation technique to further characterize products. Figure 11.2 is an example of precolumn EC oxidation of tamoxifen. With the EC cell at 600 mV, the total ion chromatogram (TIC) shows two primary peaks corresponding to the starting material (tamoxifen) and, at an earlier retention time, an N-demethylated product. With the precolumn EC cell at 900 mV (Fig. 11.3), additional peaks are evident, including possible aromatic hydroxylation and dealkylation products. This example shows that product formation can, to some extent, be manipulated through simple control of EC cell potential and that several of these EC oxidation products correspond to biological metabolites or degradants of tamoxifen [22]. It should be noted that EC oxidations lack the steric control of enzyme-catalyzed reactions and may also involve different reaction mechanisms (see Section 12.3.6 for additional details). Therefore, the relative abundance of products generated by EC is not expected to correlate with that of biological systems. EC generation of metabolites is thus considered to be a qualitative technique.

The examples in Figs. 11.2 and 11.3 illustrate on-line generation and analysis of EC products using LC-MS conditions that are typical of metabolic studies (e.g., *in vitro* microsomal analysis). Serial EC/LC-MS can thus be used with “neat” parent compound solutions for preliminary optimization of LC and MS conditions for subsequent metabolite analysis in biological samples. By using identical conditions, EC data may then be used as an input to automated metabolite identification software to aid in finding metabolites present in more complex biological matrices [23].

11.3.4 Semipreparative EC Synthesis

When the data from an EC-generated product corresponds to that of a biological metabolite, the EC technique may then be viewed as a selective and rapid synthetic

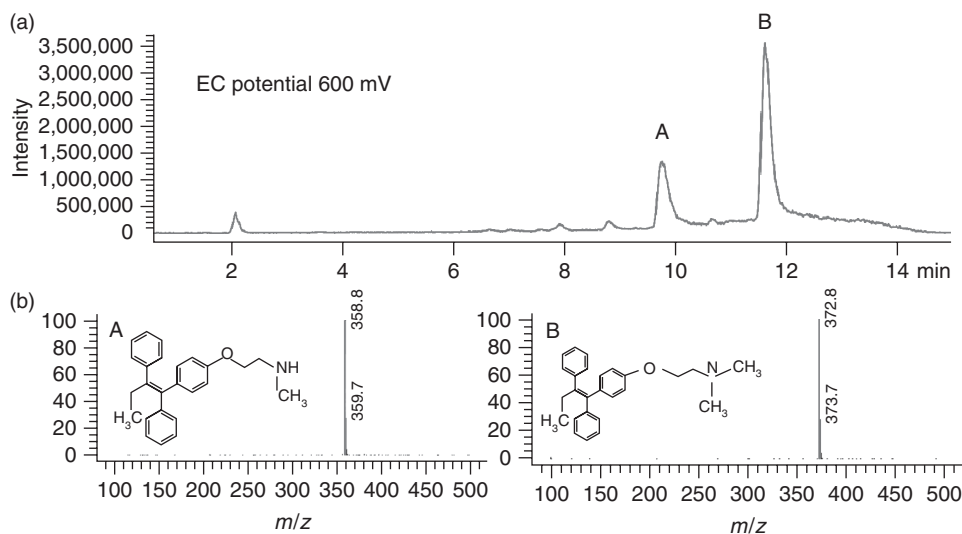


Figure 11.2 On-line oxidation of tamoxifen (50 μL of 20 $\mu\text{g/mL}$ diluted in mobile phase) using precolumn ESA Model 5021 EC cell (600 mV vs Pd). (a) Total ion chromatogram and (b) full-scan mass spectra of chromatographic peaks labeled A and B. Conditions: binary gradient from 16% to 80% acetonitrile over 10 min, 20 mM ammonium acetate as supporting electrolyte. Shiseido C18MG 3 μm 75 $\times$ 4.6 mm i.d. column, 1 mL/min, 200 $\mu\text{L/min}$ split to MS. Agilent 1100MSD single quad, positive ESI (electrospray ionization), scan 80 to 500 m/z . Fragmentor 70, gain 1.0, threshold 150, step 0.25, drying gas flow 12 L/min, nebulizer pressure 35 psig, drying gas temperature 350°C, and capillary voltage 3500 V.

route to small quantities of this metabolite. This can then be used to facilitate structural elucidation or, more generally, to produce additional chemical entities. Earlier studies demonstrated the feasibility of scaling up on-line EC techniques to produce sufficient quantities for structural confirmation by NMR [24]. Tahara *et al.* [25] have more recently described the EC production in milligram quantities and subsequent structural elucidation of a troglitazone metabolite. By using nonaqueous conditions, Tahara and coworkers [26] furthermore demonstrated the direct EC production of a reactive metabolite of troglitazone, which was useful to investigate its mechanisms of hepatotoxicity. As discussed in the following section, the ability to use a purely instrumental technique to quickly generate EC products can provide significant advantages for studying certain reactive species where nonspecific binding (i.e., interaction with endogenous biochemicals) may prevent detection in biological systems. Madsen *et al.* [27] have also recently described a method for EC synthesis of a glutathione (GSH) conjugate of clozapine, which made it possible to obtain structural information of this potentially toxic species by NMR. Moreover, the recent development of higher capacity coulometric cells (Models 5125 and 5150 Synthesis Cells, ESA, A Dionex Company) provides the ability to produce even larger quantities of material. The simplicity and speed of on-line EC/LC-MS may therefore provide an effective means of characterizing some of the many unknown metabolites, including reactive species, encountered in multivariate profiling studies.

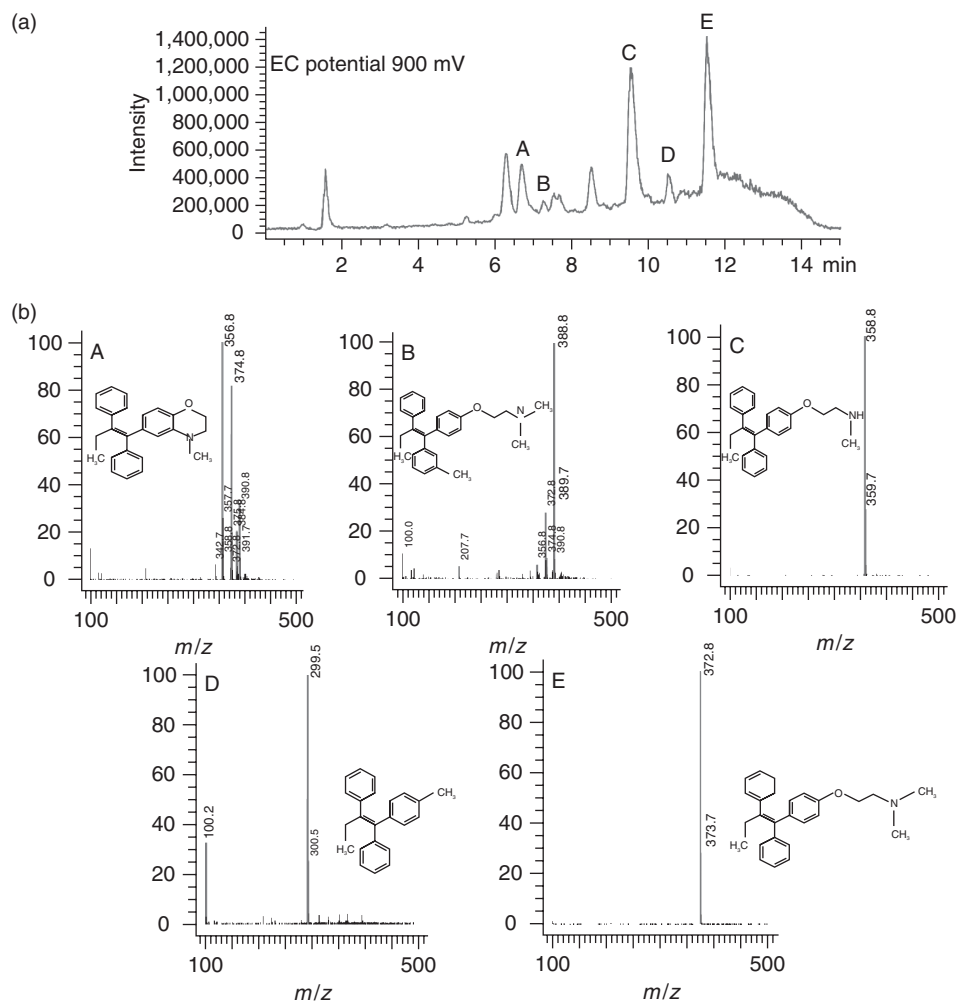


Figure 11.3 On-line oxidation of tamoxifen with precolumn EC cell at 900 mV versus Pd. Remaining conditions are as in Fig. 11.2. (a) Total ion chromatogram and (b) full-scan mass spectra of chromatographic peaks labeled A through E.

11.3.5 EC Generation and Analysis of Reactive Intermediates

Many studies suggest that redox metabolism of a wide range of chemical structures leads to the formation of reactive electrophiles that participate in a diverse array of toxic processes that typically involve covalent binding or other modifications to small and large molecules (e.g., DNA, proteins, peptides, and lipids), redox cycling, antioxidant/scavenger depletion, and other elements of oxidative stress [4–28]. The propensity of compounds to undergo redox-based metabolic activation to form reactive electrophilic species is therefore a major consideration in pharmaceutical development [29,30]. The topic of bioactivation and reactive metabolite assays is further discussed in the chapter titled *Bioactivation and Reactive Metabolite Assays* of this volume. Several reports have shown EC/LC-MS useful in the

study of reactive intermediate metabolites [21–35]. A variety of experimental configurations have been described that incorporate a nucleophilic trapping agent (typically, GSH), by either coinjection or infusion, with FIA, precolumn EC, or postcolumn EC. Lohmann *et al.* [36] have recently described EC simulation of the oxidative metabolism of paracetamol, amodiaquine, and clozapine. Interestingly, by using a reaction coil between the EC cell and HPLC injector, they were able to demonstrate adduct formation of the reactive metabolites with the proteins β -lactoglobulin A and human serum albumin. The formed drug–protein adducts were characterized with on-line time-of-flight MS, and the modification site was localized using FTICR-MS (Fourier transform ion cyclotron resonance-mass spectrometry). The authors describe this approach as a simple and fast method for the rapid risk assessment of covalent protein binding as well as for the synthesis of covalent drug–protein adducts in high purity and high yield. FIA and precolumn EC have been used to analyze neat drug solutions in order to investigate the susceptibility of a parent drug to EC oxidation, to characterize the reactive intermediates formed, and to study the chromatographic and MS behavior of trapped species. Postcolumn configurations have also been used to analyze biological (microsomal) preparations to investigate individual metabolite susceptibility to EC oxidation and to identify which metabolites form reactive species.

Figure 11.4 provides a basic example of precolumn EC oxidation using the widely studied compound, acetaminophen (APAP). Oxidative metabolic activation of APAP to form *N*-acetyl-*p*-benzoquinoneimine (NAPQI) is widely regarded as an essential component of its hepatotoxic effects in humans [5]. MS data indicate that EC oxidation of APAP in the presence of GSH (coinjected) resulted in two separate peaks corresponding to monogluthathionyl conjugates and one peak indicative of a digluthathionyl conjugate. These results are comparable to those reported nearly two decades ago by Getek and colleagues [31] using coulometric EC cells with thermospray MS. Several other “model” compounds have been studied using these techniques, including 4-aminophenol, BHT (butylated hydroxytoluene), estradiol and metabolites, phenacetin

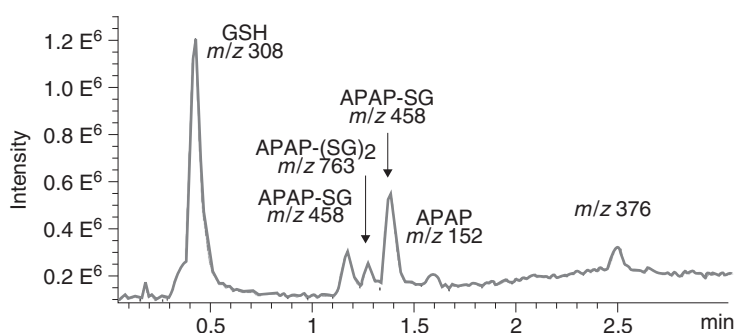


Figure 11.4 Precolumn oxidation of APAP with GSH as trapping agent. 10 μ L of 20 μ g/mL APAP, 1 mM GSH mixture with precolumn ESA Model 5021 cell at 500 mV versus Pd. Binary gradient elution from 1% to 80% acetonitrile (ACN) in 5 min, 20 mM ammonium acetate as supporting electrolyte. Shiseido C18 MG 3 μ m 50 $\times$ 4.6 mm i.d. column, 1 mL/min, 200 μ L/min split to MS. MS conditions are as in Fig. 11.3, except scan range that is 80–1000 m/z .

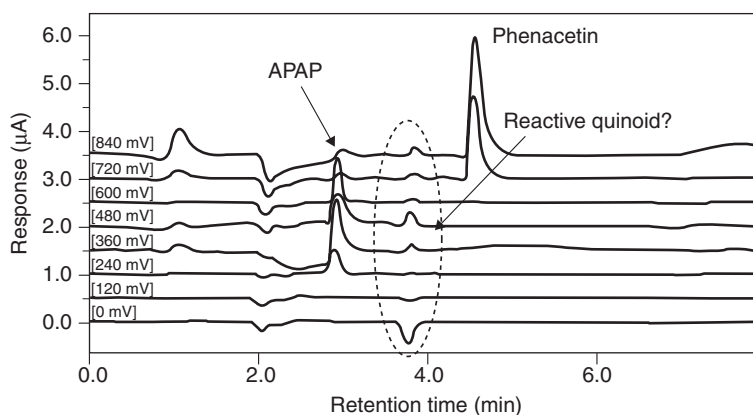


Figure 11.5 Precolumn oxidation of phenacetin. Conditions are as in Fig. 11.2, except LC detector that was an eight-channel EC-Array (CoulArray, ESA Biosciences, Inc., Chelmsford, MA, USA) with Model 6210 EC cell potentials of 0–840 mV versus Pd in 120-mV increments.

[21], and clozapine [33]. While the products expected from the known systems are generally formed and can be interrogated to reveal structural information, the EC oxidation sometimes results in a wide array of oxidation products not always seen in biological systems. It has been recognized that nonmicrosomal and nonenzymatic redox reactions may play a role in toxic processes [37], and further study is required to assess the significance of these observations. These data do, however, highlight the usefulness of EC/LC-MS as a rapid and efficient means of generating trapped products of reactive intermediates for structural characterization by LC-MS/MS from both parent drug and *in vitro* metabolites.

Figure 11.5 illustrates the precolumn oxidation of a phenacetin solution with LC-EC-Array detection (discussed in more detail in the following section). A peak, which eluted at 3.8 min, shows a characteristic voltammetric profile (i.e., reduction followed by oxidation) of a quinone species. On the basis of EC-Array and MS data (not shown), this peak has been identified as NAPQI, the expected reactive intermediate. This peak was not evident in a microsomal incubate of phenacetin analyzed using the same conditions (not shown). A possible explanation for this is that nonspecific binding of this reactive species occurred in the biological preparation. Thus, an additional advantage to the use of on-line EC/LC-MS includes the ability to examine short-lived species that, although potentially relevant, may not as readily be observed in biological systems. Many other pharmaceutically relevant applications of EC-MS have been described and are beyond the scope of this discussion. These applications include EC derivatization to enhance MS ionization [38], mass tagging [39], and protein cleavage techniques, several of them are discussed in a recent focus issue on EC combined with MS [1].

11.3.6 EC “Mimicry” of Metabolism

There are many examples, as above, which have demonstrated that the EC oxidation products for a given compound often coincide with metabolites observed in biological assays [31–33,40–42]. These and other EC oxidation products may also be observed in purposeful degradation or stability studies. It must be recognized, however, that

enzyme-catalyzed, chemical, and EC oxidations are often very complex and involve different phenomena. Studies conducted by Jurva and colleagues [43,44] provide very useful insight into the mechanistic aspects of EC and enzymatic oxidative reaction pathways, particularly in the context of cytochrome P450 (CYP450) metabolism. They have shown that EC oxidation using porous carbon WE generally leads to the formation of similar products in those enzyme-catalyzed reactions that are supposed to proceed through a mechanism initiated by a one-electron transfer oxidation. Examples of CYP450 reactions mimicked by carbon-based WE include dehydrogenation, N-deacetylation, N-dealkylation, S-oxidation, and aromatic O-dealkylation. Reactions initiated by H-atom abstraction, such as aliphatic C-oxidation and hydroxylation of aromatic rings without electron-donating groups, are, however, generally not mimicked by EC oxidation. There are several examples of alternative or modified WE materials and reaction conditions to affect specific reactions. This includes the immobilization of enzymes [45] and biomimetic redox indicators [46] on solid electrodes and solution-phase conditions such as those described for EC-assisted Fenton reactions [47]. Johansson *et al.* [48] and Lohmann and Karst [49] have recently studied the extent to which EC oxidation, electrochemically assisted Fenton chemistry, and synthetic metalloporphyrins can be used in combination to mimic a large range of CYP450-catalyzed oxidations. While these approaches are extremely useful for many applications, EC is generally considered to be a complement to, rather than a substitute for, a given *in vitro* assay. A similar argument applies to chemical oxidative degradation studies, which are discussed in a later section.

11.4 EC-ARRAY TECHNIQUES

11.4.1 General Considerations

The general concepts of EC-Array are described in detail elsewhere [7,50]. Briefly, this technique employs up to 16 series EC cells (Fig. 11.6) with porous graphitic carbon-based WE, similar in concept to those described in the previous section. Each cell is typically poised at a different fixed potential, thereby spanning a wide potential window to allow detection of a broad scope of redox-active analytes during the transit of a single injected aliquot through the cells. Efficient electrolysis obtained with high surface area coulometric WE allows for selective detection and resolution of coeluting analytes, on the basis of differences in their relative ease of oxidation and/or reduction. Stated differently, each analyte in a coeluting band will demonstrate signal dominance

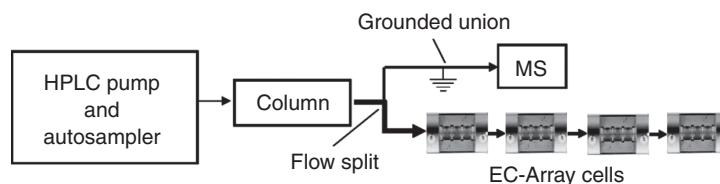


Figure 11.6 Representative configuration of LC with postcolumn flow passively split to EC-Array and MS. Model 6210 EC cells were controlled with a 16-channel CoulArray (ESA Biosciences, Inc., Chelmsford, MA, USA). *Source:* Reprinted from Ref. 75 by permission of Elsevier, Inc.

on a different WE based on their relative ease of oxidation or reduction. In general, EC reactions are typically observed according to the following general rank order (by relative ease of oxidation): *o*, *p*-quinol and *o*, *p*-aminophenol > tertiary amine < *m*-quinol $\approx$ phenol $\approx$ arylamine > secondary amine $\approx$ thiol > thioether $\neq$ primary amines and aliphatic alcohols. HDVs for each redox-active metabolite are obtained from the response across adjacent EC-Array sensors. These data are a reflection of the kinetic and thermodynamic components of electron-transfer reactions. Since chemical structure is a critical determinant of an analyte's redox behavior, the intrinsic generation of an HDV with EC-Array provides qualitative information for each species.

11.4.2 Applications Using Flow Injection Analysis

The use of FIA with a single EC cell in series with MS was described above to study compound susceptibility to oxidation. This requires multiple injections for each compound. An alternative approach is to use EC-Array (without MS) to generate voltammetric data from a single injection. This has the advantage of providing higher throughput but lacks the structure–activity and product information provided by MS. MS is not used downstream of EC-Array since the products that are selectively formed at upstream electrodes may further react at downstream electrodes, thus preventing their detection by MS. Many laboratories use EC-Array to generate voltammetric data to study oxidative stability, largely based on the pioneering work of Lombardo and Campus [51]. Chemical oxidation, a common mode of degradation for active compounds and drug products [3], is a significant concern at all stages of drug discovery and development. The relative tendency for compounds to undergo chemical oxidative degradation via electron-transfer mechanisms is closely related to their EC redox potentials [3]. As described in the previous section, oxidation reactions are often complex phenomena with a variety of mechanisms not necessarily modeled by a given EC technique. EC is typically used as part of a suite of techniques (e.g., oxygen/radical initiator, photolytic) for comprehensive study.

Representative data from FIA with 16-channel EC-Array detection is shown in Fig. 11.7. Using the described conditions, voltammetric data obtained for several model

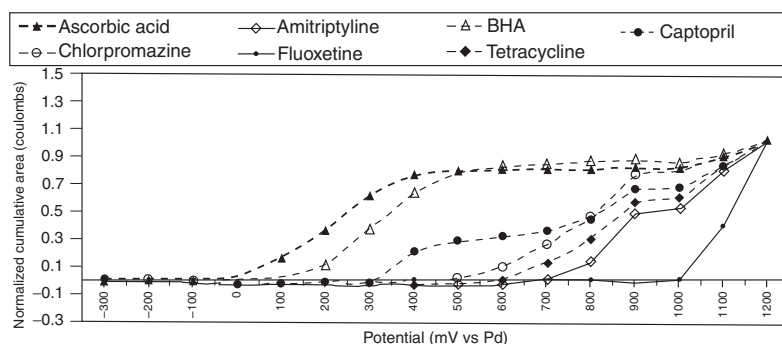


Figure 11.7 Voltammetric plots for seven model compounds representing normalized cumulative peak area. Dashed lines correspond to those compounds reported as oxidatively unstable; solid lines are indicative of oxidative stability.

compounds demonstrated that the most stable compounds oxidized at the highest potentials, while the least stable compounds oxidized at the lowest potentials [52]. While FIA was suitable for this pilot study, the approach was found to be inadequate for high throughput library-stage screening, particularly when considering the possible presence of electroactive impurities. Therefore, Lombardo and Campus [51] developed chromatographic methodology that used a short (3 cm) C18 column and isocratic elution in a strong (90% organic) eluent. This together with the inherent selectivity of EC-Array and the appropriate choice of signal threshold has allowed the generation of voltammetric data with a typical throughput of eight 96-well plates per week per instrument. This work, now applied to more than 30,000 compounds, was used to develop alerts for oxidatively unstable compounds, thus reducing the potential for later-stage issues. Furthermore, there are several additional potential uses of this approach, which are currently under investigation. These uses include the ability to assess the potential for degradation on library storage, to develop predictive structure–stability relationships, and to examine excipient compatibility. Webster *et al.* [53] have recently described the use of this technique in antioxidant selection for pharmaceutical formulations.

11.4.3 Quantitative Bioanalysis of Complex Matrices

The more traditional use of EC-Array is with LC separation for multicomponent quantitative and qualitative analyses. The primary advantages of this technique include two-dimensional (i.e., chromatographic and voltammetric) resolution, femtomole sensitivity, and data-dependent acquisition (autoranging), which facilitates use with gradient elution and provides a 10^5 dynamic response range [50,54]. LC-EC-Array has been widely used for routine analysis of redox-active substances with primary *in vivo* application to clinical chemistry [50,55,56], neurochemistry [57–59], and redox biochemistry [60–66]. Many pharmaceutical laboratories have adopted EC-Array for LC analysis of degradants and metabolites (i.e., related substances). A majority (estimated >85%) [67] of pharmacologically active small molecules possess one or more “EC-active” moieties (see above). The sensitivity (typically 10- to 1000-fold greater than UV–vis absorbance detection) [50,54], selectivity, and qualitative information obtained can provide significant advantages for a variety of applications. There are many examples of the use of EC-Array, specifically for bioavailability and pharmacokinetic studies [68–74]. The coupling of EC-Array as a parallel detector with MS is a logical extension of these approaches and is discussed below.

11.4.4 Metabolomics—Use of EC-Array in Parallel with MS

A major aspect of our recent work has been to couple EC-Array as a parallel detector with MS for metabolomic studies [75]. The comprehensive study of small molecules in living systems, that is, metabolomics, has several challenges, including analyte number and diversity, range of concentrations, and complexity of sample matrices. A variety of techniques exist that are each effective in studying certain classes of molecules within well-delineated concentration ranges; however, no one technique can overcome all the field’s inherent challenges. For instance, NMR is able to detect any molecule that contains an active nuclide (e.g., ^1H and ^{13}C); however, its detection is limited to microgram quantities and does not apply to certain functional groups such as amines and sulfates. MS is very versatile but relies on the ionizability of the compound. EC techniques,

specifically EC-Array, are extremely sensitive detectors of redox-active compounds that, in many instances, are not observed with MS. The parallel use of EC and MS provides the capability to obtain more information from a given metabolite and to extend the number of metabolites and range of chemical classes that can be effectively studied.

A representative chromatogram of rat urine analyzed by parallel EC-Array-MS is shown in Fig. 11.8. As expected, the MS base peak chromatogram shows relatively few, directly visible, metabolite peaks. Mass spectral data from full-scan exploratory studies are typically processed by extracting discrete signals each defined by a particular retention time and m/z and using algorithms to help distinguish analytical

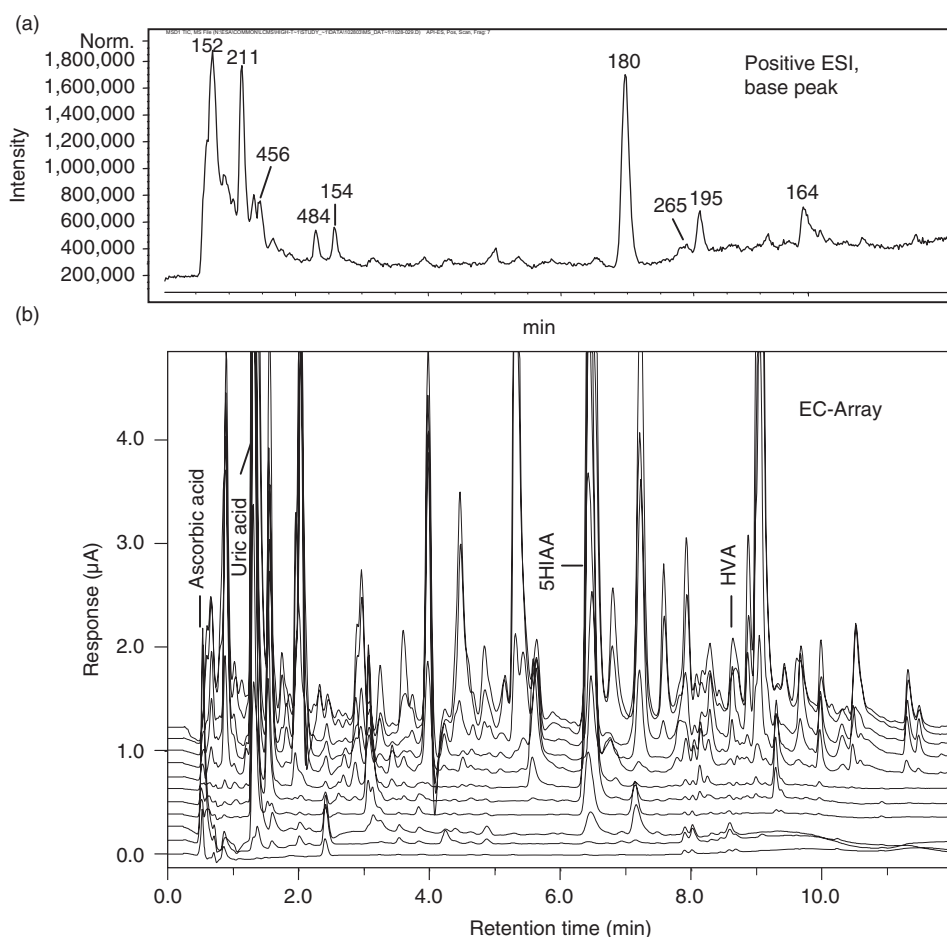


Figure 11.8 (a) MS base peak chromatogram labeled with base peak m/z , and (b) EC-Array multichannel chromatogram (11 of 16 channels shown for clarity). Analytical conditions: 20 μL of 10-fold diluted urine. Gradient elution 1–100% aqueous acetonitrile with 10 mM ammonium formate and 50 mM formic acid; flow rate 1.5 mL/min; Shiseido C18, 3 mm, 75 mm $\times$ 4.6 mm i.d. column; and 4:1 passive postcolumn flow split to EC-Array/MS. EC-Array potentials 0–1050 mV in increments of 70 mV. ESI positive mode, capillary voltage 3500 V, fragmentor voltage 70 V scan range m/z 50–850, and scan speed 1.2 s per cycle. *Source*: Reprinted from Ref. 75 by permission of Elsevier, Inc.

signal from background noise [76]. The resulting data, often consisting of hundreds of discrete signals, are then typically processed by chemometric techniques such as principle component analysis (PCA). Chromatographic variability, ionization suppression, adduct formation and in-source oxidation with MS, and electrode adsorptive and other non-Faradaic processes with EC are important complicating factors to consider in these multivariate analyses. Also, as evidenced in Fig. 11.8, a significant challenge in metabolomics studies is to identify the many unknown peaks that are often observed in a typical biological sample. Our data show that the concurrent acquisition of EC-Array and MS data for each metabolite peak helps to address these potential issues. For example, the observation of a particular redox-active metabolite peak allows the analyst to conduct a more informed and targeted interrogation of the corresponding MS data. Furthermore, specific MS data are useful to normalize for retention time variability observed with both MS and EC-Array data. In addition, our results indicate that many redox-active urinary metabolites exist as solution-phase neutral species under a variety of reversed-phase chromatographic conditions; for example, peaks annotated in Fig. 11.8 are ascorbic acid (AA), uric acid (UA), 5-hydroxyindoleacetic acid (5HIAA), and homovanillic acid (HVA). Of these metabolites, only UA was detected from extracted ion chromatograms (above baseline noise) as its protonated or adducted molecule [e.g., $M + X$ ($X = H^+, Na^+, K^+, NH_4^+$)]. The combined detection scope of MS and EC-Array thus provided higher coverage in a single analysis of the wide dynamic range and broad chemical diversity of urinary metabolites.

In a model study of APAP-induced hepatotoxicity, results from PCA of EC-Array data showed consistent differentiation between experimental groups of animals receiving high dose APAP (200 or 300 mg/kg, 0–8 h collection), low dose (20 mg/kg) APAP, high dose (200 mg/kg) acetylsalicylic acid, and controls. Figure 11.9 shows some of the individual redox-active components that, as indicated from PCA, largely contributed to the observed differentiation. This includes both endogenous (E1–E4) and xenobiotic (M1, M3, and M4) metabolites. These data further demonstrate the complementary nature of EC and MS detection. For example, MS data, along with prior knowledge of a parent compound's analytical behavior and informed prediction of biotransformations, was used to distinguish xenobiotic and endogenous metabolites. This allowed more direct study of changes in endogenous metabolite profiles by PCA. Also, EC-Array data showed clear evidence of an APAP metabolite that was not detected by MS (peak M1). Furthermore, MS data indicated that the peak M3 consisted of two major components having m/z 313 and 232. These m/z values are consistent with the commonly observed O-sulfated metabolite and the less frequently observed mercapturic acid metabolite of APAP, respectively. The similarity in voltammetric response between parental APAP and the peak corresponding to m/z 232 suggests that the easily oxidized 4-amidophenol group is intact. These data are consistent with the commonly observed ring thioether metabolites associated with high dose APAP [77]. Since APAP-mercapturate is a recognized marker of the so-called toxic pathway of APAP, the described approach of exploratory multivariate analysis and targeted interrogation of EC and MS data were an efficient and effective way to determine relevant changes associated with high dose APAP toxicity and to provide insight into the chemical identification of potential biomarkers. These results demonstrate that the combined use of EC-Array and MS may provide significant advantages over either individual technique in addressing the complex nature of metabolomic studies.

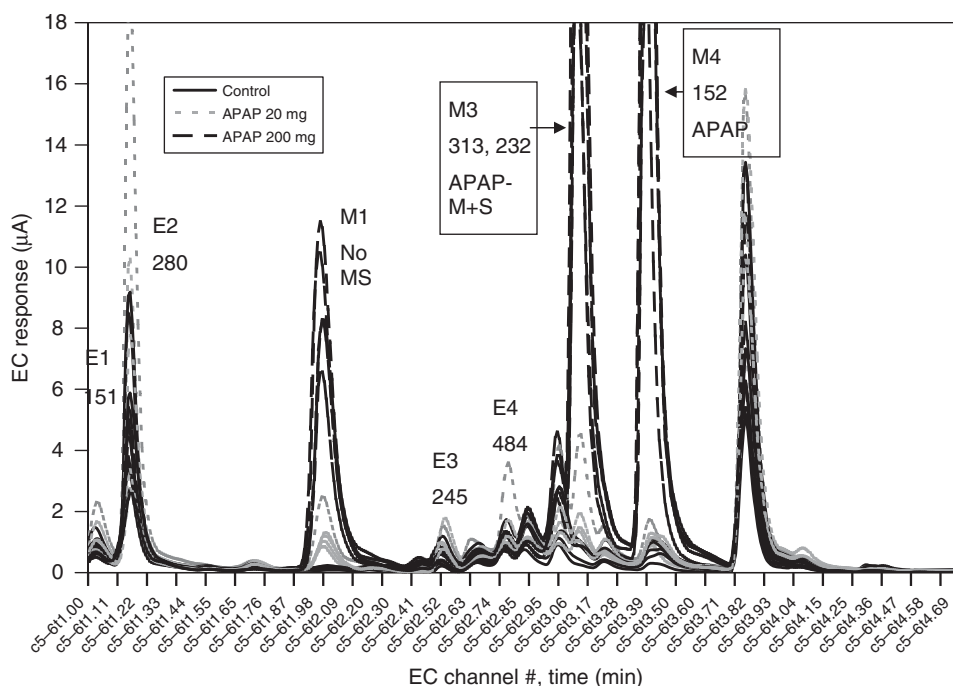


Figure 11.9 Overlay of EC-Array data from urine of rats administered (i.p.) vehicle 20 mg/kg or APAP 300 mg/kg ($n = 5$ in each group). Peaks labeled E indicate endogenous metabolites, while peaks labeled M indicate drug metabolites. Base peak m/z ratios as determined from corresponding MS data are shown. APAP-M + S indicates coelution of sulfate and mercapturate metabolites of APAP. *Source:* Reprinted from Ref. 75 by permission of Elsevier, Inc.

11.5 CONCLUSION

EC cells may be combined with LC-MS in a variety of experimental configurations that provide analytical utility and insight into drug discovery and development. This includes their use as reaction devices in series with MS for studies relevant to oxidative metabolism, degradation, and reactive species formation. EC-Array in parallel with MS provides the capability to increase the detection scope of LC-based multivariate profiling with high sensitivity, selectivity, wide dynamic range, and complementary qualitative data. The usefulness of these hyphenated techniques and the data generated for drug discovery and development are largely dependent on the challenges of a particular program and should be enhanced by their informed strategic implementation.

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12 Nuclear Magnetic Resonance Spectroscopy for the Study of Drug Metabolism

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12.1	Summary	1
12.2	Introduction	2
12.3	NMR spectroscopy—the good, the bad, and the ugly	3
12.4	NMR experiments	8
12.5	Hyphenated techniques	11
12.6	Equipment considerations for drug metabolism	13
12.7	Sample preparation	15
12.8	Discovery applications	16
12.9	Development applications	20
12.10	Stereochemical considerations	24
12.11	Conclusions	24
	Acknowledgments	25
	References	25

12.1 SUMMARY

The importance of a detailed understanding of drug metabolism for maximizing safety and differentiation has encouraged the development of numerous analytical tools including approaches based on liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). The former is the front-line tool for drug metabolism because of its sensitivity and ability to readily characterize structural changes in multiple molecules in real time as they are separately eluted off the LC column. Localization of metabolic modification can be achieved through designed fragmentation such as provided by MSⁿ experiments. Complementary information provided by NMR is required when the precise localization of sites of metabolism is necessary and when they cannot be assessed through MS-based experiments, a situation that is not uncommon. This chapter provides an overview of the specific

structural information that NMR can provide on drug metabolites in solution, such as sites of modification at the atomic level, regio- and stereoisomer characterization, and dynamic physical properties, and how these parameters are measured. Throughout, the complementarity of NMR-derived information with that provided by LC-MS experiments is pointed out. Limitations and costs associated with NMR are discussed in some detail as are considerations for ideal NMR equipment for drug metabolism studies, including a discussion of hyphenated methods such as LC-NMR. NMR is nonselective, and hence the importance of sample preparation for NMR cannot be underestimated and some attention is given to this topic. Somewhat different approaches required in discovery and development applications are discussed and illustrated through many examples in separate sections and finally a brief discussion of stereochemical measurement by NMR is provided.

12.2 INTRODUCTION

From the earliest structure–activity relationships (SARs) in discovery through the latest phases of clinical development, detailed knowledge of how a drug molecule is transformed by the body is crucial for ensuring that the most effective and safest pharmaceutical agents are brought to the patient. The quest for this knowledge has encouraged the advancement of numerous analytical fields and challenged modern biotransformation scientists to also be multifaceted technology experts [1]. Currently, the most popular tool is, and will likely be for the foreseeable future, high performance liquid chromatography coupled to a mass spectrometric detector (HPLC-MS or LC-MS). Mass spectrometry is, of course, a logical tool for studying biotransformation, as most metabolic modifications involve a change in the mass of a parent molecule, and powerful LC-MS configurations are available at moderate cost. However, unfortunately, a change in mass is frequently not sufficient for identifying the exact location of the metabolic modification and, unless appropriate standards are available, LC-MS is difficult to use quantitatively. This is where NMR steps in. NMR spectroscopy is a quantitative, nondestructive technique that provides atomic resolution information about chemical modifications and conformational changes on molecules in solution, thereby providing exactly the information that is required to fill this gap. As a result, NMR has become invaluable for drug metabolism studies, along with many other pharmaceutical research applications. Although NMR's utility in drug metabolism was recognized early (see the short review article [2] and references therein), the relative insensitivity of the method limited its utility to abundant metabolites. In the past three decades, however, advances in NMR technology have slashed sample requirements from hundreds of milligrams to submicrogram levels, availing the technique to a much broader range of biologically interesting applications, including the ability to resolve complex structural modifications that result from an organism's attempt to eliminate foreign molecules through biotransformation. NMR is the only technique able to routinely identify the exact atom in a molecule that is modified through metabolism. Although its utility is still hampered by poor sensitivity relative to many other analytical techniques, the usefulness of NMR is evidenced by its widespread use. When one does a literature search on "NMR and drug metabolism" over 4000 hits are recorded, going back as far as 1968, and maintaining a steady level over the past 30 years (Fig. 12.1). A limited number of reviews have been published on the use of NMR

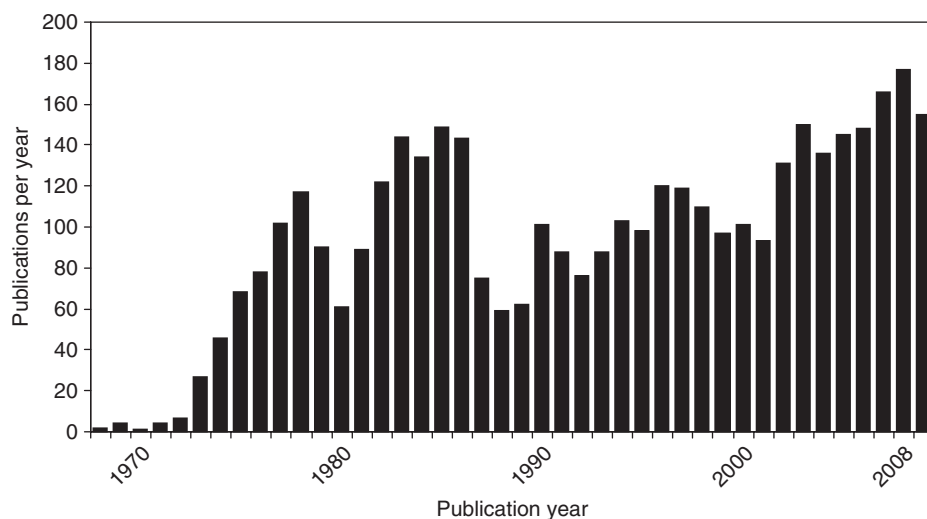


Figure 12.1 Annual publications related to the use of NMR in drug metabolism. Bars represent the number of hits returned by searching www.scopus.com using the query: ‘[(“drug metabolism” OR biotransformation) AND (NMR OR “nuclear magnetic resonance”) AND NOT (microbial OR microbiotic OR bacterial)]’ against abstracts, titles, and keywords.

in the context of drug metabolism including reviews that are general in nature [3,4], those aimed at hyphenated NMR techniques [5] and with regulatory focus [6,7]. The basic principles of NMR in the context of metabolite analysis have also been reviewed [8,9]. In this chapter, we provide a background on NMR spectroscopy aimed at those not intimately familiar with it, discuss types of NMR experiments most useful in drug metabolism studies and criteria for when to include them, discuss the strengths and weaknesses of the technique vis-à-vis other analytical approaches (especially hyphenated mass spectrometric techniques), and provide specific examples of its utility from the literature and our own laboratories.

12.3 NMR SPECTROSCOPY—THE GOOD, THE BAD, AND THE UGLY

NMR spectroscopy is the most powerful analytical technique for providing atomic level structural and dynamic information on complex molecules in solution. There are a number of readily obtainable parameters that make NMR spectroscopy such a useful tool for the study of drug metabolites. These parameters include the chemical shift, scalar (through-bond) coupling, dipolar (through-space) couplings, intensity, and relaxation properties. Nevertheless, there are some limitations to NMR, both moderate and severe, that need to be considered when assessing its applicability to any particular study. These strengths and weaknesses are discussed in this section.

12.3.1 The Good

The frequency at which a particular atomic nucleus resonates, or chemical shift, is expressed in parts per million (ppm) with respect to the nucleus’s resonant frequency, ω_0 , and is dependent on the applied magnetic field (see below) as well as the nucleus

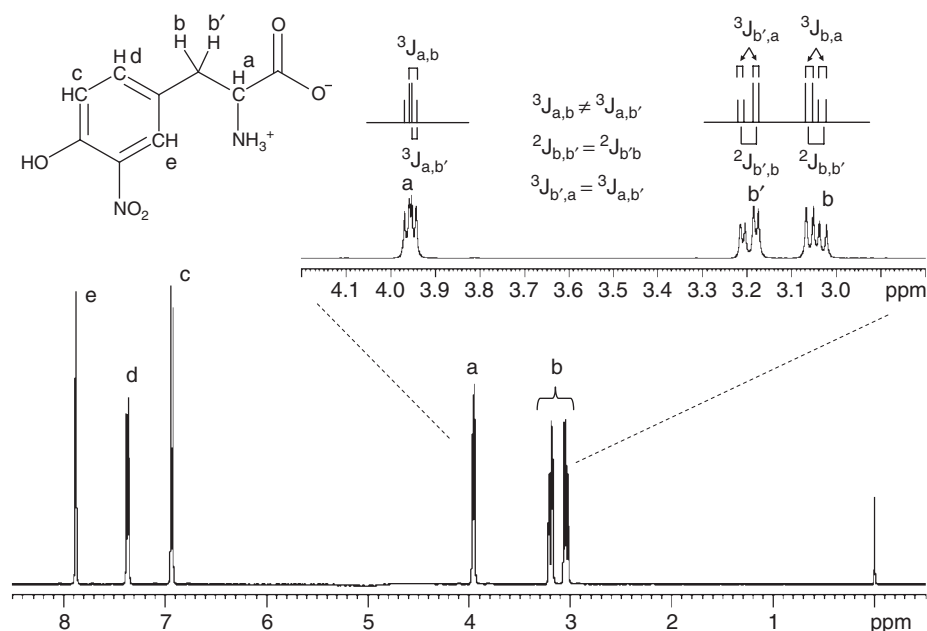


Figure 12.2 The 500-MHz NMR spectrum of 10-mM 3-nitrotyrosine dissolved in 66-mM sodium phosphate buffer, pH 7.4. The buffer contains 0.33-mM trimethylsilyl [2,2,3,3- $^2\text{H}_4$] propionic acid sodium salt (TSP) as an internal chemical shift standard. Labels assign specific protons in the molecule to specific NMR signals in the spectrum. In aqueous solvents, the amino and carboxylate protons are generally exchange-broadened and not observed. Inset: expansion of the 500-MHz ^1H NMR spectrum of 3-nitrotyrosine showing J-coupling splitting patterns in the aliphatic region. The superscript denotes the number of bonds between the coupled nuclei. Note that the different magnitudes for the three-bond couplings is a direct result of differential orbital overlap (and hence geometry) between the methine proton (a) and the two magnetically nonequivalent prochiral methylene protons (b, b'). *Source:* Reproduced from Ref. 9 with the permission of Informa Medical and Pharmaceutical Science Books.

under investigation and the electron clouds that surround it. Thus, the exact nature of the chemical bonds and nonbonded interactions that are experienced by an NMR-active nucleus within an atom will contribute to its characteristic chemical shift. If we take nitrotyrosine (the product of the endogenous amino acid and reactive nitrogen species) as an example, each nonexchangeable hydrogen nucleus (proton) will have a unique chemical shift, see Fig. 12.2. This results in a distinctive NMR fingerprint for virtually every molecule, including geometric isomers of the same molecule. Because the nucleus is shielded from the bulk environment, the NMR chemical shift is largely (although not entirely) insensitive to the surrounding analytical matrix. Hence, while changes in the matrix can cause small perturbations to an atom's chemical shift, metabolic modifications that change the nature of the chemical bonds, such as oxidation, have profound and interpretable effects. Since ionization state has a large influence on the electronic environment local to the ionized atom, the pH dependence of chemical shifts, or changes thereto, is also useful for characterizing biotransformational changes in a molecule. For example, a metabolite of cinafloxacin known to be a glucuronide based

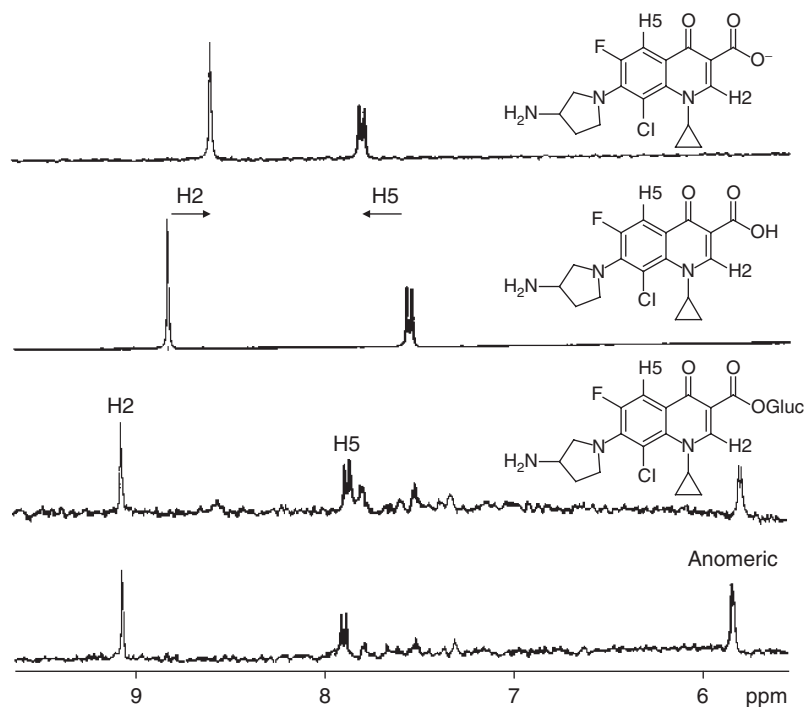


Figure 12.3 The use of pH dependence of NMR chemical shifts to evaluate site of glucuronidation in clinafloxacin. Downfield region of the 500-MHz NMR spectrum shows the clinafloxacin H2, H5, and anomeric glucuronide protons. From top to bottom: Clinafloxacin, pH 6.2; clinafloxacin, pH 1.8; clinafloxacin glucuronide, pH 7.8; and clinafloxacin glucuronide, pH 2.0. In clinafloxacin, the changes in chemical shift for H2 and H5 result from ionization of the carboxylate group. The absence of pH dependent shifts in the glucuronide is evidence that the carboxylate is the site of conjugation, rather than the amine.

on LC-MS data was shown to be conjugated on the carboxylate group and not the amino group based on the observation that aromatic protons the carboxyl group were pH independent between pH 7 and 2, indicating that the acid moiety was blocked by glucuronidation (Fig. 12.3) [10].

Scalar coupling arises from cross talk between NMR-active nuclei through shared circulating electrons in overlapping atomic orbitals. Such interactions generally occur over two to three bonds (rarely more than five bonds) and result in a splitting of coupled NMR resonances as illustrated in the nitrotyrosine example (see inset, Fig. 12.2). This “J-coupling” varies with the extent of overlap of the electron orbitals and is therefore highly dependent on molecular geometry. This fine structure within the spectrum makes each NMR molecular fingerprint even more unique than on the basis of chemical shift alone. An additional internuclear interaction phenomenon is dipolar coupling, and it is manifested through the direct mutual influence of proximal nuclear dipoles. Since it is not mediated by electron density and the magnitude of the interaction is inversely proportional to the inverse of sixth power of the distance between the dipoles, this parameter can provide internuclear distance information through the nuclear Overhauser effect (NOE) even in atoms that are far removed from each other in a structure or are

not in the same molecule at all [11]. Both of these coupling phenomena form the basis for numerous powerful two-dimensional (2D) experiments, which are discussed below.

The NMR phenomenon lends itself to quantitative analysis. The integrated NMR signal area, I_x , is proportional to the number of nuclei generating the measured signal, N_x and is given by

$$I_x = k_S N_x \quad (12.1)$$

where k_S is a factor that consists only of parameters associated with the instrument, the nuclear gyromagnetic ratio (γ , a constant characteristic of each nucleus) and the applied static magnetic field, B_0 [12]. Since the k_S is the same for all like nuclei in a given sample, the NMR signal intensity is linear, directly proportional to the concentration over many orders of magnitude and independent of matrix or molecular characteristics. This differentiates NMR from many other spectroscopic and spectrometric techniques where response is strongly influenced by the constitution of the sample and needs to be empirically evaluated for each analyte and set of conditions. As such, NMR is nonselective, and to a first approximation, anything in the sample above the limit of detection with an NMR-active nucleus on it will be observed. If the experimental parameters are selected appropriately, then I_x is directly proportional to the absolute concentration of the molecule in the sample, a property that has led to the development of so-called quantitative nuclear magnetic resonance or qNMR [13] and which can be utilized to positive end in drug metabolism studies [6] without the use of radioisotopes.

Finally, NMR can provide dynamical information on molecular diffusion, conformational exchange, orientational correlation times, and intermolecular interactions in solution. In terms of the relevance of these to drug metabolism studies, qualitative information about metabolism-induced perturbations to molecular geometry can be obtained. For example, hydroxylation of a cyclohexane ring can stabilize or destabilize the internal conformational energetics of the ring, resulting in a measurable effect on the cyclohexane NMR peak shape (and chemical shift), which can then be interpreted in a structural context [14]. As molecular correlation times increase, more efficient relaxation mechanisms lead to line broadening, an effect that is roughly proportional to the molecular weight. Thus, when small molecules, such as drug metabolites, bind to large ones, such as proteins, the relaxation and line width depend on the binding kinetics and the size of the macromolecule. This phenomenon has been used to characterize protein binding and has been applied to CYP450 SAR [15] and sialic acid biosynthesis [16].

12.3.2 The Bad

Even with the highest available magnetic fields, NMR spectroscopy suffers from having only a very small percentage of nuclear spins are in the low energy state relative to the high energy state, that is, a very low Boltzman distribution. The net result of this is that NMR is an extremely insensitive technique relative to many other analytical methods. While LC-MS can, under favorable conditions, detect femtomoles of a given analyte, even the most modern NMR equipment requires several nanomoles of material for analysis in reasonable times. Virtually all modern high resolution NMR spectrometers are pulsed Fourier transform instruments that simultaneously excite all nuclei of a given type and collect raw data in the form of a free induction decay (FID) as they relax back

to ground state (typically within a few seconds). This makes it possible to add together multiple FID transients to improve the spectrum signal-to-noise ratio (S/N), since the signals will co-add and the noise will only increase in proportion to the square root of the number of scans [17]. The S/N therefore improves with the square root of the number of scans. Such signal averaging is always necessary in drug metabolism studies and typically from a few dozen up to several thousand transients must be summed to achieve necessary S/Ns. Thus, poor sensitivity has been (and will continue to be) the Achilles heel of bioanalytical uses of NMR and increasing NMR sensitivity has been the focus of most of the technical developments that have occurred over the past four decades. Over this time, the availability of increasing powerful magnets has been driven by the proportionality between S/N and $B_0^{3/2}$ (factored into Eq. 12.1). As a matter of convention, magnet field strength is customarily expressed as the frequency at which protons resonate:

$$\omega_{\text{OH}} = \gamma_{\text{H}} B_0 \quad (12.2)$$

For example, a 14-T magnet corresponds to a proton field strength of 600 MHz. Increased resolution is another benefit to higher magnetic fields and is proportional to B_0 . Another major improvement in sensitivity was realized near the turn of this century with the development of NMR probes that have cryogenically cooled receiver coils and/or preamplifiers. These so-called cryoprobes achieve their high sensitivity by significantly reducing thermal noise contributions from these components and offer improvements so significant that they are almost considered required equipment for sample-limited work.

Confounding NMR's low intrinsic sensitivity is its nonselectivity. What was mentioned as an advantage above can also be a nuisance. Scarce metabolites, although above the limit of detection, may be fully or partially obscured by interfering components in the sample, which are present at similar or higher concentrations. These can include contaminants arising from the isolation matrixes, other molecules that coisolate, solvents, buffers, and so on, all of which can obstruct important regions of the NMR spectrum. Of course, chemically equivalent nuclei in different molecules, as in the case of enantiomers, or nuclei that are coincidentally magnetically equivalent will be indistinguishable, and conditions must be manipulated to tease apart differences in such cases. Some of these procedures are described below.

12.3.3 The Ugly

All of the tremendous advantages outlined above for NMR spectroscopy do not come without a price. An ideally equipped modern 600-MHz NMR spectrometer with all of the accessories necessary to carry out drug metabolism studies will, as of the time of this writing, cost ~\$1.3 million. Upgrade this to an 800-MHz system and the price goes up to ~\$2.8 million. If one wants the highest resolution and most sensitive commercially available magnet (950 MHz), the price tag goes up to ~\$8.8 million. These estimates do not include space considerations that also go up disproportionately with field strength. If such funds are not readily available, however, most medium to large size pharmaceutical companies have existing NMR instrumentation and expertise that can be engaged in a drug metabolism structure elucidation strategy with little or no capital investment required.

12.4 NMR EXPERIMENTS

12.4.1 Useful Nuclei

Many nuclei have a nuclear spin and therefore can be monitored using NMR spectroscopy; the ones of primary interest in drug metabolism are ^1H , ^3H , ^{13}C , ^{15}N , ^{31}P , and ^{19}F . Because proton is highly sensitive and hydrogen is present in nearly every drug metabolite of interest, ^1H NMR spectroscopy is by far the most common in drug metabolism studies. The NMR-active isotopes of carbon and nitrogen are useful as well and can occasionally be utilized without enrichment [18], but normally are not directly observable because of their low natural abundance (1.1% and 0.3%, respectively). An advantage of this is that they can be useful “tracer” nuclei that can monitor the metabolic fate of artificially labeled precursors [19,20]. By far, the most valuable use of ^{13}C and ^{15}N is as passive nuclei in proton-observe 2D experiments, which are described later in the chapter. At nearly 100% natural abundance, ^{13}P has been used extensively for studying energy metabolism, nucleic acids, and other naturally occurring phosphates and phosphate esters [21,22], and has potential for studying the fate phosphate-containing drugs and prodrugs [23]. For drugs that contain fluorine, ^{19}F can be used to monitor metabolic fate with little concern for contaminating background signal [23–25]. Tritium is not only a frequently used isotope of hydrogen for mass-balance studies and has properties similar to proton NMR but also has the advantage of having no background signal. However, ^3H NMR is not widely used because it requires special NMR equipment and high levels of radioactive nuclide incorporation [26]. Regardless of the nucleus chosen to observe, there are a plethora of experimental approaches that exploit the various NMR parameters and can be applied to biotransformation studies. These are broken down by their frequency dimensionality and are either one-dimensional (1D) and produce a single frequency (chemical shift) axis or two-dimensional (2D) and produce two frequency axes.

12.4.2 Solvent Suppression

As we saw above, a simple 1D proton NMR spectrum is very rich in structural information, providing chemical shifts, coupling information, and integrals. The primary advantage of 1D NMR is that it is faster than 2D methods and often provides enough structural information to answer the question at hand. One consideration when analyzing drug metabolites is that of solvent suppression. Metabolites of interest from *in vitro* incubations and *in vivo* sources usually contain large amounts of solvents that present very large signals in comparison with the metabolite. These can either be protonated residuals from deuterated solvents or carryover from isolation solvents and can, especially in proton NMR, create a significant dynamic range problem as shown in Fig. 12.4. Furthermore, the solvent signals obscure important metabolite signals in the spectrum. These problems can be partially ameliorated by the application of solvent suppression during the data acquisition. There are a wide variety of solvent suppression pulse sequences that are applicable for NMR-based metabolite ID. These have recently been reviewed [27], and so what follows is a brief description of a few relevant approaches. Application of a selective radio frequency pulse exactly at the solvent frequency (or frequencies) during the interpulse delay is effective for eliminating single or a limited number of solvent peaks, while retaining a uniform excitation across

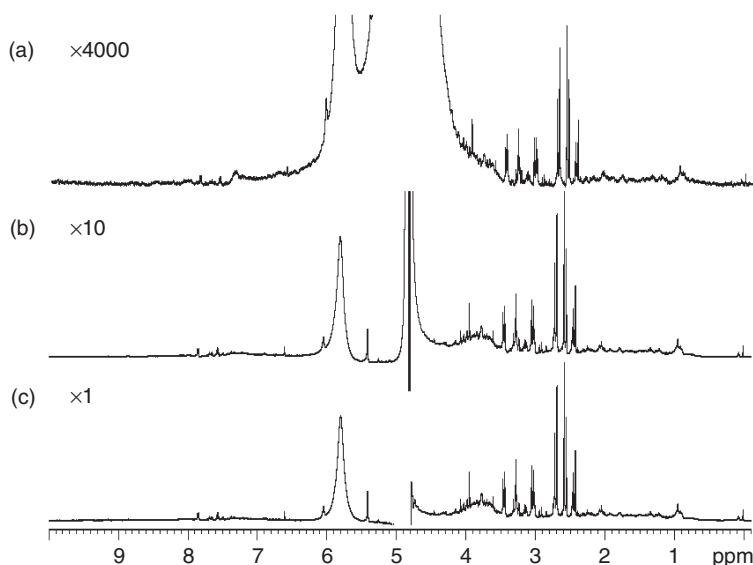


Figure 12.4 Demonstration of the importance of good water peak suppression for ^1H NMR spectra of aqueous solutions. All spectra were recorded at 500 MHz on a sample of dilute rat urine in 20-mM phosphate buffer, pH 7.4. (a) simple single-pulse ^1H NMR spectrum with the vertical scale expanded 4000-fold higher than the height of the water peak. (b) same experiment as top trace with the addition of a selective 100 mW irradiation of the water resonance for 1 s before data acquisition. This serves to equalize the spin population of the water protons so that they do not contribute to the NMR spectrum, and is referred to as *presaturation*. (c) One-dimensional version of the NOESY experiment with 100 ms mixing time and presaturation of the water peak. The 100-ms delay and phase cycling utilized in the pulse sequence cancels out broad components from the intense water signal arising from inhomogeneous parts of the sample and results in better overall water suppression than presaturation alone. *Source*: Reproduced from Ref. 9 with the permission of Informa Medical and Pharmaceutical Science Books.

the entire spectrum. This is simple to implement in almost any NMR experiment, but will also saturate analyte peaks near or coincident with the solvent signal or peaks in rapid chemical exchange with solvent hydrogens (e.g., NH and OH protons exchanging with H_2O). Solvent suppression approaches that exploit the different relaxation properties of solvent and analyte (e.g., WET) [28] destroy the solvent magnetization using pulsed field gradients, and do not suppress exchangeable proton resonances, but may not provide uniform solvent suppression or excitation in all cases. Various other solvent suppression schemes that create excitation nulls at solvent frequencies have also been widely used, but also result in nonuniform excitation. A particularly useful incarnation of this, called *excitation sculpting*, minimizes nonuniform excitation and produces flat baselines [29]. Solvent suppression approaches that exploit the diffusion differences between solvent and analyte have been reported [30,31] and allow observation of analyte signals which are completely buried under solvent peaks, albeit with a significant loss in sensitivity. Because of the nature of drug metabolism work, it is almost universally true that some sort of solvent suppression will be incorporated into an NMR experimental approach.

12.4.3 Multidimensional NMR

In the study of low level isolated or partially isolated drug metabolites, spectral complexity and signal overlap in 1D NMR spectra can prevent extraction of necessary structural information. One approach for improving spectra dispersion is through the use of 2D methods. The pulse sequences that form the basis of such multidimensional NMR approaches not only enable increased spectral dispersion but also can have the added advantage of giving information on relationships between the various resonances in the spectrum, for example, showing which ones are coupled to each other by scalar or dipolar coupling. These methods have been comprehensively described both in general [32] and in the context of drug metabolite studies [8], so the following is a brief description of the concept and the most important 2D methods for drug metabolism researchers.

The general approach taken for 2D NMR is to apply a series of excitation pulses and incremented delay periods to the sample such that there are two independent variable time intervals in the pulse sequence. One of these is the acquisition time, denoted by t_2 and equivalent to the time domain of a 1D spectrum, and the other is related to the incremented delay and denoted by t_1 . If an NMR FID is acquired for a period t_2 for each of a set of t_1 values, the NMR signal intensity (S) will be a function of both t_1 and t_2 giving a time matrix of data $S(t_1, t_2)$. A Fourier transformation with respect to both t_1 and t_2 produces a frequency matrix, $S(\omega_1, \omega_2)$. This is the 2D spectrum and is simply NMR signal intensity as a function of two frequency axes. The delay t_1 and how and what nuclei are manipulated in the pulse sequence will define exactly what the two frequency axes will represent. The most common class of proton 2D NMR experiment provides information on connectivity between nuclei that are scalar or dipolar coupled to each other. Manifestations of this “correlated” type of 2D experiment include those where the two nuclei are identical (homonuclear) and those where they are different (heteronuclear).

The proton homonuclear experiment, termed *COSY* (*correlation spectroscopy*), is used to show which resonances in a proton spectrum are scalar coupled to each other, that is, generally two or three bonds apart. The resulting spectrum has the conventional 1D NMR spectrum along the diagonal and off-diagonal peaks at chemical shifts corresponding to pairs of coupled nuclei. This is exemplified by the contour plot of the 2D COSY spectrum of 3-nitrotyrosine given in Fig. 12.5, where intensities are represented as contours in the 2D map. A related and very powerful experiment, called *TOCSY* (*total correlation spectroscopy*), provides information on unbroken chains of scalar-coupled protons in one 2D NMR spectrum. Information from these two experiments is often used together as the basic structural elucidation tool. The analogous experiment that exploits dipolar rather than scalar coupling is based on the nuclear Overhauser enhancement spectroscopy (NOESY) (and the closely related rotating-frame Overhauser enhancement spectroscopy, ROESY). The NOESY spectrum looks similar to that of the COSY, except that the off-diagonal peaks are at the chemical shifts of protons which have a mutual NOE and are thus close in space (generally $<5 \text{ \AA}$), but not necessarily close to each other in the molecule.

Although, in principle, heteronuclear 2D NMR can be between any dissimilar pair of NMR-active nuclei, in practice, sensitivity dictates that proton is usually one of the pair and the one that is in the detected (t_2) domain. In drug metabolism, the other (undetected or passive) nucleus is usually ^{13}C or less frequently ^{31}P , ^{19}F , or ^{15}N , and

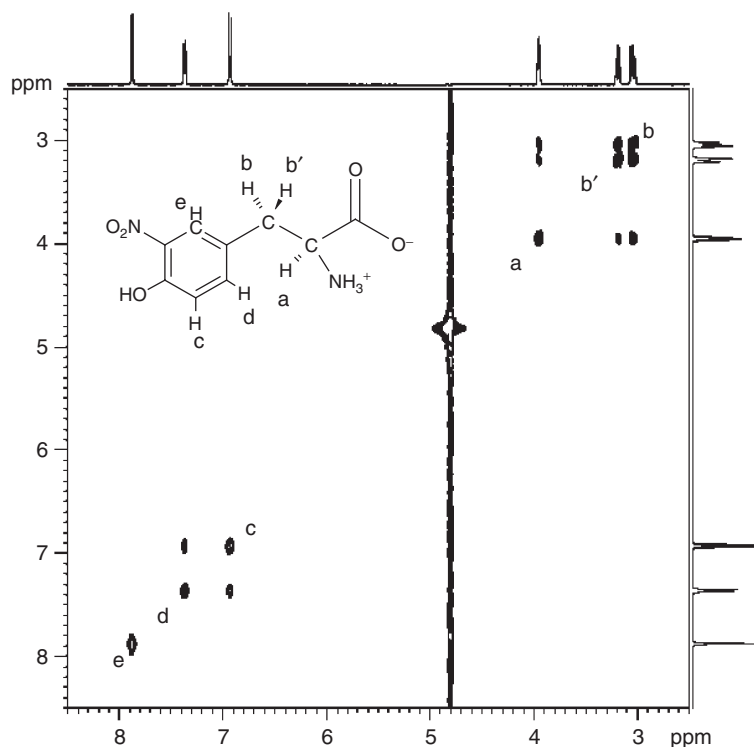


Figure 12.5 Contour plot of the ^1H - ^1H two-dimensional 500-MHz COSY NMR spectrum of 3-nitrotyrosine. Peaks labeled on the diagonal correspond to the one-dimensional proton NMR spectrum and off-diagonal peaks correlate nuclei that are spin-coupled to each other. *Source:* Reproduced from Ref. 9 with the permission of Informa Medical and Pharmaceutical Science Books.

the experiments are divided into one-bond and multiplebond types. The two principal one-bond experiments are heteronuclear multiple quantum coherence (HMQC) [33] and related heteronuclear single quantum coherence (HSQC). These rely on the strong one-bond coupling between the proton and its attached heavy atom or “heteronucleus,” which in the case of ^{13}C is usually $>100\text{ Hz}$. Analogous pulse sequences typified by the heteronuclear multiple bond correlation (HMBC) exist based on long-range ^1H -X couplings, which can then give connectivity information for heteroatoms that are two to five bonds apart [34]. The ^1H - ^{13}C HMQC spectrum of nitrotyrosine is shown in Fig. 12.6. This presentation, unlike the homonuclear correlation experiments, has no diagonal peak and the axes correspond to the appropriate chemical shift ranges of the ^1H and ^{13}C nuclei, with each peak arising from a C-H pair in the molecule.

12.5 HYPHENATED TECHNIQUES

A technological development that has received a lot of attention in the study of drug metabolism is HPLC directly coupled to an NMR spectrometer. In this hyphenated

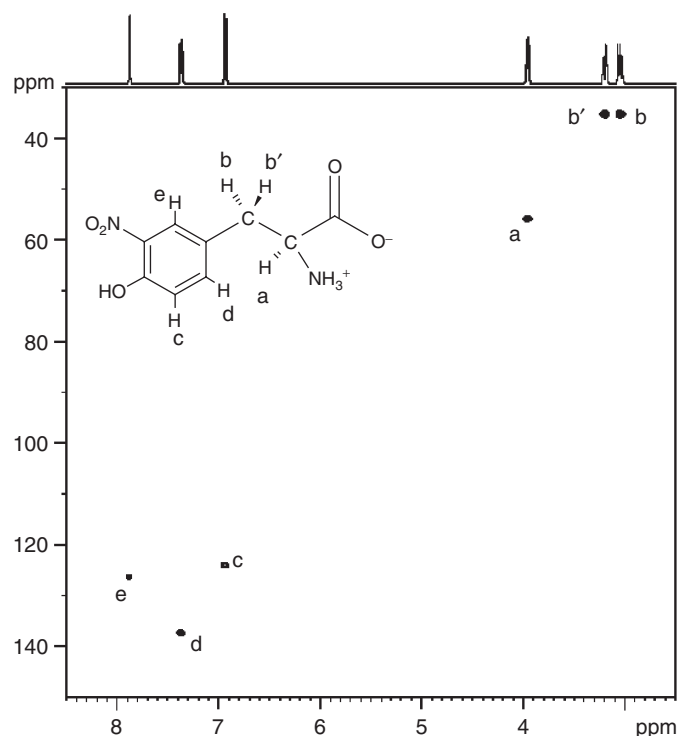


Figure 12.6 Contour plot of the ^1H – ^{13}C two-dimensional HMQC NMR spectrum of 3-nitrotyrosine. Each peak identifies a single C–H pair at the intersection of the ^{13}C (vertical axis) and ^1H (horizontal axis) chemical shifts for all CH and CH_2 groups in the molecule. *Source:* Reproduced from Ref. 9 with the permission of Informa Medical and Pharmaceutical Science Books.

LC-NMR approach, an unprocessed or partially processed sample containing the drug metabolite(s) is injected onto a chromatography system, such as an HPLC, and NMR spectra are recorded as the eluant passes through the spectrometer (on-flow mode) or with a chromatographic peak stopped in the flow cell (stopped flow mode). The analytes need not have a chromophore or ionizable group for mass spectrometric detection, because of the nonselective nature of the NMR spectrometer, nor does separation need to be complete, as the inherent frequency resolution of the NMR spectrum makes it possible to analyze many components in a single spectrum. In the case of on-flow mode, the result is a pseudo-2D NMR experiment, where the ω_1 dimension is replaced with the chromatographic retention time. More commonly, however, stopped flow mode is used, where an eluted chromatographic peak is parked with its apex at the center of the flow cell and a series of extended NMR experiments are conducted. As a result, the most highly concentrated part of the chromatographic peak is measured, which should be the purest, given adequate separation from interfering peaks. On the other hand, as the entire peak is normally not in the flow cell, the hyphenated method is usually not as sensitive as isolate-and-concentrate approaches (see below). Another useful hyphenation adds mass spectrometry to create LC-NMR-MS, an adaptation

that allows mass-directed selection of what peaks one might want to acquire NMR spectra on [35] or simply to provide a more complete analytical data set in one experiment [36].

The use of LC-NMR was first reported in 1978 [37] and based on publication records, interest peaked in about 2003, with a much smaller but steady number of reports appearing annually since. Nonetheless, numerous examples of its utility argue for inclusion of this technology in the modern biotransformation tool kit. While proton-observed LC-NMR is normally utilized for sensitivity reasons, ^1H - and ^{19}F -detected LC-NMR has been used to characterize human immunodeficiency virus (HIV)-1 reverse transcriptase inhibitors and glucuronide metabolites [38,39]. LC-NMR has also been used to effectively study unstable metabolites and those recalcitrant to isolation, such as acyl glucuronides [40,41]. A recent review of LC-NMR methods in drug metabolism [5] compares conventional NMR with the hyphenated approach, highlights numerous impactful examples of the latter, and also critically discusses disadvantages and situations where they would be of less utility (mainly based on lower inherent sensitivity). While hyphenated systems will not replace conventional isolation and NMR analysis, they clearly have a niche in drug metabolite characterization. In particular, LC-NMR is most useful when there is a large amount of the desired analyte on column and the analyte is not stable to isolation. Since most flow cells (30–120 μL) will only hold a fraction of a typical HPLC peak, a great deal more material needs to be injected than might be needed if the peak is isolated conventionally. For example, 10 μg on column might only yield 2–3 μg in the active part of the flow cell. The use of ultra high pressure HPLC could more closely match peak volumes to that of the flow cell, but column loading may be limiting with these systems. The biggest advantage of LC-NMR for metabolite ID is when dealing with reactive metabolites or those that are either unstable over time or decompose on drying. In the absence of a stability issue, it is almost always better to isolate more material and reconstitute it in an NMR-friendly solvent to get the most useful NMR data.

Another noteworthy technique inserts an automated solid-phase extraction (SPE) step, between the HPLC and the NMR [42]. The HPLC-SPE-NMR approach incorporates trapping of HPLC peaks containing metabolites onto small SPE columns, which can then be dried and subsequently eluted with high quality deuterated solvents either directly into a flow cell or into NMR tubes for conventional analysis in a fully automated manner [43]. This approach allows for the accumulation of low abundance metabolites on individual SPE cartridges through repeated HPLC injections as demonstrated for human metabolism of phenacetin [44]. This technique avoids many of the unattractive aspects of direct LC-NMR, including the requirement for large amounts of expensive deuterated solvents and limited analyte mass, while allowing the most efficient use of valuable NMR acquisition time.

12.6 EQUIPMENT CONSIDERATIONS FOR DRUG METABOLISM

Although NMR equipment continues to evolve, the cost setting up a laboratory is quite high, as alluded to earlier. As such, it is highly desirable to fully utilize such equipment, especially in today's cost conscious environment. Indeed, in our laboratories, biotransformation scientists normally do their own HPLC and LC-MS studies and collaborate closely with the analytical NMR group when the additional structural information is

required. This is beneficial in that it allows optimum utilization of expensive capital and ensures that domain experts are employed at what they do best. In such cases, it may be that a nominal investment in certain accessories for an existing high field NMR system avoids the cost of setting up a \$2 million laboratory. The following discussion focuses on the ideal equipment for a drug metabolism NMR system and indicates those accessories that are considered required and those that are nice-to-haves.

12.6.1 The NMR Spectrometer

The core component in any NMR spectrometer is the magnet. This component will typically last upward of 20 years and will survive many cycles of upgrades to other parts of the system. This, combined with the sensitivity and resolution advantages of higher fields mentioned above, dictates the use of the strongest magnet that the budget will allow and ideally no less than 600 MHz. Nearly all systems that can be purchased today are only available with self-shielded magnets which greatly minimize space requirements and reduce the distance from which accessories (e.g., chromatography systems) can be located. The footprint of such a system is dramatically smaller than with a conventional magnet, which will reduce facility costs. The spectrometer should be equipped with a minimum of two excitation channels—one for proton and one for heteronuclei (broadband), both of which should have the capability of producing shaped pulses. Optionally, a system that can observe ^{19}F simultaneously with proton can be used to monitor metabolism of fluorine-containing drugs. The spectrometer should also have a Z-axis gradient amplifier capable of producing a gradient across the sample of at least 30 G/cm (and preferably 60 G/cm) with rapid recovery time (<1 ms), enabling newer solvent suppression techniques and diffusion editing experiments to be employed. Finally, the system should have a sample temperature control unit capable of maintaining a uniform temperature across the sample of $\pm 0.1^\circ\text{C}$ or less.

12.6.2 The Probe

For most drug metabolism studies, the actual amount of sample is limited, and in such cases the smallest volume probe is usually the best [45]. Ideally, the small sample probe would be cryogenically cooled; however, this can be a significant cost factor. For example, at the time of this writing, adding a small volume cryogenic probe to a system already equipped with one will cost about \$100,000, whereas purchasing a complete cryoprobe accessory and all of the associated hardware costs between \$250,000 and \$350,000. Currently, the smallest commercially available tube-type NMR probes are 1.0 and 1.7 mm for room temperature and cryogenically cooled probes, respectively with the latter being three- to fourfold more sensitive. All things considered, if budget is limited and access cannot be had to an NMR system with an existing cryogenic platform, the best bet would be the 1.0 mm room temperature probe. If costs were no object or if an NMR system with the cryogenic platform was being accessorized, the slightly larger (1.7 mm) cryogenically cooled probe would be the best choice and the current optimum for sensitivity. For hyphenated systems, the ideal probe is a small volume flow cell, matched as closely as possible to the typical peak volume of the attached chromatographic system. Commercially available probes that are cryogenically cooled and can operate in flow or tube mode are currently available in 30–120 μL active volumes (flow mode), corresponding to 3–5-mm tube diameters. Regardless of

the choice of probe, it should have a Z-axis gradient capability and be capable of observing proton and exciting ^{13}C , ^{15}N , ^{31}P , etc.), again with simultaneous ^1H and ^{19}F capabilities nice-to-have.

12.7 SAMPLE PREPARATION

It was recognized as early as the 1960s that the resolving power of NMR dramatically lowers the purity requirements for getting useful information [46]. Indeed, early examples of NMR-based drug metabolism studies conducted directly on unprocessed urine from animals dosed with ampicillin [47] and humans with acetaminophen [48] demonstrate the power of NMR to measure drug-related compounds in complex media. Benefiting from very high tissue concentration and no competing background signal, metabolites of 5-fluorouracil have even been measured in live animals using ^{19}F magnetic resonance spectroscopy (MRS) [49]. Applications of this approach for other anticancer drugs using ^{13}C enrichment have been reported (see the review in Ref. 50 and references therein). Such cases will be the exception, however, and will work only when high levels of metabolites are present in the biofluid or tissue, or in cases confirming known metabolites. Microbial metabolism has been used to scale-up metabolites observed in higher species to provide sufficient materials for NMR studies [51]. More typically, application of NMR to drug metabolite identification involves extraction of the metabolites from the tissue, fluid, or incubation medium of interest, purification by some sort of chromatography, liquid–liquid or SPE, then redissolving the dried down isolate in an NMR-suitable solvent thus achieving higher sample concentration and reduced solvent interference. The concentrated sample is placed into an NMR tube and data are collected and interpreted.

Because of the nonselectivity of NMR, sample preparation is the single most important step for the success of NMR-based drug metabolite structure identification. Any left-over trace of the original matrix, be it protein, buffers, or residual solvents can introduce NMR signals that are orders of magnitude greater than the metabolite signals. It is frequently the case that such interferences are NMR specific and not obvious in an HPLC chromatogram or an MS analysis. We have recently introduced methodology that shows advantages in improved sample quality, reduced sample preparation time, and the results in lower sample requirements [52]. This robust methodology was established for rapid structure determination of microgram quantity drug metabolites by using high field NMR equipped with a cryoprobe. In this new approach, drug candidate metabolite formation occurs in a single 10–30 μM , 2–5 mL incubation of microsomes, hepatocytes, or recombinant drug-metabolizing enzymes (typically cytochrome P450s and UDP-glucuronosyl-transferases). Following precipitation of proteins and solvent removal, the reconstituted metabolite-enriched solution is repeatedly injected onto an HPLC/MS system and fractionated. Fractions are collected into a 96-well plate and dried. On the basis of the MS profile, selected fractions are then reconstituted in just enough deuterated solvent to fill the NMR tube, typically 30 μL for a 1.7-mm tube. NMR spectra of these isolated metabolites are then acquired on a 500 or 600 MHz spectrometer equipped with a cryogenic probe. The methodology has been successfully employed as an extension of HPLC/MS/MS-based metabolite identification. When metabolites are present in 0.5–10 μg quantities, 1D ^1H -NMR and homonuclear 2D NMR experiments with satisfactory S/N can be routinely obtained.

12.8 DISCOVERY APPLICATIONS

It is no surprise to individuals currently engaged in the field of pharmaceutical research that performance expectations are increasing along with shrinking timelines. Discovery programs have aggressive and hard deadlines and hence information delivered too late is of little use. This is no less true for drug metabolism studies in early phase programs, where minimizing metabolic liabilities is an intricate part of selecting the right candidate to move forward. This has fostered a heavy reliance on modern LC-MS-based approaches that can quickly analyze metabolites from an *in vitro* incubation and frequently provide enough information to advance the program. Sometimes, however, crucial information, such as exactly which atom in a particular part of the molecule is modified cannot be answered even with advanced MS techniques. Fortunately, the rich information content of a simple 1D ^1H NMR spectrum can often provide sufficient information to remove that ambiguity. There are various examples in the literature where simple one-dimensional ^1H NMR was sufficient to provide the ancillary information needed to yield an unambiguous structure for metabolites under study and thus have programmatic impact [8,53]. This type of “good enough, proceed onward” approach is appropriate when rapid decision making takes precedent over absolute accuracy.

A recent example of the power of the new rapid throughput NMR method described in the previous section is the rigorous analysis of oxidative metabolites and glutathione (GSH) conjugates of the antidepressant and anxiolytic drug, trazodone. In this study, 8 metabolites were quickly characterized from samples generated in single 2 mL incubation of NADPH- and GSH-supplemented human liver microsomes at 30 μM drug substrate concentration [52]. Incubation conditions, post-incubation workup, and sample preparation by HPLC closely resembled a typical biotransformation experiment. The accurate mass, MS/MS, and NMR spectral data characteristics of seven of these metabolites are summarized in Table 12.1, and the aromatic region of the ^1H -NMR spectra for each are shown in Fig. 12.7. Combined analysis of these results allowed elaboration of the metabolic fate of trazodone under these conditions as depicted in Fig. 12.8. Among the metabolites detected, the structure and regiochemistry of four metabolites, Met1a, Met3, Met4, and Met5, were unambiguously characterized using ^1H -NMR, ^1H -COSY, and ROESY techniques on total amounts of individual metabolites ranging from 1 to 10 μg . Regiochemistry assignments for the other three metabolites (Met2, Met6, and Met7) were proposed on the basis of the observed chemical shifts and similarities to other metabolites. A minor metabolite, Met1b, was not structurally defined beyond the gross structure assigned from MS/MS data primarily because of insufficient quantity. It is important to point out that the execution time for this entire workflow was a few consecutive days, during which time sample stability was closely monitored.

In addition to trazodone, a large number of unpublished examples from our laboratories have proven the value that this rapid microgram-level NMR method adds by avoiding time-consuming preparative-scale metabolite generation and purification and circumventing the technical complications associated with online LC-NMR. The turnaround time of metabolite structure determination using the approach described here synchronizes NMR-based structural elucidation with the typical compound optimization cycle times, during which medicinal chemists modify the identified metabolic soft spots while performing other iterative lead optimization activities. Thus, timely

TABLE 12.1 Characterization of Trazodone Metabolites and Glutathione Conjugates in Human Liver Microsomes Based on Integrated Data Analysis of HPLC-MS/MS and NMR

Met#	RT (min) ^a	MH ⁺ <i>m/z</i> (Δ, ppm)	Key MS/MS Fragment Ions (<i>m/z</i>)	Empirical Formula	Estimated Mass (μg)	Key NMR Information	Combined Data Interpretation on Metabolite Structure	Regio- chemistry by NMR
1a	11.7	262.1661 (0.5)	197,176,165,148	C ₁₃ H ₁₉ N ₅ O	1.8	H5, H6, H7, and H8 resonances of TAP moiety	N-dealkylation of CP ring	Yes
1b	11.7	693.2203 (1.9)	675,564,420, 388,176	C ₂₉ H ₃₈ O ₈ N ₈ ClS	—	—	GSH conjugate on CP	No
2	16.5	695.2358 (2.1)	677,659,566, 422,237	C ₂₉ H ₄₀ O ₈ N ₈ ClS	6.6	H5, H6, H7, and H8 on partially reduced TAP moiety from 1D NMR and COSY	7-OH, 8-GSH conjugate on partially reduced TAP	Yes ^b
3	17.2	197.0850 (5.0)	154	C ₁₀ H ₁₄ ClN ₂	9.4	Intact CP ring, but no proton signals due to TAP	1-(3'-CP) piperazine or m-CP	Yes
4	18.2	388.1531 (0.9)	350,251,176, 148,133	C ₁₉ H ₂₂ O ₂ N ₅ Cl	9.9	H18, H21, and H22 on CP ring, H18-H15, and H22-H15 NOEs in ROESY	<i>para</i> -Hydroxyl (20-OH) on CP	Yes

(continued overleaf)

TABLE 12.1 (continued)

Met#	RT (min) ^a	MH ⁺ <i>m/z</i> (Δ, ppm)	Key MS/MS Fragment Ions (<i>m/z</i>)	Empirical Formula	Estimated Mass (μg)	Key NMR Information	Combined Data Interpretation on Metabolite Structure	Regio- chemistry by NMR
5	20.2	406.1634 (1.6)	386,235,210, 192,182,164	C ₁₉ H ₂₄ O ₃ N ₅ Cl	1.0	H5, H6, H7, and H8 assigned to partially reduced TAP from 1D NMR and ¹ H– ¹ H COSY	7-OH, 8-OH dihydrodiol on TAP	Yes
6	21.3	406.1637 (0.7)	386,210,192,182	C ₁₉ H ₂₄ O ₃ N ₅ Cl	0.8	¹ H-NMR similar to Met5	7,8-Dihydrodiol on TAP and a diastereomer of Met5	Yes ^b
7	21.6	388.1530 (1.2)	253,192,176, 148,133	C ₁₉ H ₂₂ O ₂ N ₅ Cl	5.0	¹ H NMR similar to Met4, but H18, H20, and H21 on CP different from that of Met4.	22-OH on CP	Yes ^b

Abbreviations: TAP, triazolopyridinone; CP, chlorophenyl; RT, room temperature.

^aRetention time; trazodone had an HPLC retention time of 22.5 min.

^bProposed.

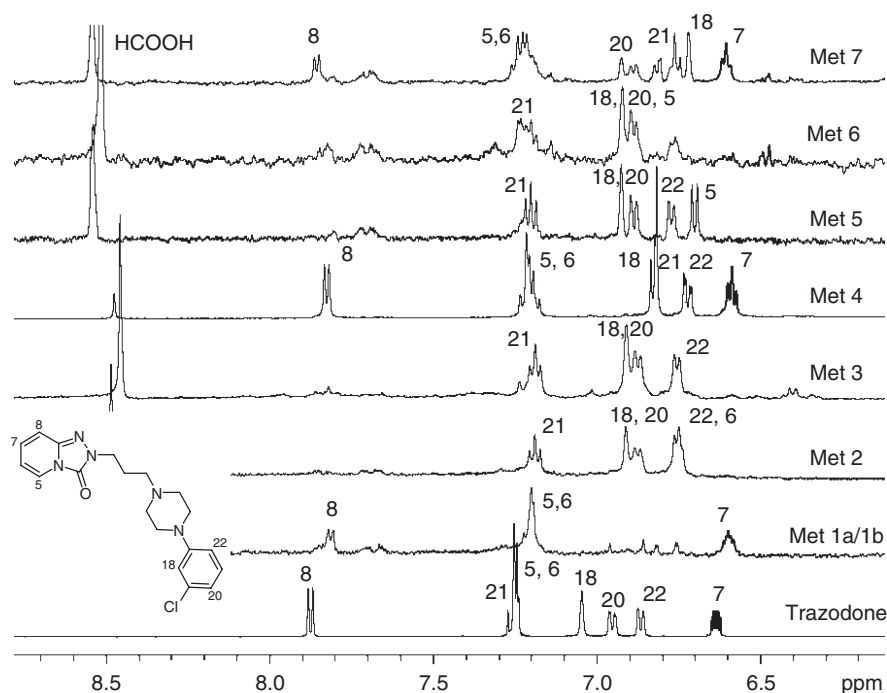


Figure 12.7 Aromatic regions of the 500-MHz ^1H -NMR spectra of aromatic region of trazodone metabolites in human liver microsomes. Assignments are indicated relative to the parent molecule structure.

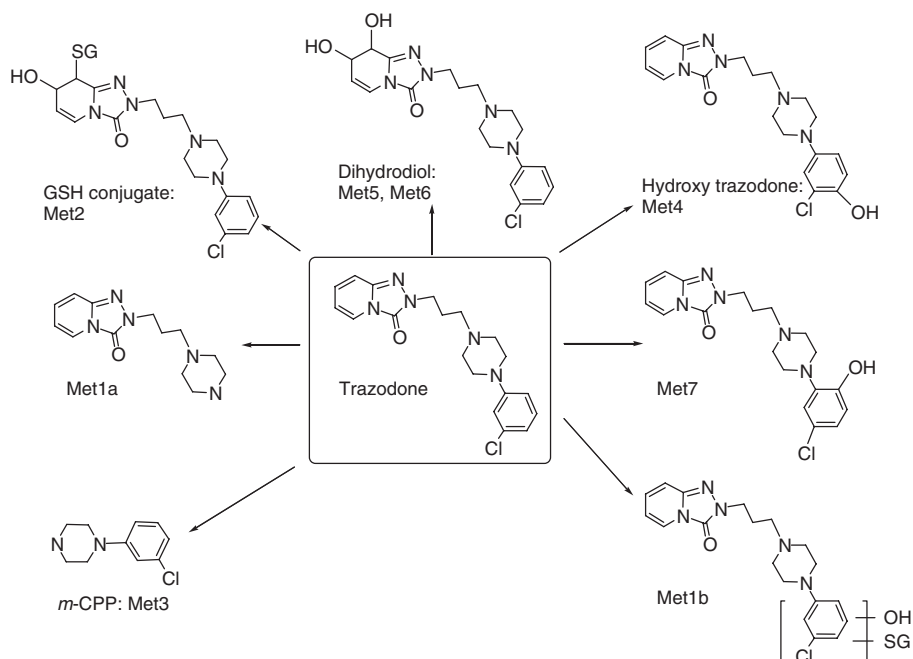


Figure 12.8 Trazodone metabolites in human liver microsomes identified by a combination of rapid, microgram-scale NMR, and LC-MS experiments.

and rigorous structural characterization of drug metabolites can still have a maximal impact on the drug discovery process.

12.9 DEVELOPMENT APPLICATIONS

The characterization of a drug's metabolites continues to play a critical role in the development process. As a drug proceeds from discovery into development, the emphasis shifts from speed to completeness and absolute accuracy takes precedent over expediency. As such, considerably more time and resources are applied to exhaustively characterize the metabolic products of a drug and greater efforts are put into isolating sufficient quantities of important metabolites for full structural characterization. Major drug metabolism-related tasks in development involve metabolite profiling, determination of metabolite exposures, and assessment of metabolic clearance pathways in humans and toxicological species [54,55] for gaining an understanding of the metabolites' contributions to on-target pharmacology and off-target receptor-mediated toxicology. As such, metabolite identification studies remain an integral part of these preclinical and clinical drug development activities. The recently issued guidance on Safety Testing of Drug Metabolites, also commonly referred to as *Metabolite in Safety Testing (MIST)* provides a framework for completion of the metabolite characterization goal [56,57]. The guidance provides recommendations on when and how to identify, characterize, and evaluate the safety of unique human metabolites and major circulating metabolites that are present in disproportionate levels in humans as compared to preclinical species used in toxicology studies. The industry and regulatory focus on these activities underscores the importance of detecting and characterizing drug metabolites despite the often complex metabolic profiles, multiple enzymatic pathways, and generation of numerous metabolites of varying concentrations from the parent drug compounds.

Frequently, metabolite characterization at the drug development stage will involve the application of two-dimensional NMR experiments that provide the needed information for unambiguous structure determination [58]. A recent example illustrates the unambiguous structural elucidation of major metabolites from medroxyprogesterone acetate using 2D NMR [59]. According to the authors, this work circumvented the need to chemically synthesize authentic compounds to confirm the metabolism. The sensitivity of modern NMR equipment makes routine acquisition of 2D NMR on low microgram quantities possible, allowing NMR to become a mainstream analytical tool for metabolite ID in drug development. This is exemplified in recent work from our laboratory, where two major circulating metabolites in dog (M21 and M22) of BMS-275183, a taxane analog being evaluated for its potential anticancer properties, were characterized from samples containing $\sim 74 \mu\text{M}$ ($\sim 10 \mu\text{g}$) of each metabolite [60]. Proton NMR spectra, acquired on a 700 MHz cryoprobe system, revealed impurities in the sample that prevented definitive structure characterization from the ^1H spectrum alone. Various 2D-NMR experiments, including homonuclear COSY and heteronuclear ^1H - ^{13}C HSQC and HMBC data provided complete proton and carbon chemical shift assignments of M22, and all but four-carbon assignments of M21. Together with key HMBC H-C correlations (Fig. 12.9), these provided definitive metabolite structure information. These experiments took a total of about 72 h to complete with a cryoprobe, without which this analysis would have been time-prohibitive. Prakash *et al.*

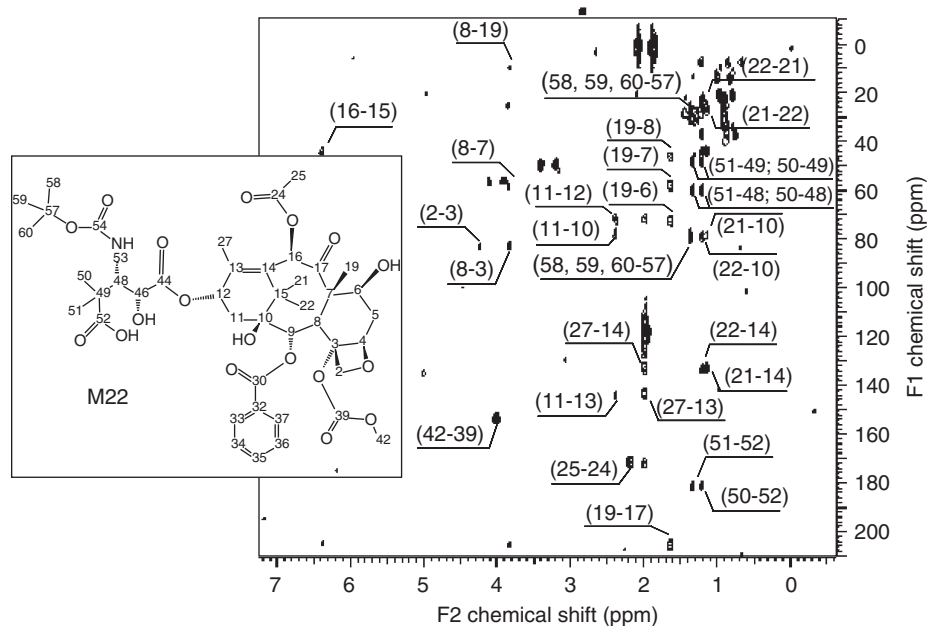


Figure 12.9 Region of the ^1H – ^{13}C HMBC 2D NMR spectrum of a 74 μM sample of M22 of BMS-275183 at 700 MHz. Crosspeaks are labeled with atom numbering indicating the H–C correlations that they correspond to in the structure.

[61] have recently used LC-NMR, 1D, and heteronuclear 2D NMR to provide critical structural information for the definitive identification of a novel quinoline metabolite (M4) and a sulfite conjugate (M28) of Torcetrapib and two unusual metabolites resulting from C-demethylation of CP-533,536 [62]. Multidimensional NMR was critical in differentiating N- and O-glucuronidation of Maxipost and elaborating the mechanism of N-glucuronidation in support of clinical trials [63].

The unique molecular/spectroscopic fingerprints provided by NMR allow discrimination of complex mixtures of geometric and regioisomers, decomposition products, and of impurities unrelated to the drug product, all within a single sample. This, in turn, places NMR in a unique and favorable position to be useful even in the case where high purity and isolation cannot be achieved chromatographically or isolated samples decompose over time. As previously noted, characterization of multiple metabolites in a single sample is feasible. While isolation of individual metabolites is usually the goal in development studies, the resolving power of NMR has been applied to the characterization of mixtures of coeluting metabolites. Analysis of chromatographic isolates of human urine by NMR spectroscopy was used to demonstrate that in human, 1-hydroxytacrine coeluted with 3-hydroxytacrine. In preclinical metabolism studies with tacrine, the 3-hydroxy metabolite was not observed and thus NMR provided the first clues to a novel human metabolite [64]. More recently, coeluting regioisomeric glucuronides of BMS-690514 [65] have been identified. A peak containing the M8 metabolite of BMS-690514 was isolated from rat bile after administration of [^{14}C]BMS-690514. Positive mode electrospray ionization (ESI) LC-MS/MS analysis indicated that M8 had an m/z at 561 (192 Da higher than BMS-690514), suggesting it was a glucuronide

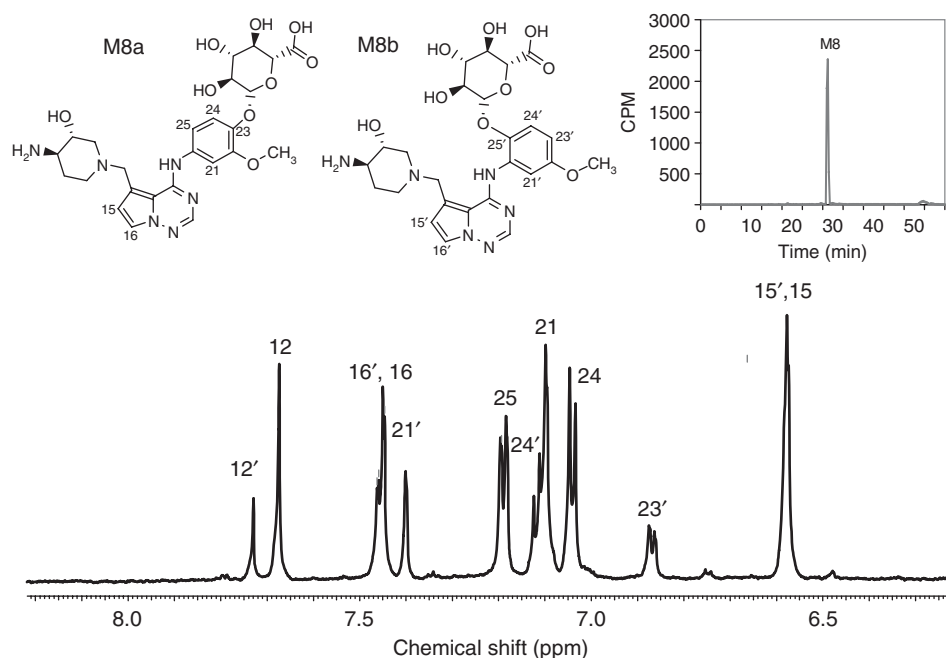


Figure 12.10 The frequency resolving power of NMR permits structure ID on two components in a mixture. The radiochromatogram of purified M8 of BMS-960514 (top right) shows the presence of a single peak and proton spectra with assignments of this isolated M8 peak (bottom), showing that it consists of a mixture of M8a and M8b metabolites.

conjugate of monohydroxylated BMS-690514. NMR analysis revealed that the isolated M8 peak contained a major (M8a) and a minor (M8b) metabolite at a 3:1 ratio. Both metabolites were successfully characterized within the mixture using 1D and 2D NMR methods. For M8a, glucuronidation occurred at the hydroxyl in position 23. For M8b, glucuronidation occurred at the hydroxyl group on position 25' (Fig. 12.10). In both these examples, mass spectrometric evaluation was not sufficient to reveal the presence of multiple metabolites in single chromatographic peaks.

Isotope labeling techniques have assisted in the study of drug metabolism, distribution, excretion, and the identification of metabolites from drugs of interest [19]. A number of these labels can be measured directly or indirectly by NMR. For example, the replacement of a proton within a drug molecule by deuterium (^2H) or tritium (^3H) can be assessed by acquiring a ^1H spectrum and observing a loss of signal of the resonance containing the label. Similarly, a deuterium or tritium NMR spectrum of the sample can be acquired in cases where one of those hydrogen isotopes is incorporated into the molecule. This presents unique opportunities for quantitating how much of the label has been incorporated, as well as following the metabolic fate of the label. Simultaneously, this makes NMR a powerful quality control and mechanistic tool in isotope-assisted metabolism studies. The formation of a metabolite from BMS-690514, an oral oncologic agent, is a great example of the utility of isotope labeling and NMR. BMS-690514 contains a pyrrolotriazine group subject to oxidation at carbon 16, Fig. 12.11, resulting in the formation of metabolite M1, a primary hydroxylated

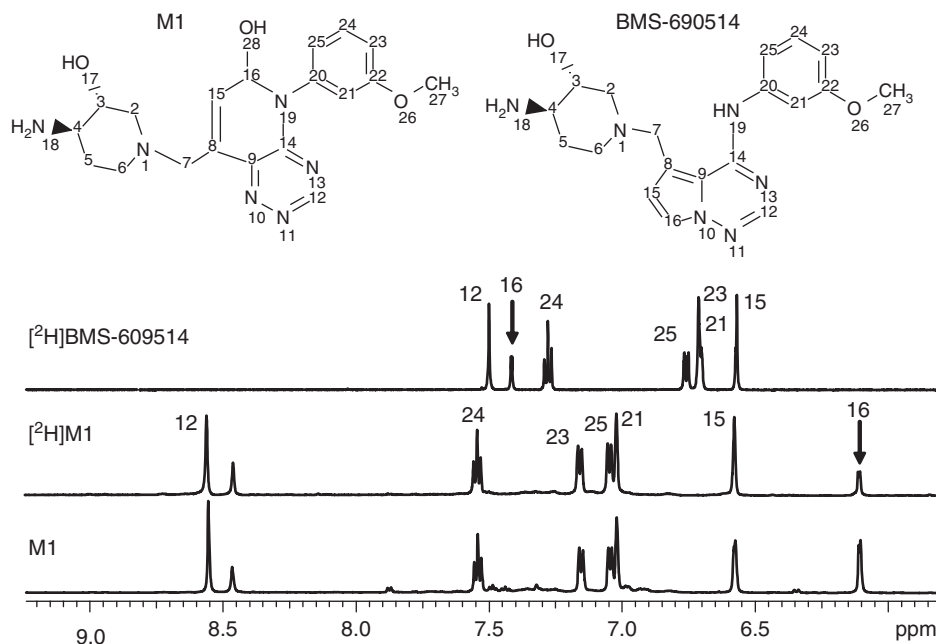


Figure 12.11 ^1H NMR spectrum of M1 (bottom), deuterated M1 at position 16 (middle), and deuterated parent at position 16 (top). The reduced intensity of the 16 proton in parent and metabolite indicates the amount of deuterium incorporation and the fact that this level is preserved during biotransformation.

metabolite identified *in vivo*. NMR data indicated that M1 underwent a structural rearrangement. To gain insight into this rearrangement $[16\text{-}^2\text{H}]\text{BMS-690514}$ was prepared and shown to contain the deuterium on the expected position 16 (Fig. 12.11). Integration of H16 in the ^1H NMR spectrum of $[16\text{-}^2\text{H}]\text{BMS-690514}$ revealed that approximately 40% was protonated, and therefore the percent of deuterium label incorporation at this position was $\sim 60\%$. Subsequently, M1 generated from human liver microsomes (HLMs) incubations with $[16\text{-}^2\text{H}]\text{BMS-690514}$ was characterized by NMR. Integration of H16 in the ^1H NMR spectrum of $[\text{2H}]\text{M1}$ indicated that the deuterium label was quantitatively preserved at position 16 (Fig. 12.11), significantly limiting the number of possible mechanisms for the formation of this metabolite [73].

Finally, the quantitative aspects of NMR described above make it a potentially valuable tool for use in compliance with MIST guidance. In this capacity, quantitative NMR measurements of biologically generated drug metabolites can subsequently be used as reference standards for quantitative work using LC/MS [6]. In this approach, ^1H NMR is applied as the method of choice, as it is both a qualitative and a quantitative tool, allowing structure determination, purity check, and quantitative measurement of the isolated metabolites. Another approach is to assimilate data from NMR spectroscopy and liquid chromatography-ultraviolet-mass spectrometry (LC-UV-MS) with plasma pooling methods to generate reliable area under the curve (AUC) values for metabolites present in the plasma of preclinical species, followed by the translation of this information to quickly assess their relationship to human-produced metabolites as dictated by the MIST guidance [66].

12.10 STEREOCHEMICAL CONSIDERATIONS

In the quest for novelty and optimum potency, chirality is frequently engineered into drug molecules. Metabolism can confound this already complex situation by introducing additional stereochemical modification. In these cases, absolute stereochemistry determinations become an essential part of characterizing the metabolite. Of course, X-ray crystallography and vibrational circular dichroism (VCD) have been used to solve such problems [67,68], but are not routinely useful for metabolites because they require many milligrams of material. NMR cannot directly distinguish enantiomeric compounds, but NMR-based methods for the determination of absolute stereochemistry have been developed, which rely on interactions between the molecule under study and various chiral reagents to form *in situ* diastereomers that *can* be differentiated (for reviews, see Refs 69 and 70). This can be achieved through covalent chemical derivatization, for example, formation of Mosher esters [71] or through transient interactions with chiral solvents or solutes [70]. The success of these methods lies in the ability to correctly predict chemical shift or coupling constant differences that arise from these interactions or chemical modifications. A covalent derivatization approach using nonchiral reagents was recently used to assign the stereochemistry of a major circulating hydroxylated metabolite of a corticotropin releasing-factor receptor antagonist [72]. The metabolite's stereochemistry was revealed by extrapolating the NOE-derived conformation of its cyclic oxazolidine derivative back to the underivatized metabolite. While not a panacea for absolute stereochemical determination, with the limited number of tools available, the NMR approaches described above can be brought to bear on important stereochemical problems that crop up in drug metabolism.

12.11 CONCLUSIONS

NMR spectroscopy has been and will continue to be a significant part of the drug metabolite structure elucidation toolbox. The type of information it provides exquisitely complements that obtained from the mainline analytical tool used for drug metabolism structure work, LC-MS. Frequently, it makes the difference between drawing a box around a large part of the molecule to indicate a region of metabolism and identifying the exact atom which is modified, and this information is more often than not crucial for guiding chemistry efforts to mitigate metabolically labile "soft spots" in a molecule. The qualitative and quantitative information obtainable from NMR provides a unique advantage when attempting to unravel extensive and complex metabolism. By optimizing isolation, sample preparation, and NMR parameters, it is possible to generate useful NMR data coincident with need to meet the demands of drug discovery and development. Additionally, NMR can deliver information on absolute metabolite levels and the fate of incorporated stable isotopes and reveal new metabolites through mixture analysis, which is difficult or impossible to accomplish with any other technique. Constantly pushing against sensitivity limitations, technology developments have led to the current state of NMR's ability to obtain interpretable 1D ^1H -NMR spectra of drug metabolites in a time and cost-effective manner with submicrogram quantities of material. When 2D NMR experiments are needed, sample requirements increase to $\sim 5\text{--}10\mu\text{g}$ depending on the complexity of the structure (e.g., conjugates) and NMR properties of the sample. Continued enhancements to sensitivity through

foreseeable future developments in probe technology and perhaps less tractable access to higher magnetic fields will further lower the sample requirements and make NMR spectroscopy even more useful.

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13 Whole-Body Autoradiography and Microautoradiography in Drug Discovery and Development

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13.1	Summary	1
13.2	Introduction to tissue distribution assays	2
13.3	Organ dissection, homogenization, and liquid scintillation assays	4
13.4	Whole-body autoradiography	6
13.5	Microautoradiography	23
13.6	Conclusions	28
	References	29
	Further Reading	34

13.1 SUMMARY

Tissue distribution studies, which are part of a suite of experiments known as *absorption, distribution, metabolism, and excretion (ADME)* studies, are performed to understand the disposition of xenobiotics and biotherapeutic drugs after administration to animals and humans. This chapter describes the following three approaches used to study the tissue distribution of chemical and biological compounds in laboratory animals: (i) organ collection, homogenization/combustion, and liquid scintillation counting (LSC) analysis; (ii) whole-body autoradiography (WBA), which includes quantitative whole-body autoradiography (QWBA); and (iii) microautoradiography (MARG), which qualitatively resolves the localization of compounds to the cellular level in a histological preparation. LSC, WBA, QWBA, and MARG studies require the use of a radiolabeled chemical or biological test compound, which provides for the tracking and quantification of both the parent molecule and potential metabolites that might be formed. A radiolabeled test drug is administered to laboratory animals, which are later euthanized at different time points. Samples of the animal's entire body, organs, tissues, and excreta are then analyzed to determine the concentrations of the test-drug-derived radioactivity in the various biological matrices. Tissue concentration versus time profiles can then be constructed to provide data for organ- or tissue-specific pharmacokinetic (PK) compartmental analysis. The animal tissue distribution data are then

used to help evaluate pharmacology and toxicology findings and to predict human exposure to drugs and metabolites.

Dissection and LSC techniques were the prevalent methods for evaluating drug distribution up until about 1995, when QWBA began to be used. This technique entails the removal of organs followed by further processing and analysis of solubilized organ homogenates and/or the combustion of the organ and/or homogenates. The resulting drug-derived radioactivity is then quantified using LSC to determine the radioactivity concentration in the organ.

QWBA is an imaging technique that provides both spatial and quantitative radioactivity concentrations in tissues of intact laboratory animals. The main advantages are minimal sample processing/alteration and provision of true tissue concentrations. This technique begins when each animal is euthanized at their respective time point post dose, and the carcass is quickly frozen. The frozen carcass is then embedded in a frozen block of supporting media and cryosectioned to obtain representative *in situ* samples of all organs, tissues, biological fluids, ingesta, and excreta. Whole-body sections are then dehydrated, exposed to phosphor imaging plates along with radioactive image calibration standards, and the resulting digital images are analyzed to determine tissue concentrations and to show detailed spatial distribution patterns. Sven Ullberg pioneered the WBA technique in 1954, and the technique was made quantifiable by Luckey in 1975 when he developed phosphor imaging technology or radioluminography. QWBA limitations include (i) tissue concentrations determined by QWBA (or LSC) can include parent drug, plus its metabolites, and/or degradation products and (ii) it is difficult to evaluate short-lived isotopes.

MARG provides pharmaceutical scientists with a high resolution tool to investigate spatial localization of radiolabeled drugs at a tissue and cellular levels. The basic procedure requires dosing of animals with a radiolabeled substance, followed by the removal and freezing of organs/tissues, cryosectioning and section mounting, and exposure to nuclear photographic emulsion. The tissue sections are then developed, stained, and evaluated microscopically to evaluate distribution at the cellular level. There are several examples in the literature of investigators attempting to obtain quantitative data from MARG preparations; however, MARG is prone to many artifacts and calibration standards are not applied, which makes true quantitation problematic. This chapter discusses the history, advantages, limitations, and examples of the application of each technique.

13.2 INTRODUCTION TO TISSUE DISTRIBUTION ASSAYS

Tissue distribution studies, which are part of a suite of experiments known as *absorption, distribution, metabolism, and excretion (ADME)* studies, are performed to understand the disposition of xenobiotics and biological drugs after administration to animals and humans. The goal of these studies is to determine how the body absorbs drugs and then how the drug is distributed throughout the body, metabolized, and then eliminated from the body. Regulatory agencies around the world require that this information be determined for the registration of new drugs. This chapter focuses on three approaches used to study the tissue distribution of chemical and biological compounds (small and large molecular weight entities) in laboratory animals and how they are used in preclinical drug research. The three approaches discussed are (i) organ collection,

homogenization/combustion, and LSC analysis, which is commonly referred to as the *cut and count* procedure; (ii) WBA, which includes QWBA; and (iii) MARG, which qualitatively resolves the localization of compounds to the cellular level in histological preparations.

LSC, WBA, QWBA, and MARG studies require the use of a radiolabeled chemical or biological test compound. Small molecular entities (chemical compounds that have molecular weights of <600) Dalton and large molecules (e.g., peptides, proteins, enzymes, oligonucleotides) need to be radiolabeled with an appropriate isotope [most often carbon-14 (^{14}C), hydrogen-3 or tritium (^3H), sulfur-35 (^{35}S), or iodine-125 (^{125}I)] so that the compound can be accurately imaged/identified, localized, and quantified in various tissues, fluids, and excreta. The radiolabeled test drug is synthesized in a radiochemistry laboratory, and the position of the radiolabel on/in the molecule is usually known for small molecules (but may not be for large molecules). The radiolabel provides a method for tracking both the parent molecule and metabolites that retain the labeled portion of the molecule. The radiolabel also enables the specific identification and quantification of metabolites that contain the radiolabel, using radio-high performance liquid chromatography (HPLC) techniques and mass spectroscopy.

In general, small organic molecules are labeled with ^{14}C , ^{35}S , or ^3H [1], and larger molecules, such as proteins and peptides, are commonly labeled using ^{125}I (and sometimes ^{35}S). ^3H is often used in drug discovery because it can usually be done relatively quickly, and it is easier to label compounds with ^3H than with ^{14}C . However, it is important to verify the stability of the ^3H label since ^3H -labeled xenobiotics are known to sometimes undergo hydrogen exchange with water [2]. The extent of *in vivo* stability can be monitored by determining the concentration of radioactivity in fresh (wet) and evaporated samples of plasma and/or urine obtained from the animals to be used for the study. The radioactive counts obtained from the wet and dried samples may be compared to see if there is a difference. A similar situation exists for large molecule peptide and protein drugs that are radiolabeled with ^{125}I . In this case, the protein-bound ^{125}I often becomes dissociated from the test article *in vivo*, and this must be characterized to interpret tissue concentration data. This is discussed further in Section 14.4.2.10.

The study designs for examining tissue distribution are varied, but a few basic designs are currently being used in the pharmaceutical industry. In the most basic design, a radiolabeled test drug is administered via a relevant dose route [e.g., orally, intravenously (IV), topically, inhalation, ocular] to laboratory animals. At various time points after dosing, subjects are euthanized, and samples of the animal's organs, tissues, and excreta are then analyzed to determine the concentrations of the test-drug-derived radioactivity [e.g., organs, tissues, plasma, bile, intestinal contents, urine, feces, cerebrospinal fluid (CSF)]. Tissue concentration versus time profiles can then be constructed to provide data for tissue-specific PK compartmental analysis.

Drug tissue distribution studies are routinely conducted in rodents, rabbits, canines, and nonhuman primates. The choice of the animal test model depends on the purpose of the study. In most cases, the animal model will mimic the expected PK in humans, but it may answer specific questions (e.g., toxicology, pharmacology, oncology, genetic modifications). The number of time points used and the overall duration of the study are also important variables to be considered. Time points should be designed to obtain useful and reliable tissue concentration–time profile data. The following tissue PK parameters can then be determined: maximal drug concentration reached (C_{max}); time

of maximal concentration ($T_{\max}$); area under the time–concentration curve (AUC), which describes tissue exposure to a drug; and terminal elimination half-life ($T_{1/2}$), which describes how long it takes for a tissue to eliminate a drug. The choice of time points to be used for the study will determine how reliable the PK data will be, and it requires at least 6, but 10 usually provide enough to enable the determination of reliable PK parameters for many tissues. To obtain reliable tissue PK parameters, there must be enough time points to capture the $T_{\max}$ and establish a reliable $T_{1/2}$. Statistically speaking, the portion of the time–concentration curve used to determine the $T_{1/2}$ for each tissue must have an r^2 value of 0.8 or higher [3], and there should be at least 3 or more time points in that part of the curve. If reliable animal tissue PK parameters are not obtained using enough time points then predictions of human tissue PK exposure must be treated with caution.

Tissue distribution study designs have changed over the years in response to new technology and ethical animal treatment concerns. Most notable has been the change from using three animals per time point and six to eight time points to using one animal per time point and more time points. Before the implementation of QWBA, when organ homogenization and LSC was used to examine tissue distribution, studies were designed to use three animals per time point and six to eight time points because of high variability observed in the organ concentrations. This variability was presumably due to inconsistent effects of organ exsanguination, cross-contamination of organs by body fluids, and inconsistencies associated with organ collection, homogenization, and further sample processing (sample combustion and/or solubilization and LSC detection efficiency). However, as QWBA techniques began replacing the LSC method, many investigators found relatively lower variability in tissue concentrations among animals at the same time point [4]. This was believed to be due to analysis of intact animal carcasses that were snap frozen on termination, thus preserving a “near-*in vivo*” snapshot of drug distribution over time. This also eliminated the variable effects of exsanguination, organ homogenization/processing, and/or the possibility of organ cross-contamination during organ removal and sample handling. Early investigators who developed QWBA techniques also recognized that the use of more time points resulted in more reliable tissue PK parameters [5]. In this way, QWBA has provided the ability to produce the highest quality, and true, “tissue” concentration data (as opposed to organ homogenate data), as well as the most conservative and ethical use of laboratory animals. Nevertheless, organ dissection/homogenization and LSC are still used today by some pharmaceutical companies that have not yet realized the benefits of and/or accepted QWBA as the method for conducting drug distribution studies. To those ends, this chapter describes the techniques of “cut and count” LSC organ analysis and autoradiography (ARG) tissue analysis and how they are used in drug discovery and development.

13.3 ORGAN DISSECTION, HOMOGENIZATION, AND LIQUID SCINTILLATION ASSAYS

Until the early 1990s, organ dissection, homogenization, and LSC techniques were the prevalent methods for evaluating drug distribution. The technique [6] begins with the process of euthanasia. Typically, animals are deeply anesthetized, and blood is

removed via cardiac puncture, followed immediately by the dissection of the animal, where organs of interest are removed, trimmed, rinsed, weighed, and samples are obtained for homogenization, solubilization, and/or oxidation. Investigators should be aware that exsanguination effects many physiological processes [7] that can alter drug distribution, and so tissue concentration data obtained from tissues removed from a carcass need to keep that in mind when interpreting data obtained in this manner. Technicians performing the necropsy are normally required to wash their instruments between individual organ collection to reduce the possibility of cross-contamination of organs during removal. Technicians must assure that all connective tissue, fat, and other fluids are consistently removed from each organ. The number of organs removed can vary considerably, but normally, ~25–30 organs are removed, which include adipose (abdominal and brown) tissue, adrenal gland, blood, bone, bone marrow, brain, cecum (and contents), epididymis, esophagus, eyeball, Harderian gland, kidney, large intestine (and contents), liver, lung, lymph nodes, myocardium, ovary, pancreas, pituitary gland, prostate, salivary gland, seminal vesicles, skeletal muscle, skin (nonpigmented and pigmented), small intestine (and contents), spinal cord, spleen, stomach (and contents), testis, thymus, thyroid, urinary bladder (and contents), and uterus. Organs are then prepared for homogenization and/or combustion (or oxidation). Organs with a heterogeneous tissue composition, such as bone/bone marrow, pituitary gland, spleen, liver, kidney, brain, and muscle, are usually homogenized using a probe homogenizer and/or manual mincing/crushing before sample aliquots are obtained for drying and combustion. Small organs (usually weighing <1 g) such as adrenal glands, pituitary gland, and small samples of tissues for combustion may be placed directly on combustion cones. All samples are allowed to dry to improve complete and uniform combustion. Often triplicate aliquots (0.100 g) of the homogenized sample or an entire organ will be analyzed to monitor/control variability. Each sample aliquot for analysis must be weighed before combustion in the sample oxidizer to the determination of the concentration of drug-derived radioactivity in the sample. Each sample is individually placed into a combustion instrument and then burned completely. The liberated $^{14}\text{CO}_2$ or ^3H gas is then trapped in a solution, and the radioactivity is determined by LSC. Blank samples that contain a known amount of radioactivity are often oxidized and compared to noncombusted standards to determine the percentage of recovery and efficiency of the combustion process, although it must be realized that each tissue may have different oxidation efficiencies and this is not usually determined due to the significant amount of extra work involved. Nevertheless, the efficiency of sample oxidizers must be verified before and during sample oxidation because the performance of these instruments can vary as samples are being processed. Furthermore, the potential exists for carry-over of radioactivity from one sample to another, which, of course, could alter the concentration data. Most sample oxidizers require loading and unloading and careful monitoring by a technician.

An alternative to sample combustion is sample solubilization, where organ homogenates and/or entire small organs are solubilized using a strong caustic reagent. Aliquots of the solubilized sample are then bleached to remove any color that can impede the efficiency of the LSC instrument, and this is typically done by adding peroxide to the sample before scintillation fluid is added and the samples are counted by LSC.

LSC of the resulting samples relies on the performance of the scintillation analyzer. The LSC analyzer detects photons of light that are produced as the radioactive β

particles emitted from decaying isotopes, such as ^{14}C or ^3H , excite the scintillant. The photons emitted are detected by photomultipliers and the signal is translated into a “count” of the radioactivity. The radioactivity [i.e., counts per minute (cpm)] in each sample is then converted to disintegrations of radioactivity per minute (dpm) by means of an external standardization and a quench curve that are part of LSC analyzer protocols. Quenched standards are typically used to calibrate the liquid scintillation counter on each day of analysis, and samples are typically counted for at least 5–10 min each.

The LSC data (dpm) is then used to determine the concentration of drug-derived radioactivity in the sample by dividing the decay per minute by the weight of the sample, which if diluted during homogenization, must be corrected using a dilution factor. It is important for researchers to recognize that the concentration data obtained this way is from organ homogenates and is not true tissue concentration. All organs are composed of several different tissue types, and each tissue of an organ can have vastly different concentrations of the test drug. The ability to determine organ concentration enables researchers to determine the percentage of the radioactive dose that has been taken up in an organ because the total organ weight was obtained at necropsy. This percentage of radioactive dose per organ per time point is usually determined and reported.

In conclusion, exsanguination, organ dissection, homogenization, oxidation or solubilization, and LSC techniques offer pharmaceutical researchers a method to determine organ concentrations; however, true tissue level concentration determinations cannot be obtained. Nevertheless, LSC techniques have been a valuable tool to study the ADME properties of drugs, and it will undoubtedly be used in the future as an alternative method to WBA.

13.4 WHOLE-BODY AUTORADIOGRAPHY

Today, most tissue distribution studies used to support new drug registrations are performed using QWBA techniques [8], which is the imaging and quantitation of drug-derived radioactivity concentrations in tissues of preclinical species. QWBA has numerous advantages over “cut and count” LSC analyses. The key advantages are tissue, rather than organ, concentrations are obtained; there is no cross-contamination from tissues; and the variability due to exsanguination is eliminated. As previously mentioned, QWBA typically uses one animal per time point, and each animal is euthanized and then the carcass is rapidly snap frozen typically in a dry ice/hexane bath (Fig. 13.1a). This process eliminates redistribution of compounds post death. A small blood sample is normally obtained before freezing and can be collected via cardiac puncture or alternative methods. Plasma may then be prepared from a portion of the blood sample. The frozen carcass is then embedded in a frozen block of supporting media (Fig. 13.1b) and precision cryosectioned (Fig. 13.1c) in a sagittal orientation at 40 μm thickness using a special macrotome to obtain representative *in situ* samples of all organs, tissues, biological fluids, ingesta, and excreta. Approximately 5–10 whole-body sections at various depths through the animal are necessary to collect the typical set of 40–50 tissues analyzed by this method. These samples, collected on adhesive tape, are then dehydrated (approximately two days in a cryomacrotome or more rapidly using a lyophilizer). Phosphor imaging has replaced traditional film ARG

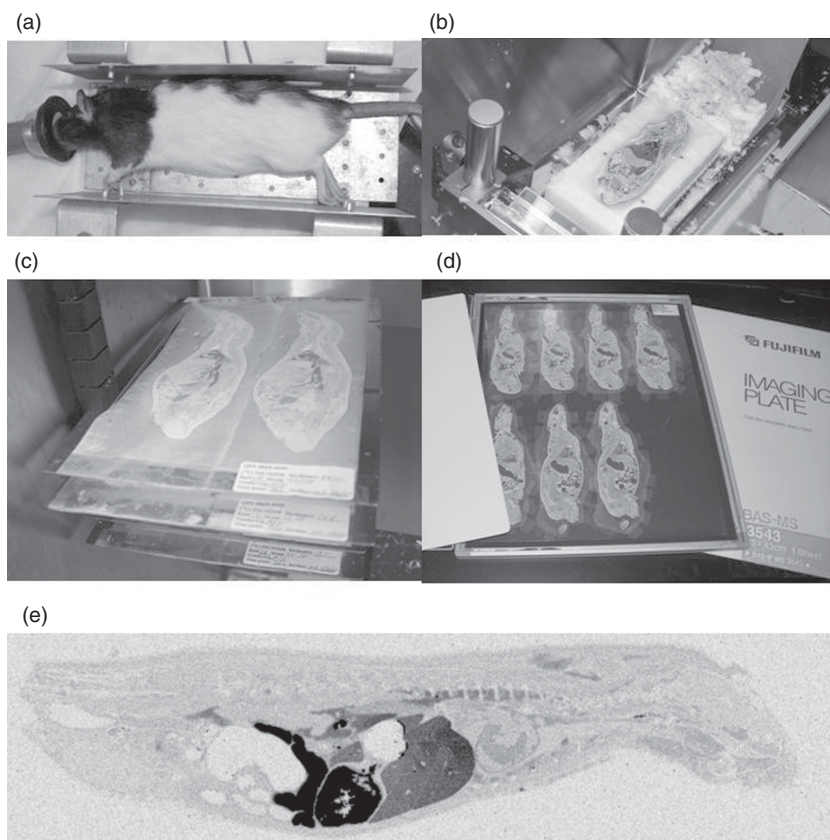


Figure 13.1 Whole-body autoradiography methods. (a) The carcass is frozen in a hexane-dry ice bath, (b) whole-body sections are dehydrated, (c) sections are exposed to phosphor imaging plates along with radioactive image calibration standards, (d) the imaging plates are scanned by a phosphor imaging plate scanner, and (e) grayscale digital images of the radioactivity in the sections are obtained for image analysis to determine tissue concentrations. (See color insert.)

because of its larger linear range and shorter exposure times. Samples are opposed to phosphor imaging plates along with radioactive calibration standards (Fig. 13.1d). After an appropriate exposure period (1–14 days, depending on the isotope and/or amount of radioactivity present in the sample), the imaging plates are scanned by a phosphor imaging plate scanner, and grayscale digital images of the radioactivity in the sections are obtained (Fig. 13.1e). The digital images are then calibrated using image analysis software so that different grayscale levels, which are assigned by the image analysis software, are interpolated to provide a quantitative value of radioactivity in all tissues imaged. Data can be reported as nanocuries (nCi) or microcuries (μCi) of radioactivity per gram of the tissue or in terms of drug equivalents per gram of tissue (or matrix). Digital QWBA images typically have pixel resolutions of $100\mu\text{m}^2$; therefore, accurate measurements can be obtained even for small tissues; however, today's phosphor and direct nuclear imagers often offer pixel resolutions down to 10 and/or $25\mu\text{m}^2$, which are among the highest resolving powers available.

13.4.1 History, Strengths, and Limitations of Whole-Body Autoradiography

The eloquent method of QWBA came from crude beginnings in the 1800s. In 1867, Niepse de Saint Victor first described the phenomenon of ARG as the “persistent activity due to an unknown chemical radiation” [9,10]. This observation lead another scientist named London to perform an experiment in which an autoradiographic image of a frog treated with radium was first produced in 1904 [11]. To that end, ARG is truly the first molecular imaging technique used for the localization of radioactivity in biological specimens. Fifty years later, Dziewaitkovski used β radiation to investigate the localization of compounds in biological samples [12]. This was followed by the development of the WBA technique by Ullberg in 1954 [13], who pioneered the technique by administering ^{35}S -penicillin to mice followed by freeze embedding them in water-soaked cotton using dry ice. He then sectioned their entire body bodies using a microtome in a walk-in freezer. The whole-body sections he produced were then exposed to X-ray film, which produced the autoradiographs that revealed the tissue distribution of drug-derived radioactivity. Methods for sectioning the whole bodies of laboratory animals were also developed and included the use of dry-ice-cooled microtomes [14], grinding down frozen carcasses [15], abrasion of resin-embedded carcasses [16], thick sectioning using a circular saw [17], and, finally, the development of a large microtome held inside a chest freezer [18]. Leica Microsystems, Inc. (Nussloch, Germany) began manufacturing commercially available large format cryomicrotomes and remains the leading provider of large cryomicrotomes used for WBA.

Quantitation of autoradiographs was the next challenge for pioneering macroautora-diographers. Berlin and Ullberg [19] and Kutzim [20] made the first attempts to quantify tissue concentrations in autoradiographs using early image analysis techniques. Unfortunately, the results achieved were only semiquantitative, but in the following years (1974–1987), several investigators [21–26] researched methods to better determine quantitative data from autoradiographs with limited success because of the inherent nonlinearity of film. Also during this time, Schweitzer [27] developed an image calibration method using ^{14}C -spiked blood standards at concentrations that bracketed the sensitivity of phosphor imaging plates. This robust technique continues to be used by many investigators today. Luckey revolutionized WBA in 1975 by developing and patenting phosphor imaging technology or radioluminography [28], which provided digital images from whole-body sections within days. Most importantly, these images enabled the direct determination of quantitative tissue concentrations over four to five orders of magnitude. Technical validation of the phosphor imaging instrument and QWBA methods, which more completely described the principals, specifications, and limitations of the instrumentation and QWBA, was published in 2000 [29].

In 1994, a group of autoradiographers in the pharmaceutical industry formed the Society for Whole-Body Autoradiography (SWBA) whose mission was to promote the use of QWBA over traditional organ dissection homogenate methods to determine tissue distribution of new drugs. Further work parameterized quantitative aspects to meet stringent bioanalytical expectations [30–35]. In 1990, a Japanese collaboration of >20 companies proposed that QWBA should replace the use of traditional dissection and LSC assay to determine true tissue distribution during the drug development [36]. Dr. Yasuo Ohno of the National Institute of Health Science (Tokyo, Japan) concluded his presentation at the 1997 meeting of the SWBA by stating that the Japanese Ministry of Health and Welfare would accept QWBA data in lieu of traditional organ

dissection distribution studies for the approval of new drugs as long as the procedures were appropriately validated. Today, pharmaceutical companies have almost entirely eliminated the use of dissection studies to determine the definitive tissue distribution of new pharmaceuticals, and these studies are now routinely submitted to regulatory agencies.

In addition to ARG and autoradioluminography, direct nuclear imaging technologies were also developed. These instruments utilize ionization chambers and different imaging technologies [e.g., scintillating sheet, charge coupled device (CCD) camera] and were first developed by Jeavons in 1983 [37]. Today's instrument consists of a parallel plaque avalanche chamber, which is based on the invention by Charpak in 1989 [38]. These instruments (Beta Imager and Micro Imager), which are currently sold by Biospace Lab (Paris, France), also image radioactivity in whole-body and individual tissue sections and have the ability to acquire quantitative images in real time. These instruments have proved most useful in drug discovery because they are capable of dual isotope analysis and provide data relatively quickly. However, relatively few sections can be analyzed at one time, and the instruments require regular and careful maintenance.

The key strength of the QWBA technique is its ability to provide true tissue distribution of radiolabeled test articles in a relatively unadulterated, *in situ* sample. Phosphor imaging has also been shown to be a very robust yet sensitive technology for the quantification of radioactivity in whole-body sections. Its wide linear detection range (four to five orders of magnitude) and sensitivity that can reliably quantify ~45 dpm distributed over an area of 0.5 cm² far exceed those achieved by LSC for similar sized samples. Phosphor imaging is also able to image the relatively weak energy of ¹⁴C and ³H, which, fortunately, are also long-lived isotopes, so that drugs and metabolites with very long half-lives can be tracked in the body of animals over years. This is not possible using the relatively short-lived isotopes used for *in vivo* positron emission topography (PET) and single photon emission computed topography (SPECT) imaging, which utilize special isotopes with half-lives generally much less than one day. In general, QWBA provides a much higher quality data set than that yielded by organ dissection and homogenate assay by providing concentration data on all tissues, not just those chosen for typical dissection studies, which eliminates the possibility of missing organs that may have high concentrations. The images can also be reviewed and analyzed at any time so that if an issue arises after the tissue distribution has been completed then investigators can go back, review, and quantify the nonroutine tissue(s) of interest.

QWBAs does, however, have several limitations that need to be considered. One of the major limitations of QWBA is its inherent nonselectivity, which is true for all radiolabeled studies. The actual molecular identity of the radioactivity signal detected may be due to unchanged parent drug, one of many metabolites that have retained the radiolabeled portion of the molecule, and/or degradation products. In addition, QWBA tissue sections are typically dehydrated to facilitate further processing, which results in the loss of volatile metabolites. Another limitation of QWBA is that it is difficult to evaluate short-lived isotopes, such as those used for radiopharmaceuticals, due to the processing time required, although it is possible to alter the processes to obtain data more quickly, but it requires special attention. For example, when using ⁹⁰Y, ¹¹C, or ¹⁸F as the radiolabel, which have half-lives of 2.67 days, 21 min, and 60 min, respectively, the frozen "wet" sections may need to be exposed to imaging plates

immediately and while under freezer conditions. Despite these technical challenges, Kaim *et al.* [39] were able to demonstrate that the uptake of ^{18}F -fluoroethyl-L-tyrosine (^{18}F -FET) in nonneoplastic inflammatory cells in an experimental soft tissue infection model was lower than that of ^{18}F -fluorodeoxyglucose (^{18}F -FDG), and they predicted a higher specificity for the detection of tumor cells using ^{18}F -FET.

Cost of instrumentation is another limitation. The instrumentation for QWBA is relatively expensive, and highly trained technicians are needed to perform the processing and analysis. QWBA is also limited, in that it cannot adequately provide data at the microscopic level because of the freezing technique. Despite the “snap freezing” techniques used, the freezing of all tissues is too slow to prevent cellular damage due to ice crystal formation. This makes histological receptor localization/staining and cellular identification difficult. Therefore, alternative methods are needed to evaluate cellular distribution and receptor occupancy.

Other general considerations for both dissection and QWBA tissue distribution studies include the radiochemical purity of the test compound, the radioactive dose, and methods of euthanasia. Generally, impurities should account for less than 5% of the material, and so the radiopurity of test articles should be greater than 95%. The use of anesthesia and the method of euthanasia can also affect the tissue distribution of any test article and should be carefully considered. For example, it is commonly thought that CO_2 alters the permeability of the blood–brain barrier (acidosis) and therefore alters normal brain penetration. Thus, euthanasia by CO_2 inhalation may not be a good technique to use if the objective is to study brain penetration. Likewise, euthanasia by exsanguination will undoubtedly have an effect on tissue distribution due to the massive fluid changes that take place in the body during this process [7].

13.4.2 Whole-Body Autoradiography in Drug Discovery

New initiatives in research, such as combinatorial chemistry, expanded compound libraries, parallel syntheses, and high throughput biological screening, have resulted in the generation of large numbers of compounds as putative drug candidates at pharmaceutical companies. The net result has been and will continue to be an increase in the number of drug candidates recommended for successful development. However, to increase the likelihood of developing a new drug, adequate screening for metabolic and PK liabilities during the early discovery process have become mandatory and efforts and cost to evaluate relevant metabolic characteristics of each new chemical entity (NCE) have increased substantially. It is, therefore, critical to reevaluate the current utilization of new technologies and the role of drug metabolism studies to support the discovery of NCEs. More sensitive and novel detection systems have made this process less cumbersome than in previous years. PK screening, drug stability studies, evaluation of metabolites, cytochrome P450 (CYP) involvement, enzyme induction and inhibition, and excretion studies play a major role in the drug discovery process. However, notably absent were tissue distribution studies, especially those conducted using WBA, which before the early 1980s demanded many resources and required a long time to achieve results (4–10 weeks). Quantitation of the autoradiographic images was also difficult because of the limited linear response of the films used to capture the autoradiographic images. However, within the last 15 years, the established methods of WBA have been combined with phosphor imaging technologies, which now provide reliable, quantitative tissue distribution information for

radiolabeled drug discovery compounds. This information can often be obtained within two weeks of dosing. WBA has also been used to answer pivotal questions regarding tissue PKs, routes of elimination, drug–drug interactions, drug localization, clearance, tissue targeting, solubility and formulation issues, routes of administration, tumor and brain penetration, interspecies comparisons, and tissue retention.

13.4.2.1 Brain and Cerebrospinal Fluid Penetration. A common clinical surrogate marker for central nervous system (CNS) penetration of drugs is the plasma to CSF concentration ratio [40]. However, unless this model is validated for each compound, it can be misleading when predicting the extent of CNS penetration of a particular compound. Discrepancies between CSF and CNS penetration can result from functional and structural differences between the blood–CSF and blood–brain barriers (i.e., permeability and surface area differences), variable diffusion of compounds from CSF into CNS tissue, and bulk CSF flow kinetics [41]. Numerous studies performed by preclinical PK laboratories have shown that many drugs get distributed differently into CSF and the brain [42,43]. Despite the evidence, which has demonstrated clear differences between CSF and CNS drug levels, the HIV literature contains many comparisons of CNS permeability of various therapeutic agents based on their relative CSF concentrations. Figure 13.2 shows the WBA results obtained using two different compounds that were being developed to treat HIV-1 [40]. The images clearly show different tissue distribution patterns between brain and CFS levels of drug-derived radioactivity. Thus, drug-A-derived radioactivity distributed preferentially in the CNS, whereas drug B distributed into the CSF but not in the brain tissue. This comparison revealed that there was no correlation between the distribution of drug-derived radioactivity in CSF and brain levels. Although the radioactivity observed in both compartments does

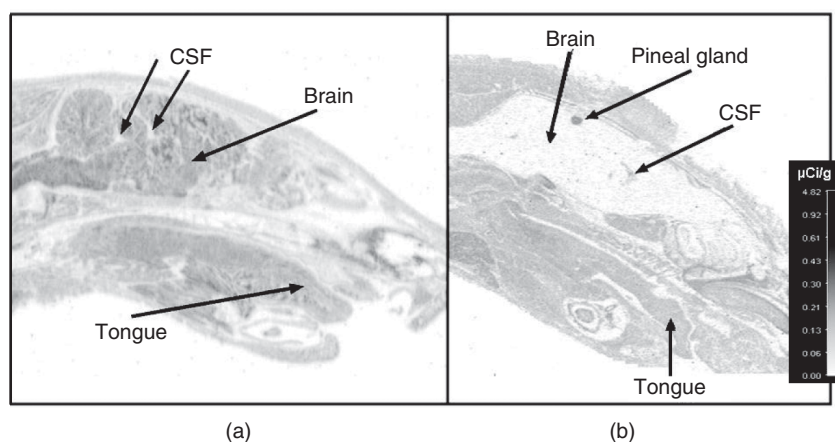


Figure 13.2 Cerebrospinal fluid (CSF) and brain penetration comparison in rats. Whole-body autoradiographs reveal differential distribution of drug-derived ^{14}C between brain and CSF of rats treated with different compounds. The comparison shows that drug levels measured in the CSF do not necessarily predict brain penetration and such models should be appropriately validated before making this assumption. (a) A brain with high concentrations of drug-derived radioactivity in the CSF but absent in the brain tissue. (b) An opposite situation to that in (a) Both are small molecule drugs that were expected to penetrate the brain because of high lipophilicity.

not discriminate between parent drug and/or its metabolites, the unequal distribution observed in all cases strongly suggested that each compound needed to be individually evaluated before making the assumption that levels of parent compound are distributed equally between CSF and brain.

13.4.2.2 Drug–Drug Interactions. Drug–drug interactions are a concern to drug developers whenever the potential exists for the coadministration of two drugs. It is, therefore, often valuable to know whether or not the drug(s) in question will change the disposition of the coadministered drug. An example is presented in Fig. 13.3, where a ^{14}C -labeled compound being developed to treat cancer was examined using WBA [40]. In this study, a single dose of the compound was administered alone (Fig. 13.3a) or coadministered with either 1-aminobenzotriazole (ABT) (Fig. 13.3b) or LY335979 (Fig. 13.3c). ABT is a known inhibitor of the cytochrome P450 metabolizing enzymes [44], the latter of which is responsible for the metabolism of the cancer drug being studied. LY335979 is a potent inhibitor of the transporter protein P-glycoprotein (Pgp) [45] for which the cancer drug was a substrate. Figure 13.3b shows that the pretreatment of animals with ABT resulted in reduced levels of radioactivity in the brain, whereas the use of LY335979 resulted in an enhancement of the radioactivity (Fig. 13.3c) in the brain as compared to the control (Fig. 13.3a). The results of this study suggested that the metabolites, but not the parent compound, of the drug contributed most to the amount of drug-derived radioactivity observed in the brain. This type of finding could have significant impact on the interpretation of *ex vivo* receptor autoradiographic binding assays performed to investigate the pharmacology of the compound in the CNS. Additionally, the study revealed that brain, and theoretically tumor penetration, could be improved by using LY335979. Similar studies with amprenavir [46] revealed an enhancement of concentration of the radioactivity in the brain of mice pretreated with GF120918, which is another potent Pgp inhibitor [47]. These examples show the potential of using known drug–drug interactions to study all types of toxicological, physiological, and pharmacological mechanisms of action, as well as PKs.

13.4.2.3 Melanin Binding. Melanin is a compound responsible for coloration in pigmented animals but is absent in albino animals, such as the Sprague Dawley (SD) rat, which is widely used in toxicology studies. Melanin is found in pigmented skin; choroid plexus and meninges of the CNS; the pigmented epithelial layer of the retina of the eye, such as in Long-Evans (LE) rats [48]; and in nerve cells of primates [49] and amphibians [50]. Many lipophilic drugs with pK_a values above 7 bind to melanin; however, the toxicological consequence of this binding has been debated for many compounds in the literature [51]. Structure–activity relationship studies using WBA for melanin binding as a function of various other physicochemical characteristics showed a correlation with volume of distribution, $\text{Log } P$, pK_a , and binding energy in pigmented rats. Strongly basic structures such as piperidine and piperazine moieties and other amines showed potential for retention in the ocular melanin [52]. Chloroquine, ofloxacin, norephedrine, and diazepam bind to synthetic melanin with varying affinity [53]. Diazepam, flunitrazepam, and Ro-5-4864 are known to induce melanogenesis [54]. Covalent binding of drugs to melanin is rare; however, its polyanionic nature and high content of carboxyl and semiquinone subunits facilitates noncovalent binding with many drugs. Although melanin binding of NCEs is not predictive of toxicity, it may prohibit the conduct of human radiolabeled studies where prolonged exposure of

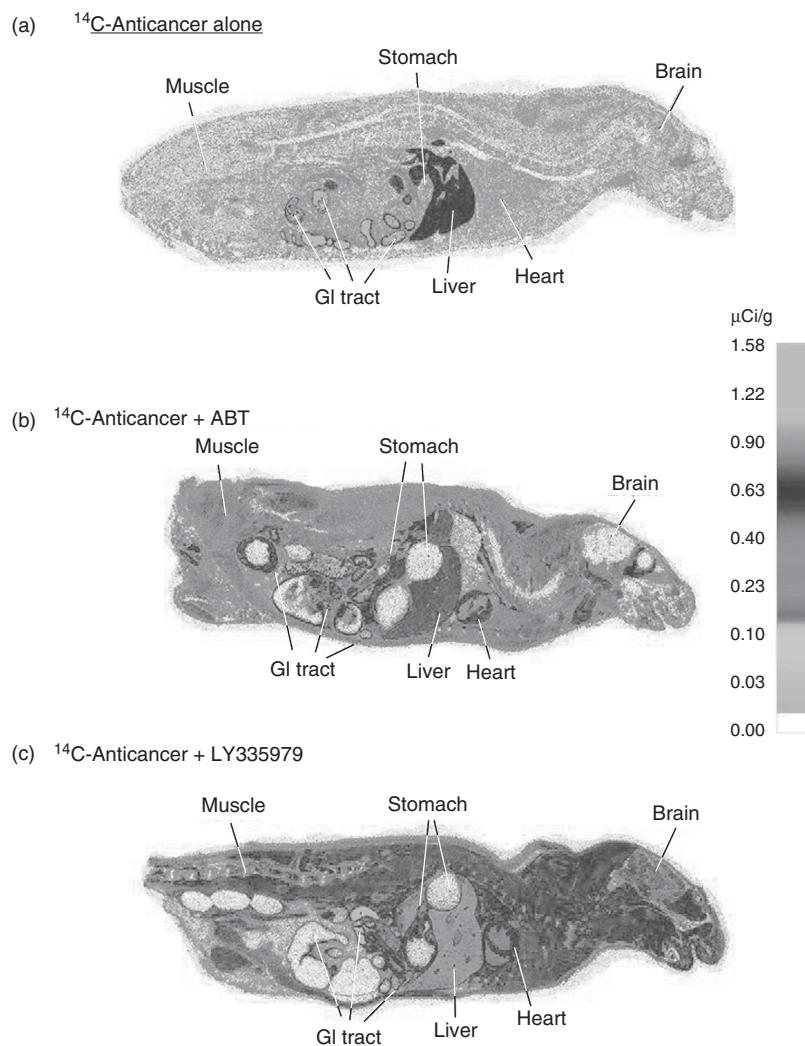


Figure 13.3 Tissue distribution of an anticancer drug after the application of known drug–drug interactions. (a) The tissue distribution of drug-derived radioactivity after a single oral dose of an anticancer drug. (b) The effect of the coadministration of 1-aminobenzotriazole, which is a potent inhibitor of the CYP450 class of metabolizing enzymes, on the tissue distribution of the drug-derived radioactivity after a single oral dose of an anticancer drug. (c) The effect of the coadministration of LY335979, which is a potent P-glycoprotein inhibitor, on the tissue distribution of the drug-derived radioactivity after a single oral dose of an anticancer drug. (See color insert.)

pigmented tissues, especially the eye, to radioactivity could pose a health risk [55]. QWBA in pigmented rats often provides the first evidence of melanin binding of new drugs because melanin binding is not routinely assayed by other means in drug discovery and development. Figure 13.4a shows a SD rat autoradiograph obtained at 2 h after a single administration of a ^{14}C -labeled compound and shows wide tissue distribution of the drug. Figure 13.4b, which is an autoradiograph of an albino rat that

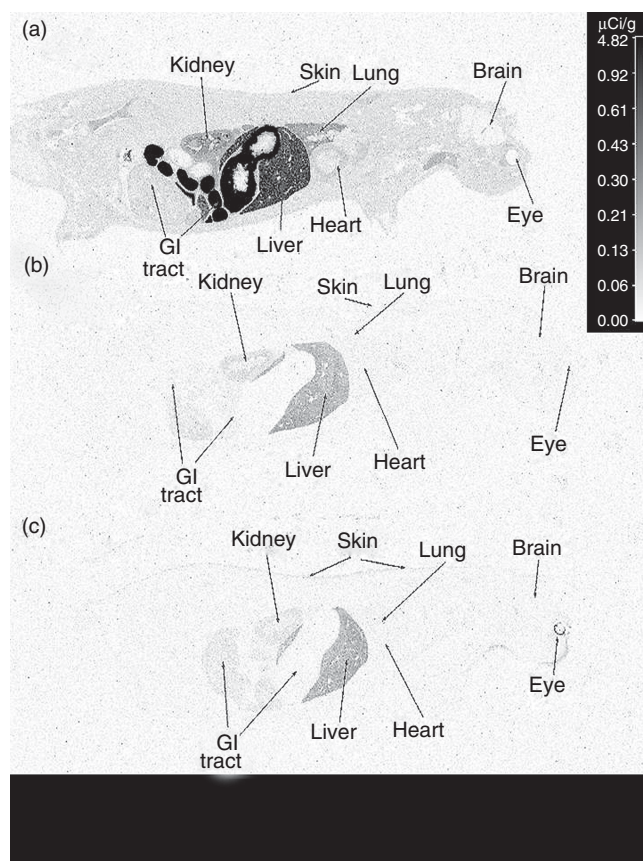


Figure 13.4 Melanin binding. Whole-body autoradiographs reveal tissue distribution of a ^{14}C -labeled drug in albino and pigmented rats. (a) A Sprague Dawley rat at 2 h post dose shows wide distribution of drug-derived radioactivity. (b) A Sprague Dawley rat at 96 h post dose shows limited distribution of drug-derived radioactivity and, notably, no radioactivity in the eye. (c) A pigmented Long-Evans rat at 72 h post dose shows limited tissue distribution of drug-derived radioactivity and, however, a high concentration of radioactivity in the eye, which is due to melanin binding.

was prepared for WBA at 96 h after a single dose, shows the absence of drug-derived radioactivity in the ocular tissues. Figure 13.4c shows the binding of drug-derived radioactivity in the pigmented skin and uveal tract of the eye of a pigmented LE rat at 72 h after a single oral dose. Although this compound did not produce any toxicity related to melanin binding, the predicted exposure to drug-derived radioactivity in humans during a proposed human ^{14}C metabolite identification study was shown to be a cause for concern.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective destruction of melanin-containing monoaminergic neurons of the brains in primates [49] and amphibians [50]. MPTP is metabolized by monoamine oxidase type B to 1-methyl-4-phenylpyridine (MPP^+), which is known to bind to neuromelanin with high affinity and causes cell death and neurochemical features of Parkinson's disease.

The antimalarial drug chloroquine, which also has high affinity for melanin, competitively inhibits the binding of MPP^+ to neuromelanin in monkeys and provides a protective effect against motor abnormalities [56]. Such distribution studies could be monitored easily by WBA. MPTP-related toxicities were not seen in rodents that lack neuromelanin. The pigmented cells acted as depots for drug and/or metabolites, which results in toxic cytoplasmic concentrations causing nerve cell death. Similar melanin-binding studies have also been reported for ^{14}C - N - N' -dicyclopropyl-methyl-piperazine-dichlorohydrate in cynomolgus monkeys [49] and ^3H -aflatoxin in pigs [57].

13.4.2.4 Enzyme Induction/Inhibition. The induction and/or inhibition of drug-metabolizing enzymes by NCEs is a concern in drug development because it can effect the disposition and thus activity of the drug being developed and/or coadministered drugs [58]. The enzyme induction or inhibition caused by a compound may be a benefit, liability, or nonissue to the clinical use of the drug. For this reason, the drug's ability to induce or inhibit metabolism must be considered. WBA has been used to investigate the effects of enzyme induction by the compound on the tissue distribution and PKs of drugs [40]. Figure 13.5 demonstrates the effect of enzyme induction on the tissue distribution of a ^{14}C -labeled drug 24 h after a single oral administration (Fig. 13.5a) and 24 h after the last day of five days of single daily oral administration (Fig. 13.5b). In this instance, enzyme induction was known to occur *in vitro* and *in vivo*, but the actual effect on targeted tissues was unknown. The levels of drug-derived radioactivity in all tissues were lower in the induced animals (multiply dosed) than those of single-dosed animals. Apparently, induction of CYPs led to increased metabolism and excretion of metabolites via bile. This type of study can provide investigators with

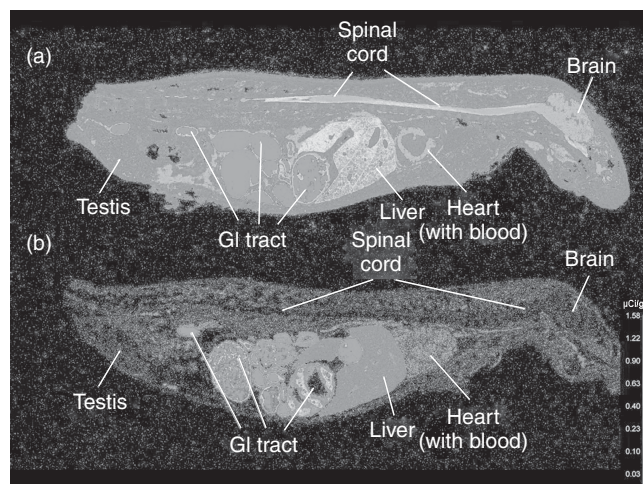


Figure 13.5 Enzyme induction/inhibition and tissue distribution and PK. (a) Whole-body autoradiograph of drug-derived radioactivity in a Sprague Dawley rat at 24 h after a single oral dose of a ^{14}C -labeled drug. (b) Whole-body autoradiograph of drug-derived radioactivity in a Sprague Dawley rat at 24 h after the last of five daily oral doses of the same drug (days 1–4 administered unlabeled drug, day 5 administered ^{14}C -labeled drug). The data obtained at 24 h post dose showed that the elimination after multiple dosing was more rapid than that after a single dose and thus tissue exposure was notably lower. (See color insert.)

important information regarding exposure to target organs/tissues after multiple daily dosing and, in some cases, may require alteration in the dosing regimen in the clinic. The effect of enzyme inhibition on the tissue disposition of NCEs can also be studied by observing the effects that coadministered drugs have on the distribution of NCEs.

13.4.2.5 Tumor Penetration. WBA has been an invaluable tool in cancer research for its ability to provide a visual depiction of tumor penetration, as well as providing a snapshot of general tissue distribution in both tumor and toxicological animal models. Figure 13.6 shows the distribution of a ^{90}Y -labeled anticancer agent, which was administered orally to transgenic mice that expressed human mammary tumors. This study, which was performed very quickly (within three days) because of the short half-life of the radioisotope, showed excellent tumor penetration as well as retention in the tumor versus normal tissues [40]. Importantly, since this technique involves examining all

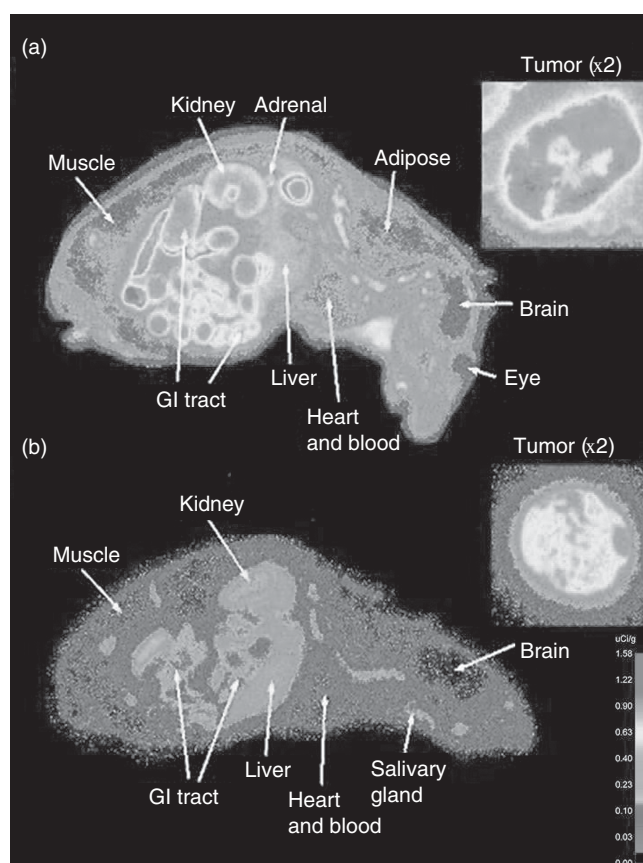


Figure 13.6 Tumor penetration model. Whole-body autoradiographs of tumor-bearing mice that were administered a single IV dose of a ^{90}Y -labeled anticancer drug and sacrificed at (a) 2 h or (b) 24 h post dose. Insets show close-ups (enlarged $\times 4$) of the tumor in each mouse. WBA revealed that high levels of drug-derived radioactivity remained in the tumors for up to 24 h post dose, while radioactivity in normal tissues declined to lower levels. (See color insert.)

other major tissues, distribution of the compound to other potential target organs, such as pancreas, liver, and stomach, can be monitored.

In other oncology studies conducted, mice implanted with ovarian Oca-1 tumors and dosed with ^3H -paclitaxel (TXL) in Cremophor EL and alcohol vehicle or ^3H -poly(glutamic acid)-paclitaxel (PG-TXL) showed that the better efficacy of PG-TXL over TXL in inhibiting tumor growth due to the broader distribution of TXL and/or its metabolites in tumors when dosed as the macromolecular conjugate [59]. The concentration of free and conjugated TXL was determined by excision and processing of tumors in mice in a parallel study. By WBA, the distribution of radioactivity in the tumor was determined to be initially located in the periphery of tumors on day 1 and was homogeneously distributed into the center of the tumor by day 6. Similarly, a comparison of intratumoral injection of mitomycin C and its dextran conjugate in rats bearing Walker 256 carcinosarcoma, by WBA, showed that while mitomycin C cleared the tumor rapidly, the intratumor clearance was greatly retarded when it was conjugated with dextran [60].

13.4.2.6 Fetal Penetration. WBA can also be used to address questions about placental transfer and tissue retention because clear pictures of the distribution to various organs within the fetus can be investigated. For example fetal penetration of [^3H]deramciclane, an anxiolytic agent, after IV and oral administration in rats was not observed [61], which was a useful finding in animals and was used in the package labeling to support safe administration in pregnant women. On the other hand, [^{14}C]olanzapine [62], *Fusarium* mycotoxin zearalenone [63], and chloroacetonitrile [64] have been shown to cross the placental barrier in rats by WBA, which posed a potential safety liability for development of these drugs.

13.4.2.7 Formulation Selection. Formulation changes are sought to bring about desired alterations in the absorption and tissue distribution and also to avoid certain side effects. QWBA was used to determine that a submicron lipid emulsion of the drug tirilazad over an aqueous solution helped to avoid venous irritation without sacrificing PKs and tissue distribution in rats [65]. WBA of holmium-166 (^{166}Ho) chitosan complex, a radiopharmaceutical for cancer therapy, after intrahepatic administration to rats and mice, showed that the drug was not distributed to tissues but was retained at the site of administration [66].

13.4.2.8 Metabolism/Covalent Binding. The use of WBA coupled with other procedures can be adopted for studying metabolism and covalent binding of compounds to specific tissues that show retention of radioactivity. Notable examples include the use of (i) whole-body sections where specific areas of the sections were punched to obtain tissue samples that were subsequently extracted and analyzed for metabolites [67] using mass spectroscopy analysis and (ii) freeze-dried tissue sections from a lamb, which was dosed with ^3H -aflatoxin, were extracted successively with 5% trichloroacetic acid (TCA), 50% ethanol, 99.5% ethanol, heptane, and water and then dried and imaged to study the distribution of nonextractable radioactivity [68]. These studies enabled the differentiation of metabolites and tightly bound compounds in tissues. New technologies such as the Advion Nanomate[®] instrument that facilitates the extraction and collection of compounds from whole-body sections can make this process easier. There are other examples of similar analysis that used the residual frozen animal carcasses after WBA samples were collected [64,69,70].

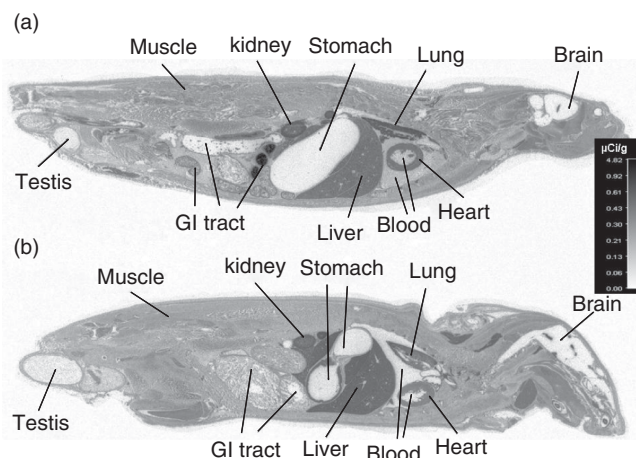


Figure 13.7 Retention in rat tissues. Whole-body autoradiographs of two rats given similar IV doses of a ^{14}C -labeled compound and were sacrificed at (a) 0.5 and (b) 48 h post dose. Autoradiographs showed little or no decline in the amount of drug-derived radioactivity at 48 h post dose, which suggested that the compound was retained in the tissues of the rat.

13.4.2.9 General Tissue Retention. WBA can also be used to determine sites of retention of drug-related material when the mass balance of administered material in excreta samples is low and/or when unusual plasma PK is observed. In an early mass balance study in our laboratories, the total radioactivity of a new compound was not recovered from rats 24 h after IV administration of the compound, with only small amounts of radioactivity recovered in urine, feces, and bile [40]. QWBA was used to identify the unknown sites of possible tissue retention, and it showed that the compound was widely distributed at both 0.5 and 48 h post dose (Fig. 13.7) and tissue levels of radioactivity were essentially unchanged from 0.5 to 48 h post dose. Data suggested that drug-derived radioactivity was retained in the tissues of the rat, while being rapidly cleared from the systemic circulation. The observed tissue retention raised concerns of possible toxicological problems, especially in liver, kidney, heart, and lungs where concentrations were highest. In another study, IV administration of [^{14}C]-D- or -L-serine to rats led to the detection of accumulation of radioactivity in the corticomedullary areas of kidneys, which suggested a possible link between the observed acute necrosis of the renal proximal tubules and administration of large doses of D-serine [71].

13.4.2.10 Application of QWBA to Therapeutic Peptides and Proteins. ^{125}I -labeled proteins and peptides are being used for QWBA analyses to study tissue distribution and PKs in the biotechnology arena. Increasingly, regulatory agencies are asking for this information, as data gleaned from these studies have helped them to better understand and select their compounds in drug development. Although the *in vivo* stability of ^{125}I on most large molecule xenobiotics is difficult to assure, the data generated from these studies can be useful. Semiquantitative data can be obtained with confidence when the *in vivo* stability and amount of free ^{125}I circulating in the body is characterized.

Currently, ^{125}I ARG is most often used to provide a quantitative method for determining tissue distribution of large molecules because it is either not possible or extremely expensive to radiolabel these molecules with ^{14}C or ^3H . Although ^{35}S can

be used in some instances to label large molecules, the synthesis and location of the radiolabel can be difficult, but the resulting stability of the radiolabel, image resolution, and quantification are an improvement over ^{125}I labeling, which is much more instable. The stability of ^{125}I labeling on large molecules is subject to cleavage *in vivo*, which often results in relatively high concentrations of free ^{125}I . The determination of drug concentrations in tissues using ^{125}I labeled molecules must be considered as semiquantitative because of the inevitability of measuring free ^{125}I along with the test article. Thus, care must be taken when interpreting tissue concentrations of ^{125}I -labeled compounds for the thyroid, stomach, kidneys, mammary gland, salivary gland, thymus, epidermis, and choroid plexus [36]. These organs contain a sodium-iodide symporter that are involved in the organification and/or elimination of free ^{125}I [72,73] and often have very high concentrations of free and/or organified ^{125}I , which is not drug related and thus provide misleading results. Most iodopeptides are absorbed intact through the intestinal tract, but once in the blood stream, deiodination can occur to varying degrees depending on the labeling [72,74]. Iodide has a volume of distribution that is about 38% of body weight and is mostly extracellular. In humans, total plasma iodide clearance is about 12% per hour (rate = 45–60 mL/min) and thyroid clearance varies widely depending on dietary intake and can be as low as 3–4 mL/min [73,74]. These data must be considered when considering tissue quantification and imaging with ^{125}I -labeled biotherapeutics. Finally, regulatory agencies have begun requesting that biotechnology companies show the ADME characteristics of large molecules. This can be a challenge in some cases because large molecules are usually not easily labeled with conventional more stable isotopes (e.g., ^{14}C) and their metabolism is often similar to that of endogenous proteins and peptides.

Administration of nonradiolabeled sodium iodide to test animals before giving the ^{125}I test is one way to reduce the uptake of free ^{125}I by shunting free ^{125}I to the kidneys to increase the rate of clearance and reduce the effect on tissue quantitation. Furthermore, the concentrations of free ^{125}I in the body must be characterized to better understand the true drug-derived concentrations in tissue and this can be done by using protein precipitation with TCA protein precipitation of plasma and determining the ratio of protein-precipitable ^{125}I versus free ^{125}I [73]. This ratio can then be used to correct tissue concentrations obtained using QWBA. Further analysis to determine the radiostability of ^{125}I -labeled compounds in tissues may be done using gel electrophoresis, thin-layer chromatography, ELISA, and/or specialized mass spectroscopy techniques. To these ends, tissue quantitation of drug-derived radioactivity using ^{125}I and QWBA or gamma counting techniques can, at best, be considered as a semiquantitative technique, albeit one of the only practical techniques currently available.

Skofitsch *et al.* [75] used ^{125}I -galatin to identify specific binding sites in rat brain using ARG, which is one of the earlier examples of the use to study large molecule distribution. As many types of antibodies are being developed to treat cancer and other diseases, ^{125}I -labeled antibodies have been used increasingly to evaluate tumor penetration and localization. Figure 13.8 shows such an example where QWBA demonstrated localization in the highly vascularized portions of the tumor. Targeting of drugs to necrotic portions of a tumor can be studied by QWBA since differences in uptake between different regions of tumors can be observed, whereas this information could not be obtained through a typical excision/homogenization experiment. The increased interest in delivering oligonucleotide therapeutics including small interfering RNAs [small nuclear ribonucleic acid (snRNA)] to their desired site of action has prompted

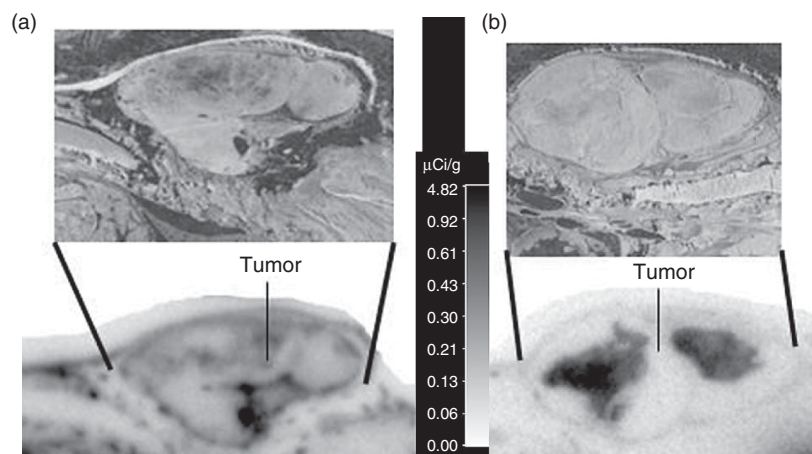


Figure 13.8 Tumor penetration and distribution. ^{125}I -Peptide distribution in different tissue regions of tumors are shown. Note the higher concentrations (darker regions of autoradioluminograph) in the more vascularized areas of the tumor (a) as compared to the regions that show a high concentration in the solid tissue regions (b). (See color insert.)

distribution studies of these entities. ^3H or ^{14}C labels can be incorporated for QWBA investigations.

13.4.3 Whole-Body Autoradiography in Drug Development

Most often, QWBA is used during the drug development stages and to support regulatory submissions. The data are used to show tissue PKs and to predict human radiation dosimetry for the human radiolabeled ADME studies. A routine tissue distribution performed using QWBA typically evaluates 35–40 tissues using 6 or more time points designed around known plasma or tissue PKs.

13.4.3.1 ADME Regulatory Studies. The design of tissue distribution studies for drug development and regulatory submission has changed since QWBA has been implemented [36]. In the past, the number of time points used was rarely sufficient to determine reliable tissue PK parameters. Most notably was the determination of the $T_{1/2}$ of drugs in individual tissues. This has implications for predicting the tissue PK and pharmacodynamics of drugs as well as the exposure of human tissue to radiation during human radiolabeled studies conducted to determine human excretion patterns and identify metabolites. Albino strains are most commonly used for whole-body autoradioluminography (WBAL) because they are used most often as the toxicology model; however, pigmented animals are often included in studies conducted to support human radiodosimetry predictions in order to gain exposure information in pigmented tissues such as the skin and eye. When pigmented and albino rats are used, investigators must be aware of strain differences, especially to physiological differences related to drug metabolism and brain physiology [76,77]. Large animals such as rabbits, dogs, and monkeys are also frequently performed for drug development purposes, especially when the species demonstrates plasma kinetics similar to humans. Furthermore, samples of blood, plasma, bile, urine, and feces may be collected from the animals on the QWBA

studies and analyzed by other means such as liquid chromatography/mass spectrometry (LC/MS) or radio-HPLC to provide information regarding metabolism. Investigators and regulatory agencies have embraced these approaches and currently recommend that tissue distribution studies be conducted using validated QWBA methods [78].

Another important consideration is the choice of using a single dose or multiple doses, and a uniform guidance regarding this has been issued by worldwide regulatory agencies [78,79]. They state repeated dose distribution studies may be appropriate when

1. A single-dose distribution study suggests that the apparent half-life of the test article and/or metabolites in organs/tissues significantly exceeds the elimination half-life in plasma and is also more than twice the dosing interval of toxicology studies.
2. Steady state concentrations of the compound/metabolite in circulation of PK or toxicology studies are higher than those predicted by a single-dose kinetic study.
3. Critical histopathological findings of safety studies are observed that would not be predicted by a short-term toxicology and/or single-dose distribution or pharmacology study. The focus of the distribution study should be on those target organs.
4. The pharmaceutical is being developed for site-specific targeted delivery.

The regulatory guidelines and requirements for the designs are less specific but state that the study design should utilize radiolabeled compounds or alternative methods with sufficient sensitivity and specificity to provide quantitative data. They also suggest that metabolites be surveyed as well as the parent molecule [80].

13.4.3.2 Studies for Human Radiation Dosimetry Predictions. Human radiolabeled drug studies are often performed as part of phase II clinical trials to determine human metabolism and PKs of new drug entities. ^{14}C - and ^3H -labeled compounds are routinely used in human beings to determine ADME characteristics of NCEs in human subjects. In order to perform these studies, a sponsor needs to provide a prediction of radiation exposure to tissues and/or organs in the body that might result from the administration of a radiolabeled drug. Clinical investigators must assure that the dose of radioactivity associated with the radiolabeled drug will not be harmful to volunteers and falls within acceptable exposure ranges before radiolabeled drug studies can be performed in humans. The Food and Drug Administration (FDA) also requires that pharmaceutical companies perform a prediction of human exposure to radioactivity as a result of the administration of radiolabeled test compounds.

Dosimetry predictions rely on mathematical models and known radioactive tissue/organ concentration profiles and/or excretion patterns, which are obtained from QWBA or “cut and count” tissue distribution studies. The FDA and the International Commission on Radiological Protection (ICRP) have set allowable limits for exposure of radiation involving the administration of single doses of radionuclides to human volunteers. The FDA has limits of 3 rem, which is a measurement of equivalent radiation exposure to whole body and critical organs, and 5 rem to other organs. The ICRP Category IIa effective dose upper limit has been set at 1 mSv, which is another unit of radiation dose equivalent. Recommendations for the methods to determine human and dosimetry predictions have been published by the FDA and ICRP. However, the calculations and data obtained from the various methods can produce different predictions

of radiation exposure and can present problems for clinical review boards considering the approval of proposed human radiolabeled drug studies [81].

Several mathematical prediction methods and calculations have been proposed over the years, but most pharmaceutical companies rely on guidance from the ICRP and those proposed by the Medical Internal Radiation Dose (MIRD) Committee of the Society for Nuclear Medicine [82]. Representatives of the FDA have also published a guidance on the prediction [56]; however, the FDA initiated an updated guidance in 2009 [78]. Additionally, several textbooks have been written over the years, and the basic calculations have not changed much since the early 1960s [83]. However, the equations suggested by the MIRD Committee have been revised over the years and two software packages called MIRDose and OLINDA, which were based on the MIRD equations and developed by the Oak Ridge National Laboratory, were created to aid in dosimetry predictions. These softwares utilize mean residence times (MRTs) obtained from the animal tissue/organ distribution information obtained from earlier studies to predict the radioactive exposure to humans given a radiolabeled NCE or other radioactive substances. MIRDose provides predictions for whole-body exposure and major organs only. MIRDose does not calculate the exposure for many other tissues, and for this reason, its utility for dosimetry is limited.

The equation referenced by Dain *et al.* [56] was originally developed by Marinelli [84] and then refined, to account for animal data, by Hendee [85]. The calculations and methods for predicting human dosimetry presented by the ICRP appear to be related more to industrial radiation workers and the general population and not necessarily focused on clinical radiolabeled drug studies.

The literature on radiation exposure predictions often refer to and consider the $T_{1/2}$ and/or MRT of the isotope used to label the test compound but not necessarily the biological $T_{1/2}$ or MRT of the test article, which may vary considerably from the isotope. This has presented a challenge for pharmaceutical researchers who need to determine what calculations and methods can be used along with the detailed tissue distribution data offered by QWBA.

Anecdotal evidence suggested that there are misunderstandings about dosimetry and different dosimetry methods provide different estimates. A rodent tissue distribution study, which was designed to provide reliable tissue PK parameters by Solon [81], tested this hypothesis by choosing the following three sets of recommended calculations: (i) “Hendee–Marinelli” equation [56]; (ii) MIRD Committee, MIRD equation [86]; and (iii) ICRP recommendations (weighting factors for whole-body exposure [87]). The results showed that each method produced a different prediction of tissue and whole-body exposure. It was also observed that some equations (e.g., MIRD) were best designed for organ homogenate data, which are obtained from organ dissection/homogenate analyses, and do not readily account for the use of actual tissue concentrations. The equations also did not adequately consider allometric scaling from rodents to humans. Furthermore, the use of weighting was not appropriate for use on individual tissues but for determining the contribution of tissues to whole-body exposure only. A common practice among laboratories is also to use PK parameters that have been obtained from studies where reliable parameters cannot be obtained because of study designs that do not adequately capture time points. This results in unrealistic $T_{1/2}$ and AUC values that can grossly over or underestimate exposures. QWBA results have repeatedly shown that tissue concentrations often vary considerably within organs, thus predictions obtained by using organ homogenate data can greatly

under- or overestimate the actual tissue exposure to radioactivity (e.g., concentration found in the cortex and medulla of the kidney). This finding suggests that the current dosimetry methods may need to be revised to better utilize true “tissue” concentration data from QWBA studies for determining the tissue level exposure. This study showed the need to reexamine in detail the guidelines and methods being used to determine human ^{14}C dosimetry from administered radiolabeled drugs for human studies.

13.4.3.3 Postapproval Studies. QWBA has also been used to examine drugs after they have been approved for use to support marketing efforts and/or to address postapproval issues. Schweitzer *et al.* [88] investigated the distribution of [^{14}C] diclofenac sodium (Voltaren[®]), which is a drug that has been marketed for years, after a single oral administration in rats. These investigators showed that diclofenac preferentially distributed into the inflamed tissues and achieved exposures that were 26- and 53-fold higher in the inflamed neck and inflamed paws of treated animals than in control rats, while all other tissues in treated animals and control animals showed similar distribution and exposure. In this case, the study was done in response to a request from a project team and it also supported marketing efforts for the compound. It also provided for comparing the dermal penetration and distribution of future formulations that could improve exposure and efficacy.

13.5 MICROAUTORADIOGRAPHY

MARG provides pharmaceutical scientists with a high resolution tool to investigate spatial localization of radiolabeled drugs at a tissue and cellular level. MARG is especially good at providing insight regarding *in vivo* receptor binding in various cell types and has predictive value for specific drug targeting. In this respect, it has been used widely in academic settings to answer basic research questions and/or to support pharmacologic studies where it can provide important information on cellular mechanisms. MARG has applications in all areas of science, but this report discusses examples in drug metabolism, pharmacology, toxicology, and molecular biology.

The methods used by the author, which are described below, are based on the methods of Appleton [89] and Stumpf [90], but it is important to realize that there are many variations of the method presented in the literature. To begin, an animal is dosed with a radiolabeled substance (typically, ^3H , ^{14}C , ^{35}S , or ^{125}I), the animal is exsanguinated, and tissues are dissected and snap frozen in isopentane that is chilled in liquid nitrogen. The tissue is then cryosectioned at -20°C (or the optimal cutting temperature for a given tissue/organ), to obtain 4- to 5- μm thick sections. Then, under darkroom conditions, sections are thaw mounted onto dry glass microscope slides that have been precoated with nuclear photographic emulsion. The slides are placed into a light-tight box with desiccant and allowed to expose for an appropriate amount of time. The collection of sections onto dry slides is a key step developed by Appleton [91] and eliminates the possibility of diffusion of soluble compounds, which can happen during slide and section dipping into an aqueous emulsion. In contrast, the original Stumpf and Roth method [90] involved collection of the section into vials for freeze-drying, which required very careful section handling, was very time consuming, and prone to sample destruction. Following exposure, the slides are developed in a manner similar to developing photographic film before being stained using conventional histological

staining protocols. This may include immunostaining techniques that can provide positive colocalization of drug-derived radioactivity to known cell types, receptors, and/or other structures/markers for which antibody staining protocols exist [90].

There are several examples in the literature of investigators attempting to obtain quantitative data from MARG preparations. A few methods have been proposed to determine subcellular concentrations of drugs. These methods, which date back to the late 1960s, have been presented in books by Stumpf [90] and Baker [92]. One of the earliest methods, which was known as *restricted method*, was based on the analysis of structures of similar shape and size [93] and used to describe the distribution of silver grains in sections of ^3H -noradrenalin-labeled nerve terminals. A second “restricted method” was also based on the analysis of structures of similar shape but dissimilar sizes [94]. This method established a “universal curve” and required that the two-dimensional structures associated with radioactivity were of similar shape. An “unrestricted method” or “circle method” was developed by Williams in 1969 [95]. This tested the hypothesis that radioactivity was randomly distributed, and it ascribed values for relative concentrations. It also attempted to account for a lack of precision of the restricted models. A second “unrestricted method” known as the *hypothetical grain* method was developed by Blackett and Parry in 1973 [96]. This method has five stages that include (i) overlay screen preparation, (ii) collection of “hypothetical grains” and construction of a “crossfire” matrix, (iii) collection of real grain data, (iv) fitting of hypothetical and real grain data using chi-square, and (v) modification of the matrix until an “acceptable fit” is obtained. Each of these methods claimed to be able to estimate the number of molecules in subcellular regions if the specific silver grain yield (average number of disintegrations to produce one silver grain) was determined. Then, it was “theoretically” possible to determine the number of molecules in a cell or nucleus. The formulas must consider exposure time, specific activity, silver grain yield (dependant on uniform method conditions), section thickness, volume of the compartment, and “other parameters,” which are very difficult to control because of the various artifacts inherent in an inherently sensitive technique. High sensitivity techniques require extremely consistent and careful preparation, especially because the current MARG techniques rely on many manual and “artful” steps. The uniformity of the detection media (i.e., emulsion-coated slide) is unknown and not characterized in almost all examples in the literature. Tissue absorption of radioactivity is also rarely considered or characterized. The number of cells counted/quantified must be sufficient for robust statistical analysis. Background subtraction and variable background can invalidate results. There are no tissue section thickness quality control standards and/or internal calibration standards used in MARG. Changes in daily cosmic and background radiation can adversely affect an entire, several months long study. In short, most pharmaceutical researchers do not have the resources available to develop routine MARG procedures to enable and or prove that MARG is a quantitative technique, and thus its limited application to date.

13.5.1 History

The first microautoradiographic data were produced by Lacassagne in 1924 [97], which lead to further work by Bélanger and Leblond [98], who poured liquid photographic emulsion onto histological sections to reveal the location of radioactive substances in the tissues. Jofte and Warren in 1955 [99] revised that technique and dipped slides

into photographic emulsion, which gained wide use because of its ease of manipulation. This technique has survived the years and is sometimes used today. However, if diffusible radiolabeled compound are utilized, the results can be useless owing to the relocation of the radiolabeled substance, which produces telltale artifacts that can invalidate experiments and discourage investigators. During the 1940s, methods utilizing strips of dried emulsion were developed where the histological sample containing a radioactive substance was placed in direct contact with the dried emulsion strip for a finite exposure time. This method proved cumbersome because maintaining the precise position of the strip on the samples proved cumbersome during exposure, development, and staining. Often alignment could not be maintained, ending the entire process in failure. However, in 1964, Appleton [89] first developed the technique of collecting cryosections onto slides covered with strips of dried emulsion using a thaw mounting technique. This required tissue sectioning and collection to be conducted in a darkroom and under safelight conditions, which required a high level of skill.

The use of cryopreservation and cryosectioning remains critical to the study of diffusible substances because it maintains the spatial locale of the radiolabeled substance in the matrix, whereas liquid tissue fixation steps often solubilize and relocate diffusible test articles. However, when substances are tightly bound to cellular structures (e.g., receptor proteins), positive results may still be obtained from samples processed using conventional histology techniques. Further refinement of MARG techniques occurred during the 1960s by Caro [100], and shortly after that Stumpf and Roth [91] made additional improvements to establish receptor ARG as a more reliable technique. This established the basis for the current MARG techniques. Numerous elaborations on the techniques have been presented by different investigators [101], but the basic principals have remained unchanged for >40 years. Today, as in the past, the MARG technique is very difficult to master, which continues to hamper its use. Researchers must use caution when reviewing the literature and relying on articles that used the emulsion dipping technique and claim quantitative data. The conclusions may be questionable and disputed in some cases. Validation of the technique is lacking in most laboratories and results can be very subjective. This is due to the lack of thickness uniformity of both the tissue sections and the emulsion detection media used. Additionally, there are few, if any, instances where calibration and/or quality control standards have been co-exposed within the samples, which is the only way to clearly prove quantitation across different types of tissue samples.

13.5.2 Strengths and Limitations of MARG

The main strength of the MARG technique is the ability to provide researchers with a high resolution imaging tool for determining the cellular and to a certain extent subcellular, localization of radiolabeled compounds in animal tissues. It is also a very sensitive technique that can theoretically detect the actual number of molecules in a preparation [90], although those claims are difficult to substantiate, and so the technique is mostly used as a qualitative tool. ARG techniques can also be applied in specimens prepared for electron microscopy. However, because of the high degree of tissue processing, it does not lend itself to the localization of diffusible substances, which can be easily solubilized and relocated from tissue during fixation, infiltration, and embedding procedures; however, when the radiolabeled substance is stable in the tissues, such as DNA, RNA, structural proteins, and/or covalently or tightly bound substances, then

the technique can be very powerful and useful. *In situ* hybridization [102] is a good example of how molecular biologists have applied ARG techniques to reveal many aspects of cellular processes, which has been used for many years. Another strength of MARG is limited tissue samples processing and thus accurate capture of the localization of test articles in space and time. The main assumption is that exsanguination and dissection of the tissue samples used do not adversely effect localization of the radiolabeled test substance. Tissues can be removed and frozen relatively quickly and so qualitative localization is believed to be accurately reflected.

Several limitations have impeded the progress and wider use of MARG in drug discovery and development. These include the high rate and ease of artifact production and difficulties of tissue section collection under darkroom conditions. The processing time to obtain results by MARG is difficult to gauge as each tissue must be treated and evaluated differently, depending on how much radioactivity is present. The exposure time can take anywhere from days to weeks or even months to obtain optimal results. Technology may help to solve some of these problems if the detection media (e.g., emulsions) can be more uniformly produced and made to have inherently linear quantitation. It may also help to develop easier methods of collecting uniformly thick tissue sections that can be automatically mounted onto slides for processing, although this would be quite a challenge because of the varying matrices to be sectioned (e.g., hard bone, adipose, and eyes). Dependable micro-sized calibration and quality control standards that can be co-exposed with every section would also need to be developed to assure reproducibility of quantitation. Finally, the new methods would need to enable a significant reduction in the amount and types of artifacts that are produced. Currently, the following types of artifacts [90] must be controlled: (i) effects on emulsion by slight variations in light, humidity, temperature, tissue characteristics, fixation, freezing, chemicals, pH, developer, fixer, and miscellaneous debris in developer solutions; (ii) tissue condition (e.g., freezing technique, fixation, autolysis, sectioning temperature, improper section mounting); (iii) light leaks; (iv) latent image fading; (v) folding and shrinking of emulsion; (vi) positive chemography, which is exposure of the emulsion by endogenous chemical effects such as tissue enzymes; (vii) negative chemography; (viii) deviations of pH in processing fluids; (ix) pressure artifacts; (x) ice crystals on knife; and (xi) crystalline deposits from developing process. Some of these are more easily controlled than others, but together they require a high level of skill by the analyst to overcome. The current technique will remain qualitative and too daunting for routine use in pharmaceutical discovery and development until methods and/or technologies can be developed to better control for variables and artifacts. The lack of new developments in MARG methods has continued to make MARG an under-utilized technique in drug discovery and development, but when performed correctly, the results can be of utmost value in promoting a drug candidate and in answering some pivotal questions for pharmaceutical investigators.

13.5.3 MARG in Drug Discovery and Development

Despite the difficulty in mastering the technique and high risk of artifacts, MARG has made important contributions to drug discovery and development over the years and has provided insight into the localization of pharmaceuticals to support proof of concept studies, mechanisms of toxicity, efficacy, physiology of hormone action, and cell regulation. For example, MARG is useful to study skin penetration of various

compounds and is routinely used in the cosmetic industry, physiology research, pharmacology, and safety studies. Unilever has developed MARG techniques to examine skin penetration in different *in vitro* test models in rats, pigs, and human skin sample to demonstrate efficacy for consumer skin care products using MARG coupled with confocal microscopy and other techniques (Minter 2007, Personal communication). Linoleic acid (LA) is commonly used in cosmetics, but its *in vivo* human skin penetration characteristics were not very well demonstrated. However, in 2006, Rauvast and Mavon [103] used a unique, *in situ*, “virtual” microautoradiographic slide to examine transfollicular delivery of LA in human scalp. They combined their MARG data with an *in vitro* permeation experiment and compartmental analysis to show that most of the LA was localized to the hair sheath but none was present in the dermal compartment and that 10% of the total LA recovered was found in the stratum corneum and dermis after 6 h. This supported the notion that the diffusion of LA occurred by a transfollicular route. It also demonstrated the value of high resolution MARG for providing detailed cellular localization of the molecule. Skin receptor MARG techniques have also been used to study the absorption, penetration, and cellular localization of ^3H -Maxacalcitol, which is a vitamin D analog used for the treatment of psoriasis. Hayakawa *et al.* treated the dorsal skin of rats with a ^3H -Maxacalcitol ointment and examined skin exposed for periods of 0.5 through 168 h [104]. They discovered two routes of skin penetration; one via epidermal cell layers and the other via hair follicles. They were also able to distinguish very fine regions of cellular localization, which supported theories on the mechanism of efficacy. Young *et al.* [105] used ^{14}C -iodoantipyrine as a tracer to study intrarenal blood flow in nephrectomized rats, and they used their microautoradiographs and standards to determine blood flow rates. MARG provided a means to identify the morphological location of blood flow, and they concluded “that medullary blood flow was dependent on local prostaglandin production and is also influenced by sympathetic nervous supply.” Figure 13.9 shows an example of the distribution of ^{14}C -azidothymidine (^{14}C -AZT) in the glomerulus of a rat kidney after a single IV dose [36]. This example demonstrates the fine detail of localization that can be obtained with MARG. Both renal function and localization of various substances in the kidney have been studied by MARG. Another laboratory used *in vitro* MARG to show localization and density of atrial natriuretic peptide (ANP) in nephrectomy biopsy samples obtained from patients with renal disease [106]. These investigators used [^{125}I]- α -human (1–28) ANP and found localization in the glomerulus and tubular regions in the human biopsy specimens. They also observed that density of ANP binding generally decreased in patients with renal dysfunction and hypertension. Overall, though, their study established an *in vitro* MARG method to assess ANP binding in human biopsy specimens.

More recent work by Yamamoto *et al.* [107] used MARG in combination with immunohistochemistry, macroautoradiography, and PET to examine intestinal ulceration and healing in the rat. They used ^{18}F -FDG to examine ulcerations in the small intestine of rats, which were induced using indomethacin. MARG combined with immunohistochemistry showed an accumulation of ^{18}F -FDG in inflammatory cells as well as in granular tissue-forming cells, forming granulation tissue, and around ulcers. ^{18}F -FDG was also found to be present in proliferating intestinal crypt cells and intact intestinal tissue taken from indomethacin-treated and control animals. They concluded that ulceration could be visualized early by the prominent uptake of ^{18}F -FDG by inflammatory cells and by the formation of granulation tissue by cells in and around

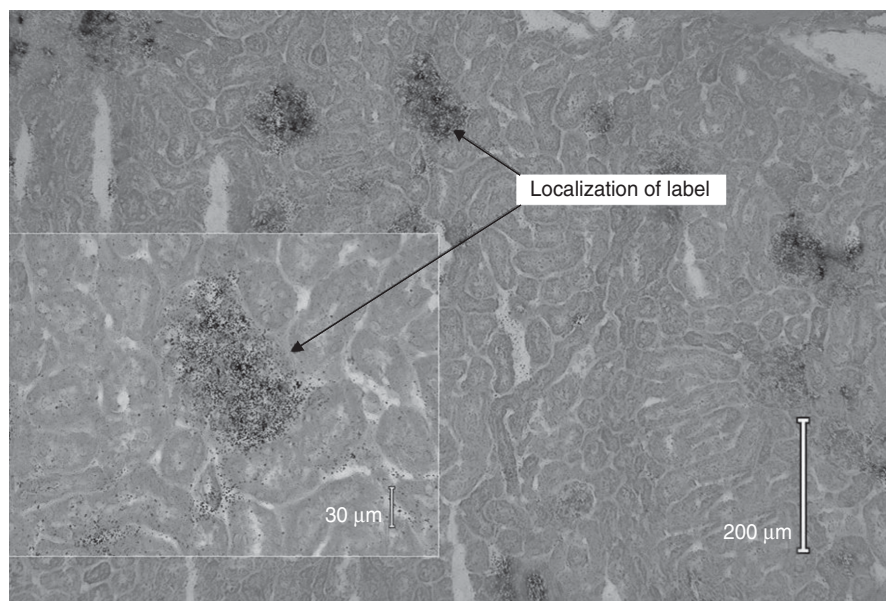


Figure 13.9 Microautoradiography results of rat kidney after a single administration of ^{14}C -azidothymidine in a rat kidney after a single IV dose. The location of the drug-derived radioactivity is made apparent by the black specks that appear over the section. In this case, a high concentration of the drug is shown localized to the glomerulus. (See color insert.)

ulcers. This work also demonstrated the value of combining both *in vivo* and *ex vivo* imaging techniques, which provided robust data sets for analysis.

13.6 CONCLUSIONS

Molecular imaging has grown dramatically over the last 50 years, and most recently, *in vivo* imaging modalities such as PET [108] and fluorescence [109] imaging has captured the attention of scientists in drug discovery and development owing to the ability to visualize organ *in vivo* distribution in preclinical species. However, these new *in vivo* modalities have their limitations, such as relying on the use of short-lived isotopes as in PET imaging, which limits the ability to study tissue PKs over days or weeks or encountering issues with reliable quantitation due to background fluorescence and poor resolution, which occurs when imaging fluorescent tags *in vivo*. Additionally, the positron-emitting isotopes for PET and the fluorescent labels used can alter the structure and/or binding capabilities of molecules, thus rendering them ineffective at providing the needed information. The inability to distinguish between labeled drug, metabolite(s), and/or degradants is also a limitation of these modalities. However, with the recent developments in matrix-assisted laser desorption ionization mass spectrometric imaging (MALDI MSI), desorption electrospray ionization mass spectrometry (DESI), and secondary ion mass spectrometric imaging (SIMS) technologies, which rely on the use of whole-body sections, the ability to distinguish between the parent molecule and metabolites is possible. Unfortunately, these technologies have not

reached the point of being quantitative, which means QWBA is expected to remain the technique of choice for tissue quantitation. To these ends, *ex vivo/in situ* imaging modalities such as QWBA and MARG will undoubtedly be relied on for many years to come to provide the kind of detailed tissue distribution information required for drug discovery and development programs.

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FURTHER READING

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14 Ligand-Binding Assays

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14.1	Summary	1
14.2	Introduction—new light through old windows	2
14.3	From history to future—technologies	3
14.4	Kinetic considerations in LBAs	10
14.5	Assay formats for LBAs	10
14.6	Immunoassay validation	13
14.7	Applications	14
	References	21

14.1 SUMMARY

Ligand-binding assays (LBAs) or immunoassays are the analytical method of choice for pharmacokinetic (PK) and immunogenicity assessment for biopharmaceuticals. As pipelines for this class of drugs are expanding, LBAs are revisited to address current scientific questions with this classic technology. Although immunoassays come in a vast variety, they share some similar concepts. Typically, the analyte of interest is captured out of a mostly unpurified solution using specific antibodies or receptors. Bound analyte is then detected with a labeled reagent either in a competitive or a sandwich format.

Since their earliest inception for the measurement of insulin, immunoassays rely on antibodies specific to the analyte of interest. These days, different capture and detection reagents are often used, for example, two different antibodies or a combination of target and antibody. In any case, reagent specificity, selectivity, and quality are keys to a successful LBA. Monoclonal antibodies and other recombinant technologies allow the generation of custom reagents to meet the requirements of most LBA methods.

Some advantages of immunoassays include their limited cost per sample, sensitivity, and lack of need for extensive sample purification. Disadvantages are the indirect readout that is inherent in the method, long lead times for optimal reagent generation, and the potential for interferences by the sample matrix, in particular, body fluids.

While the basic immunoassay schematic remained the same, recent technologic advancements addressed some of the downsides. Meso Scale Discovery (MSD)-based assays are now widely accepted for immunogenicity assessment and also make their

way into PK assessment. They potentially have lower background and less matrix interferences than traditional enzyme-linked immunosorbent assays (ELISAs). Gyrolab also has potentially lower matrix interference, while in addition, it is fully automated to reduce the need for extensive operator training. Furthermore, mass-spectrometry-based methods have undergone significant improvement in the recent years. Although no clinical application of mass spectrometry to clinical biopharmaceutical PK/PD (pharmacodynamic) analysis has yet been published, numerous feasibility studies indicate a potential future direction of the field.

Caution should be applied when comparing or extrapolating immunoassays on the basis of different formats or technologies. As they will give different results, a thorough understanding of the bioanalytical background is necessary. Bioassays focus on a functional drug, while mass-spectrometry-based assays only measure a short signature peptide. Antibody therapeutics, for example, can be measured as free, total, or partially target-engaged drug and possibly a combination thereof. The assay itself might influence the balance of these forms as well. Soluble targets are particularly challenging since their serum levels are fluctuating and they remain a part of the sample matrix in the assay.

Toxicology and clinical applications require thorough method validation. So far, no FDA guidance specific to large molecules has been published. However, the general bioanalytical guidance in combination with a series of white papers should be applied to LBAs. Many immunoassay validation parameters are similar to mass spectrometry. Some key differences include the appreciation of less accuracy and greater variability, as well as the need for more extensive selectivity testing. Applicable clinical patient matrices should be kept in mind rather than an exclusive focus on a healthy population during validation. Also, reagent lot qualification and bridging is essential in maintaining a bioanalytical.

PK and immunogenicity of biopharmaceuticals are assessed using LBAs. PK assay formats are dictated by scientific requirements as well as compound biology and program phase. “Generic” human IgG assays relying solely on commercial reagents may be deployed during a basic research stage, while drug-specific assays may be needed for toxicology and clinical applications. Immunogenicity screening assays, on the other hand, typically use a common bridge format with labeled drug, although confirmation assays can display a greater variety.

Despite or because of its age and low technology, immunoassays in their vast variety continue to shape the development of modern biotherapeutics into the foreseeable future. During recent years, advances in mass spectrometry and recombinant technologies demonstrated the feasibility for these methods to replace immunoassays in the long run. However, more method development work and general scientific acceptance are needed. Until then, LBAs will be inseparable from biopharmaceutical development.

14.2 INTRODUCTION—NEW LIGHT THROUGH OLD WINDOWS

During the last three decades, the advances in molecular biology, immunology, and protein biochemistry have led to the rise of a new class of drugs, namely, biopharmaceuticals, often referred to as *biologics* [1]. While traditional pharmaceuticals are of low molecular weight (100–500 Da) and chemically synthesized, biopharmaceuticals are high molecular weight proteins or peptides that are produced in living cells. Owing

to their structural complexity and size, these molecules cannot be readily analyzed by mass spectrometry methods. Up to now, immunoassays have been the method of choice to study the PK and immunogenicity of biopharmaceuticals.

The application of immunoassays to peptide bioanalysis has a long history that goes back to the first uses of animal-derived insulin [2]. Yalow and Berson [3] developed a competitive insulin radioimmunoassay (RIA) method in the 1960s, which was awarded the Nobel Prize in 1977. Their breakthrough demonstrated the feasibility of endogenous hormone quantification at very low concentrations [4]. The first immunoassays used polyclonal antibodies purified from human or animal serum, resulting in limited assay flexibility and reagent availability [2,3]. The monoclonal antibody technology developed by Köhler and Milstein [5] (Nobel prize in 1984) enabled the generation of highly specific, custom-made antibodies with virtually unlimited supply. As a result, antibody use proliferated in research and diagnostics immunoassays, as well as in development as therapeutic agents.

PK measurements of biopharmaceuticals rely on LBAs with high sensitivity and specificity. Antibodies specific to the drug enable quantification down to pg/mL levels in crude serum or other matrices without prior enrichment, thereby potentially reducing technical artifacts. Despite some limitations that are discussed in the following chapters, LBAs still enjoy widespread scientific and regulatory acceptance. Potential future trends including coupling to liquid chromatography-mass spectrometry (LC-MS) will be discussed.

A schematic applicable to most immunoassays is depicted in Fig. 14.1. Although competitive RIAs were the first format developed (Section 14.3.1), today dual-detection assays are the most common. They employ either two antibodies for capture and detection or a biological target in combination with a detection antibody [6]. As there are numerous variations on these themes, we have not discussed all possible scenarios. A common feature of all immunoassays is the need for a detection label. Initially, radiolabels were used allowing for unparalleled sensitivity. Later, enzymes such as horseradish peroxidase replaced the radiolabels [7–9], and most recently, acridinium, ruthenium, and lanthanide labels became available [10,11]. Further details on these methods are discussed later.

14.3 FROM HISTORY TO FUTURE—TECHNOLOGIES

As outlined above, a variety of LBA formats have been developed. Beyond their obvious differences, they share the same principle: an analyte-specific capture (antibody, target, or complementary peptide) is used to immobilize the drug of interest from a solution (matrix); for drug metabolism studies, typically plasma/serum or similar biological fluids are used as applicable. The immobilized drug is then detected with a second labeled analyte-specific reagent, which is either the labeled drug itself or, more often, another antibody. Depending on the assay format, wash steps are used to remove unbound substances.

Since biological matrices are not purified before application in the assay, the selection of appropriate reagents is key to method performance [12,13]. In particular, capture reagents must specifically recognize the analyte of interest among many endogenous proteins. Especially, closely related proteins can cause nonspecific signals that cannot be distinguished from specific binding [14]. If wash steps are used to remove unbound

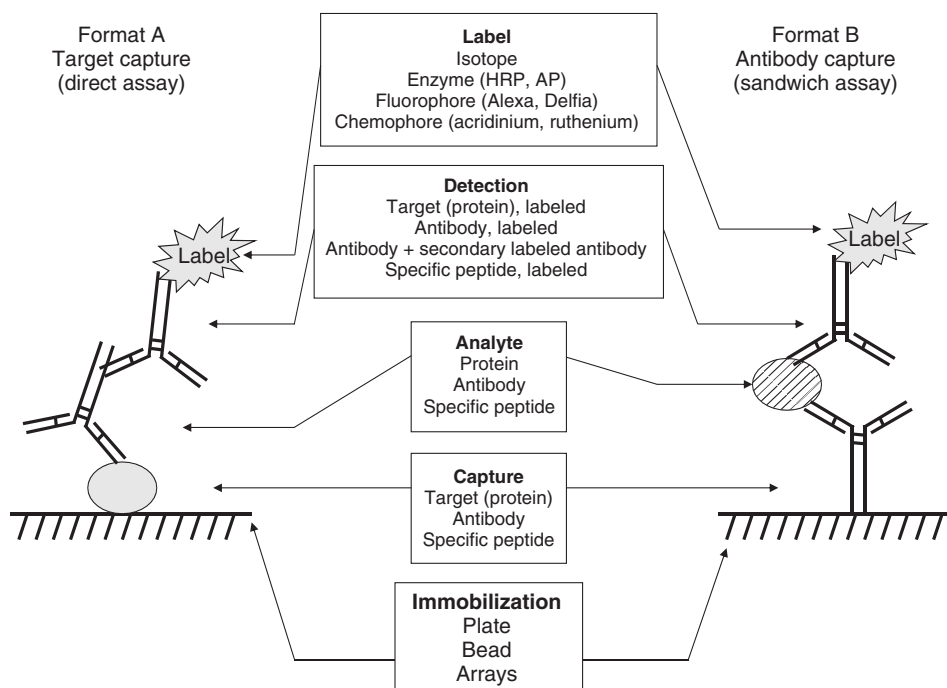


Figure 14.1 Schematic representation of ligand-binding assays. Format A: target-mediated capture with antibody detection. Format B: antibody-mediated capture and detection. Alternative reagents that can be used are listed for each step. The use of secondary anti-species antibodies or labeled streptavidin detection systems has been omitted. HRP, horseradish peroxidase; AP, alkaline phosphatase.

substances, detection can be less specific. For example, anti-species antibodies are often used to detect bound antibodies. When working with samples from the same species as the antibody of interest (e.g., when detecting human therapeutic antibodies in human serum), other antibodies in the serum sample may become nonspecifically immobilized and not completely wash away. The nonspecifically immobilized antibody will be detected and will result in higher background.

As a general rule, decreasing the background improves assay sensitivity if signal/background ratios increase. Close attention should be paid to reagent purity, as contaminating proteins can result in higher assay background or increased variability. Unpurified antisera often contain antibodies that can bind “nonspecifically” in the assay. As greater specificity and affinity for both capture and detection will result in superior assay sensitivity, immunoaffinity purification is recommended for polyclonal antibodies when high background is encountered. In addition, optimization of blocking and assay buffer composition can provide reduced background and improved signal to noise ratios.

The selection of antibodies is dependent on the method’s objectives. Polyclonal antibodies tend to offer high affinity and are less affected by slight changes in the analyte [15]. Monoclonal antibodies bind to the molecule only in a single epitope and may not bind if the epitope is altered. This can also be used as an advantage to measure full-length molecules with antibodies binding at either end (alternatively,

polyclonal antibodies generated against subunits of the analyte of interest could be used). Monoclonal antibodies can be produced without changes for decades, making them ideal for long-running methods. When analyzing antibody therapeutics, anti-idiotypic antibodies are often used for highest specificity and improved assay sensitivity [16,17]. These antibodies bind to the antigen-binding site of the therapeutic antibody of interest. The downside of using anti-idiotypic-specific antibodies and other custom antibodies is the long lead time for immunization and production.

Besides full-length, “natural” antibody reagents, numerous alternatives have been deployed for either capture or detection of analytes of interest. Advances in antibody engineering allowed for molecular design of recombinant antibodies and for phage display-derived antibodies [18,19]. Receptors have successfully been used in immunoassays, mostly for capture but sometimes also for detection [10]. Lately, aptamers have been tested in LBAs [20]. These short oligonucleotide or peptide sequences are generated to bind to a target molecule of interest similar to antibodies. They can be selected and produced relatively quickly, but their applications to the bioanalysis of therapeutics are very limited [21].

14.3.1 Radioimmunoassay (RIA)

Although immunoradiometric assays (IRMAs) employing sandwich formats have been developed [22–25], classical RIAs are competitive assays (Fig. 14.2) [26,27]. The analyte of interest is captured from biological fluids by a specific antibody. Radioactively (^{125}I) labeled analyte is added to compete with the unlabeled drug for binding to the capture antibody. The immune complexes are then precipitated, typically by the addition of protein A or anti-antibody-coated beads, although other methods such as ammonium sulfate precipitation have been used. After pelleting the immune complexes by centrifugation, excess liquid, including unbound tracer, can be removed and retained radioactivity can be measured in a scintillation counter. The amount of radioactivity is inversely proportional to the analyte concentration in the sample.

Nowadays, most RIAs have made way for nonradioactive methods described in the following paragraphs. However, they still have their place when high sensitivity is required, for example, to quantify peptides and hormones [28,29]. For historic reasons and in order to achieve greatest sensitivity, RIAs are still used for PK bioanalysis of insulin compounds such as glargine [30] and in clinical chemistry laboratories. Besides traditional RIAs, IRMAs employing two antibodies were published as well for bioanalysis of insulin and insulin analogs [23].

14.3.2 Enzyme Immunoassay (EIA)

Although the nomenclature is used interchangeably throughout the literature, EIAs (enzyme immunoassays) and ELISAs are slightly different: EIAs are a direct homogeneous format translation of RIAs into a nonradioactive setting. The radiolabel is replaced by an enzyme such as alkaline phosphatase or horseradish peroxidase, followed by a signal-producing chemical reaction (colorimetric, chemiluminescent, or fluorescent). ELISAs are heterogeneous with an immobilized capture phase (Fig. 14.2). After the analytes of interest are bound, the remaining sample is washed off and a second analyte-specific antibody is used for detection. The enzyme label can be conjugated directly to the detection antibody, to an anti-species antibody, or to streptavidin if a biotin label

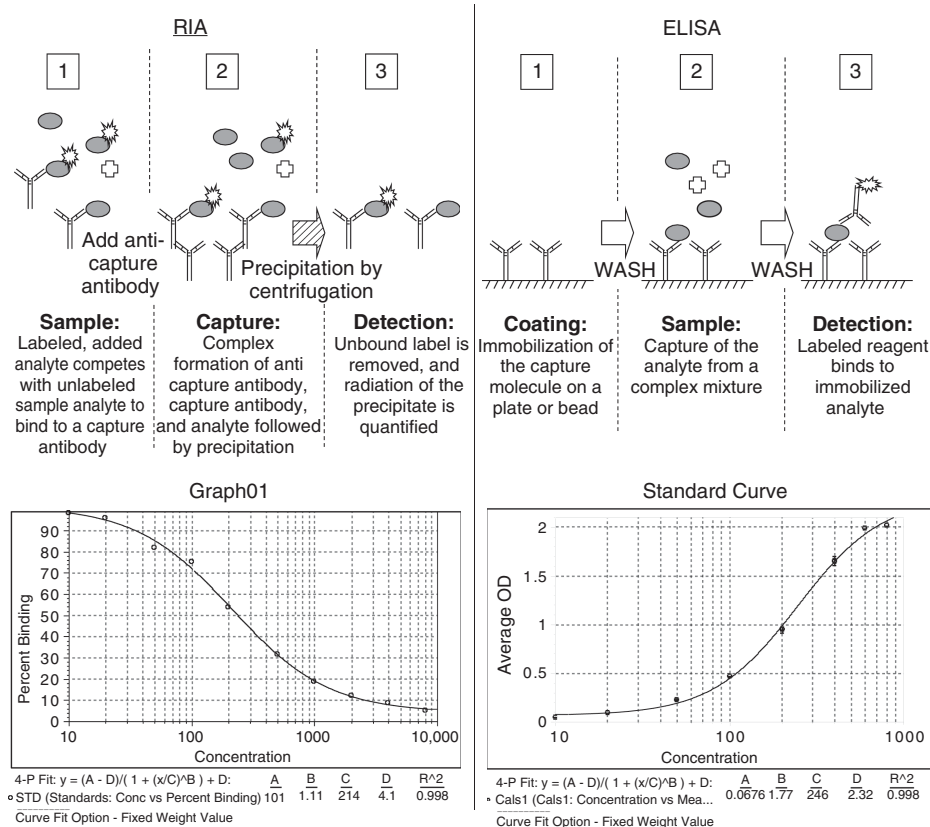


Figure 14.2 Schematic comparison of RIA and ELISA methods with example calibration curves: RIAs are competition assays. A single analyte-specific reagent (antibody) is used to capture the compound of interest. Labeled compound at a fixed concentration is added to compete with the analyte. The immune complexes are precipitated, and radiation is quantified in the pellet. ELISAs use an immobilized capture phase. Analyte is captured from the sample and detected with a second, labeled reagent. Each step is separated by washes.

is used. There are numerous variations on this basic theme, which are beyond this chapter's limits. Even though the strict use of the name ELISA implies the use of an enzyme, alternative labels can be applied, including nonenzymatic fluorophores and chemophores. Often these are referred to as *ELISAs*, although the proper nomenclature is “fluorescent immunoassay” (FIA) and “chemiluminescent immunoassay” (CLIA), respectively.

ELISAs are the most common format for large-molecule PK applications [31], with wide acceptance by regulatory agencies. Its overall simplicity and flexibility with respect to labels and procedures allow implementation in virtually any laboratory environment. However, some shortcomings exist: signal development is dependent on enzyme kinetics that is influenced, among other factors, by temperature, mixing, and substrate feedback inhibition. These are partially compensated for by the use of calibrator curves included on each assay plate, but higher data variability still is higher than that of small-molecule mass spectrometry methods. Also, the dynamic range is

rather limited even with excellent reagents. Therefore, PK study samples often have to be extensively diluted to be measurable within the assay range, introducing additional potential for analytical error. Newer technologies circumvent some of these limitations and are discussed below.

14.3.3 Other Assays

A number of immunoassay-based technologies became available in recent years. These include the following techniques.

14.3.3.1 Electrochemiluminescence. Often abbreviated as “ECL” (not to be confused with the enzyme substrate), electrochemiluminescence was commercially developed by Igen and incorporated into Roche’s line of diagnostic immunochemistry analyzers. MSD made the technology more broadly available for research laboratories. The basic format is similar to ELISA. However, the enzyme is replaced with a ruthenium label that is used to generate light on application of electric current. Special plates are required to facilitate the electric charge. Advantages of MSD assays include lower background and larger dynamic range than ELISA, thus potentially allowing for improved assay sensitivity. Enzyme-related variability is reduced as well. Since the signal reaction takes place only at the immediate proximity of the electrode, homogenous assays often can be developed without the nonspecific background issues of conventional ELISA [32,33]. The major disadvantage is the limited commercial choice, as MSD is the only supplier. However, MSD-based assays have found widespread acceptance for immunogenicity assays and increasing applications for PK assays [34].

14.3.3.2 Gyrolab. The Gyrolab (Gyros AB, Sweden) nanoscale assay platform transferred a standard fluorescence-tag immunoassay into a compact disk (CD). The entire assay only requires 5–10 μ L of sample or reagents and is fully automated. Briefly, biotinylated capture molecules are immobilized in flow-through streptavidin columns, followed by sample and a fluorophore-labeled detection antibody. Each column corresponds to a well in traditional plate-based assays. The run time per CD is ~50–60 min, but shorter runs can be accomplished using partial CDs. Advantages and disadvantages are similar to MSD assays. The small sample and reagent volume requirements as well as the automation are additional benefits of Gyrolab. We have used Gyrolab-based assays extensively in the lead identification and optimization space for PK measurements for over 14 drug discovery programs. The small sample volumes allowed for serial bleeds from transgenic mice, which are often too costly and limited to use in terminal bleed PK studies. Optimization of assay conditions was routinely finished within one to two days, and troubleshooting of any issues could be carried out with multiple runs in a single day.

14.3.3.3 Biacore. Surface plasmon resonance (SPR) is coming of age to study molecule interactions. Capture molecules are immobilized on a chip that allows detection of subsequent binding events individually through SPR effects. Wash steps are limited, and no labels are necessary. Biacore is routinely used to screen and characterize antibody reagents but has not found widespread acceptance for routine sample analysis because of limited throughput, high costs, and complex technology. Inherent

to SPR technology is limited sensitivity, particularly when studying high affinity interactions. Recently, Biacore became an important tool for immunogenicity assessment of large molecules, as it can detect low affinity anti-drug IgM that are washed away in traditional ELISA and sometimes even MSD (Section 14.7.2) [35,36].

14.3.3.4 Immuno Polymerase Chain Reaction (Immuno-PCR). This combination of traditional immunoassay and molecular biology attempts to harvest advantages from both worlds: protein-based detection and high sensitivity [37,38]. In this case, the detection antibody is labeled with an oligonucleotide that can be amplified via PCR. The immunosandwich capturing the analyte of interest is assembled following a standard immunoassay protocol. However, visualization and quantitation are accomplished by a subsequent PCR amplification of the oligonucleotide label. A number of variations have been developed on this theme, including commercial technologies [37]. Assay sensitivity, low background, and a large dynamic range are obvious advantages of immuno-PCR. On the other hand, the assay process is still rather complex and requires well-trained staff. Automated technologies are being developed to make daily applications more feasible. While there are a number of diagnostic applications, only a few PK assays are based on immuno-PCR [39].

14.3.4 Homogenous Assays

The adsorption of proteins to solid surfaces changes their tertiary and quaternary structures, potentially affecting interactions with other molecules in the assay [40]. Cancer antigen EpCAM II, for example, contains a discontinuous immunogenic epitope that is disrupted when coating the antigen directly onto microtiter plates (Verch unpublished data). Other limitations of solid-phase assays include wash steps and lengthy incubation times, making complete automation difficult. Some traditional plate-based technologies are amendable to semihomogenous formats, in particular, MSD assays or bead (flow) systems such as Luminex. Most homogenous assays, however, are performed entirely in solution in a single step without any washes. Different technologies are based on similar principles. Capture and detection molecules are labeled with complementary donor and receptor tags. On formation of the immunosandwich, energy transfers between the tags results in the generation of light or fluorescence (Fig. 14.1). Several technologies have been developed to optimize the light generation, including fluorescence resonance energy transfer (FRET), homogeneous time-resolved fluorescence (HTRF) [41], and, lately, AlphaLISA [42]. The close proximity necessary to achieve the energy transfer made widespread FRET and HTRF applications difficult, while AlphaLISA allows more flexible and larger immunosandwiches. The signal is based on chemiluminescence, which results from a cascade of several dyes [43]. Although there have been limited publications on AlphaLISA in the PK field, the technology is closely watched for its promising potential.

14.3.5 LC-MS for Proteins

Owing to their large size, proteins cannot readily analyzed by LC-MS-based methods. Large amounts of other proteins such as albumin that are present in preclinical and clinical matrices and often have high homology to the drug add to the bioanalytical complexity. However, LC-MS methods have evolved to potentially address some of

the disadvantages of ELISA [44,45]. Table 14.1 compares immunoassays to mass spectrometry methods. As a key difference, immunoassays can potentially measure entire functional molecules, while LC-MS focuses on peptide signatures after tryptic digests. If selected appropriately, these peptide signatures are unique to the analyte of interest in contrast to signals in immunoassays in which nonspecific and specific binding events are indistinguishable. While the selection of representative peptides out of a digest pool (billions of possible antibody sequences in a patient population) can be a challenge,

TABLE 14.1 Comparison of Immunoassay and Mass Spectrometry

Parameter	Immunoassay	LC-MS	Comments
Sensitivity	pg/mL range	μg/mL range	High affinity antibody reagent can improve sensitivity for both methods
Specificity	Limited (reagent dependent)	High (inherent)	—
Measured molecule identification	No	Yes	—
Accuracy/precision	Low (<20% bias and CV)	High (<10–15% bias and CV)	Both methods often perform well above expectations in the hands of experienced operators
Throughput	High	Medium to low	—
Cost/sample	Low	High	—
Assay development time	3–6 wk + 6–8 mo reagent lead	2 wk (no reagent lead unless immunocapture is used)	—
Special reagents required	Yes (specific antibodies and target)	No (except for immunocapture methods)	—
Complex sample matrix compatible	Yes	No (extraction necessary)	—
Matrix interferences	Yes	Potentially reduced	—
Internal standards required	No	Yes	Labeled standards for large molecules can be technically challenging
Free/total compound measurements (typical)	Mixture of free and total most common	Total	Method modifications can accommodate different types of measurements
Regulatory and industry acceptance	Reference method for large molecules	Reference method for small molecules	—

Abbreviations: LC-MS, liquid chromatography-mass spectrometry; CV, coefficient of variance.

in silico prediction has greatly reduced the efforts [46]. Chromatographic resolution and sensitivity are greater issues since a certain mass is necessary for detection. Combination methods of immunoaffinity analyte enrichment and LC-MS or MS-MS detection can address these disadvantages [47,48]. However, this also eliminates some of the advantages of LC-MS, that is, reduced development times and the lack of need for method-specific reagents.

A few assays have been published that apply LC-MS to PK of protein therapeutics [44,49,50], but clinical applications have lagged behind to date. Future improvements of the technology are undoubtedly still to come.

14.4 KINETIC CONSIDERATIONS IN LBAs

Immunoassays are influenced by affinities and kinetics between the different reagents and the matrix components. Binding reactions are reversible and limited by the equilibrium concentrations [51], making the application of kinetic models difficult with many unknown parameters (e.g., how much antibody/target has been immobilized in the capture phase) [52]. Affinity determinations made in buffer are not necessarily transferable to a serum matrix. Therefore, underlying principles between binding and release should be kept in mind for LBA development and data interpretation. The assay protocol is a key to understanding the data generated.

Although the distinction between bound and unbound drug is more applicable to small molecules, discussions evolved during the last few years, in particular, for antibody drugs. The so-called free antibody has at least one site available for antigen binding, whereas the “total” drug includes both forms with and without bound antigen [53]. Although some assay formats are claimed to measure free or total antibody, an LBA inevitably will have a mixture of both forms. The assay reagents used for capture and detection as well as the incubation times and conditions influence the equilibrium between free and total drug that may exist in the original samples. Despite these difficulties, some assumptions can be used to guide PK analysis: if the target is used for capture, mostly free antibody will be measured. Indirect capture systems (Section 14.5.2) or general anti-IgG assays are more likely to measure total antibody (Fig. 14.3). Owing to the complexity of the assay interactions, these assumptions should be treated with caution.

14.5 ASSAY FORMATS FOR LBAs

Immunoassays are very flexible and offer the possibility of numerous formats [10]. We have reviewed only a selected few that appear to be common and are of specific interest. Some general points should be considered for any immunoassay. Depending on the question, different assay formats may lead to different conclusions. There are also technical considerations of methods details that often are not widely appreciated. As discussed in Section 14.5.3, the sample matrix is not readily exchangeable: even seemingly small differences such as EDTA plasma versus heparin plasma or one rat strain versus an alternative strain can significantly change assay performance.

The use of commercial kits to analyze proprietary biopharmaceuticals is not always straightforward either. At minimum, the assay calibrators and quality controls (QCs)

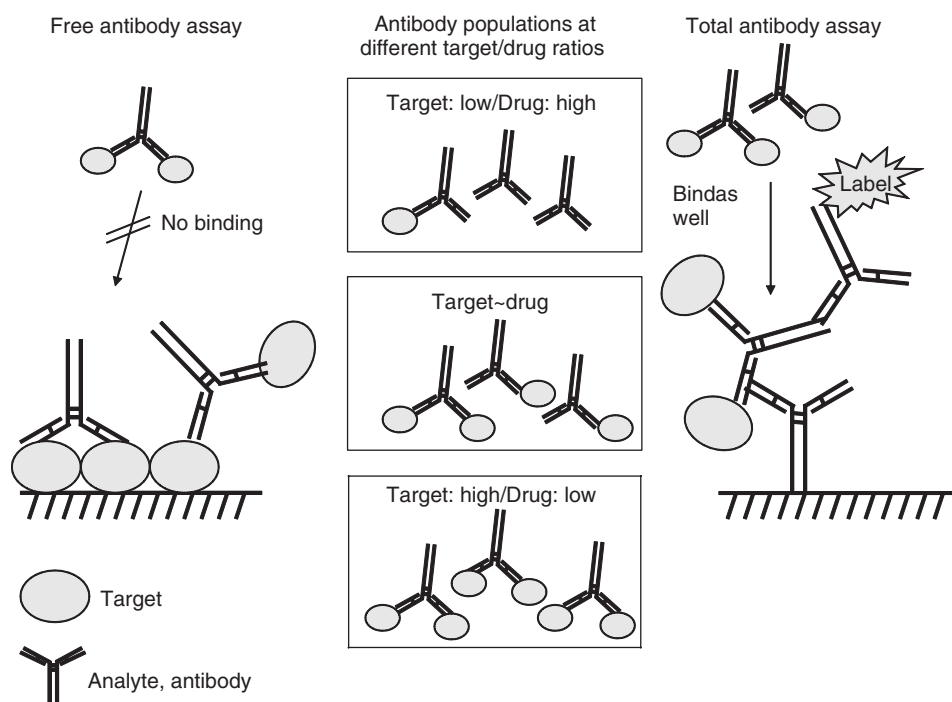


Figure 14.3 Comparison of free versus total antibody immunoassays: Free antibody assays typically use the target or anti-idiotypic antibodies for capture. If target is bound to the antibody analyte, binding sites will not be available for binding in the assay. Total antibody assays capture and detect the antibody analyte independently from target engagement. Different populations of free, partially target-engaged, and bound antibodies are present depending on the ratios of target to drug (i.e., antibody analyte).

will need to be changed to reflect the new analyte. Typically, additional optimization for the new analyte is required, although options are limited due to the kit's preset reagent titrations. When using *in vitro* diagnostics (IVDs), some manufacturers may ask for a written assurance that data are not used to make patient treatment decisions outside the IVD label.

Assay sensitivity is tightly linked to reagents. Although assay platforms (Section 14.3) have an influence, sensitivity gains usually are modest if the reagents are not optimized. A switch from anti-species-specific to anti-idiotypic-specific detection can result in up to 100-fold greater sensitivity. More details are discussed in the following sections.

In the following sections, we present more details of assay formats. Since many biopharmaceuticals under development are antibody based, we focus on these to a greater extent. However, with some extrapolation, many of the lessons may be transferable to other analytes of interest.

14.5.1 Direct Assays

Formats that capture the analyte of interest directly out of the matrix are the most common applications. Typically, the target is immobilized on the solid phase and an

anti-species antibody may be used for detection. In the case of antibody compounds, a bridge assay may be employed, with immobilized target for capture and labeled target for detection. Many assays for biopharmaceuticals employ compound-specific custom reagents, in particular, anti-idiotypic-specific antibodies against antibody therapeutics. The key to a successful method is a combination of a suitable format and the quality of the reagents. Specificity and selectivity make or break LBAs.

Using anti-species antibodies can be problematic when operating with a similar matrix (anti-human antibodies and a human serum sample). Other endogenous antibodies beyond the therapeutic are present in 1000-fold excess so that even 1% nonspecific binding can result in significant background noise. These problems are reduced when clinical antibodies are tested in preclinical species. If cross-reactivity is limited, the so-called generic anti-human antibody assays can be conducted, and they have been published [54]. Anti-human antibodies are used for capture and detection to support various animal studies.

14.5.2 Soluble Target Assays

Soluble targets represent a specific challenge: at low drug-target ratios, very few binding sites of the drug may be available to engage immobilized target in the immunoassay, whereas at high drug-target ratios the reverse effect can be observed (Fig. 14.3). These effects can impact the measured PK profile depending on the assay format. Assays measuring Remicade, for example, were reported to exhibit TNF- α interference [55]. Other soluble targets that are subject to antibody therapy include various interleukins, Dickkopf-related protein 1, and proprotein convertase subtilisin/kexin type 9. Soluble ligands may also arise from shedding of membrane-associated molecules such as in the case of CD20 [56] and erb2/Her2 [57].

It may be necessary to evaluate whether the assay measures free or total compound and evaluation in two assays may help to establish the PK picture. Different approaches may be used to develop a total compound immunoassay with minimal soluble target interference.

An indirect capture assay to address potential interference of shed Her2/neu receptor in a Herceptin PK assay has been reported [58]. As the plate is coated with a mixture of target-engaged and free anti-receptor antibody, both compound forms (bound and free) can theoretically be captured in the assay. Dissociation steps, for example, with acid, are not commonly used for PK assays but could be applied to resolve anti-drug antibody interference and soluble target interference [54,59–62]. Very high affinity anti-idiotypic-specific antibodies can potentially elevate target interference as well, if they can outcompete the target engagement.

Recently, Doucet and Avrameas [63] used enzymatic digestion to degrade interfering targets. Through careful optimization of pepsin digestion conditions, they were able to digest soluble target in samples while retaining the activity of the antibody of interest.

14.5.3 Selectivity Issues

As LBAs provide only an indirect measurement via the assessment of a reporter signal, assay selectivity is essential and numerous articles have been published on that subject. Particularly, diagnostic biomarkers are scrutinized as patient decisions are made on the basis of assay results. Without selectivity and specificity, it is not possible to

determine whether a positive assay signal is actually related to the compound rather than nonspecific binding [64,65]. While it is acceptable to use normal, pooled matrix for calibrators and QCs, selectivity validation (also known as *spike recovery*) should be undertaken with the expected clinical patient population in addition to a normal population. Commercial, diagnostic patient matrices as close to the final population as reasonable can be spiked with the compound and tested as surrogate samples in the assay. Since selectivity issues can occur in unspiked and spiked samples, both require evaluation [66,67]. In particular, samples from patients with underlying autoimmune diseases can be challenging. Human-anti-mouse antibody (HAMA) or human-anti-rabbit antibody (HARA) can potentially bind to assay reagents and result in a false signal. Additives such as heterophilic blocking reagents or animal serum in the assay diluent may address potential selectivity issues [68,69].

14.5.4 Design of Experiments (DOE)

Immunoassays are a classic example of a multifactorial system where different factors influence each other in generating the final result [70]. Traditionally, assays were developed “one factor at a time,” that is, first optimal coating, then blocking, then detection, and so on. Since each condition is dependent on the others, checkerboard systems were applied to optimized coating and detection in conjunction.

The Quality by Design (QbD) initiative from the FDA aims at improving pharmaceutical manufacturing processes [71] using design of experiments (DOE) as a central component for optimization. Although not commonly applied yet, DOE is gaining more attention in the development of various types of immunoassays [72,73]. It can be used at three stages: (i) development (screening of factors and influences), (ii) optimization, and (iii) method robustness [74,75]. While a similar result may be achieved by applying good scientific rationale and experience, DOE can speed up the development of complicated assays and better define the design space of any method. This can be advantageous when accommodating changes (reagents, equipment, environment) or when outsourcing methods in later stages of development to other laboratories. Although DOE can help significantly in assay development and the control of critical factors, it does not replace critical scientific evaluation. Experimental conditions and results need to be analyzed not only from a statistical but also, most importantly, from a scientific perspective. Mathematical models cannot replace biological understanding of what is going on in the assay.

14.6 IMMUNOASSAY VALIDATION

14.6.1 Guidelines, White Papers, and Industry Standards

The application of immunoassays in the safety assessment and clinical development space requires validation to FDA and ICH (International Conference on Harmonization) standards. The chapter titled *Analytical Method Development and Validation in Accordance to the Regulatory Guidelines* of this encyclopedia focuses on this subject in more detail, while below, we present a few basic concepts as they apply to immunoassays. Existing FDA industry guidance is mostly applicable to LC-MS methods, and only limited sections apply to immunoassays [66,76]. A series of white

papers coming out of industry–FDA workshops have been published to help with setting up suitable validation plans [67,77–79], and some key validation parameters are listed in Table 14.2. Generally, LBAs are associated with greater variability and lower accuracy compared to LC-MS methods. Prevalidation experiments including different reagent lots, if available, may avoid surprises during a critical time line.

Ruggedness is an important aspect of validation since the method might have to be transferred to other laboratories [80]. Incubation times, temperatures, and equipment minimally should be evaluated. For assays carried out on the laboratory bench, a room temperature range should be tested. Caution should be applied to changes in the laboratory environment between summer and winter, as humidity and temperature can shift drastically. Ideally, temperature-controlled incubators are used. Some assays may even be sensitive to regular fluctuations from room air handlers, for example, if low affinity IgM antibodies are used whose binding can be influenced by subtle changes.

Once the method is deployed for study support, incurred sample reanalysis (ISR) is a regulatory requirement [81]. Selected samples are reanalyzed and compared to their original test value. The two results should be within a certain acceptance range: typically at least 66% of the retests should be within 30–40% of the original test value.

14.6.2 Best Practices and Common Pitfalls

The complexity of immunoassays can result in seemingly limited method changes having major effects with—in extreme cases—the method falling apart. Thus, extensive method validation, as described earlier, is a prerequisite to generating solid data.

As reagents are essential for immunoassay performance, lot switches should be avoided or minimized. Any new critical reagent lot, for example, capture and detection reagents, needs to be qualified for assay performance at least with accuracy and precision evaluation. Obviously, the uniqueness of immunoassay reagents prohibits the use of “equivalent” reagents; for example, replacement of an anti-species detection antibody from one supplier with an antibody from a different source. Calibrators and QCs should be bridged to previous lots and trending in preparations should be monitored.

As immunoassays can exhibit significant matrix effects, calibrators and QCs should be prepared in a matrix that is reflective of the samples. Attention needs to be paid to details: for example, plasma comes in different varieties (EDTA, Heparin, LiCl) that are not interchangeable. At minimum, the QCs need to simulate actual samples appropriately even if calibrators are prepared in buffer. It is preferable to have both calibrators and QCs prepared in the appropriate matrix.

Large molecules can also display a nonparallelism phenomenon. In this case, study samples, but not necessarily *ex vivo* spiked samples, do not dilute parallel to the calibrator curve [82]. Thus, the calculated sample concentrations differ depending on what dilution is used and multiple dilutions are necessary for each sample.

14.7 APPLICATIONS

It is important to keep the bioanalytical background of each method in mind. Mass spectrometry methods, LBAs, or cell-based bioassays each measure different aspects of a molecule and, thus, will seldom result in comparable data when applied to the same sample [83,84]. While mass spectrometry focuses on purified, small fragments

TABLE 14.2 Validation Parameters for Immunoassays and Expected Performance as Outlined in White Papers^a

Validation Parameter	Expected Assay Performance	Comments
<i>Pharmacokinetic Assays</i>		
Accuracy and precision	20% Bias/CV 25% Bias/CV at the LLOQ	Higher bias/CV acceptance criteria need to be justified with performance data
Dilution linearity	Linear over the applicable sample dilution range	—
Hook (prozone) effect	None	If a hook effect is observed, samples need to be tested at different dilutions
Specificity	No cross-reactivity with other sample components	—
Selectivity	Unspiked: BLOQ Spiked: 20% bias to nominal concentration	—
Robustness	Incubation ranges (time, temperature) do not affect assay	—
Stability, freeze–thaw	20% Bias/CV ^b compared to fresh (unfrozen) baseline	—
Stability, sample handling	20% Bias/CV ^b compared to baseline	Test conditions should cover sample processing during the assay
Stability, sample storage (long term)	20% Bias/CV ^c compared to baseline or nominal	Time should cover the storage time of the samples and beyond for retest options
Stability, reagents	No effects on assay performance as tested by accuracy and precision	—
<i>Immunogenicity Assays</i>		
Accuracy and precision	CV: <20%	High variability affects the normal cutoff
Negative sample cutoff	5% False-positive rate	False positives are used to demonstrate the method that can detect bridging
Positive control sensitivity	Preclinical: 1 µg/mL Clinical: 250–500 µg/mL	—

(continued overleaf)

TABLE 14.2 *(continued)*

Validation Parameter	Expected Assay Performance	Comments
Drug interference	No interference with the expected drug concentrations at the sampling time point	Immunogenicity samples should be taken when little or no drug is on board
Robustness	Loss of sensitivity of samples spiked with positive control ^c : <20% bias	—
Stability, freeze–thaw	Loss of sensitivity of samples spiked with positive control ^c : <20% bias	—
Stability, sample handling	Loss of sensitivity of samples spiked with positive control ^c : <20% bias	—
Stability, sample storage	Loss of sensitivity of samples spiked with positive control ^c : <20% bias	—
Stability, reagents	No effects on assay performance as tested by accuracy and precision	—

Abbreviation: CV, coefficient of variance; LLOQ, lower limit of quantification; BLOQ, below the LLOQ.

^aRefs 62,63,72–74, and 79.

^bAlternative: bias/CV within the method acceptance criteria for accuracy/precision.

^cActual positive samples may be used if available.

of large-molecule digests, bioassays measure functional aspects that often require a complete protein. Depending on the assay format, LBAs might measure subparts or complete and functional analytes.

Different immunoassay formats do not necessarily give similar results [84,85]. The PK parameters of gentamicin derived from RIA and ELISA displayed significant differences in distribution volume, clearance, and half-life despite good bioanalytical correlation [86]. Similar issues were encountered for a PK rituximab assay [87]. Therefore, a thorough understanding of bioanalytical assay background is essential to analyze PK data for large molecules [88].

Interestingly, to characterize abnormal PK bioanalytical data caused by anti-drug antibody responses interfering with LBAs, another immunoassay is used to assess immunogenicity.

14.7.1 Pharmacokinetics

Most if not all biotherapeutics that are marketed established label PK parameters by immunoassay, although the assays rarely are published in detail [31]. Their applications stretch through the drug development process from lead optimization of optimal PK factors, over toxicology studies to assess the compound's safety profile to determination of final PK for the drug label. Basic information derived from bioanalytical immunoassay data includes area under the curve, half-life, and maximum and trough concentrations, while more complex modeling allows for human dose prediction.

As assay development can be time consuming and format switches can lead to changes in measurements, a suitable PK strategy should be considered early enough to avoid spending resources on molecules that will not see the development space. Well-designed and characterized LBAs can sometimes replace *in vivo* studies for early compound screening. Binding of antibodies to the neonatal Fc-receptor (FcRn) can be assessed *in vitro* in a preliminary fashion [89]. Ideally, the assay format remains the same from discovery to clinical so that bioanalytical data can be easily compared. As this is not realistic under a drug development paradigm, a staged approach can be pursued: a basic assay with minimal qualification can be established with commercial reagents initially to carry the molecule through discovery and candidate selection. Limited toxicology studies potentially could still be supported by an early format if it will be validated. In the case of antibody therapeutics, a "generic" anti-human IgG assay sometimes is used throughout the preclinical space [54,90]. Clinical assays, on the other hand, often require compound-specific assays for regulatory compliance, and the assay should be set up more robustly with tight control over assay reagents. Typical development points where bioanalytical assays can be easily switched are before toxicology and clinical programs. Whenever format changes are involved, extrapolations of PK data should be approached, with close consideration of the bioanalytical background.

Since preclinical studies including toxicology are limited in scope and time frame, novel assay technologies can easily be implemented. On the other hand, clinical studies can last for years and often are outsourced as a commodity. Therefore, clinical assay development needs to take equipment and capabilities of contract research organizations (CROs) into consideration. In addition, clinical assays need to be robust enough to withstand reagent lot, operator, laboratory site, and other changes. If necessary, a qualification strategy should be put in place to address these issues. Abbreviated

accuracy and precision evaluations typically are used. To avoid a need for assay redevelopment and bridging in the middle of a clinical program, novel technologies with limited vendor and CRO support should be avoided.

14.7.2 Immunogenicity

Assessment of anti-drug immune responses is gaining increased attention driven to a large extent by the development of anti-erythropoietin- α antibodies in patients receiving recombinant erythropoietin products. Between 1998 and 2000, ~ 13 patients with pure red cell aplasia were reported in France compared to 3 cases in the previous 10 years, ultimately accumulating to 191 cases throughout Europe [91]. This sharp increase was linked to anti-drug antibodies (ADAs) against recombinant erythropoietin α therapy leading to an autoimmune reaction against the patients' endogenous protein. The investigation of the case displayed well how different immunoassay formats and technologies can monitor various aspects of an issue. Initial radioimmunoprecipitation assays and ELISAs were able to detect high affinity IgG only. Later investigations with Biacore revealed the presence of temporary IgM against erythropoietin α [36,92]. These low affinity interactions had been overlooked as the stringent conditions required for ELISA/RIA-washed IgM out before detection. The elimination of secondary detection antibodies also made detection of subclasses other than IgG more feasible.

Bridge immunoassays using labeled drug are a standard format to screen sera for potential, with various technologies being deployed. RIAs and ELISAs are widely spread, but in recent years, MSD-based assays have been favored, as they allow for greater sensitivity [93]. They combine plate-based assay advantages such as high throughput and limited costs with Biacore/SPR advantages including the detection of low affinity IgM antibodies [94].

Immunogenicity assessment should not be based on a single assay, but it requires a more comprehensive strategy [95–98]. The initial screening assays take the variability of an undosed patient population into account to statistically set up a false-positive rate at 5%. This serves as a demonstration that the assay can recognize potential immune responses against the drug among a high background of nonresponders. Since factors other than specific ADA has the potential to form a bridge in the screening assay, caution should be applied when interpreting potential positive results [60]. Confirmation assays are necessary, which often include immunoprecipitation and/or titration of the response. Only a positive signal in screening and confirmation assays can be acknowledged as true ADA in order to be characterized further, for example, through isotyping or evaluation of neutralizing ADA activity. In recent years, replacement of cell-based functional assays by LBAs has been discussed. Although used occasionally, regulatory agencies are not quite convinced by this approach.

During drug development, PK and ADA analyses go hand in hand. A sudden decline of drug concentrations in serum beyond the molecule's expected PK is a good indicator of ADA interfering with PK measurements. Since animal immunogenicity is not predictive of human immunogenicity, the PK profile may be sufficient during early compound development. Toxicology studies eventually will require at least a screening assay to identify potential ADA-positive sera. Clinical immunogenicity assessment is a multilayered approach consisting of a screening assay, a confirmatory assay, and characterization assays. The immunogenicity assay strategy will closely define the patient's immune responses that can be detected [98,99].

14.7.3 Pharmacodynamics

While PK and ADA analyze the effects of the body onto the drug, PD measures the effects of the drug on the body. Biomarkers are increasingly used to assess biotherapeutics efficacy and have become a focus of regulatory agencies throughout the world [100]. The chapter titled *Bioactivation and Reactive Metabolite Assays* of this encyclopedia discusses the subject in more detail. Here, we focus on some bioanalytical examples where immunoassays play a role again.

Three basic assay categories can be distinguished: (i) standard clinical chemistry such as cholesterol and blood protein assessment; (ii) target monitoring, for example, the Her2/neu assays for Herceptin treatment; and (iii) complex multiplex assays monitoring marker panels, for example, for cancer risk prediction (Fig. 14.4).

14.7.3.1 Clinical Chemistry Assays. Changes in blood proteins often are useful biomarkers that do not require extensive assay development. Diagnostic assays are available for most serum components and routinely deployed in clinical reference laboratories using automated immunoanalyzers. These machines can analyze hundreds of patient samples per hour from beginning to end, requiring only sample and reagent loading by the operator. The assay principles are the same as discussed earlier, with an immunosandwich being assembled on a bead surface followed by detection with a label. However, assays specific for drug development often are difficult to establish due to the closed platform design of immunoanalyzers.

14.7.3.2 Target Monitoring. Biotherapeutics typically have a well-defined target pharmacology that can be used to develop biomarkers or companion diagnostics.

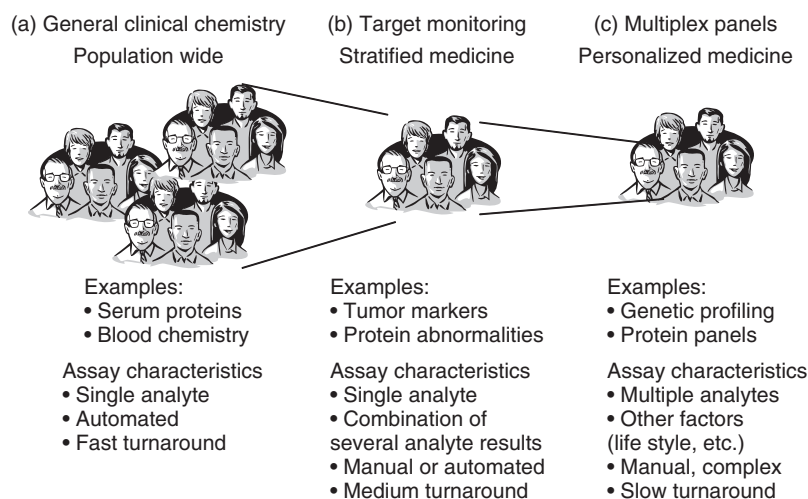


Figure 14.4 Schematic overview of (a) general, (b) stratified, and (c) personalized medicine. Immunoassays measuring standard blood chemistry and components typically are high throughput, automated assays that can be applied to the general population. Assays measuring drug targets can be either manual or automated and are used to identify patient subsets with specific features, for example, potential responders to therapy. Personalized medicine assays tend to be complex and carried out manually to date. Results are different for each person in the ideal case.

Herceptin is a good example for biomarker and assay development. This therapeutic antibody is used to treat Her2/neu-receptor-positive breast cancer [101]. Starting in early clinical trials and all the way to marketing of the drug, the target has been used to select patients who can benefit from the drug [102]. Initially, an immunohistochemistry assay and a fluorescence *in situ* hybridization (FISH) assay were available for patient selection. Recently, ELISAs measuring serum Her2/neu concentrations have become available. It is important to note that the different assay types also differ in their claims: patient selection for drug therapy can be based on FISH and immunohistochemistry results, as these are FDA-approved companion diagnostics [103]. ELISA measures a different aspect of the target pharmacology and is only cleared for cancer monitoring. Similar to the discussion of PK assay formats, Her2/neu biomarker assays are not interchangeable.

14.7.3.3 Multiplex Analysis. The trend to personalized medicine requires more extensive biomarker analysis. Instead of single target measurements, panels of multiple markers are combined to diagnose or predict disease. Most of the research focuses on pharmacogenomics, but some protein panels are being developed as well. When dealing with a small number of protein biomarkers, individual immunoassays can be carried out. But complex panels may be too costly and cumbersome for single assay analysis, and multiplex assays are more efficient. Going beyond single Her2/neu tests, the next generation of assays is on the horizon, combining multiple analytes to a single patient score [104]. The diagnosis of ovarian cancer also exemplifies the development from single biomarker tests over “oligoplex” to multiplex assays. Initially, CA125 serum concentrations were measured by RIA and ELISA to monitor cancer progression [105]. Recently, combinations with HE4 serum concentrations indicated a diagnostic benefit [106], and current trends indicate multiplex immunoassays on the horizon [107–109].

14.7.3.4 LBA Technologies for PD Assays. While some LBA technologies discussed previously can be modified for multiplex analysis, additional options are available to parallelly measure several analytes. MSD offers multiarray plates where up to nine different analytes are spot coated onto a single microtiter plate well. Signals from each spot can be analyzed individually.

Time-resolved ELISA and AlphaLISA can accommodate up to four multiple analytes by using different detection labels in the same assay. Thus, the analytes are only resolved by the detection side of the LBA but not at the capture as in MSD’s plates.

Bead-based arrays as offered by Luminex and its partners as well as by BD Biosciences have advanced into diagnostic applications already. The immunosandwich is assembled with capture reagents immobilized on dye-coded beads combined with a fluorescently labeled detection antibody. The dual signal of bound detection antibody together with each bead dye is measured individually in a flow-through system. Up to 100 analytes can be distinguished through the bead colors on Luminex systems. A number of other multiplex options are available, which are beyond the scope of this chapter [110,111]. The main obstacles for multiplex immunoassays are reagent selectivity and cross-reactivity, as well as potentially increased variability of multiplex test systems.

If patient treatment decisions and/or drug label claims are made based on biomarker analysis, thorough assay validation is required. While many parameters are similar to

PK assay validation (see above), more stringent standards are applied to diagnostic biomarkers as outlined in guidelines by the ICH [112,113].

Since a detailed discussion of PD markers and assays are beyond the scope of this, the above-listed examples represent only a very limited view of a vast and rapidly expanding field.

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15 Micellar Electrokinetic Chromatography

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15.1	Introduction	1
15.2	Migration patterns and resolution in MEKC	2
15.3	Control and prediction of retention in MEKC	5
15.4	Pseudostationary phases in MEKC	6
15.5	Characterization and classification of pseudostationary phases	11
15.6	Detection and sensitivity enhancement	12
15.7	Microfluidic MEKC	15
15.8	Two-dimensional MEKC separations	17
15.9	Conclusions	19
	References	20

15.1 INTRODUCTION

In capillary electrophoresis (CE), mixtures of charged compounds migrate under the influence of an electric field in a capillary tube and are separated according to their differences in charge-to-size ratio. The capillary is typically made of fused silica with typical dimensions of internal diameter (i.d.) $<100\mu\text{m}$ (most commonly used are 50 and $75\mu\text{m}$ i.d.) and length smaller than 100 cm. The advantage of performing electrophoresis in capillary tubes is the effective dissipation of joules heating that is generated as a result of the application of the electric field. A number of key advantages of CE have been demonstrated over the past three decades, including very high resolving power due to high peak efficiency, fast analysis time, small sample size, feasibility and flexibility of manipulating chemical selectivity, broad scope of samples and applications, high throughput capabilities (especially with microfluidic platforms), and so on. There are four main CE-based techniques: capillary zone electrophoresis (CZE), capillary isotachopheresis (CITP), capillary isoelectrofocusing (CIEF), capillary gel electrophoresis (CGE), and electrokinetic chromatography (EKC). These methods differ with respect to migration and separation mechanisms and subsequently the scope of applications. Micellar electrokinetic chromatography (MEKC) is the only CE technique that is capable of separating uncharged and charged compounds [1–5].

Terabe *et al.* [6] extended the scope of CZE to separate uncharged molecules by incorporating charged micelles in the aqueous buffer solution. The technique, named MEKC, uses the same instrumental setup as CE. Charged organized media such as micelles are incorporated in the buffer solution and serve as the separation medium for uncharged solutes. The charged micelles serve as pseudostationary phases as they provide sites of interactions for solutes in the sample mixture (similar to stationary phases in chromatography). For the MEKC separation of uncharged solutes, the pseudostationary phase must be charged; thus, nonionic and zwitterionic surfactants can only be used in mixed micelles along with a charged surfactant.

Hydrophobic interaction plays a predominant role in solute partitioning into micelles and consequently retention in MEKC, while hydrogen-bonding and dipolar interactions contribute to selectivity. MEKC is very similar to reversed-phase liquid chromatography (RPLC) with regard to the scope of applications. This is due to highly similar underlying types of interactions that control retention and selectivity in both techniques. MEKC can be viewed as a hybrid of RPLC and CZE as the separation process incorporates hydrophobic and polar interactions, a partitioning mechanism, and electromigration. However, MEKC offers a combination of unique features of CZE and RPLC such as high efficiencies, rapid analysis, small sample size, small solvent consumption, and versatility of incorporating chemical selectivity in the separation process. It offers higher efficiency than RPLC and capillary electrochromatography (CEC). The number of theoretical plates in an MEKC separation can be 10 times or higher than that in RPLC. Another major advantage over conventional chromatographic techniques as well as CEC is the flexibility and ease of changing chemical composition of the pseudostationary phases. For example, the type and/or composition of the micellar solution can be easily modified or replaced by simply rinsing the capillary with a new type of pseudostationary phase. The equilibration times are often very rapid. This inherent flexibility of controlling key parameters leads to enhanced separations and greatly facilitates the process of method development. MEKC is mostly suited for analysis of small molecules and is the only CE technique that is capable of separation of both charged and uncharged compounds as well as structural, positional, and chiral isomers with applications in analysis of pharmaceutical, clinical, environmental, biological, and chemical samples [1–5].

15.2 MIGRATION PATTERNS AND RESOLUTION IN MEKC

15.2.1 Uncharged Solutes

Figure 15.1 illustrates the migration pattern and separation of two hypothetical uncharged solutes in MEKC using a fused silica capillary with negatively charged walls. The mixture is introduced at the anode inlet of the capillary and migrates with the electroosmotic flow (EOF) toward the cathode, where the detector is located. The negatively charged micelles migrate toward anode in the electric field at an electrophoretic mobility (μ_{mc}) that is proportional to their charge-to-size ratios. Anionic micelles migrate in the opposite direction of the EOF in an uncoated capillary. Typically, the EOF velocity is stronger than the electrophoretic velocity of anionic micelles under “normal” conditions (e.g., an uncoated capillary and pH > 6). As a result, the anionic micelles are carried toward the cathode. Using cationic micelles, the capillary wall is coated with the positively charged surfactants and often times it

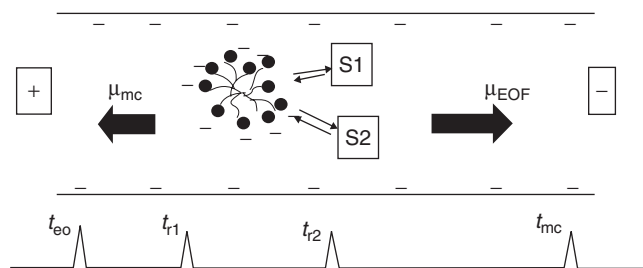


Figure 15.1 Schematic diagram of migration of uncharged compounds in MEKC with anionic pseudostationary phases. Separation of solutes S1 and S2 is achieved due to their differential partitioning into the pseudostationary phase. The uncharged solutes are eluted within an elution window (t_{mc}/t_{eo}).

leads to a reversal in the direction of the EOF. It is therefore necessary to reverse the polarity of the electrodes in the CE setup to ensure the elution of the cationic micelles and consequently the uncharged solutes through the detection window.

The two uncharged compounds, S1 and S2, are separated due to differences in their water–micelle partition coefficients, K_{mw} . Charged micelles migrate in the electric field and elute at a certain time (t_{mc}). Subsequently, MEKC has a limited migration window between the elution time of an unretained solute, t_{eo} , and the migration time of micelles, t_{mc} (Figure 15.1). The elution window is defined by two types of solutes. First are the unretained solutes that do not interact with micelles ($K_{mw} \sim 0$) spend all of their migration times in the bulk aqueous phase and migrate at the electroosmotic mobility. These are typically uncharged polar molecules such as methanol or acetonitrile that are EOF markers and elute at t_{eo} . The other end is defined by the elution of analytes that interact so strongly with the micelles ($K_{mw} \sim \infty$) that they spend all of their migration time with micelles. The t_{mc} markers are typically very hydrophobic compounds that are sparingly soluble in the aqueous media, reported examples being Sudan III and dodecanophenone. The elution times for these analytes coincide with the micellar migration time, t_{mc} . The existence of an elution window limits the peak capacity in MEKC as all uncharged solutes are to be separated between the migration time of an unretained solute, t_{eo} , and a fully retained solute, t_{mc} . The size of the elution window varies with the type and concentration of the charged pseudophases, the presence of organic modifiers, mixed micelles, or capillary wall modification [1–5].

Like chromatography, retention factor in MEKC is defined as the ratio of the number of moles of solute in micellar pseudostationary phase, n_{mc} , and that in bulk aqueous phase, n_{aq} . Retention factor is directly proportional to the micelle–water partition coefficient, K_{mw} , and phase ratio, Φ as follows:

$$k = \frac{n_{mc}}{n_{aq}} = K_{mw} \Phi \quad (15.1)$$

Retention factor in MEKC can be determined from migration time data using the following equation:

$$k = \left\{ \frac{t_r - t_{eo}}{t_{eo} \left[1 - \left(\frac{t_r}{t_{mc}} \right) \right]} \right\} \quad (15.2)$$

This is very similar to the equation for retention factor in conventional chromatography with the exception of the additional term $(1 - t_r/t_{mc})$ in the denominator. This term indicates the existence of an elution window due to the fact that the “stationary phase in MEKC is actually mobile.” If t_{mc} approaches infinity (i.e., stationary micelles), the extra term in the denominator is omitted and the retention factor equation becomes the same as that in conventional chromatography.

15.2.2 Ionizable Solutes

In addition to partitioning into micelles and migrating at the micellar mobility, charged compounds possess electrophoretic mobilities of their own in the bulk aqueous solvent. As a result, the observed retention time also includes the time that solute migrated electrophoretically in the bulk aqueous phase. Migration patterns for ionizable solutes (weak acidic and basic compounds) are considerably more complicated than uncharged compounds in MEKC due to existence of acid–base and ion-pairing equilibria in the aqueous solution and [7–9]. For separation of ionizable solutes in MEKC, control of buffer pH (in addition to type and concentration of micelles) would be critical in method development and optimization of separation. Quantitative models have been developed that would allow prediction of migration patterns of ionizable solutes and subsequently rapid optimization of separation conditions based on limited initial experiments [9].

15.2.3 Resolution in MEKC

Equation 15.3 shows the resolution equation for uncharged solutes in MEKC:

$$R = \left(\frac{N^{1/2}}{4} \right) \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k_2}{1 + k_2} \right) \left(\frac{1 - \left(\frac{t_{eo}}{t_{mc}} \right)}{1 + \left(\frac{t_{eo}}{t_{mc}} \right) k_1} \right) \quad (15.3)$$

As can be seen, Equation 15.3 has the same format as that for conventional chromatography as it indicates that resolution depends on three terms related to efficiency, selectivity, and retention [6]. The fourth term is unique to MEKC that represents the existence of an elution window.

Again, if micelles were truly stationary (i.e., $t_{mc} \sim \infty$), the fourth term would drop out and the equation would be identical to that in conventional chromatography. The size of the elution window has a significant impact on MEKC separations. Likewise, peak capacity, n , can also be increased with wider elution windows as follows:

$$n = 1 + \frac{\sqrt{N}}{4} \ln \frac{t_{mc}}{t_{eo}} \quad (15.4)$$

Like chromatography, better resolution is achieved at higher efficiency. The disadvantage of the limited elution window is mostly compensated by the large number of theoretical plates that are routinely achieved in MEKC. The concentration and type of surfactant (pseudostationary phase) should be optimized to enhance resolution due to their influence on at least three of the parameters (k , α , and t_{mc}/t_{eo}).

15.3 CONTROL AND PREDICTION OF RETENTION IN MEKC

The primary role of surfactant concentration is to adjust retention factor to within the optimum range in order to achieve better resolution. The relationship between retention factor, k , and surfactant concentration can be described as in the following equation [6]:

$$k = \frac{v(C_{\text{sf}} - \text{CMC})}{1 - v(C_{\text{sf}} - \text{CMC})} K_{\text{mw}} \quad (15.5)$$

where the first term is the phase ratio, ϕ , defined as the volume of the micellar pseudostationary phase over that of the bulk aqueous phase (i.e., $V_{\text{mc}}/V_{\text{aq}}$) and is related to v (surfactant molar volume), C_{sf} (total surfactant concentration), and critical micelle concentration (CMC). K_{mw} is the partition coefficient of a solute between an aqueous phase and micelles. In general, retention factor increases linearly with surfactant concentration, as Equation 15.5 can be simplified to, because the denominator approximately equals to one under typical operating surfactant concentrations as follows:

$$k = K_{\text{mw}}v(C_{\text{sf}} - \text{CMC}) \quad (15.6)$$

A unique advantage of MEKC is that the phase ratio can be determined accurately (first term in Eq. 15.5) and will not change from capillary to capillary or with time as is the case for chromatography columns. The phase ratio in MEKC is related to two intrinsic properties of the surfactant: partial specific molar volume (v) and CMC, as well as surfactant concentration. This provides a unique opportunity to predict retention factor in MEKC for solutes with known micelle–water partition coefficients without any prior experiments. Unfortunately, there is a lack of extensive and reliable K_{mw} databases.

Additivity-based approaches have been developed to calculate micelle–water partition coefficients from solute structure [10,11]. Micelle–water partition coefficients can be described as the sum of the partition coefficients of the constituent atomic/molecular fragments, measured by fragmental constant values, f_i , as well as correction factors to account for various “intramolecular effects” that cause deviations from the predicted partition coefficients as follows [10,11]:

$$\log K_{\text{mw}} = \sum_{i=1}^n a_i^* f_i + \sum_{i=1}^m k_i^* C_m \quad (15.7)$$

where f_i is the fragmental constant and a_i is the frequency of occurrence of each fragment found in the solute structure, k_i is a constant that varies with the type of “intramolecular effect.” This equation is based on the Rekker’s fragmental constant approach for calculation of octanol–water partition coefficients. The fragmental constants of 41 fragments in the sodium dodecylsulfate (SDS) micelle system have been determined. Using Equation 15.7, the SDS micelle–water partition coefficients and subsequently their retention factor, and retention times for 170 aromatic solutes were successfully predicted in MEKC using SDS micelles. The linear regression of the predicted versus observed retention times resulted in $R^2 = 0.971$ and slope of 0.992.

15.4 PSEUDOSTATIONARY PHASES IN MEKC

In addition to micelles made of synthetic surfactants above their CMCs [1–6], a wide variety of pseudostationary phases have been used that has significantly extended for the applications of the technique. Some examples of pseudostationary phases are ionic polymers [12] cyclodextrins (CDs) and other chiral reagents [13], fullerenes and carbon nanotubes (CNTs) [14], vesicles [15,16], liposomes [17–20], dendrimers, and others.

15.4.1 Micellar Pseudophases

Micelles have been the most commonly used pseudostationary phase in EKC. Anionic alkyl chain surfactants, especially SDS, have been the most widely used surfactant type. The popularity of SDS can be attributed to the high aqueous solubility, low CMC, low Kraft point, low UV molar absorptivity even at low wavelengths, availability, and cost. Serendipitously, SDS might provide the “right” type of selectivity for many solute mixtures. Anionic alkyl chain surfactants, especially SDS, have been used in a variety of applications such as MEKC separations of phenols and other acidic solutes, pharmaceutical amines, organic solvents, keto compounds and carbohydrate derivatives, fungicide and phytotoxin, caffeine metabolites in human urine, DNA adducts, pharmaceuticals such as antibiotics, vitamins, sulfonamides, xanthines, peptides and proteins, organometallic and ligands, amino acids, sulfur and nitro compounds in environmental applications, flavonoids in foods, and nucleic acids constituents in body fluids [1–5].

As an alternative to SDS, the use of bile salt surfactants in MEKC separations has become popular due to their different selectivities as compared to SDS. Bile salt surfactants have very different aggregation properties and structures from SDS micelles. They can tolerate relatively higher concentration of organic modifier (30% organic solvent) than SDS micelles without a disruption of their structural integrity. Bile salts have been used in a variety of MEKC separations such as steroids, amino acids, benzodiazepines, synthetic colors, bioactive compounds, environmental analysis, proteins and peptides, bilirubin, organic acids, and hydrophobic compounds, including polycyclic aromatic hydrocarbons, chiral and diastereoisomers, and anti-HIV agents [1–5].

The use of fluorinated surfactants in MEKC separations has been very limited. This has been mostly due to the lack of availability of MEKC compatible fluorocarbon surfactants in high purity. Ye *et al.* [21] reported the first application of the fluorocarbon micelles of lithium perfluorooctane sulfonate (LiPFOS) for the MEKC separations of a group of small peptides and reported very different selectivity and elution patterns as compared to SDS. LiPFOS micelles are stronger hydrogen-bond donor (HBD) than other micellar systems that have been studied thus far [22,23].

Cationic surfactants generally interact with the negatively charged silica capillary wall and reverse the direction of the EOF. As a result of the reversed EOF, the polarity of the electrodes would have to be reversed in order to elute solutes through the detector window [1–5]. Cetyltrimethylammonium bromide (CTAB) micelles are among the strongest hydrogen-bond acceptors (HBAs), thus have a very different interactive behavior than SDS or LiPFOS micelles [22,23]. The usefulness of cationic surfactants has been explored in a number of MEKC applications. A few examples are phenolic carboxylic acids, charged molecules with nearly identical electrophoretic mobilities such as bis(amidinohydrazones), nucleic acid constituents, cocaine and illicit drugs, adrenergic blocking agents, glycosylated compounds, and aromatic hydrocarbons [1–5].

Surfactants with a zero net charge such as nonionic and zwitterionic surfactants can be used along with an ionic surfactant as mixed micelles. In order to separate uncharged molecules in MEKC, charged surfactants must be used. They can also have a great influence on the MEKC separation of charged molecules. Both nonionic and zwitterionic surfactants have been used alone for the separations of charged compounds such as amino acids and polypeptides. Nonionic surfactants and mixtures of anionic/nonionic surfactants have been shown to provide different selectivities, reduced currents, and changes in elution window sizes as compared to anionic surfactant systems. Zwitterionic surfactants have been used alone and in conjunction with SDS in MEKC. Because these surfactants do not increase the conductivity of the buffer, they can be used at high concentrations, while still allowing the use of high voltages and large i.d. capillaries.

15.4.2 Polymeric and Nanoparticle Pseudostationary Phases

At least five different classes of polymeric phases have been reported, including polymer micelles where surfactant monomers are polymerized and covalently bonded together [12], cascade macromolecules such as starburst dendrimers, and ionic block copolymers [1–5], fullerenes, and nanoparticles [24,25]. Figure 15.2 illustrates the structures of some polymeric surfactants. Initially, polymeric pseudostationary phases (PSP) were used for the MEKC separation of highly hydrophobic compounds that would require use of relatively high concentrations of organic modifiers in the aqueous buffer. One of the shortcomings of the micellar aggregates is their incompatibility with the presence of higher than 20–30% organic cosolvents. In addition, the high molecular weight of the PSP makes them more compatible with MS detection, which

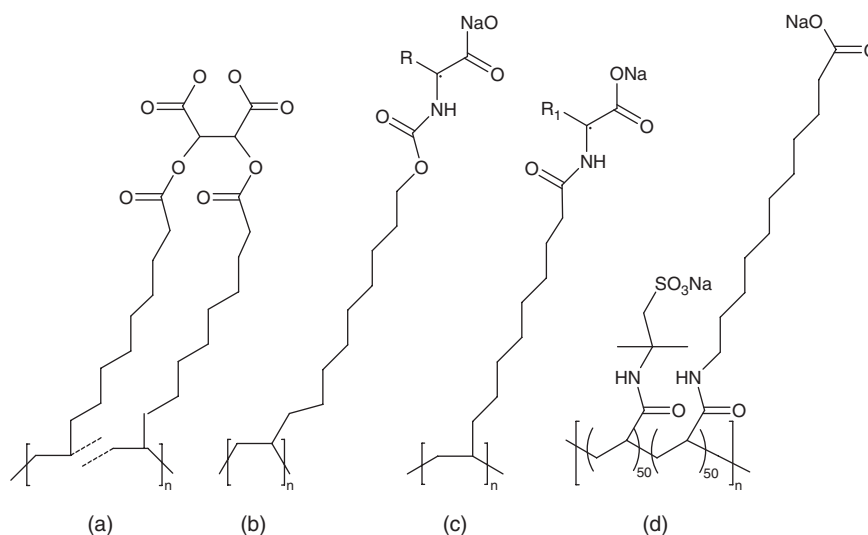


Figure 15.2 Structures of polymeric surfactants. (a) Gemini surfactant polymer poly(sodium diundecenyl tartrate), (b) poly(sodium undecenoxy carbonyl amino acidate), (c) poly(sodium undecenyl amino acidate), and (d) poly-(sodium 2-(acrylamido)-2-methylpropanesulfonate/11-(acrylamido)-undecanoic acid). *Source:* Palmer CP. *Electrophoresis* 2007;28:164–173; reprinted with permission.

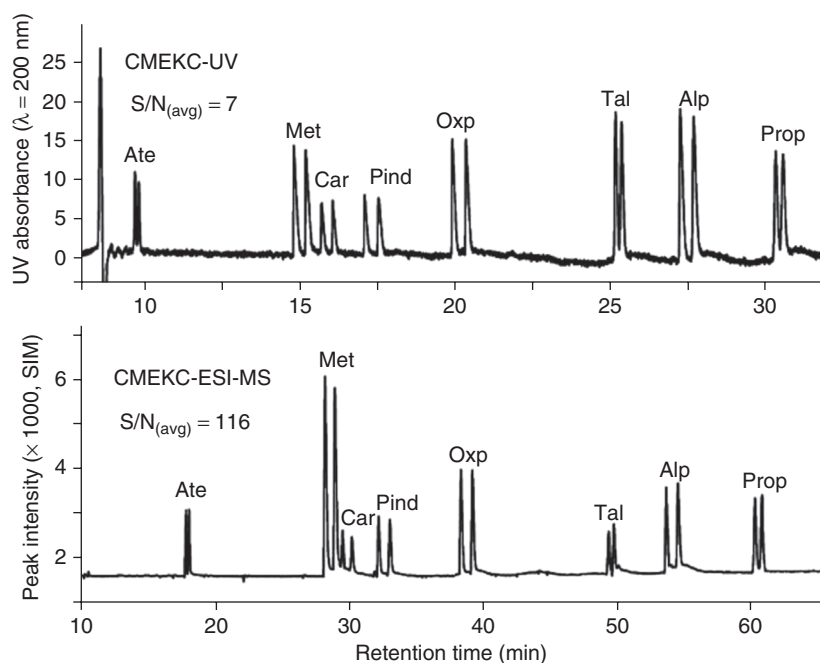


Figure 15.3 MEKC separation of β -blockers with UV and MS detection; MEKC buffer composition: polymeric pseudophase poly-L-SUCL at 15 mM in 25 mM ammonium acetate/25 mM triethylamine at pH 8.0. Peak i.d. Ate, atenolol; Met, metoprolol; Car, carteolol; Pind, pindolol; Oxp, oxprenolol; Tal, talinolol; Alp, alprenolol; and Prop, propranolol. *Source:* Akbay C, Rizvi SAA, Shamsi SA. *Anal. Chem* 2005;77:1672–1683; reprinted with permission.

is a significant potential advantage. Figure 15.3 illustrates the MEKC separation of an enantiomeric mixture of β -blockers with both the UV and MS detection using a chiral PSP (poly-L-SUCL) (poly(sodium N-undecenoxy carbonyl-L-leucinate)). Some PSP are not fully compatible with UV detection and lower efficiencies could be observed with certain PSP due to higher polydispersity and slow mass transfer kinetics. Another potential advantage of PSP is the possibility of extending the range of selectivity through control of the chemical composition and structure of the polymers (see below) [1–5,12].

The use of nanoparticles as PSP in MEKC has only been investigated in recent years. For examples, applications of polymeric, molecularly imprinted, silica, gold nanoparticles as well as CNTs have been reported. The CNT would have to be functionalized (e.g., with carboxylate groups) or be dispersed in SDS solutions to be used as PSP in EKC. Several recent publications have reported the applications of CNT and fullerene PSP for the separation of caffeine and theobromine, purine and pyrimidine, DNA fragments, anti-inflammatory drugs, enantiomers, and polyaromatic hydrocarbons (PAH) [26–31]. An example of the application of CNT is shown in Fig. 15.4.

Suspensions of silica nanoparticles in EKC have been reported for the separation of phenolic compounds, polyaromatic hydrocarbons, steroids, and aromatic acids [26–33]. Gold nanoparticles have been used in polymeric solutions of poly(ethylene oxide) (PEO) for the separation of double-stranded DNA fragments up to 48.5 kbp [34,35]. Note that the separation mechanism in this case is molecular sieving, which is different

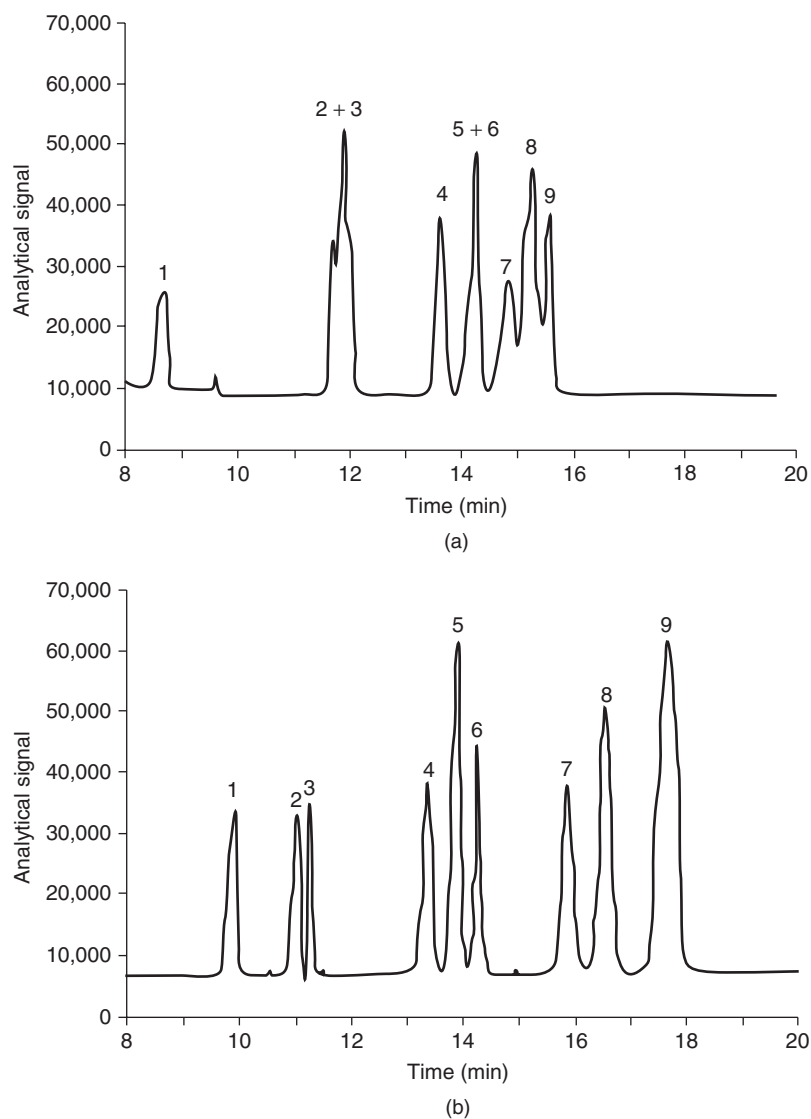


Figure 15.4 EKC separation of chlorophenols with carbon nanotubes. In (a) absence and (b) presence of surfactant-coated single-walled carbon nanotubes (SC-SWNTs) (3.2 mg/L). The background electrolyte (BGE) was 25 mM sodium hydrogen phosphate, 15 mM sodium tetraborate, 100 mM SDS, and 6% ethanol at pH 7.95. Other operating conditions: capillary 30 cm_75 mm i.d., 15 kV, 201 C. Peaks: (1) 2,6-dichlorophenol; (2) 3,5-dichlorophenol; (3) 2,3-dichlorophenol; (4) 2,5-dichlorophenol; (5) 3,4-dichlorophenol; (6) 2,3,5-trichlorophenol; (7) 2,4,5-trichlorophenol; (8) 2,3,6-trichlorophenol; and (9) pentachlorophenol. *Source*: Ref. 29; reprinted with permission.

from EKC, where differential solute partitioning into a pseudostationary phase is the main driving force for the separation process.

15.4.3 Mixed Micelles

For certain complex mixtures of structurally similar solutes, one might not be able to find a suitable surfactant type that provides adequate resolution. This is typically due to a lack of selectivity and/or narrow elution window. In these situations, the use of mixed micelles (or mixed surfactant–polymer) can lead to enhanced separations. Mixing surfactants with different interactive properties can lead to great changes in selectivity for a given mixture. Selection of optimum composition would then be crucial in improving the quality of separation. The size of the elution window for certain mixed micellar systems is often larger than that for the individual constituent surfactants. Retention factor and selectivity change with the mole fraction of surfactants in the mixed micelles. Efficiency of the mixed micellar systems is often not much different from those of the single surfactant systems; however, in a few cases, loss of efficiency has been observed. Various combinations of different surfactants such as bile salt/anionic, anionic/zwitterionic, cationic/zwitterionic, fluorocarbon/anionic, anionic/nonionic, cationic/cationic, anionic/cationic, bile salt/bile salt, and nonionic/nonionic (for charged solutes) have been used in MEKC [1–5].

15.4.4 Organic Modifiers

Modifiers such as organic solvents, CDs, and urea are typically incorporated in the aqueous buffers of MEKC in order to reduce the retention factors of strongly bound solutes to micelles. Their presence can also lead to wider elution ranges and/or higher selectivity. Their use in MEKC separations, however, has been mostly for improving the separations of hydrophobic compounds that interact strongly with micelles and elute at or near migration time of micelles. The main role of organic modifiers in MEKC has been to reduce retention factors of highly hydrophobic solutes to within or near the optimum range. Typically, inclusion of organic solvents leads to an increase in the size of the elution window. As a result, resolution in MEKC can be enhanced at the expense of longer analysis times. The concentration of organic solvents would have to be limited (typically $\leq 20\%$) in order to maintain the integrity of micelles. The use of polymeric phases will provide an opportunity to investigate the role of organic solvents over a wider range of concentrations.

In addition to typical organic solvents, the use of other modifiers such as urea and glucose has also been reported. Urea reduces the interactions of hydrophobic compounds with micelles by increasing their solubility in the aqueous solutions. Retention factors of hydrophobic compounds in micellar solutions are therefore decreased dramatically. The size of the elution window has also been shown to increase with the addition of urea to MEKC systems. Another type of modifier that has been used in MEKC is CDs. In CD-modified MEKC (CD-MEKC), the hydrophobic cavity of CDs provides an alternative site of interaction to micelles for the hydrophobic solutes. Since uncharged CDs migrate at the EOF velocity and in the opposite direction of anionic SDS micelles, the net retention factor of solutes decreases in the presence of CDs. As a result, hydrophobic solutes that would otherwise elute with micelles can be better

separated. In addition, CDs introduce a shape selectivity effect that is beneficial for the separation of structural isomers [1–5].

15.5 CHARACTERIZATION AND CLASSIFICATION OF PSEUDOSTATIONARY PHASES

The first attempt for characterization and classification of the chemical selectivity of pseudostationary phases in MEKC was reported in 1990's using linear solvation energy relationships (LSERs) [36–38]. Since then, the LSER methodology has been extensively used for achieving a better understanding of the underlying interactions that control retention and selectivity of various types of micelles, block copolymers, polymerized micelles, mixed micelles, organically modified micelles, as well as vesicular and liposomes. The retention factor, k , in MEKC can be described by the general LSER model as follows:

$$\log k = c + vV + bB + aA + sS + eE \quad (15.8)$$

The LSER models quantitatively describe the contributions of hydrophobic/dispersion (vV term), type A hydrogen bonding (bB term, micellar phase as a HBD), type B hydrogen bonding (aA term, micellar phase as a HBA), dipolarity (sS term), and polarizability (eE term). Solute properties are described by the McGowan volume (V), HBA strength (B), HBD strength (A), dipolarity (S), and polarizability (E). The interactive properties of the micellar pseudophase are described by coefficient v , a measure of cohesiveness and dispersion interactions, coefficient b that is a measure of the HBD strength, coefficient a that is proportional to the HBA strength of the micelles, s that describes dipolarity, and e that is a measure of the affinity of micelles for σ and π electrons of solutes, and c is the regression constant. The LSER analysis clearly indicates that while the hydrophobic interaction (as quantified by the vV term) is the main driving force for retention in MEKC, the main source of selectivity variations between pseudophases is their hydrogen-bonding donor strength (the b coefficient) followed by hydrogen-bonding acceptor strength and dipolarity/polarizability interactions.

Fu and Khaledi have developed a novel micellar selectivity triangle (MST) to characterize and classify the chemical selectivities of pseudophases in EKC [22,23]. The MST scheme was devised based on the LSER parameters for construction of new, normalized scales for hydrogen-bonding (donor and acceptor) strengths, dipolarity, and polarizability of pseudophases and MST plots can be used to readily visualize the relative strength and classification of pseudophases based on their interactive properties. Figure 15.5 illustrates the comparison of the selectivities of over 70 pseudophases in terms of their hydrogen-bonding basicity, hydrogen-bonding acidity, and dipolarity and shows that the MEKC pseudophases clustered into four general groups. Group A consists of the SDS micelles and its analogs plus some mixed micelles containing SDS; group B consists of stronger HBD pseudophases such as LiPFOS micelles in single and mixed format with other surfactants and organic modifiers; group C consists of HBAs such as CTAB micelles, bile salts, microemulsions, as well as biphasic partitioning system of octanol–water; and group D, which is slightly stronger acceptor

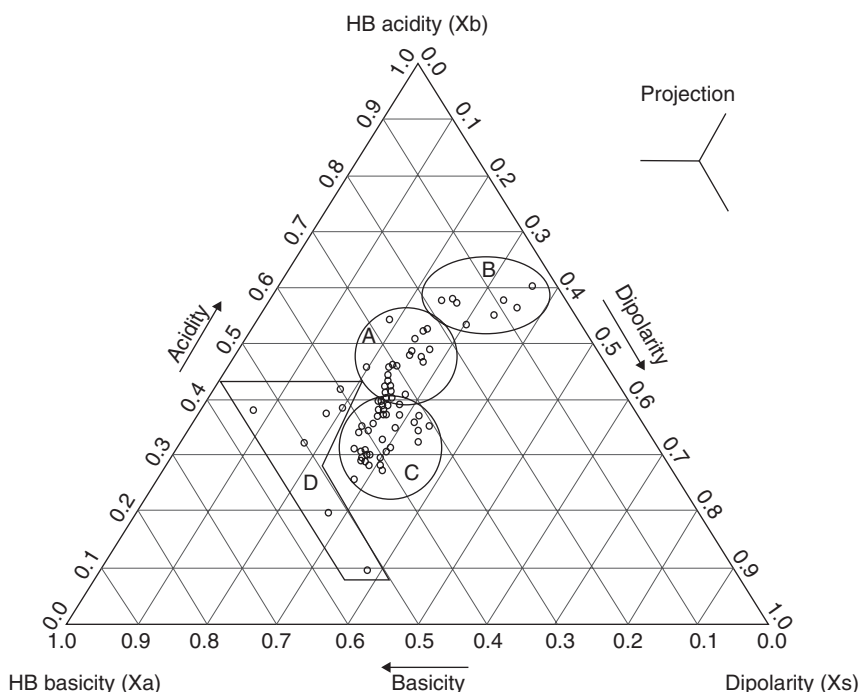


Figure 15.5 Micellar selectivity triangle for characterization and classification of various micellar, polymeric, vesicular, and mixed pseudophases. *Source:* Ref. 22; reprinted with permission.

and has wider dipolarity range, consists of polymeric micelles of AGENT as well as some CTAB-modified micelles.

An advantage of MEKC is the feasibility and flexibility of controlling selectivity through adjusting the composition of the pseudophase. The MST scheme facilitates the selection of optimum pseudophases based on their selectivities. For example, Fig. 15.6 shows that solvation properties of SDS, CTAB, and LiPFOS micellar systems could be significantly changed through addition of organic modifiers hexafluoroisopropanol (HFIP) (a strong HBD) and pentanol (HBA). The two organic modifiers, PeOH and HFIP, mainly affect the hydrogen-bonding capability of micelles; thus, it is interesting to monitor the migration behaviors of HBA and HBD solutes in the selected micellar phases. Naturally, all HBD solutes have measurable HBA capability as well. Figure 15.7a–c shows the changes in retention patterns on addition of HFIP and PeOH to SDS micelles. As illustrated, on the addition of HFIP, the SDS micelles become a stronger HBD and have stronger interaction with solutes that have HBA groups, while addition of PeOH would enhance the HBA strength of the micelles and result in stronger interactions with HBD solutes.

15.6 DETECTION AND SENSITIVITY ENHANCEMENT

The detection systems for MEKC (and CE in general) are very similar to those used in HPLC. Absorbance detection is the most commonly used due to convenience, capability of detecting a wide range of compounds, feasibility of operation for on-column

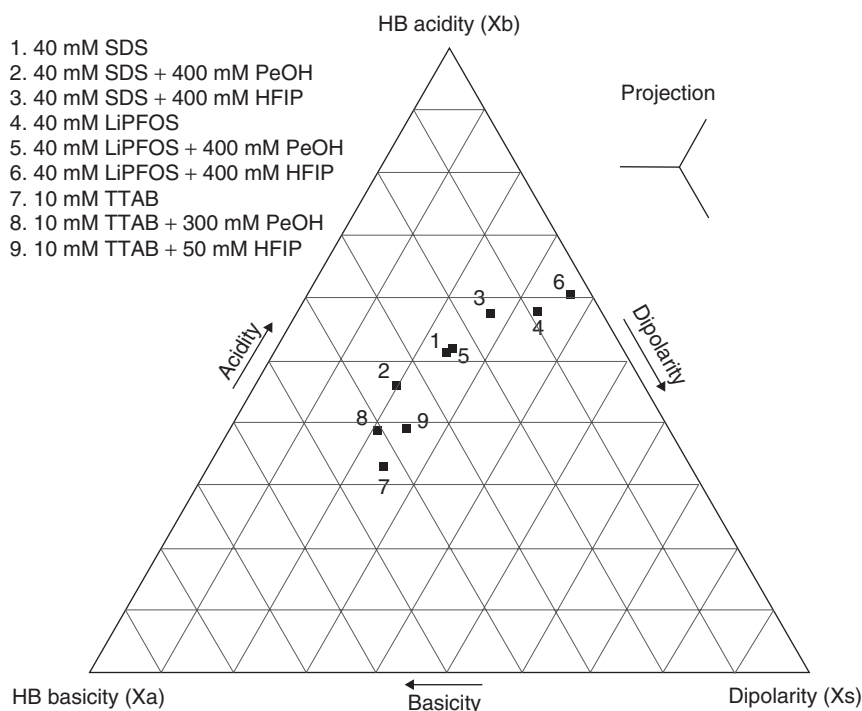


Figure 15.6 MST for three micelles and six modified micellar systems. *Source:* Ref. 22; reprinted with permission.

detection, robustness, and good linearity. However, due to the exceedingly small peak volume (in nanoliter range), peaks must be detected in the capillary (i.e., on-column detection). Subsequently, the detection sensitivity of absorbance detection is modest (in micro-molar range) due to small light path length (which is the capillary diameter, typically 50–75 μm) and very small injection size. Detection path length (thus sensitivity) could be enhanced through the use of bubble cell [1–5]. Laser-induced fluorescence (LIF) has significantly better detection sensitivity (in subnanomolar range), albeit narrower scope of application as fewer classes of compounds are inherently fluorescent. Attachment of various fluorophore moieties to the parent compound would extend the range of applications of LIF detection of wider classes of compounds. Electrochemical detection is readily compatible with the CE techniques and offer great sensitivity for certain groups of compounds such as neurotransmitters. Unfortunately, MEKC has limited compatibility with MS detection due to the presence of high concentrations of surfactants in the buffer solution. The use of partial filling method, fluorinated surfactants, or polymeric pseudophases has been reported to improve the situation [1–5].

Owing to poor concentration sensitivity (LOD) in MEKC (and CE in general), on-line concentration of samples before the separation process is necessary for trace analysis. The two widely reported methods for sample preconcentration are based on stacking and sweeping mechanisms [1–5,39].

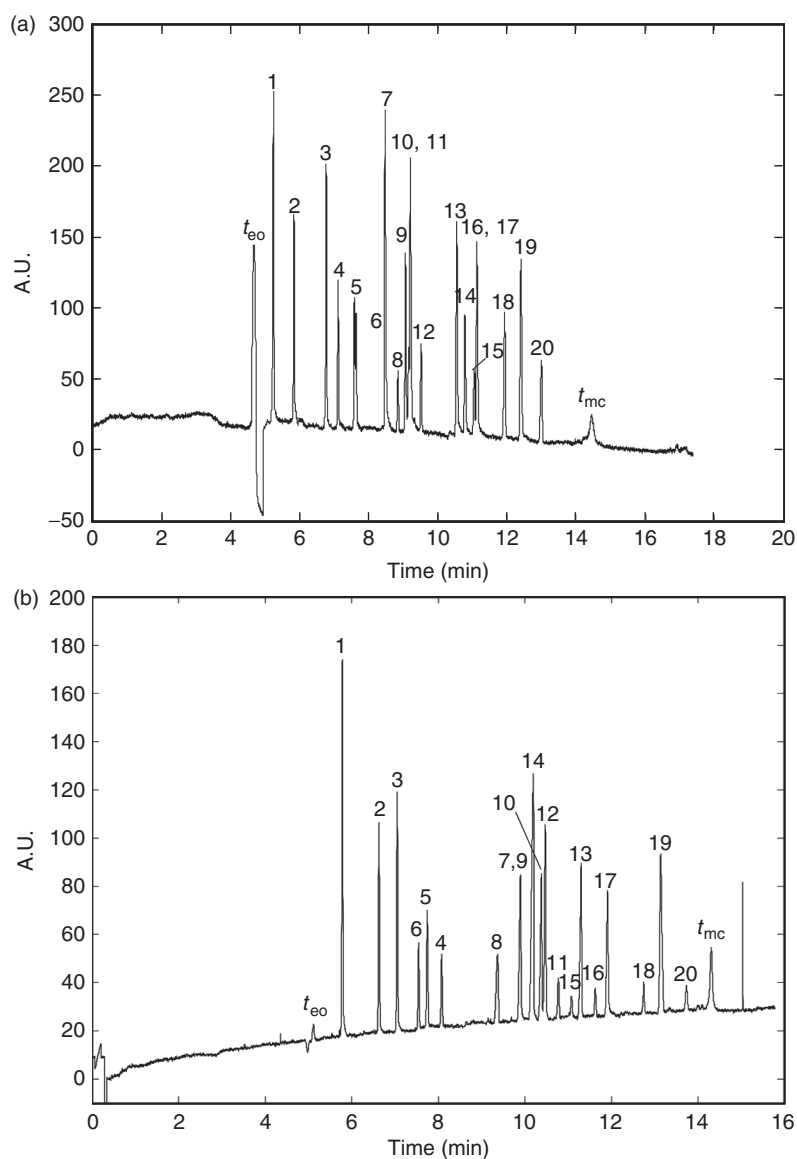


Figure 15.7 Chromatograms of the separation of 20 aromatic solutes composed of hydrogen-bond donors (HBDs): (1) resorcinol; (2) phenol; (7) 3-chlorophenol; (10) 4-ethylphenol; (12) 3,5-dimethyl phenol; and (15) 4-iodophenol. Hydrogen-bond acceptors (HBAs): (3) phenethyl alcohol; (5) nitrobenzene; (6) acetophenone; (8) methylbenzoate; (9) benzyl chloride; (14) 4-chloroacetophenone; and (16) methyl-2-methyl benzoate. Nonhydrogen-bond donors (NHBDs): (4) benzene; (11) toluene; (13) chlorobenzene; (17) ethylbenzene; (18) 4-chlorotoluene; (19) naphthalene; (20) propylbenzene. Micellar phases IDs: (a) 40 mM SDS, (b) 40 mM SDS/400 mM MPeOH (4.34% v/v), and (c) 40 mM SDS/400 mM HFIP (4.16% v/v). *Source:* Ref. 22; reprinted with permission.

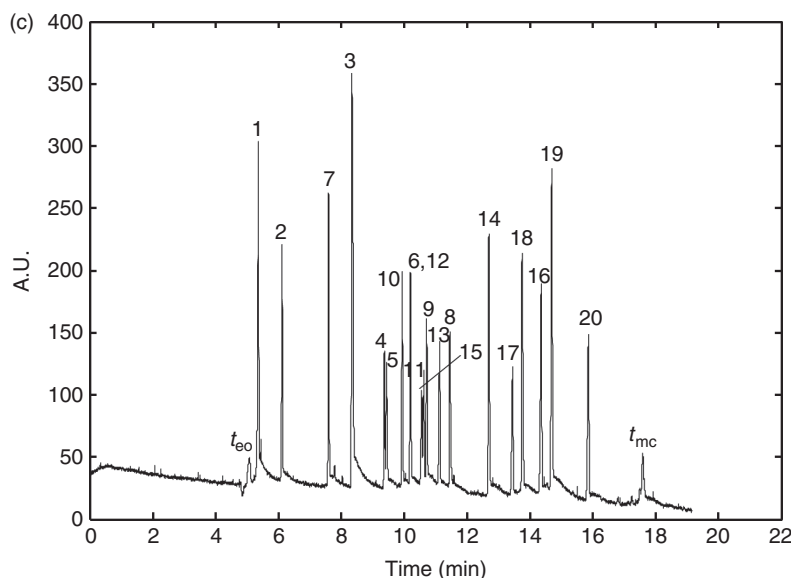


Figure 15.7 (continued)

15.7 MICROFLUIDIC MEKC

Microfluidic platform offers unique advantages over capillary tubes in MEKC (and CE) separations. The exceedingly smaller dimensions of separation channels in microchips as compared to capillary tubes are much more effective in dissipation of joules heating, thus allowing the use of much higher electric field strength and require significant reduction in the sample injection size. This results in higher separation efficiencies as well as significant reduction in analysis times from minutes to seconds. In addition, the microchip platform greatly facilitates designing parallel analysis (or separation) for increasing sample throughput, integration of sample manipulation and separation, as well as combining orthogonal separation techniques for two- or multidimensional separations for enhancement of peak capacity for resolving complex samples [40].

Ramsey's group first reported MEKC separations on microchips and investigated the effects of geometry and dimensions of separation channels. Using the straight channel design would allow a short separation length where high field separations can be performed. For example, a mixture of three coumarins were separated in <3 s using an electric field strength of 500 V/cm over a distance of 1.3 cm [41–44]. While the shorter separation channels and higher electric field strengths are desirable for achieving very fast separations of relatively simple mixtures, longer separation channel lengths are needed to generate larger absolute efficiencies for more complex and challenging mixtures. Increasing channel lengths on microchips can be achieved by incorporating turns in channel designs such as the use of serpentine and spiral geometries. However, it was determined that the turns in the separation channels could introduce additional band dispersion through “racetrack effect” as the molecules on the inside wall travel a shorter distance than those on the outer walls. New changes in microchip design

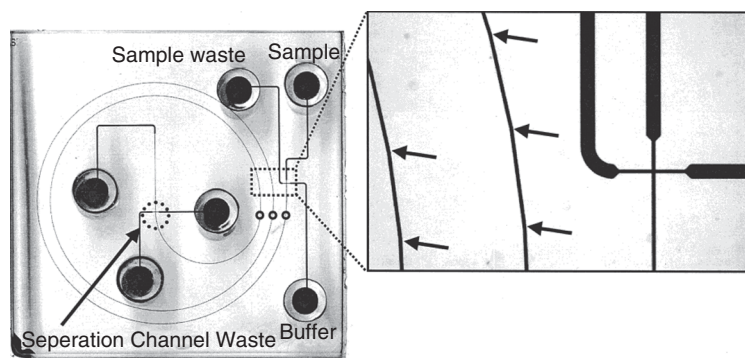


Figure 15.8 Image of a microchip with a spiral separation channel. The reservoirs on the interior of the spiral were replaced by a single reservoir located at the injection cross (dashed circle) for the experiments reported here. The small circles are the locations of the detection points from outside to inside 0.25, 11.9, and 22.2 cm. The inset is a magnification of the injection cross and parts of the spiral channel. The buffer, sample, and sample waste channels are 219- μm wide (half-depth) for all their length except for the last 500 μm before the injection cross where they narrow to only 40 μm (half-depth). The separation channel was 24.9-cm long and 40- μm wide (half-depth). The black arrows on the inset indicate the vertexes of the polygonally shaped channels. *Source*: Ref. 43; reprinted with permission.

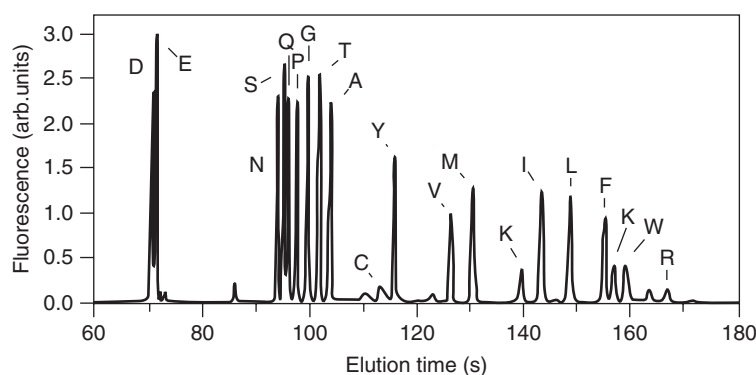


Figure 15.9 MEKC separation of 19 TRITC-labeled amino acids in a 10 mM sodium tetraborate/50 mM SDS buffer with 10% (v/v) 2-propanol. The field strength was 770 V/cm, and the detection point was 11.87 cm from the injection cross. The peak locations of the amino acids are indicated by their standard one-letter abbreviations. *Source*: Ref. 43; reprinted with permission.

can be incorporated to minimize this geometrical band broadening effect; for example, by reducing the width of the channel in the turn or increasing the radius of the turn. Figure 15.8 illustrates the microchip spiral design with a separation length of about 25 cm. Figure 15.9 shows the MEKC separation of 19 tetramethylrhodamine isothiocyanate (TRITC) amino acids in <3 min using the spiral microchip with a field strength of 770 V/cm and channel length of 11.87 cm between the injection and detection. The separation efficiency is over 280,000 with a minimum resolution of 1.2 between adjacent peaks.

15.8 TWO-DIMENSIONAL MEKC SEPARATIONS

In multidimensional separations, different techniques with orthogonal separation mechanisms are combined to enhance peak capacity and achieve the necessary resolving power for complex mixtures as in proteomics applications. Theory predicts that the peak capacity of a multidimensional separation method equals the product of the individual peak capacities of the constituent orthogonal methods [45–51]. There are three main orthogonal mechanisms based on solute hydrophobicity (using RPLC or MEKC), charge (ion exchange LC or CZE), and size (size exclusion LC or capillary sieving electrophoresis). The widely used conventional two-dimensional (2D) gel electrophoresis is a powerful separation methodology with high resolving power where solutes are

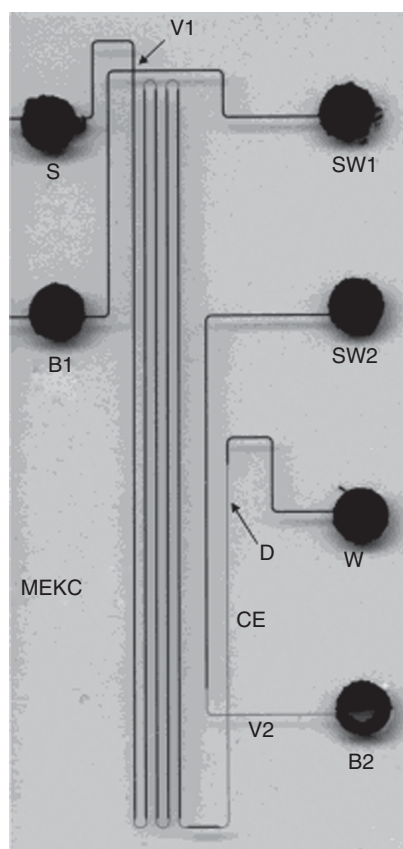


Figure 15.10 Image of a microchip with a serpentine channel for two-dimensional separations. Injections were made at valve 1 (V1) for the first-dimension MEKC separation and at valve 2 (V2) for the second-dimension CE separation. The sample was detected 1-cm downstream from V2 at point D using laser-induced fluorescence. The reservoirs are labeled sample (S), buffer 1 (B1), sample waste 1 (SW1), buffer 2 (B2), sample waste (SW2), and waste (W). The channels and reservoirs were filled with black ink for contrast. *Source*: Ref. 49; reprinted with permission.

separated based on charge and size in the two dimensions. However, the technique has the disadvantages of being slow, labor intensive, and not quantitative. Over the past two decades, researchers have investigated 2D methods by combining a variety of HPLC and/or CE techniques in series, where the sample bands from the first dimension are transferred sequentially into a second dimension. A great majority of the 2D separations have involved the combination of different LC methods or LC in one dimension and an electrophoretic technique (IEF or CE) in the other dimension. There are only a few 2D separation reports where both dimensions are CE methods. In a handful of reports, MEKC has been used as one of the dimensions where solutes are separated based on their hydrophobicity.

Sheng and Pawliszyn [46] designed a 2D system by combining MEKC as the first dimension and CIEF as the second dimension using a 10-port valve as an interface for the separation of protein digests [46]. Hu *et al.* [47] analyzed protein expression in single mammalian cell using a 2D system with capillary sieving electrophoresis in the first dimension for size-based separation and MEKC in the second dimension and LIF for sensitive detection [47].

A major challenge in performing 2D capillary-based CE separations is interfacing the capillaries and independently controlling the electric fields in each dimension. On the other hand, microchip platform is much more suitable for performing two (and multi)-dimensional electrophoretic separations, as different channels can be readily connected and voltages at various locations can be easily monitored to control the flow of solutions and sample zones between different channels. Ramsey and coworkers reported 2D separation of protein digests using microfluidic devices where peptides were separated by MEKC in the first dimension followed by zone electrophoresis (CE) in the second dimension [48,49]. The microchip design for 2D MEKC–CZE separation of protein digests is illustrated in Fig. 15.10, where a 19.6-cm-long serpentine channel is used for MEKC separation followed by rapid CE separation in the second dimension using a short 1.3-cm-long straight channel. A peak capacity of 4200 was observed for the separation of the bovine serum albumin (BSA) tryptic digest mixture (shown in Fig. 15.11). Note that the timescale for the MEKC dimension is <15 min, while it is only around 1 s in the CE dimension. The electric field strength for MEKC was 200 and 2400 V/cm for the second CE dimension. The combination of short channel length and high electric field strength ensures rapid separation to avoid remixing of separated zones in the second dimension.

Soper's group reported 2D separations of proteins on microchips made of poly(methyl methacrylate) (PMMA) with SDS-PAGE for size-based separation in the first dimension and MEKC separation in the second dimension [50]. The proteins were labeled with a fluorescent tag and LIF was used for high sensitivity detection. Initially, they used the 2D system to separate a sample of 10 proteins with a molecular weight range of 38–110 kDa. The separation was completed in 12 min and the method provided a peak capacity of 1000. More recently, they reported comprehensive 2D SDS-CGE $\times$ MEKC separation of a complex protein mixture in fetal calf serum on a cross-design PMMA microchip (Fig. 15.12). The separation was completed in <30 min with an average peak capacity of 2600. The results were compared with that using conventional 2D gel electrophoresis, which is a combination of IEF and SDS-PAGE. The 2D microchip separation was 60 $\times$ faster with a peak capacity three times of the conventional method.

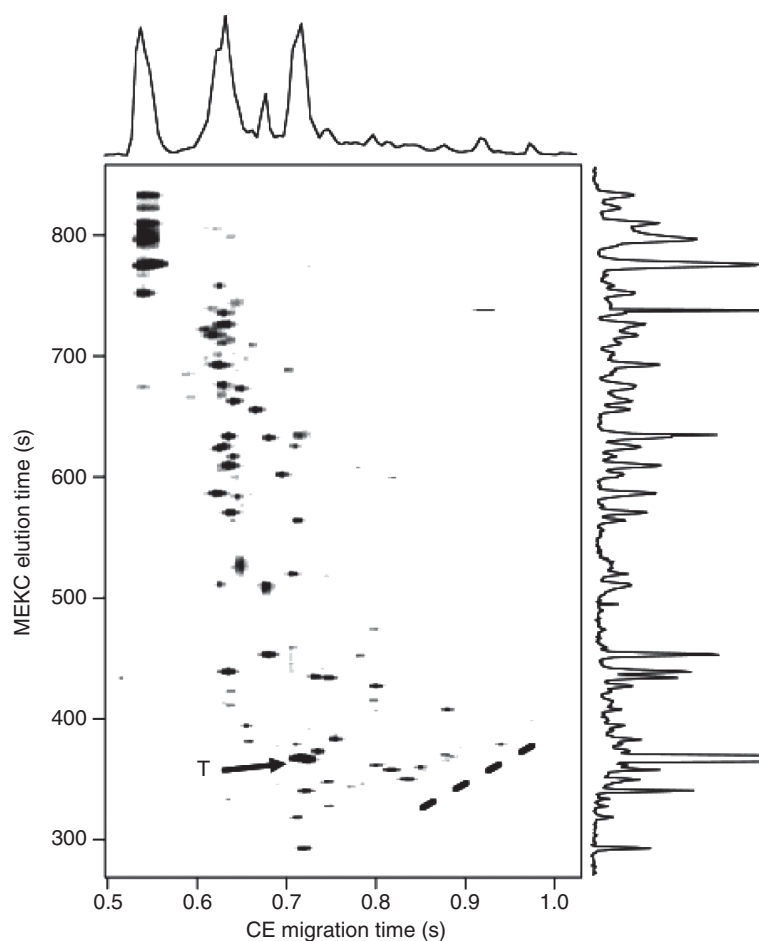


Figure 15.11 Two-dimensional separation of a bovine serum albumin tryptic digest. The projections of the two-dimensional data onto each axis are shown. Spots marked with a “T” represent unreacted 5-TAMRA dye. The dotted line illustrates correlation in the separation mechanisms. *Source:* Ref. 49; reprinted with permission.

15.9 CONCLUSIONS

EKC has matured into a microscale separation technique since its first introduction in 1984. The technique offers unique capabilities such as high resolving power, fast analysis time, availability of a wide range of pseudophases, and flexibility of rapidly adjusting the pseudophase composition for optimizing separation selectivity and enhancing resolution. In addition, the possibility of calculating micelle–water partition coefficients from solute structure and subsequently retention factor in MEKC is quite powerful for rapid optimization and enhancement of separation of complex mixtures. Significant progress has been made in synthesizing new pseudophases (especially polymeric and nanoparticles), and this trend will likely continue in the coming years. The wide range of selectivities in MEKC would be a great asset in 2D separations.

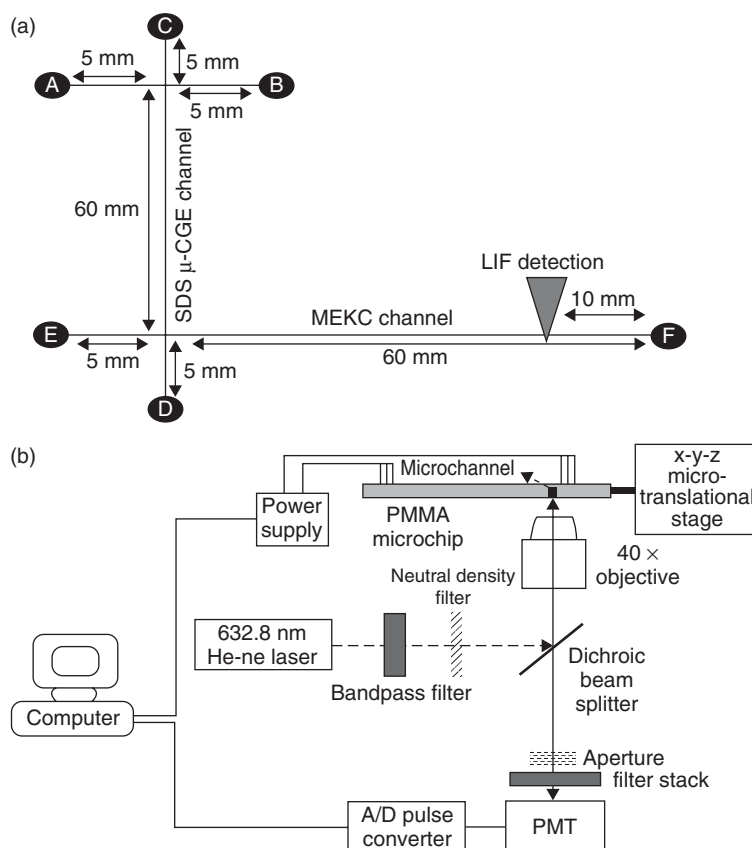


Figure 15.12 (a) Topography of the 2D microchip used for these studies. The channels were 50- μ m deep and 20- μ m wide in all cases. The first- and second-dimension channels were 7 cm (filled with gel media) and 6 cm (filled with MEKC buffer), respectively, in terms of their total column lengths. The effective column lengths for the first and second dimensions were 6 and 5 cm, respectively. (b) Diagram of the in-house constructed LIF system used for the separation. *Source*: Ref. 51; reprinted with permission.

The microfluidic platforms are ideal for interfacing two or more orthogonal electromigration techniques. Finally, much progress has been made in interfacing MS detection with MEKC. However, more work is needed before MEKC-MS comes to maturation for use in routine analytical applications.

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16 Direct Biofluid Analysis Systems

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16.1 Summary	1
16.2 Introduction	3
16.3 On-line SPE	3
16.4 RAM	8
16.5 Monolithic chromatography	11
16.6 Turbulent-flow chromatography	13
16.7 Conclusions and future directions	18
Acknowledgments	19
References	19

16.1 SUMMARY

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based methodology provides the most sensitive and specific methodology available for the analysis of small molecule drugs and endogenous metabolites in biofluids such as serum and plasma. However, the extraction and concentration of samples before the analysis represents a major hurdle for the implementation of routine assays and for assays when there are a large number of samples such as in drug disposition studies. This chapter provides a survey on the use of four different direct injection techniques that have been employed over the last decade for the analysis of biofluid samples. Specifically, background information, together with applications of solid-phase extraction (SPE), restricted access media (RAM), monolithic chromatography, and turbulent-flow chromatography have been reviewed. One of the major techniques for direct biofluid analysis utilizes on-line SPE. This is because a major drawback of off-line SPE procedures is that they often require many steps before reaching a concentrated extract suitable for the analysis, of which only a small portion is actually injected onto the LC column. In contrast, on-line SPE offers numerous advantages including the ability to readily automate the analyses. SPE sorbents include traditional materials, such as chemically bonded silica, ion-exchange and carbon-based materials, and some novel sorbents, such as RAM, molecularly imprinted polymers, immunosorbent, and monolithic material. Interfering substances present in the biofluids are flushed to waste while the analytes are retained

on the bonded phase of the SPE cartridge. Small molecule analytes are then eluted on-line, through a switching valve onto the LC analytical column. Simultaneously with the analytical separation, an exchange of the cartridge or reconditioning of the precolumn can take place.

The term *RAM* was introduced to describe a group of phases that allow direct and repetitive injection of untreated biological samples into reversed-phase LC systems. Macromolecules present in the biofluid samples cannot access the adsorption sites of porous supports because of their molecular size and so they elute in the void volume of the column. The RAM supports permit the separation of analytes through a combination of size-exclusion and conventional hydrophobic or ion-exchange interactions, which allows the passage of small molecules and restricts the access of macromolecules such as proteins. Two of the most popular RAM stationary phases are those employing internal surface reversed phase (ISRP) and alkyl-diol-silica (ADS) phase and there is an extensive literature describing the use of these columns. An interesting relatively recent development has involved the preparation of RAM using immobilized antibodies for the rapid and selective capture of small analytes by immunoextraction, giving rise to materials referred to as *immunoaffinity (IA)-RAM*. To make the required IA-RAM, an antibody for the desired target was first immobilized onto porous silica, with antibodies at or near the outer surface of the support then being treated with papain (or a related agent) to release and remove their binding domains. This resulted in a RAM support in which only antibodies deep within the pores remained intact and able to bind to the target analyte. This IA-RAM approach could potentially be used with many different antibodies to small molecule analytes and so could be useful for the rapid separation of drugs from drug-protein complexes.

Monolithic stationary phases comprise porous channels rather than beads found in conventional stationary phases employed for LC separations. They are typically prepared using a simple molding process carried out within the confines of a capillary. Monolithic stationary phases provide high rates of mass transfer at lower pressure drops, which enables much faster separations to be conducted when compared with conventional stationary phases. In addition, the nature of the pores in the monolithic phase allows easy permeability for large molecules. This means that monolithic columns can be used for direct biofluid analyses either as conventional LC columns or as sorbents in SPE columns for the extraction and concentration of small molecules. Turbulent-flow methodology was introduced specifically for the direct injection of biological samples without previous extraction or treatment onto an LC column packed with large particles. These large particles have an additional level of selectivity through different stationary phase chemistries (such as reversed phase or ion exchange) that can be added. Conventional LC methodologies employ parabolic flow profiles, while turbulent-flow LC (as its name implies) promotes turbulence in a packed column bed. The stream of mobile phase in the column moves so rapidly that it creates vacillating eddies within the stationary phase, which then cause flow equalization across the column. This results in a mobile phase front profile that is more like a plug than the parabola found in conventional LC separations. The eddies are also able to promote cross-channel mass transfer by convection within the interstitial spaces of the packed column bed. The use of fast mobile phase flow rates coupled with a plug flow profile results in an increase in the rate of change of the sample concentration gradient, thus increasing the effective diffusion rates within the pores. This turbulent-flow motion supports a more uniform velocity profile across the diameter of the column and controls effective

diffusion of the solutes. Therefore, analyte molecules can move quickly into and out of the pores and packing particles, which means that rapid chromatography can be conducted. The impressive sensitivity that has been attained with two-dimensional (2D) turbulent-flow LC-MS/MS suggests that it will be a powerful method for future routine applications for direct plasma and serum analyses of drugs and their metabolites, as well as endogenous metabolites.

16.2 INTRODUCTION

There is a compelling need for rapid, sensitive, and specific methods to analyze small molecule drugs, their metabolites, and endogenous metabolites for pharmaceutical development, as well as biomarker, therapeutic, and metabolomic applications [1–5]. The analysis of drugs in biofluids such as serum and plasma represents an important component in the determination of the physiological performance of a drug [6,7]. Therefore, a substantial amount of research has been conducted to develop rapid high throughput methods for the direct analysis of drugs and their metabolites in biofluids. Stable isotope dilution LC-MS/MS provides powerful bioanalytical methodology that combines high specificity with high sensitivity [8–10]. In spite of many advantages that have accrued due to recent advances and innovations in the area of instrumentation and dedicated software support, analysis of biofluid samples by LC-MS can be challenging and very time consuming [11]. One approach for improving sample throughput is to conduct direct on-line analysis of biofluid samples with no off-line extraction and purification. Such methods offer the advantage of reducing sample preparation steps and enabling effective preconcentration and cleanup of biological fluids. These procedures can also be readily automated, which reduces the requirements for handling potentially infectious biomaterial, improves reproducibility, minimizes sample manipulations, and limits potential contamination [12]. This chapter provides an overview on the use of four different direct injection techniques that have been employed over the last decade for the direct analysis of biofluid samples. Specifically, underlying principles involved in the application of SPE, RAM, monolithic chromatography, and turbulent-flow chromatography for direct analyses are addressed. A major issue that arises from direct analyses is the propensity for components of the biofluids to either suppress or enhance ionization in the analyzer region of the mass spectrometer [13]. Therefore, methodology for assessing such effects on different commonly used LC-MS ionization techniques is discussed [13,14]. A number of excellent review articles have appeared over the last decade describing the application of various methodologies for the direct analysis of biofluid samples [11,12,15–20]. Therefore, this chapter is focused primarily on recent advances that have been made in the application of the various direct on-line methods.

16.3 ON-LINE SPE

16.3.1 Background

Sample preparation is a major task in both regulated and unregulated bioanalytical laboratories. Sample preparation procedures significantly impact on assay throughput, data

quality, and analysis cost. Therefore, selecting and optimizing an appropriate sample preparation method is essential for successful method development using direct biofluid analysis methods [11]. Two of the major techniques that are employed utilize off-line SPE or on-line SPE. Significant drawbacks of off-line SPE procedures is that they can require many steps before reaching a concentrated extract suitable for the analysis, of which only a small portion is actually injected onto the LC column (Fig. 16.1) [21,22]. In contrast, on-line SPE offers numerous advantages including the ability to completely automate the analyses [22–24]. The on-line SPE approach involves the injection of a biofluid sample directly into an SPE cartridge, which retains the relevant analytes. SPE sorbents include traditional materials, such as chemically bonded silica, ion-exchange and carbon-based materials, as well as some novel sorbents, such as RAM, molecularly imprinted polymers, immunosorbents, and monolithic material [25]. Interfering substances present in the biofluid are flushed to waste, while the analytes are retained on the bonded phase of the SPE cartridge. They are then eluted on-line, through a switching valve onto the LC analytical column [23]. Simultaneously with the analytical separation, an exchange of the cartridge or reconditioning of the precolumn can take place.

Direct analysis of biofluid samples using SPE-based methodology has a long history because it can significantly increase sample throughput [12]. The overall methodology for LiChrograph OSP-2 (On-line Sample Preparation Unit, Merck, Darmstadt, Germany) Microlab SPE (Hamilton, Reno, NV, USA), and the widely used PROSPEKT (Programmable On-line Solid Phase Extraction Technique, Spark Holland BV, The Netherlands) systems have been discussed extensively in previous review articles [26–28]. Therefore, this chapter provides an overview of the symbiosis system developed by Spark Holland BV [29,30]. The system allows the use of

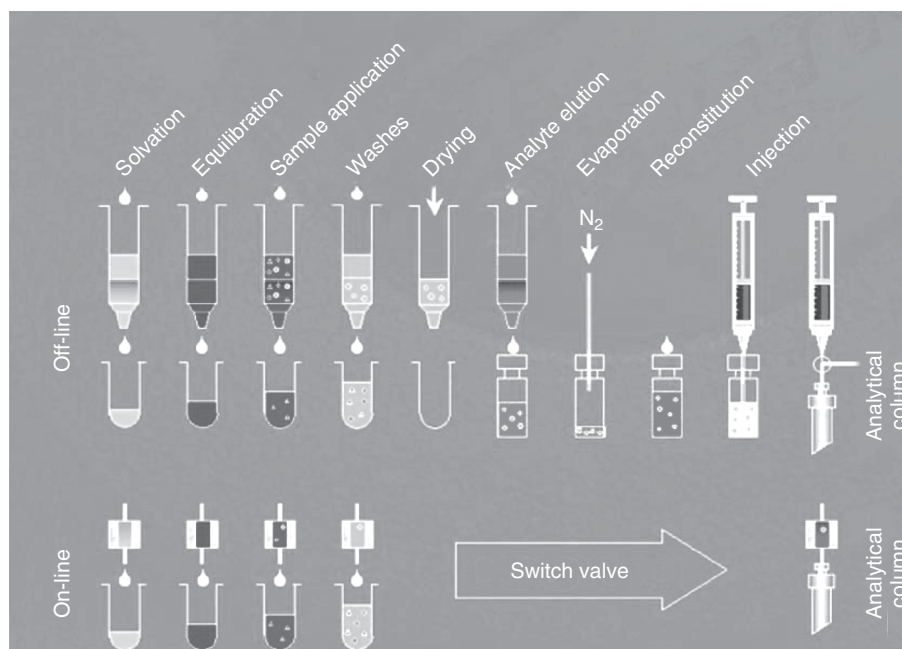


Figure 16.1 Comparison between on-line and off-line SPE methodology. *Source:* Reprinted with permission from Mitchell *et al.*, 2010 [22]. (See color insert.)

two different extraction mechanisms on two separate disposable SPE cartridges in one sample run, which can significantly improve upon methods that employ single SPE cartridges. Disposable cartridges help to eliminate sample-to-sample carryover problems that can sometimes be observed when columns are reused, particularly for low level analytes. The Symbiosis system can handle sample volumes from 10 μ L to 10 mL and can add, mix, and dilute samples before the analysis. The principles of extraction are essentially the same as off-line SPE. However, the system design allows the SPE eluate to be directly injected onto the chromatography system, thus removing the need for any evaporation (Fig. 16.1). By avoiding evaporation steps, it is possible to significantly cut down on the amount of time required for the sample analysis. In addition, the ability to use two separate liquid flows for the extraction and chromatography steps significantly reduces the potential for carryover from sample to sample. This can be a particular problem when analyzing large concentration ranges of drugs and endogenous metabolites that are present in plasma and serum samples. Importantly, there is a large variety of stationary phases available for the SPE cartridges, which can help in the development of highly selective on-line extraction methodology.

The development of analytical methodology for sophisticated on-line SPE extractions requires a substantial amount of time to optimize all of the conditions. A particular advantage of the Symbiosis system is that the SPE cartridges can be washed with heated solvents. This can be useful when washing with organic solvents is precluded by the polarity of the analytes. Parameters such as elution time, elution flow rate, solvent polarity, and type of stationary phase have to be modified for different analytes. A recent study demonstrated that the performance of assays with on-line extraction was essentially identical with that obtained by off-line SPE extraction [22]. Therefore, the benefits generally only accrue for large sample batches. Consequently, off-line SPE extraction methodology generally remains the method of choice for small numbers of samples. However, for volatile or unstable samples, on-line methodology is preferable even for small sample numbers [29]. Both on-line and off-line SPE-LC-MS configurations can result in significant matrix effects, which depend on whether electrospray or atmospheric pressure chemical ionization (APCI) is being used and the particular extraction support that is utilized [31]. Therefore, it is necessary to assess the amount of suppression or enhancement of ionization that is occurring with different batches of plasma or serum samples [13].

16.3.2 Applications of On-Line SPE

A validated method for on-line SPE coupled with LC-tandem mass spectrometry (MS/MS) for analysis of the anxiolytic drug bromazepam in human plasma provides an illustration of how direct biofluid analyses can be conducted [32]. The method involved addition of carbamazepine internal standard, vortex-mixing, centrifugation, and injection of 100 μ L of the supernatant. Analytes were ionized using positive electrospray mass spectrometry then detected by multiple reaction monitoring/MS. The calibration curve was linear from 1 ng/mL (limit of quantification) to 200 ng/mL with LC retention times for bromazepam and carbamazepine of 2.6 and 3.2 min, respectively. This automated method was successfully applied in a bioequivalence study of two tablet formulations. The comparison of different experimental conditions for establishing a dissolution profile *in vitro*, along with the bioavailability data that were generated,

made it possible to propose rationally based experimental conditions for a dissolution test of bromazepam tablets.

In the field of environmental toxicology, SPE-LC-MS/MS was employed for quantifying trace levels of 18 perfluorinated chemicals (three perfluorosulfonates, eight perfluorocarboxylates, and seven perfluorosulfonamides) in serum [29]. After dilution with 0.1M formic acid, an aliquot of 100 μ L of serum was injected into a commercial column-switching system that allowed for concurrent SPE and LC-MS/MS analyses. Analytes were concentrated on a C18 SPE column and the eluates then automatically injected on a reversed-phase C8 analytical LC column. Excellent recovery was achieved for all analytes including the volatile sulfonamide derivatives, which could not be analyzed using traditional off-line SPE methods. The high throughput and low limits of detection (0.05–0.8 ng/mL) using a small sample volume and isotope dilution quantification make this method suitable for large-scale epidemiological studies.

A novel approach for on-line introduction of internal standard for quantitative analysis using LC-MS/MS was developed for use with on-line SPE methodology [33]. In this approach, the analyte and internal standard were introduced into the sample injection loop in different steps. The analyte was introduced into the injection loop using a conventional autosampler (injector) needle pickup from a sample vial, whereas the internal standard was introduced into the sample injection loop on-line from a microreservoir containing the internal standard solution. As a result, both analyte and internal standard were contained in the sample loop before the injection into the column. This new technique was applied for direct analysis of model compounds in rat plasma using on-line SPE-LC-MS/MS quantification. The assays yielded accuracy (85–119%), precision (2–16%), and analyte recovery comparable to those obtained using off-line internal standard introduction. Furthermore, on-line internal standard introduction allowed for nonvolumetric plasma collection and direct analysis without the need of measuring and aliquoting a fixed sample volume before the on-line SPE-LC-MS/MS analysis. Therefore, this methodology enabled direct plasma analyses to be conducted without any sample manipulation and preparation. On-line SPE coupled with capillary LC-ESI-MS was employed to improve the sensitivity for quantification of the antidepressant and anorectic drug fluoxetine (valium) in human plasma [34]. Before injection, the plasma was spiked with metronidazole (internal standard) and mixed with ammonium formate buffer for effective chloroform liquid–liquid extraction. The method was validated in the range 5–60 ng/mL fluoxetine. The method was then used to determine the amount of fluoxetine in a healthy male 14 h after an intake of one capsule of flutrin, which contains 20 mg fluoxetine/capsule. The use of capillary LC increased the sensitivity by a factor of similar to 100 when compared with conventional LC systems.

The analysis of endogenous metabolites in plasma by direct SPE-LC-MS is exemplified by the studies conducted on the analysis of steroids in serum [35]. Following protein precipitation of 100 μ L serum, on-line SPE and chromatographic separation were performed for 13 steroids in 1.8 min. Analyte identities were confirmed by the characteristic fragmentation patterns observed by MS/MS analyses in a quadrupole ion trap. The total run time of the method was 4 min. Limits of quantitation ranged between 50 pg/mL for testosterone and 48 ng/mL for dehydroepiandrosterone sulfate. The method was linear up to 7000 ng/mL for dehydroepiandrosterone sulfate, 500 ng/mL for cortisol, 125 ng/mL for 11-deoxycortisol, and 25 ng/mL for aldosterone, 17-hydroxyprogesterone, progesterone, testosterone, androstenedione, and β -estradiol, respectively. Accuracy ranged between 80% and 114%. Between-day variance at three

different concentration levels was $<15\%$. Excellent correlations with immunoassays were observed for testosterone, cortisol, and β -estradiol. Therefore, this novel on-line SPE-LC-MS/MS platform offers a very fast and reliable method for the quantification of steroid patterns for relatively routine laboratory applications. However, the limits of detection would need to be substantially lower for research applications such as the determination of breast and prostate cancer risk [3,36].

Recently, an on-line SPE-LC-MS/MS method was optimized for quantification of the anti-HIV (human immunodeficiency virus) peptide sifuvirtide in human plasma [37]. The SPE sorbents, loading buffer composition, and other aspects of the on-line SPE column were investigated in detail for efficiently extracting the interesting peptides and simultaneously discarding the large amount of proteins from the plasma. Method validation revealed a linear calibration curve that covered a wide range of 6.1–6250 ng/mL, with excellent correlation coefficients. The lower limit of detection with a signal-to-noise ratio >10 was 6.1 ng/mL. Impressively, more than 900 samples from a clinical trial were analyzed in a single week because of the rapid run time of 6.5 min for individual samples.

16.3.3 Background and Applications of Miniaturized SPE (μ -SPE) Cartridges

μ -SPE (micro solid-phase extraction) cartridges are available as a component of the RapidFire System (BIOCIUS Life Sciences, Wakefield, MA), which also uses innovative microfluidics technology. This system is emerging as a useful approach to direct biofluid analysis. Its utility was demonstrated by comparing a high throughput RapidFire MS System with a scintillation proximity assay [38]. The RapidFire System allows continuous SPE extractions to be performed in 5–10 s time intervals [39]. Thus, this technology performs rapid SPE purification of biofluids in 96 or 384 well plates followed by MS-based analysis [40,41]. Extracted analytes are rapidly eluted to a mass spectrometer after every SPE step, and analytes that are present in each well are quantified sequentially. Initial high throughput applications have focused on the analysis of enzyme incubation mixtures [38,41–43]. For example, a series of 120 compounds was evaluated for potential drug–drug interaction by incubating over a range of eight test concentrations and against a panel of six cytochrome P450 (CYP) enzymes, 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4 [43]. The on-line SPE-MS/MS system reduced analysis time to <15 min per 96-well plate, translating to a 15-fold time savings compared to the 2-min LC/MS/MS method. There was an excellent correlation between this new rapid high throughput method and previous methods for the individual CYP enzymes.

The success of direct analyses of biofluids from enzyme assays using μ -SPE cartridges has encouraged investigators to test more challenging biofluids such as plasma. A presentation at the recent Applied Pharmaceutical Analysis meeting in Baltimore, MD explored the use of μ -SPE-based methodology for the high throughput direct analysis of drugs in plasma samples [44]. Encouragingly, the assay results from RapidFire-MS/MS analysis correlated well with the results from conventional LC-MS/MS analyses. Use of stable isotope labeled and structural analog internal standards effectively compensated for matrix effects. When the ion source conditions were optimized to minimize interference from an acyl-glucuronide metabolite, parent drug concentrations were comparable to those obtained from LC-MS/MS system. Therefore, co-eluting metabolites had a negligible impact on quantitation. Even though there were variable amounts of sample-to-sample carryover, no significant

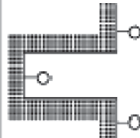
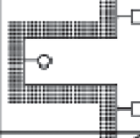
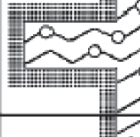
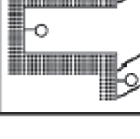
impact on quantitation results was observed. Therefore, the μ -SPE-based RapidFire System might be a useful tool for high throughput MS quantitation of *in vivo*-derived biofluid samples in situations where large sets of samples justify the up-front burden of method development [44].

16.4 RAM

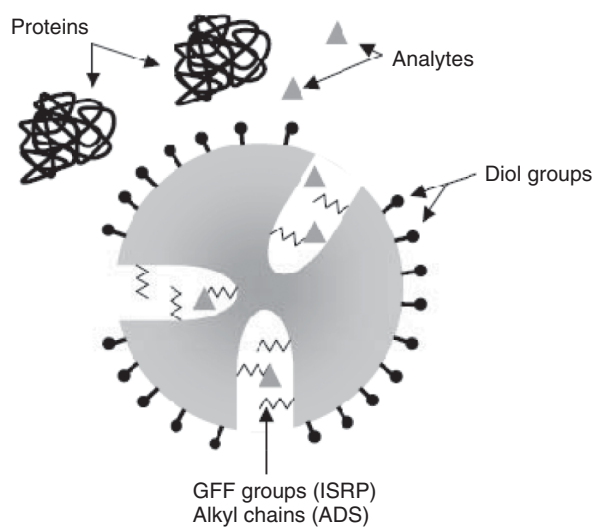
16.4.1 Background

The term *RAM* was introduced by the Desilets *et al.* to describe a group of phases that allows direct and repetitive injection of untreated biological samples into a reversed-phase LC systems [45]. Macromolecules present in the samples cannot access the adsorption sites of porous supports because of their molecular size and so they elute in the void volume of the column [46]. RAM supports permit the separation of analytes through a combination of size-exclusion and conventional hydrophobic or ion-exchange interactions, which allows the passage of small molecules and restricts the access of macromolecules such as proteins [47]. Macromolecular exclusion can be performed using a physical diffusion barrier based on the size of the pore or by a chemical diffusion barrier created by a network covering the surface of the support, which consists of covalently or adsorptive-bonded synthetic polymers, natural polymers, or proteins at the outer surface of the silica. It has been suggested that RAM sorbents can be divided into four groups: physical diffusion barriers with either unimodal, bimodal phases, chemical diffusion barriers with either unimodal, or bimodal phases (Fig. 16.2a) [28,47]. RAM stationary phases such as ISRP (Fig. 16.2b), semipermeable surface, ADS (Fig. 16.2b), shielded hydrophobic phase, mixed-function phase, and protein-coated silica have all been employed for LC-MS-based direct plasma analyses [46]. Commercial columns are available for each of these applications. The two most popular RAM stationary phases are those employing ISRP and ADS. A large number of applications, together with the commercially available columns that were used, have been described in the excellent extensive review by Souverain *et al.* [48].

An interesting relatively recent development has involved the preparation of RAM using immobilized antibodies for the rapid and selective capture of small analytes by immunoextraction, giving rise to materials referred to as *IA-RAM* [49]. To make the required IA-RAM, an antibody for the desired target was first immobilized onto porous silica, with antibodies at or near the outer surface of the support then being treated with papain (or a related agent) to release and remove their binding domains. This resulted in a RAM support in which only antibodies deep within the pores remained intact and able to bind to the target analyte. This IM-RAM approach could potentially be used with many different antibodies to small molecule analytes and so could be useful for the rapid separation of drugs from drug-protein complexes. It could also be particularly valuable for separating endogenous DNA bases such as 2'-deoxy-guansoine (dGuo) from 7,8-dihydro-8-oxo-dGuo (8-oxo-dGuo) in plasma samples. This would help prevent the artifactual formation of 8-oxo-dGuo when there is an excess of dGuo present [50]. The need for RAM capillary columns that could be used for low flow applications that enhance detection sensitivity was recognized by Jarmalaviciene *et al.* [51]. They have developed different strategies for the preparation of novel shielded polymeric reversed-phase monolithic material in the presence of different numbers

RAM phase	Diffusion barrier	Surface topochemistry	Type
	Physical	Homogeneous	A
	Physical	Heterogeneous	B
	Chemical	Homogeneous	C
	Chemical	Heterogeneous	D

(a)



(b)

Figure 16.2 (a) Classification of RAM supports. *Source:* Reprinted with permission from Cassiano *et al.* [47]. (b) Schematic representation of internal surface reversed-phase (ISR) with GFF groups and alkyl-diol-silica (ADS) with alkyl chains. *Source:* Reprinted with permission from Souverain *et al.*, 2004 [48].

of reactive groups and concentrations of the coating polymer. Inverse size-exclusion chromatography was used for investigation of the pore structure properties of the beds. The resulting RAM capillary columns were then applied for nanochromatography of biological fluids containing a mixture of the sedative benzodiazepine drugs nitrazepam and medazepam.

16.4.2 Applications of RAM

As might be predicted, RAM can be used as the solid support for both off-line and on-line SPE of small molecules in plasma as well as the stationary phase for LC separations [28,48]. There are many applications appearing in the current literature that use the RAM for this purpose rather than conventional LC procedures. A recent application described the use of an LC system that was equipped with an on-line dilution system for on-line SPE analysis [52]. Use of a six- to eightfold on-line dilution ratio for plasma samples resulted in almost quantitative recovery of both acidic and basic drugs from plasma. It was found that the relationship between the on-line dilution times and drug recovery efficiencies from plasma could be explained in terms of the binding constant between the drug and albumin. The applicability of this column-switching LC with an on-line dilution system and the effectiveness of the extraction procedure were confirmed by a simultaneous determination of the basic compound, ER-118585, and its metabolites in canine plasma.

Another interesting example of on-line SPE analysis involved the extraction of the proton-pump inhibitor, lansoprazole from plasma using an octyl RAM bovine serum albumin column (C-8 RAM BSA) [53]. Enantioselective LC separations were performed on an amylose tris(3,5-dimethoxyphenylcarbamate) chiral column. The method was applied to the analysis of the plasma samples obtained from nine volunteers who received a 30-mg oral dose of racemic lansoprazole and was able to quantify the enantiomers of lansoprazole in all the samples that were analyzed. RAM-based SPE extraction methodology has proved to be particularly useful for the analysis of the radioactive components in plasma taken during positron emission tomography (PET) measurements using [^{11}C]-labeled radiopharmaceuticals [54]. Plasma samples were analyzed directly, following a simple filtration, by the use of a small RAM SPE column, combined with a monolithic LC analysis in a column-switching system. Up to 4 mL of plasma could be analyzed by this method within 4.5–7 min in a fully automated process. A large number of samples could be analyzed during a 90-min PET scan. This method made it possible to conduct accurate determinations of the radioactive components in plasma even at 90 min after injection of a [^{11}C]-labeled radiopharmaceutical.

An important aspect of on-line RAM SPE for routine analyses is the ability to concentrate the sample into a small volume. Santos-Neto *et al.* found that the peak-focusing efficiency of the RAM SPE column was more effective in back-flush compared to fore-flush mode [18]. Their system was able to concentrate polar drugs and their metabolites reaching quantifiable results as low as 1 ng/mL utilizing a sample volume of only 333 nL of plasma. New column hardware was developed to circumvent clogging problems that were experienced with plasma injections. The glass fiber filter frit, which is commonly used, was replaced with a short piece of 20- μm internal diameter fused silica capillary tubing. The RAM SPE columns were then able to handle up to 60 injections without any deterioration in separation efficiency. The columns also had a

high loading capacity, making the saturation of the MS detector, the limiting factor on the linear dynamic range. Using this method, it was possible to simultaneously separate and detect 10 drugs and metabolites within 8 min.

A simple and rapid RAM-based LC-MS method was used for direct analysis of the antibiotic rifaximin in rat serum [55]. Separation of rifaximin from serum was achieved by the injection of rat serum onto a Supelco LC-Hisep LC column, which contains a shielded hydrophobic RAM stationary phase. The linear range of the assay was a 0.10–20 ng/mL of serum. The validated RAM-based LC method was successfully applied to pharmacokinetic studies of rifaximin in serum after oral administration to rats. RAM LC has also been employed for coupled-column separations [56]. A RAM bovine serum albumin RAM LC column was used in the first dimension in order to exclude macromolecules and retain small molecules. An amylose tris[(*S*)-1-phenylethylcarbamate chiral column was used in the second dimension. This fully validated method has provided the first example of the rigorous analysis of both enantiomers of the analeptic drug modafinil and its two major metabolites in plasma. The coupled RAM-chiral column method showed good linearity, precision, accuracy, sensitivity, and selectivity, allowing it to be used for pharmacokinetic studies. Importantly, the quality of the performance of both columns was maintained with over 280 plasma injections.

16.5 MONOLITHIC CHROMATOGRAPHY

16.5.1 Background

Monolithic stationary phases comprise porous channels rather than the beads found in conventional stationary phases employed for LC separations [12,16]. They are typically prepared using a simple molding process carried out within the confines of the capillary [57]. Monolithic stationary phases provide high rates of mass transfer at lower pressure drops, enable much faster separations and the nature of the pores allows easy permeability for large molecules (Fig. 16.3) [58]. This means they can be used for direct plasma analyses either as conventional LC columns [59,60] or as sorbents in SPE columns for the extraction and concentration of small molecules [20]. Silica-based monolithic stationary phases are prepared from tetraalkoxysilane by

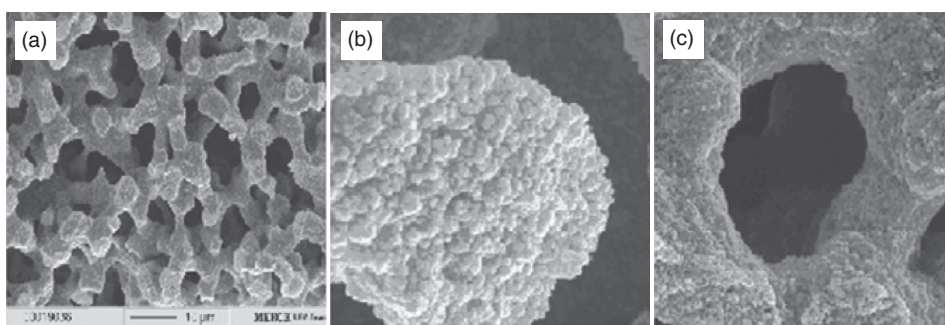


Figure 16.3 (a) SEM-picture of the typical porous structure of monolithic silica columns. (b) The mesoporous structure of the silica skeleton. (c) The macropores or through-pores. *Source:* Reprinted with permission from Hayes *et al.*, 2000 [58].

a sol-gel method. This can provide either micrometer-size through-pores or high specific surface areas that are well suited for small molecules in LC modes. In contrast, organic polymer-based monolithic stationary phases including monolithic molecularly imprinted polymers have been applied to LC analysis of macromolecules and they have also been employed as sorbents for both on-line and off-line SPE extraction of plasma. The most commonly reported organic polymers are based on polystyrenes, polymethacrylates, and polyacrylamides. Monolithic phases have a number of potential advantages over more conventional silica-based particulate materials, as outlined in the excellent review by Saunders *et al.* [61]. Polymer monoliths can be adapted to many bioanalytical situations such as sample preparation and concentrations as well and capillary LC separations, which explains their increasing use for direct plasma analyses. These materials have some interesting properties when compared to more traditional particulate materials, such as their high permeability for liquids and biological samples, making them ideal for SPE applications.

16.5.2 Applications of Monolithic Chromatography

Many new applications on the monolithic SPE in sample extraction and concentration (as a sample extraction and concentration tool before more conventional LC separations) have appeared over the last decade [10,25]. A comprehensive review of the use of monolithic chromatography has appeared recently [20]. Although many of the

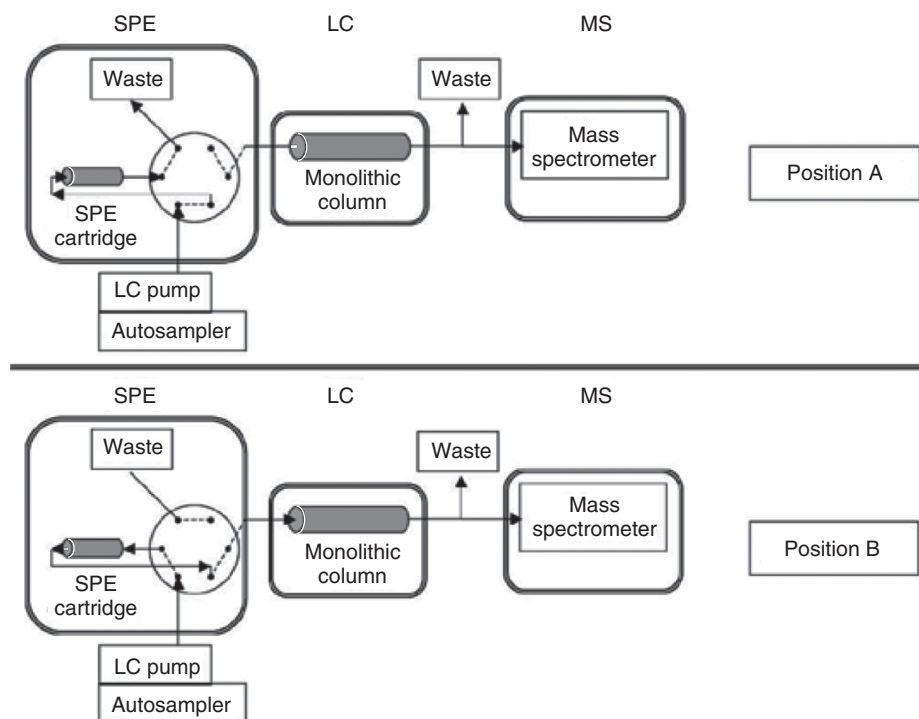


Figure 16.4 Schematic diagram of the on-line SPE monolithic LC/MS/MS configuration. Source: Reprinted with permission from Zang *et al.*, 2005 [62].

applications do not relate specifically to direct plasma analyses on a monolithic column itself, the examples that have been provided can be used as the basis for embarking on the use of this type of methodology. In an interesting example, Zang *et al.* developed on-line SPE LC/MS/MS assay using a monolithic LC column for the direct analysis of plasma samples containing multiple analytes [62]. A simple column-switching configuration that required only one six-port valve and one LC pumping system was employed for on-line plasma sample preparation and subsequent gradient LC separation using a monolithic Chromolith Speed ROD RP-18e (4.6×50 mm i.d., Merck) (Fig. 16.4). The method was found to perform satisfactorily for direct plasma analysis with respect to assay linearity, specificity, sensitivity, precision, accuracy, carryover, and short-term stability of an eight-analyte mixture in plasma. Gradient LC conditions were applied in order to separate the eight analytes that could not be readily differentiated by MS/MS analysis. With a run time for every injection of 2.8 min, a minimum of 300 direct plasma injections were made on one on-line SPE column without noticeable changes in system performance. Owing to the ruggedness and simplicity of this system, the authors suggested that generic methods could be easily developed for the analysis of a wide variety of small molecules in plasma in a high throughput manner without laborious off-line sample preparation.

A particularly good example of direct plasma analysis using monolithic LC-MS/MS is provided by the method developed by Hsieh *et al.* [15]. The method was employed for high-speed direct simultaneous determination of a drug discovery compound and its major circulating metabolite (M-72) in rat plasma. This methodology made use of flow programming and an alkyl-bonded silica rod column for fast macromolecule removal and chromatographic separation without the need for significant sample preparation. After 200 plasma injections on the monolithic silica column (50×4.6 mm i.d.), consistent column efficiency of approximately 39,000 theoretical plates per meter could be obtained together with reproducible retention times. The apparent on-column recoveries of 12 test compounds in rat plasma samples were $>90\%$. The direct plasma injection method was tested over a three-day period, where inter-day coefficients of variation of $<15\%$ were observed for both analytes.

16.6 TURBULENT-FLOW CHROMATOGRAPHY

16.6.1 Background

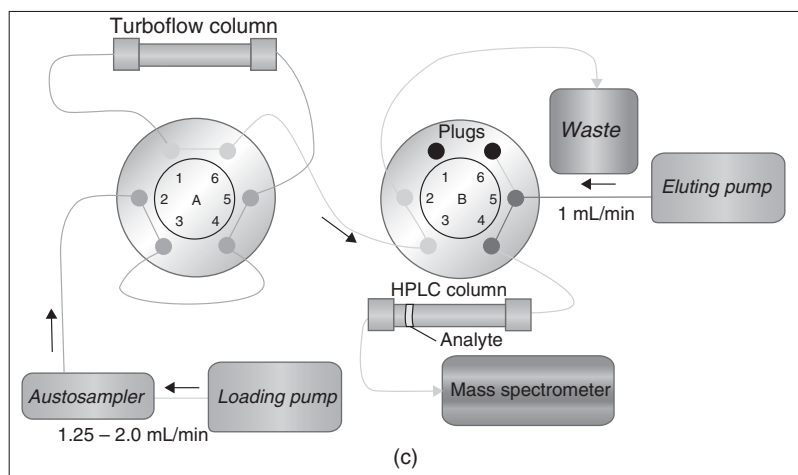
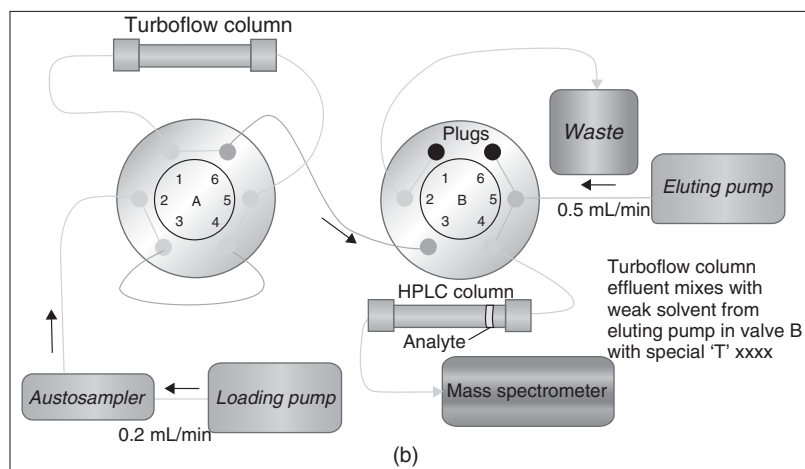
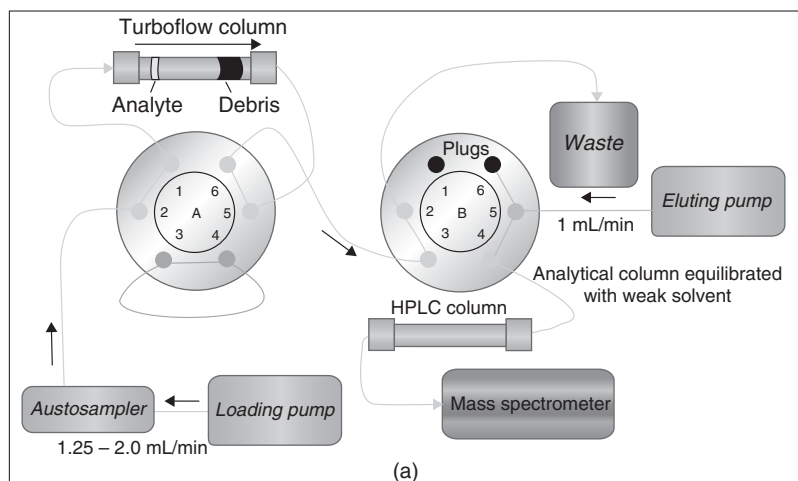
Turbulent-flow methodology was developed by Quinn and Takarewski specifically for the direct injection of biofluids without previous extraction or treatment onto an LC column packed with large particles [19]. These large particles have an additional level of selectivity through different stationary phase chemistries (such as reversed phase or ion exchange) that can be added. Conventional LC methodologies employ parabolic flow profiles, whereas turbulent-flow LC (as its name implies) promotes turbulence in a packed column bed. The stream of mobile phase in the column moves so rapidly that it creates vacillating eddies within the stationary phase, which then cause flow equalization across the column. This results in a mobile phase front profile that is more like a plug than the parabola found in conventional LC [63]. The eddies also promote cross-channel mass transfer by convection within the interstitial spaces of the packed column bed [63]. The use of fast mobile phase flow rates coupled with a plug flow

profile results in an increase in the rate of change of the sample concentration gradient, thus increasing the effective diffusion rates within the pores. This turbulent-flow motion supports a more uniform velocity profile across the diameter of the column and controls effective diffusion of the solutes. Therefore, analyte molecules can move quickly into and out of the pores and packing particles, which means that rapid chromatography can be conducted.

Typically, a biofluid sample is injected onto a turbulent-flow LC column at flow rates of between 1.5 and 5.0 mL/min. Initially, aqueous buffers are used as the mobile phase, so that small molecules are retained on the column through diffusion into the particle pores. The proteins that are present in the sample rapidly elute from the column and are washed to waste. Once the small molecules have been extracted from the biofluid into the stationary phase, they are then either eluted from the turbulent-flow column to a conventional analytical LC column in a 2D approach as described by Jemal [64], Wu *et al.* [65], and Chassaing *et al.* [66], or they are analyzed directly on the same column as described by Ayrton *et al.* [63]. Not surprisingly, the 2D approach has become the method of choice because it allows for much more selectivity as well as improved sensitivity through the use of low flow rates on the analytical column. For use of the turbulent-flow column as the initial extraction method, it has been recommended that the small molecule analytes should be eluted onto the analytical column using solvent that has been stored in a holding loop [19]. The holding loop should have a volume at least 10 times that of the turbulent-flow column and is typically filled with organic mobile phase (for reversed stationary phase) or pH buffered solutions (for ion-exchange phases) [19]. The use of switching valves to obtain optimal system performance is shown in Fig. 16.5.

A major issue with all biofluid assays is the degree of suppression or enhancement of ionization that can occur with LC-MS-based methodology [67]. This issue is extremely important when nonidentical or deuterium labeled standards are employed because there can be significant separation between the analyte and its relevant internal standard [68]. Suppression effects can potentially be problematic even with [^{13}C] and [^{15}N]-labeled internal standards because they can significantly affect the lower limit of quantitation. The current regulatory requirements include the need for the assessment and elimination of the matrix effect in bioanalytical methods that are submitted to the Food and Drug Administration [13]. These effects can be determined by infusing the analyte and observing the suppression and ionization caused by the LC effluent [14]. A robust procedure has also been described in which five different plasma lots are analyzed [13]. This makes it possible to determine the absolute effects for particular plasma samples or the relative effects with different plasma samples. The magnitude of matrix suppression and enhancement effects on ionization efficiency appears to be dependent on the actual mechanism of ionization. Under otherwise identical sample

Figure 16.5 (a) Loading step: turbulent-flow removed proteins from the plasma through the turbulent-flow (Turboflow) column while small molecules are retained. (b) Transfer step: flow from both pumps is combined through the T-rotor seal, which allows the loop contents to transfer the analytes retained on the turbulent-flow column into a stacked band on the analytical column. (c) Elution step: the analytes are eluted from the analytical LC column using an isocratic or gradient mobile phase. The turbulent-flow column is washed and the loop is filled and closed in preparation for the next injection. *Source*: Reprinted with permission from Chassaing and Robinson, 2009 [19].



extraction and chromatographic conditions, the matrix effect was absent when APCI was employed but very significant with electrospray ionization [13]. Therefore, a thorough consideration of the ionization technique that is employed can be an important component of the analytical development procedure. A fully automated turbulent-flow method for the analysis of antidepressants in serum investigated the suppression of plasma constituents [69]. This study revealed that all of the suppression occurred before the relevant analytes eluted from the column and illustrated the power of turbulent-flow extraction methodology (Fig. 16.6).

16.6.2 Applications of Turbulent-Flow Chromatography

The quantification of Vitamin D in serum and plasma is an important diagnostic test of nutritional status that is often performed by radioimmunoassay procedures. Stable isotope dilution LC-MS/MS is in principle more accurate and specific and can be employed for multiple vitamin D metabolites [2]. However, time-consuming methods are required for extraction and purification of vitamin D and its analogs, which have restricted the use of LC-MS approaches. The analysis of plasma and serum vitamin D is very challenging because the concentrations are often in the low ng/mL (low nM) range [2]. A turbulent-flow on-line extraction-based 2D method was reported recently for the analysis of 25-hydroxy vitamin D3 (25OHD3), and 25-hydroxy vitamin D2 (25OHD2), the two most important vitamin D metabolites [70]. The second dimension was conducted using a Hypersil gold aQ LC column (Thermo Fisher) coupled with APCI-MS. Assays were linear from 3.0 to 283 nmol/L for 25OHD3 and from 4.6 to

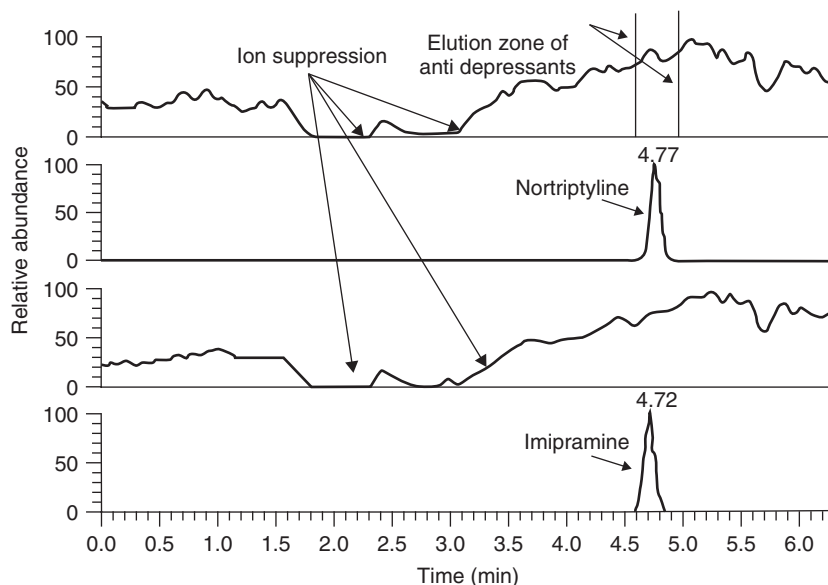


Figure 16.6 Study of the ion suppression phenomenon by means of continuous infusion of 20 antidepressants at 100 ng/mL and parallel injection of extracts of blank serum samples: the example of the nortriptyline and imipramine extracted-ion chromatograms. *Source:* Reprinted with permission from Sauvage *et al.*, 2006 [69].

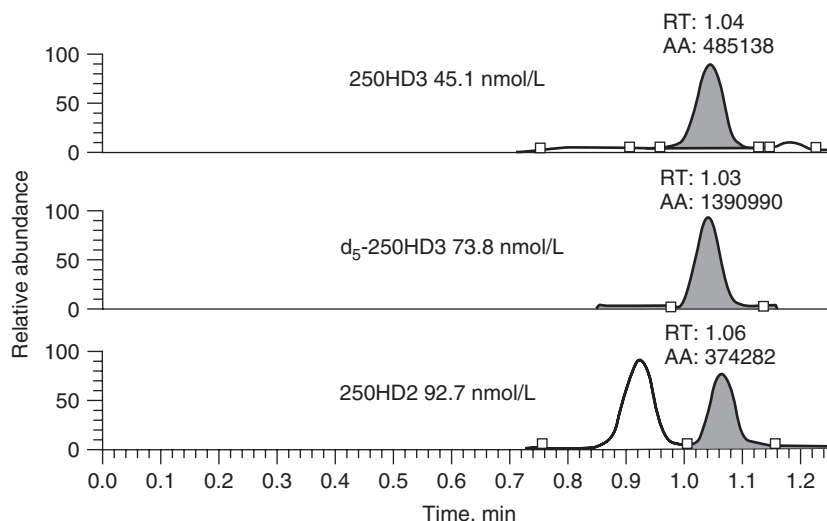


Figure 16.7 Chromatogram from 2D turbulent-flow LC-MS analysis of a patient's serum sample with mid-levels of 25OHD2 and 25OHD3. *Source:* Reprinted with permission from Bunch *et al.*, 2009 [70].

277.9 nmol/L for 25OHD3 with chromatographic run times of only 1 min (Fig. 16.7). Importantly, no carryover or ion suppression was observed, lending further credence to the concept that APCI is less susceptible to matrix effects than other ionization techniques [13].

An area that offers significant future applications is in the field of metabolomic analyses because it is very difficult to analyze the polar metabolites without causing significant suppression of ionization as well as contamination of the ion source from plasma constituents [8]. A recent study has compared the use of protein precipitation with a 2D turbulent-flow LC-MS/MS approach [5]. This comparison has shown that turbulent-flow chromatography could be effectively used with a substantial reduction of the time required for sample handling. Furthermore, there were substantial differences in the overall metabolite profiles for the two methods. This was ascribed to greatly reduced amounts of phospholipids (ca. 10-fold reduction) for the turbulent-flow methodology compared with protein-precipitated samples. Interestingly, the majority of ions were detected in the mass range of m/z 200–400 compared with m/z 400–600 for the protein-precipitated samples. It was suggested that this could have been due in part to the loss of small molecules bound to proteins in the turbulent-flow method and suggest that a combination of both methods could maximize differences in metabolomic characterizations.

An impressive example of the sensitivity that can be attained with 2D turbulent-flow LC-MS/MS methodology was reported recently for the analysis of plasma testosterone [71]. Total analysis time was 1.15 min per sample when using the multiplexing system that was developed. Testosterone concentrations were measured directly from 150 μ L of serum or plasma without derivatization or liquid–liquid extraction. The lower limit of quantification was 300 fg/mL and the assay was linear up to 200 ng/mL. Furthermore, the method compared very well with an established radioimmunoassay. Therefore,

this high throughput method appears to be suitable for quantifying the expected low-testosterone concentrations seen in women, children, and hypogonadal males and for monitoring testosterone suppressive therapy in prostate cancer patients.

In the field of drug disposition, a recent article described a fully automated turbulent-flow 2D LC-MS/MS method for the detection of tricyclic antidepressant drugs (amitriptyline, desipramine, imipramine, and nortriptyline) in serum [72]. Human serum and an internal standard were injected directly onto a turbulent-flow Cyclone-P on-line SPE column (0.5×50 mm, Thermo Fisher). Following removal of serum proteins and other components the analytes were transferred to a Hypersil Gold C-18 (50×3 mm, Thermo Fisher) analytical column. Gradient elution was performed with water and acetonitrile each containing 0.1% formic acid. The antidepressant drugs were ionized and detected over a 3.5 min analysis time using electrospray-ionization/MS. Matrix effects were characterized and carryover, precision, linearity, recovery, and limits of detection and quantitation were evaluated. The limits of detection and quantitation for all drugs were <3 ng/mL and <20 ng/mL, respectively. Recoveries were between 97% and 114%. On the basis of the validation data, this specific, sensitive fully automated method can be employed for rapid quantitation of tricyclic antidepressants in serum.

16.7 CONCLUSIONS AND FUTURE DIRECTIONS

Over the last decade, the direct analysis of biofluid samples has become a realistic alternative to time-consuming off-line extraction and purification procedures [11,12,15–20, 65]. This evolution has resulted in essentially two different approaches (SPE and turbulent-flow chromatography) for use in combination with MS instrumentation. On-line SPE coupled with conventional LC-MS has become a very mature method with the ability to conduct efficient on-line purification coupled with disposable columns [11,29,30]. The solid-phase sorbents can be of the conventional modified silica supports as well as RAM and monolithic sorbents. Intriguing future directions of this methodology will be the application of IM-RAM sorbents for the highly selective analysis of small molecules [49] and the use of μ -SPE-columns [38,40,41].

Turbulent-flow chromatography has become a flexible high sensitivity system, which employs a 2D LC-MS/MS approach [19]. The turbulent-flow component is employed to concentrate the sample and to remove proteins and phospholipids [5]. The resulting extract is then separated on an analytical column, which provides enormous flexibility in terms of flow rate and column type. The analytical column is then linked to a suitable ionization technique for high sensitivity MS/MS analysis. A series of novel applications has appeared recently for the analysis of both drugs and endogenous metabolites, suggesting that this methodology could hold the key for future complex metabolomic applications [5]. A particularly attractive feature of the turbulent-flow 2D LC-MS/MS system is the ability to remove constituents from the biofluid that cause suppression or enhancement of ionization [69]. This makes it a realistic proposition to conduct analyses in the subpicogram range [70], which raises the possibility that turbulent-flow methodology can be employed in the future for the routine direct analysis of plasma and serum for low abundance endogenous metabolites such as vitamins and steroids [70,71]. Similarly, the recent application of 2D turbulent-flow LC-MS/MS to the analysis of tricyclic antidepressants in serum [72] suggests that this could be a very

useful technique in the future for the direct analysis of drugs and their metabolites in plasma and serum.

Recently, a novel system incorporating an automated direct analysis in real time (DART) ionization source coupled with a triple-quadrupole mass spectrometer was developed and evaluated for direct analysis of drugs in biofluids [73]. This system can potentially eliminate the need for sample cleanup and chromatographic separations. Additional pumping of the mass spectrometer ion source was employed to compensate for the increased vacuum load from the high flow helium introduced by the DART. This resulted in an improvement of detection sensitivity by a factor of 10- to 100-fold and minimized matrix effects on a diversified group of analytes. The system was employed for the direct analysis of biofluid samples with similar results to those obtained with conventional LC-MS/MS methods. Therefore, this new automated DART-triple quadrupole mass spectrometer system has significant potential future for high throughput direct biofluid analyses [73]. Finally, the μ -SPE-based RapidFire System might be a useful tool for high throughput MS quantitation of *in vivo* biofluid samples in situations where large sets of samples justify the up-front burden of method development [44]. Clearly, direct analysis of biofluid samples has come of age and is now a realistic alternative to more time-consuming off-line purification procedures particularly when large numbers of samples are involved.

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17 Analytical Method Development and Validation in Accordance to the Regulatory Guidelines

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17.1	Summary	1
17.2	History of regulated bioanalysis	4
17.3	Response calibration	7
17.4	Matrix effects	14
17.5	Recovery	16
17.6	Carryover	18
17.7	Dilutions	18
17.8	Stability	19
17.9	Specificity and selectivity	22
17.10	Contamination	23
17.11	System suitability and response changes	25
17.12	Sample reanalysis	26
17.13	Multiple analyte assays	29
17.14	Assay transfers and changes	33
17.15	Documentation	34
17.16	Instrument qualification	35
17.17	Exploratory clinical studies	35
17.18	Larger molecules	36
17.19	Dried blood spots	39
17.20	Conclusions	40
	References	40

17.1 SUMMARY

Present requirements to perform bioanalysis for submission to regulatory agencies have evolved as the result of bioanalytical workshops held in 1990, 2000, and 2006. Following the second meeting, the FDA issued its guidance in 2001. The European

Medicines Agency (EMA) has issued draft guidance, which will be issued as final guidance in February 2012.

Setting the assay range includes assessment of the lower limit of quantification (LLOQ), which is related to precision and accuracy of the lowest standard, as determined in replicate measurements, and is required to be within 20%. Chromatographic assays generally yield linear or quadratic response over a broad range of concentrations. Multiple days of validation data are needed to establish reproducibility of the regression model. The correlation coefficient of the standard curve must be >0.99 . When validating in multiple species, a mixture of full and partial validations is common. As each matrix is unique, full validations should be undertaken whenever the matrix is changed.

For regulatory bioanalysis, matrix of the standards must match study samples. Included with each analytical batch are blank matrix, a sample with only internal standard (IS), and a minimum of six nonzero calibration standards. The absolute difference between the back-calculated and nominal concentration for each calibration standard should be within 15% for all standards, except at the LLOQ where $\leq 20\%$ is acceptable. A minimum of 75% of the standards should be within these limits. Values falling outside these limits can be discarded, starting with the most deviant value and iteratively reregressing until all results are acceptable or the run fails. In cases where it is not possible to obtain the matrix in sufficient quantity to prepare standard and QC (quality control) samples, a surrogate matrix may be used. While it is preferable to have a certificate of analysis, some compounds available in very small amounts require only less qualification.

QC samples should be positioned evenly through the batch, reflecting the concentration of study samples. QC samples measured at a minimum of three concentrations in duplicate (or at least 5% of the unknown samples) are required. Spacing is within $3 \times \text{LLOQ}$, one near the center and one near the upper boundary (top quartile). Wider-range assays should include the addition of more QC levels to better represent study data. At least two-thirds overall and 50% at each level must be within 15% of their nominal value. When performing an assay validation, more replicates and additional QC samples are required to assess accuracy and precision at the LLOQ and to test dilutional linearity. A minimum of five determinations at each QC concentration are required. Additional QC samples include the LLOQ QC whose intraday and interday precision and accuracy needs to be within 20%. Dilution QC samples should test the maximum range over which the dilution is anticipated.

Matrix effect is the suppression or enhancement of ionization of analytes by components in the biological samples and is measured by comparing the response from a sample of blank matrix to which an equivalent amount of analyte was added after extraction to an equivalent amount in mobile phase. ISs in MS (mass spectrometry)-based assays can minimize the impact of matrix effects by normalizing response ratios. Having a highly variable matrix effect in individual subjects may cause irreproducibility. Absolute and IS-normalized matrix effects for six individual matrix lots are tested, and variability should be within 15%. When using stable isotope IS, this determination is unnecessary.

To accurately assess the recovery of analyte or IS from the sample matrix, response from an extracted sample is compared to a sample of blank matrix to which an equivalent amount of analyte was added after extraction. Apart from the criteria that recovery of analyte and IS be consistent, precise, and reproducible, there is no required minimum.

Carryover in an HPLC system is contamination by the preceding sample. If carryover is more than 20% LLOQ, its acceptance should be justified and appropriate precautions used to minimize its impact. In contrast to carryover, contamination is unpredictable and may occur during any part of the study (dosing, sample collection, or analysis).

Guidance does not mandate that within a study dilution QC samples are used, provided dilutions are conducted with like matrix and tested within validation. A preferable approach is to include dilution QC samples whenever study samples require it.

Procedures must evaluate the stability of analytes during sample collection and handling. The effect of different anticoagulants on stability should also be tested. Use of selective inhibitors is preferred since these can be added in smaller quantities and can result in minimum change to the matrix. Nonspecific binding of drug to containers also needs to be considered. In addition to stock solution stability, assessments in the unaltered matrix of qualifying QC samples include long-term stability under storage conditions, freeze–thaw cycles, bench-top (room temperature and/or ice processing), processed sample or extract stability, and reinjection integrity.

Specificity should ensure that background response from six independent lots of blank matrix is <20% of the response from the LLOQ sample and <5% of the IS. Selectivity is defined as “the ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present.” Guidance requires that “each blank sample should be tested for interference, and selectivity should be ensured at the LLOQ.” An assay should be tested whenever clinical studies are performed, where there is the potential for concomitant medication or when used in drug–drug interaction (DDI) studies.

Only qualified and properly maintained instruments should be used for drug analysis. To qualify instruments at time of use, a system suitability test may be used. This generally includes replicate testing of the analyte for sensitivity and precision near the LLOQ before starting an analytical run. Similar tests following the run may be used to confirm instrument stability.

A means to judge individual results, based on the response of ISs, is often used. Rules must be in place that allow *a priori* decisions to reject sample results that show aberrant IS response. Use of a stable label rather than a structural analog as IS is always preferable, as, once equilibrated within the matrix, it compensates for any changes.

Samples may be reanalyzed for an assignable cause such as a technical error, poor chromatography, unacceptable IS response, or evidence of carryover. The original result is discarded, and the sample is reanalyzed in singlet. The acceptable reanalysis value is reported. Samples may also be reanalyzed for anomalous results or for pharmacokinetic (PK) reasons. Typically, confirmatory reanalysis is performed in replicate and the original result is either confirmed or replaced.

There are a number of situations where the performance of QC samples may not adequately mimic that of study samples from dosed subjects (incurred samples). Recent studies have required the assessment of incurred sample reanalysis (ISR). Randomly selected subjects include a minimum of 10% of the total analyzed samples. For larger studies, 5% of the total sample size is allowed. The difference between the initial and reanalyzed values should be $\leq 20\%$ (small molecules) in at least 67% of reassayed samples. Unlike repeat analysis for anomalous results or for PK reasons, ISR is performed in singlet from selected samples about the $C_{\max}$ and in the elimination phase.

Simultaneously measuring multiple analytes increases the statistical probability of run failures. Results should not be rejected for the remaining analytes if one analyte

fails. Concentrations from the first accepted run should be reported. If this analyte is repeated in simultaneous assays when analyzing for different analytes, it is not necessary to quantitate the already reported analytes.

Tiered assays can be used when reference standards of metabolites are unavailable and a preliminary assessment of human exposure and safety coverage is needed. There are multiple strategies that have been used to allow this across-species comparison. Once the metabolism is fully known, a solid decision can be made regarding what regulated assay(s) would be effective in supporting later clinical drug development.

Assay transfer within a laboratory or across laboratories involves an assessment of its equivalence following the change. Depending on the nature of the change, this may involve either a partial or full validation. Conformance testing by analysis of QC samples is a good practice but not required. Cross-validation by testing study samples is unnecessary unless two different assays are used within a single study.

Proper installation and operational and performance qualification of all instruments is required before performing regulated bioanalysis. User specifications and a validation plan should be developed. System functionality can be verified and any gaps identified. User acceptance testing (UAT) should include a traceability matrix to regulations.

When unsure of clinical success, exploratory investigational new drug (eIND) studies allow the assessment of new chemical entities that have undergone limited safety testing. Provided there is a sensitive LC-MS (liquid chromatography-mass spectrometry) assay or a method such as AMS (accelerator mass spectrometry) is used, a subtherapeutic dose may afford the opportunity to quickly determine adsorption, distribution, metabolism, and excretion (ADME) properties.

Peptides, small proteins, and oligonucleotides are representative of mid-size molecules that can be directly measured using LC-MS. Larger proteins may be digested and indirectly measured by LC-MS analysis of a peptide surrogate. When using a mixture of ligand-binding and LC-MS assays, cross-validation using both QC and study samples can help to illustrate assay differences.

Dried blood spots (DBSs) offer numerous advantages, particularly from the in-life study. Bioanalytical challenges include recovering the analyte from filter paper without the introduction of matrix effects and overcoming sensitivity limitations for highly potent drugs. As more investigators explore the potential of DBSs in clinical studies, there will undoubtedly be regulatory bioanalytical guidance that comes forth.

The nature of regulated bioanalysis is ever changing. New methodology, technology, and novel drug candidates challenge past practices. Common procedures are now in place for validation and sample analysis of both large and small molecules. Drug development has integrated new cultures and perspectives into our science, making it essential that global regulatory requirements are standardized to allow for new drug submissions in all countries.

17.2 HISTORY OF REGULATED BIOANALYSIS

The term *bioanalysis* was derived in the 1970s to describe the quantification of drugs in biological fluids in support of PK studies. It evolved over the next decade to include toxicokinetics, the assessment of systemic exposures in animals undergoing toxicology studies. As many drug candidates show nonproportional dose–exposure relationships,

underpinning toxicity assessments with plasma or target-organ drug levels was a significant advance.

Problems related to preclinical research studies revealed serious issues with the conduct of safety studies submitted to the FDA. As a result, FDA published in December 1978 its good laboratory practices (GLP)s regulations. These regulations were collected in Title 21 “Food and Drugs” of the Code of Federal Regulations (CFR) as Part 58 “Good Laboratory Practice for Nonclinical Laboratory Studies” and applied to all non-clinical safety studies intended to support research permits or marketing authorizations of products regulated by FDA. For clinical *in vivo* bioavailability or bioequivalence study, 21 CFR 320.29 can be applied.

In Europe, adherence to GLPs is governed by the European Union (EU) law and, as in the United States, is applied to nonclinical safety testing. In 1981, the Organization for Economic Co-operation and Development (OECD) finalized its GLP principles. Later, the European Community adopted the OECD principles of GLP, requiring states to incorporate into their laws that nonclinical safety studies be conducted to GLP standards. ICH S3A provides limited bioanalytical guidance in the assessment of toxicokinetics, whereas ICH E6 requires documented competence in bioanalysis for clinical studies. EMEA CPMP/EWP/QWP/1401/98 states that the bioanalytical part of bioequivalence trials should be conducted according to GLP principles.

Although clinical bioanalysis is generally performed to the same high standard as nonclinical bioanalysis, it cannot be considered GLP. Methods are validated to the same standards, and there is adherence to many parts of the quality system that are fundamental to GLP regulations. The European Bioanalytical Forum has promoted the use of “regulated bioanalysis” to encompass studies that adhere to common regulatory guidance for bioanalysis rather than “GLP bioanalysis.” Both terms will be found within the literature.

Present US requirements to perform bioanalysis for submission to regulatory agencies have evolved over the last two decades. Karnes *et al.* [1] provided early bioanalytical guidance. Shah *et al.* [2] published the conference report from the first bioanalytical method workshop. This report served as the foundation for bioanalysis for several years. The workshop defined accuracy, precision, selectivity, sensitivity, reproducibility, limit of quantification, stability, and recovery. Of these, the assessment of accuracy and precision was one of the most criticized parameters [3,4]. Statistical assessments from larger data sets were proposed as an alternative to fixed acceptance rules [5]. These arguments also found favor within the large-molecule bioanalysis community where less precise and accurate immunoassays had difficulty meeting rigid requirements. However, having simple, *a priori* rules by which to judge an assay validation and its application was welcomed by those performing small-molecule bioanalysis and was readily adopted.

During this period, two other guidance documents emerged from the International Conference on Harmonization (ICH), which were approved by the EU, the United States, and Japan [6,7]. While both dealt with analytical methods for pharmaceutical products, they nonetheless provided guidance on method validation. Because the workshop report was not an official document, the FDA decided that a guidance should be developed. The draft guidance was issued in January 1999. The second bioanalytical method workshop was held in January 2000 and reported later that year [8]. In 2001, the FDA issued its final guidance on bioanalytical method validation [9]. This guidance has served the small-molecule bioanalysis community for many years.

The large-molecule (biologics) community worked from separate guidance [10,11]. It was not until the third bioanalytical method workshop in 2006 that both the small- and large-molecule communities accepted similar rules for method validation and sample analysis. Both assess accuracy and precision in a similar manner; however, acceptance criteria differ. For large molecules, accuracy and precision were both required to be within 20% or 25% at the LLOQ, whereas the small-molecule community continued to require 15% (20% at LLOQ). Having both small- and large-molecule groups define common procedures and similar acceptance criteria allowed easier assessment of data by regulatory agencies.

The third bioanalytical workshop report was issued in 2007 [12]. Apart from the inclusion of the ligand binding or large-molecule bioanalysis into a common community, the most notable change was the requirement to demonstrate repeatability from incurred (study) samples. This had been a requirement of the Health Protection Branch for Canadian submissions from 1992 until the Therapeutics Products Directorate of Health Canada revoked the policy in 2003. Under this Canadian requirement, 15% of samples were randomly selected and reanalyzed.

Since 2007, ISR has become common practice for US submissions, which has added value by uncovering errors unnoticed from QC samples [13]. Other additions included an assessment of matrix effects [14] and carryover, as well as clarifications on stability testing and use of system suitability samples. More details on documentation were also added in the conference report, aiding in transparency and giving guidance on reporting failed runs.

Overall, there were limited changes to the prior procedures and acceptance criteria for small-molecule bioanalysis. There were significant changes for ligand-binding assays of large molecules. The report recognized the inherent differences in drug product manufactured from a biological process from drug substance generated by chemical synthesis. The heterogeneity of a biologic product, often including posttranslational glycosylation or phosphorylation, requires special consideration when defining validation criteria. Ligand-binding assays are often necessary to define not just chemical stability but the biological integrity in a stability assessment. In contrast, chemical stability of small molecules is more easily determined. Reagents, especially antibodies, used in ligand-binding assays may vary more than chemicals used in small-molecule assays. For these reasons, acceptance criteria were lessened. Assessment of total error (sum of the absolute value of mean bias and precision) was required to be within 30% (40% at LLOQ). No similar requirement of total error exists for small-molecule guidance.

The EMEA issued draft guidance on the validation of bioanalytical methods in September 2009, and requests for comments ended May 2010 [15]. Within it, many of the procedures and acceptance criteria within the FDA guidance and bioanalytical method workshop reports have been adopted. However, there are some notable differences. The EMEA guidance goes further in defining when the possibility of back-conversion of metabolites to parent should be tested. More detailed procedures on assessment of the matrix effect, particularly with regard to population diversity, have also been recommended. Inclusion of testing lipemic and hemolyzed plasma in the EMEA draft guidance parallels that found within the Brazilian regulations [16]. As a result of divergence from a commonly accepted FDA guidance, pharmaceutical companies are requesting that a common standard be adopted for global regulatory submissions [17,18]. Even minor differences in procedures or acceptance criteria could make a submission unsuitable in some countries. Therefore, establishing common

global expectations for method validation and sample analysis is critical to global submissions.

This chapter discusses regulatory bioanalytical requirements in a manner that attempts to provide guidance on how to achieve acceptance criteria. Deploying assays without fully characterizing their limits can result in failure runs. This is most apparent when assays are transferred to new laboratories or are used by new analysts. Best practices often include a standardized design and testing of assays during method development to better ensure that they are relatively insensitive to small changes. Knowing critical limits helps to hold an assay in control or provides the information needed to quickly diagnose and correct failures. We focus on the use of LC-MS, particularly in combination with mass spectrometry/mass spectrometry (MS/MS), to measure drug molecules and their metabolites to regulatory standards.

17.3 RESPONSE CALIBRATION

Sensitivity is defined as *the slope of the calibration model*. It should not be confused with the ability to detect or quantify an analyte in a given matrix. The limit of detection (LOD) is related to the signal-to-noise (S/N) ratio and, at a minimum, is required to be ≥ 3 . The exact assessment may vary dependent on whether peak-to-peak or root mean squared (RMS) noise is used. The LOD is rarely used in the field of bioanalysis, except that chromatographic peaks of standards must have a signal that is fivefold greater than the background [9]. Some have proposed that the LLOQ should require a S/N ratio of ≥ 10 . This is good guidance, as it is unlikely that all samples will be as free from interference as those analyzed during the validation. The LLOQ is related to precision and accuracy of the lowest standard, as determined in replicate measurements, and is required to be within 20%.

Having an understanding of what limits quantification at the extremes of the standard curve can guide method development and allow proper decision on assay range. In an ideal situation, detector response is proportional to concentration over a wide range. Provided there are limited losses due to adsorption (nonspecific binding), testing the assay range using pure solutions can establish the optimum detection range. Under these conditions, there are no recovery, stability, or matrix effects that can either dilute analyte concentration or its response. For a pure solution near the LLOQ, electronic noise limits detection. A major objective of method development should be the extraction and separation of drug from plasma to yield response limited by ion formation, transmission, and detection. Release of adsorbed analyte from a prior injection (injector carryover) can result in greater than the proportional response at low levels, so attention should be paid to the normalized response (response factors) to make a proper assessment. Near the upper limit of quantification (ULOQ), ionization and detector saturation can result in lower than the proportional response. MS systems using pulse counting electronics may also suffer from the inability to discriminate multiple ion events within the detection time frame. Within a 10-ns counting window, ion events $> 10^8$ counts per second result in detector saturation. Analog electron multipliers do not suffer from this problem but are more limited by electronic noise at the LLOQ. With present instrumentation, ionization saturation generally occurs before detector saturation. This is typical for electrospray ionization (ESI) and is discussed later in

greater detail. Unless limited by adsorption, the assay range will be no greater when the analyte is placed into the matrix than when it is in a pure solution.

When spiked into matrix, chemical background often interferes with detection near the LLOQ. Less obvious background such as phospholipids in plasma, bile acids in bile, and salts or urinary acids in urine can modulate MS detection and are referred to as *matrix effects* [14]. Both chemical background and matrix effects can significantly impact measurements at low levels. While it may be easy to detect analyte at low levels, precise and accurate quantification is more difficult. When coeluted with analyte, the numerous and abundant components within biological fluids and tissues, compete for ionization with the analyte and affect response. If they vary significantly from the matrix used to prepare the standard curve, there is the potential for error. Use of a stable label as an IS compensates for variation in matrix effects and is a good addition to any assay. For multiplex assays, obtaining multiple stable label ISs can be difficult. Caution should be used when measuring parent drug and metabolites with only stable label IS of parent drug. High levels of parent drug can suppress ionization of its stable label IS, which, if also used to measure metabolites, can result in errors.

Unlike immunoassays, chromatographic assays generally yield linear or quadratic response over a broad range of concentrations. Method development should strive to establish the simplest fit, a linear fit with minimum weighting. Validation should strive to establish the best and most reproducible fit. The regression model must be justified and shown to be reproducible. Justification involves testing multiple regression models and different fitting parameters. These tests should use validated software applications, including the MS or LIMS (laboratory information management system) software, which will perform the sample analysis.

Linear fits are common; however, quadratic models can be used. A quadratic regression model can be used to address less than proportional response at increasing concentrations due to ionization or detector saturation. When using quadratic curves, a stable label IS is highly recommended to overcome intersample variations and their potential impact on MS response at its ionization limit. By using a stable label IS, the response ratio is better normalized, resulting in an extended dynamic range. Less than proportional response at decreasing concentration is often due to adsorption losses and associated changes in extraction recovery. This is common for hydrophobic drugs in matrices such as cerebral spinal fluid (CSF) and urine. Quadratic fitting should not be used to correct for nonlinear response at lower concentrations. Less than proportional response at low concentrations should be corrected by selecting a different container, adjusting pH to enhance solubility, or adding a solubilizing agent such as a surfactant or organic diluent. In both extremes, sensitivity is decreased as either the recovery near the LLOQ or detection at the ULOQ is impacted.

A much preferred approach is to require linear fitting of calibration curves. Linear fits can restrict the dynamic range that may require more dilution of study samples. However, a linear response model generally has better interday reproducibility. A linear fit is therefore the preferred and the anticipated response function. Deviations from a linear regression model may be challenged, particularly when fitting parameters show significant interday variations.

Weighting ($1/x$ or $1/x^2$) should be used for heteroscedastic data or whenever error is proportional to sample concentration. These corrections should be carefully scrutinized as daily changes in carryover or contamination can impact whether $1/x$ or $1/x^2$

weighting should be used. The power for weights approach evaluates the linear dependence of the logarithm of the standard deviations of peak response on the logarithm of sample concentration [19]. Other statistical approaches can also afford an unbiased selection.

Guidance requires the correlation coefficient (r^2) to be >0.99 . It is likely that many regression models will meet the guidance criteria. Examining residual errors from validation data can aid in selecting the best fit. Proper documentation of the rationale for selecting the model is critical. Multiple days of validation data are needed to establish reproducibility of the regression model.

A common mistake is to define a linear model during validation only to find, following an improvement in instrument performance, that a quadratic fit may have been better. It is possible to inject less extract or simply retune MS performance to its less sensitive, linear state. However, if a wide assay range was needed, a quadratic fit may have been a better fit. When sensitivity is decreased, a quadratic fit is reduced to a linear fit (quadratic term $\rightarrow 0$). The reverse is not possible. Linear fits are preferred and can yield assays with reasonable dynamic range, provided extract purity and separation avoids ionization saturation. It should be noted that during the establishment of the regression model, variables, including injection volume, should be controlled. It is the total amount of coeluting components that affect MS response, so changes in their absolute amount will affect ionization saturation [20].

When validating in multiple species, a mixture of full and partial validations is common. Many analysts perform full (three days or more) validations in man and the primary (rodent and nonrodent) toxicology species with partial (one day or more) validations in other species. For instance, plasma assays in rat and either dog or monkey used for long-term (definitive) toxicology studies would receive full validation. Plasma assays for mouse (carcinogenicity or pharmacology), rabbit (reproductive), and dog (safety pharmacology; if monkey is used as primary) may be performed as partial validations. Others perform full validations in all species. Unless there is a reason to suspect a gender, or population difference, validations in humans generally assume equivalence. In contrast, validations in animals are generally both species and strain-specific. Testing any differences can be performed within the full validation to demonstrate equivalence on substitution. Changes in sample anticoagulant are not assumed to be equivalent and require at least a partial validation.

Provided there are no significant differences across species, the assay range established from the full validations should be applicable to other species. This simple assumption fails when there are significant interspecies differences in sample volume, drug potency, PK properties, stability, recovery, matrix effects, and endogenous interference. It is, however, preferred to test assay applicability across species before undertaking formal validations. Having a common analytical procedure reduces complexity in drug development. Much can also be learned from discovery studies, as assays were generally applied to numerous species and matrices.

Quantification of multiple analytes (comedications, metabolites) across species multiplies challenges in defining the proper assay ranges and regression models. Species differences in metabolism are common, resulting in different ranges and regression models for parent and metabolite. Multiple component assays should therefore be undertaken with great care to avoid deploying an assay with high failure rates. Some sense of assay requirements in each species can generally be gained by reviewing prior discovery studies, and this is helpful in devising a common analytical strategy.

The analyst's ability to control both contamination and carryover will also affect decisions on the LLOQ and assay range. Bioanalytical guidance restricts contamination of blank samples to within 20% of the LLOQ. EMEA also has guidance on measuring drug levels in control animals from toxicology studies [21]. Since the bioanalyst's role is to define whether control animals were contaminated, careful consideration of the LLOQ should be made to avoid false-positive detection and quantification. Contamination, held in control by more experienced analysts, may be increased when less experienced analysts undertake the analysis. Automation and proper training serve to reduce this risk.

The regulatory expectation is that assay range should be adjusted to study needs. It is common to have a high sensitivity, wide range assay during first-in-human studies. By the end of the multiple ascending dose (MAD) study, some indication of a safe, therapeutic dose should have been established. The analyst may therefore wish, at this time, to consider whether the assay range should be adjusted. Apart from being inefficient, dilution of study samples can amplify errors. This practice is more common to highly sensitive, narrow range immunoassays. However, dilution factors as great as 1000-fold have been used when measuring wide therapeutic margin drugs in high dose toxicology studies by LC-MS. QC samples must reflect study samples, so readjusting the range or adding extra QC samples may be needed.

As each matrix is unique, full validations should be undertaken whenever the matrix is changed. When urine data is critical to interpret PK or safety data, a full validation is needed and measurements may be required in all samples. For instance, in cases of compounds that show moderate-to-high renal clearance or where the kidney is the target site of action or toxicity, an assay should be fully validated in urine and a routine urine analysis may be required. Full validations are less needed to make early clinical assessments, particularly when animal studies show little parent drug is excreted in urine. Estimating urine levels at the highest single dose before deciding on the urine assay range for multiple dose studies is one means to better judge its requirements before validation. In either case, sufficient testing must ensure that sample collection avoids losses due to instability or irreversible adsorption. Measuring renal clearance and testing its dose proportionality at steady state is generally an objective that will require a validated assay. Most would accomplish this objective within the MAD study.

There is less guidance when demonstrating penetration into remote compartments. When critical to understanding PK–PD (pharmacodynamics) relationships, a full validation should be undertaken. For instance, measuring levels in synovial fluid for arthritis [22], bone for osteoporosis [23], CSF for CNS [24], or tumor for oncology [25], drugs would support target engagement. Tissue analysis is common in discovery to ensure penetration at the site of action, but translation to human studies can be difficult. There are many practical limitations, including obtaining tissue or bone samples, finding sources or surrogates for these matrices, assessing consistency in recovery of standards versus study samples, and ensuring the proper preparation of homogenates. Within toxicology studies, staff is often skilled and can prepare homogenates for storage before shipment and bioanalysis. Storing homogenates rather than tissue allows one to conduct a proper stability campaign. In contrast, preparing homogenates at multiple clinical sites is a challenge. These objectives should be supported using a full validation.

If one wants to demonstrate penetration to numerous clinical tissues but in a limited numbers of samples, a partial validation is generally acceptable. This would be the

case of an antibiotic tissue distribution study to determine whether numerous nonsystemic levels exceeded drug potency throughout the dosing interval (e.g., $C_{\text{tissue}} > IC_{50}$). A similar penetration assessment of antiviral agents measures their intracellular drug levels in peripheral blood mononuclear cells (PBMCs). Intracellular determinations afford a better understanding of drug uptake, efflux, and intracellular metabolism [26,27]. Since only a single matrix is involved and there are no limitations on its availability, a full validation would be expected.

17.3.1 Standards

For regulatory bioanalysis, standards must match study samples, which are most frequently plasma, blood, or urine. It is unacceptable to quantify study samples using another matrix standard curve. It is acceptable to dilute study samples within another matrix, provided standards are prepared in an identical manner. This may aid quantification by using a mixed calibrator curve or by diluting samples in a less problematic matrix.

Included with each analytical batch are blank matrix (sample without IS), zero standard (sample with IS), and a minimum of six nonzero calibration standards. The absolute difference between the back-calculated concentration and nominal concentration of each calibration standard should be within 15% for all standards, except at the LLOQ where $\leq 20\%$ is acceptable. A minimum of 75% of the standards should be within these limits. Values falling outside these limits can be discarded, starting with the most deviant value and iteratively regressing until all results are acceptable or the run fails. When performing sample analysis, the exclusion of calibration standards cannot change the established model.

One standard curve is acceptable. Some prefer to use a second curve, often placing one at the front and the other at the end of the run. A significant change in sensitivity over the course of the run or a mistake in preparation will be noted by this approach. All results must be integrated into a common standard curve. Runs where the sensitivity changes over the course of the run by more than 25% will generally fail to match standards with QC samples.

It may not be possible to obtain the matrix in sufficient quantity to prepare standard and QC samples. In these instances, a surrogate matrix may be necessary. This is often the case in pediatric or human penetration studies. If unavailable, artificial CSF may serve as surrogate matrix for human CSF. Likewise, the same animal tissue from a closely related species may serve as a surrogate for human. A partial test of equivalence is always needed. Using predose or control matrix to prepare standard and QC samples is sometimes possible. Dilution using a more common matrix also helps to reduce the need for large quantities of matrix.

Although it is preferable to have a certificate of analysis for all analytes, some compounds are available in very small amounts, with less qualification. Since the issuance of the Metabolites In Safety Testing (MIST) guidance [28], the sourcing and qualification of metabolite reference standards has become increasingly important. More on this topic is presented later. Guidance requires that reference standards come from the USP (United States Pharmacopeia) compendial standards, a reputable commercial source, or are custom synthesized and qualified by an analytical laboratory or other noncommercial establishments [9]. Source and lot number, expiration date, certificates of analyses (if available), and evidence of identity and purity must be furnished.

Biosynthetic or isolated metabolites yield quantities that are insufficient to be as well characterized as synthetic reference materials. One approach isolates microgram levels of individual metabolites for identification using MS and NMR, with quantification using NMR [29,30]. NMR signal response is independent of structure, allowing isolated metabolites to be quantified by comparing their response to that of a known added amount of parent drug. In another approach, radiochemical detection has been used to calibrate stock solutions of metabolites [31]. This approach requires separation of metabolites, as, unlike NMR or MS, detection is nonspecific. Detector response serves to calibrate the concentration of each metabolite by the response to parent drug. The bioanalytical standard curve is prepared by spiking the isolate into the matrix with subsequent dilution. The value of radiochemical detection is that metabolites do not need to be isolated or identified before their quantification. However, radiolabeled drug is needed. For measurements in humans, the metabolite also must be seen in animals or from human *in vitro* experiments. Neither approach requires time-intensive synthesis. However, without synthesis, there is no confirmation of the assigned structure or supply for testing of activity or toxicity. Bioanalytical and biotransformation scientists need to carefully decide how to undertake metabolite quantification to properly integrate approaches with clinical ADME studies.

When measuring endogenous compounds such as biomarkers, the use of a surrogate analyte may be required. Removing endogenous levels by charcoal stripping can be ineffective [32]. Likewise, antibodies may be unavailable for immunoprecipitation. Addition of a stable label analog can serve as a surrogate calibrator. For instance, a tris ^{13}C stable isotope is measured for standard and QC samples. The unlabeled isotope is measured in study samples. To avoid primary kinetic isotope effects, use of a ^{13}C or ^{15}N stable label is preferred. If a ^2H stable label is used as a surrogate analyte, its response must be demonstrated to be equivalent to its nondeuterated form.

Another variation of the surrogate analyte approach is when a peptide digestion product is measured as a surrogate for a protein. Protein is indirectly determined by measuring a representative peptide following enzymatic digestion. In one example, matrix metalloproteinase-9 (MMP-9) was determined in mouse serum by capturing on magnetic beads coated with MMP-9 antibody, trypsin digestion, and LC-MS/MS analysis of the surrogate peptide GSPLQGPFILTAR [33]. Assessment of sequence specificity of the selected fragment was aided from database searching. Stable label of the peptide can serve as IS, and pure peptide is used as reference standard. Highly accurate peptide assays can be established. However, consistent capture and conversion of the protein to its surrogate peptide is needed for accurate analysis of protein. Any variation in recovery or conversion of a study sample from the standards will result in errors. Addition of IS following immunoprecipitation and enzyme digestion only serves to compensate for variations in the LC-MS analysis. Similar concerns are present when assaying antibody–drug conjugates (ADCs) for total drug [34]. A more accurate, but less feasible, approach is to incorporate stable label protein before isolation and digestion.

17.3.2 Quality Controls

QC samples should be positioned evenly through the batch, reflecting the concentration of study samples. When study data fall over a small percentage of the calibration curve, none of the QC concentrations may be near the unknown concentrations, limiting the

monitoring power of the QC samples. Adjustment of either the assay range or QC number and placement is expected.

QC samples are measured at a minimum of three concentrations in duplicate (or at least 5% of the unknown samples) are required. Spacing is within $3 \times$ LLOQ, one near the center and one near the upper boundary (top quartile). Grouping “near the center” or “midrange of the calibration curve” has afforded some latitude in mid-QC placement. Some have moved from its linear (50% of ULOQ) placement closer to a logarithmic (geometric mean) placement. This is consistent with a desire to more accurately capture the elimination phase. Depending on interpretation, there are significant differences. Table 17.1 illustrates the disproportionate gaps between QC samples when a linear placement is used instead of a geometric mean. When calculating the mid-QC, adjustment of its final concentration should be done to allow for easy preparation of this QC. Another option is to include two mid-QC samples. This has the added value of providing better coverage when study samples differ from their expected concentrations. Wider-range assays (≥ 1000 -fold) should include the addition of more QC levels to better represent study data.

During sample analysis, each batch should contain at least six (minimum 5% of total) QC samples. QC samples at a minimum of three concentrations in duplicate are added to each plate. At least two-thirds overall and 50% at each level must be within 15% of their nominal value. It is advantageous to avoid odd replicates, as at least two of three QC samples would be required to meet the 50% rule.

The analyst should make every effort to minimize the amount of organic spiking solution and to match QC and standard preparation. Study samples contain no organic solution. Even a few percent of an organic solution can denature proteins, causing a loss in enzyme activity or protein binding. The result is an inaccurate assessment of stability. Deactivation yields a false stability not seen with incurred samples. Apparent instability due to analyte entrapment within precipitating plasma proteins or instability when analyte is released from protecting proteins can also occur. Preparation by serial dilution in matrix is one effective means to reduce the organic composition in QC samples.

When performing an assay validation, more replicates and additional QC samples are required to assess accuracy and precision at the LLOQ and to test dilutional linearity.

TABLE 17.1 Dynamic Range and its Associated Gap Between QC Samples Using Either Linear (Top) or Geometric Mean (Bottom) QC Placement of Middle QC

Range	Low	Mid	High	Fold Gap (Low–Mid)	Fold Gap (Mid–High)
<i>Linear Mid-QC</i>					
100	3	20	80	5.7	3.0
250	3	50	200	15.7	3.0
500	3	100	400	32.3	3.0
1000	3	200	800	65.7	3.0
<i>GM Mid-QC^a</i>					
100	3	15.5	80	4.2	4.2
250	3	24.5	200	7.2	7.2
500	3	34.6	400	10.5	10.5
1000	3	49.0	800	15.3	15.3

Abbreviation: GM, geometric mean.

^aSquare root (low QC $\times$ high QC).

A minimum of five determinations at each QC concentration are required. In practice, many laboratories use replicates of six to better align with acceptance criteria. Additional QC samples include the LLOQ QC whose intraday and interday precision and accuracy needs to be within 20%. Dilution QC samples should test the maximum range over which the dilution is anticipated. If proven during validation, dilution QC samples do not need to be run in study sample analysis. This approach tests only dilutional linearity of the assay. It does not provide routine monitoring of the analyst's ability to execute dilutions nor does it test the ability to dilute matrix from different subjects (parallelism). For that reason, many include dilution QC samples within runs to support values reported for any diluted samples. However, neither LLOQ nor dilution QC samples need to be included within regular analytical runs.

While up to 25% of standards not meeting acceptance criteria are deactivated, QC samples should never be removed unless there was an assignable analytical cause. Measurement of the intrarun and interrune assay precision and accuracy during both validation and study sample analysis requires the entire population of QC data. Outlier tests such as Dixon's or Grubbs' [35] test may be applied, but they should be judiciously used.

QC samples are considered as a surrogate for study samples in which the true (nominal) concentration is known. Testing of incurred (study) samples reflects the observation that QC samples do not always behave the same as study samples. Some causes for this observation are discussed later. While replicate analysis of incurred samples tests interday precision, QC samples generally provide the only assessment of accuracy. Additional means to derive a true result from an incurred sample include measurements at multiple dilutions (tests parallelism) and the method of standard addition. Both require multiple aliquots of sample, are time intensive, and would need validation. For these reasons, they are not generally used. Validation establishes precision using QC samples selected by the bioanalyst, whereas ISR tests interday assay variability from the study population.

17.4 MATRIX EFFECTS

Nonselective detection of analyte signal, resulting in the apparent elevation of its true concentration, is referred to as *background* or *chemical noise*. Components within the chemical matrix can also either reduce or elevate the apparent analyte concentration by affecting detector response. The matrix effect is therefore the suppression or enhancement of ionization of analytes by matrix components in the biological samples [36]. A quantitative measure is the matrix factor (MF), which is the ratio of the analyte peak response in the presence of matrix ions to its response in the absence of matrix ions (Table 17.2). Spiking analyte into extracted blank matrix and comparing its response to an equivalent amount of pure compound is the best means to quantitatively assess this parameter [37]. The MFs of both the analyte and ISs should be determined. The relative MF is the ratio of the two and should be near 1.0.

Matrix effects are generally due to components within the sample, which are at high abundance, and yield high response. Salts or urinary acids are abundant and ionize readily in ESI, affecting analyte response in urine assays. Urinary salts or acids can also entrap drug during precipitation, so making the proper assessment is important. The high abundance, good surface properties, and ionic nature of phospholipids make them

TABLE 17.2 Determining Matrix Factor and Recovery (Extraction Efficiency)

Analyte	Internal Standard
Preextraction spike	Preextraction spike
Postextraction spike to blank	Postextraction spike to blank
Mobile phase spike	Mobile phase spike
Recovery (analyte or IS) = (preextraction spike/postextraction spike) $\times$ 100%	
Matrix factor (analyte or IS) = (postextraction spike/mobile phase spike)	

a concern in plasma assays [38,39]. Bile acids, present at millimolar concentrations in bile, can impact analyte ionization. The levels of bile acids in urine or plasma are far less and therefore not generally a problem in these matrices. Each matrix and each ionization method has its unique challenges, requiring sufficient extraction or chromatographic separation before ionization.

Ionization that occurs concurrently with desorption from a condensed phase is generally more prone to matrix effects [40]. ESI has shown greater matrix effects than atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI). The requirement to volatilize analyte before either ionization within a corona discharge (APCI) or photoionization (APPI) source effectively removes many matrix components that impact analyte ionization [41,42]. Phospholipids are not volatile and therefore do not present the same problems in APCI or APPI as they do in ESI. However, many analytes are not volatile and require ESI with proper sample processing before ionization.

Indirect detection of matrix effects by postcolumn infusion is relatively insensitive and does not identify the offending component within the matrix [36]. Having a direct and simple means to identify exact phospholipids in plasma greatly assists their monitoring and removal [43]. Positive precursor ion scan of m/z 184, positive neutral loss (NL) scan of 141 Da, and negative precursor ion scan of m/z 153 are the three widely used means. When a total assessment of phosphatidylcholines is desired, monitoring the m/z 184 $\rightarrow$ 184 transition under high source energy is most commonly used [39].

ISs in MS-based assays can minimize the impact of matrix effects by normalizing response ratios. Matrix effects observed for stable isotope labeled IS are similar to those observed for the analyte, particularly for ^{13}C or ^{15}N and less so for ^2H stable label analogs. From a practical perspective, deuterated ISs are generally more readily available and some can be prepared using single step alkylation or hydrogenation reactions. Deuterated stable labels can exhibit primary kinetic isotope effects not seen with ^{13}C or ^{15}N stable labels. They are generally acceptable, provided the label is not scrambled during MS analysis and is metabolically stable within the matrix. The introduction of higher resolution separations (e.g., UPLC (ultraperformance liquid chromatography)) and more extensive substitution can result in uncompensated matrix effects if analyte and IS elute at different times. For these reasons, some have suggested coelution is always preferred, including when using a structural analog [44].

Matrix effects are less a problem when the matrix of the study population matches that used to prepare standard and QC samples. If the analyte response is affected in study samples, it should be similarly affected in standard and QC samples. Detection sensitivity can be affected but accurate quantification would not be. That premise is

generally true for animal studies, in which strains of similar genetic and environmental background are tested.

Matrix effect measures response from a sample of blank matrix to which an equivalent amount of analyte was added after extraction (postextraction spike) to response from a sample that contains the same amount in mobile phase (no matrix). Having a highly variable MF in individual subjects would be a cause for the lack of reproducibility of analysis. It is therefore required that the absolute MF and IS-normalized MF for six individual lots of the matrix be tested and that the variability in MFs, as measured by the coefficient of variation (CV), be within 15%. However, when using stable isotope IS, it is not necessary to determine the MF in six different lots.

To test assay specificity, Brazilian requirements define that samples of the biological matrix must be analyzed from at least one lipemic and one hemolyzed sample [16]. Blank samples must be tested using the established procedure and results compared with those obtained from the aqueous solution of the analyte, in concentration close to the LLOQ. A similar assessment within the EMEA guidance requires measuring MF in QC samples prepared from both lipemic and hemolyzed plasma [15]. This assessment will test the potential for phospholipid suppression at its extreme.

17.5 RECOVERY

The introduction of requirements to test sample diversity during method development and validation is good assurance that methods either will be entirely insensitive to population variability or will define their failure points. Brazilian guidance also requires that at least one hemolyzed sample be tested for specificity in a manner similar to that noted above [16]. While spectral interference from hemolyzed red blood cells (RBCs) is common in colorimetric assays used in clinical chemistry, the specificity of LC-MS assays makes them less prone to this problem. There are cases, however, when an extensively hemolyzed sample measured using LC-MS will be affected. The cause may include instability following the release of RBC enzymes, lower recovery from cell debris, or matrix effects from RBC components. The EMEA guidance requires measuring MF in at least one low QC sample prepared from hemolyzed plasma [15]. Testing extensively hemolyzed plasma assures one that lysed blood cells or their intracellular contents do not impact analyte stability, recovery, or detection. If the assay is sensitive to hemolysis, knowing its limits would allow one to make assessments of whether data should be reported [45,46]. Since most samples are not measured for hemolysis, attention to sample conditions and good documentation is needed.

Having an assay that meets global (FDA/US, EMEA/Europe, ANVISA/Brazil, TGA/Australia, SFDA/China, and MHLW/Japan) requirements is essential to support the registration of new drug candidates in all countries. Some of the less common requirements are associated with how matrix effect and recovery are assessed. There is an extensive effort among the bioanalytical communities to ensure that common standards are adopted. Included in this effort are the Global Bioanalysis Consortium, European Bioanalysis Forum, and Global Contract Research Organization Council for Bioanalysis. Large challenges lie ahead to achieve the right balance of consensus and the appropriate level of detail in the upcoming EMEA and FDA Guidance.

To accurately assess the recovery (extraction efficiency) of analyte or IS from the sample matrix using MS, matrix effects must be eliminated. This is achieved by

comparing response from an extracted sample to a sample of blank matrix to which an equivalent amount of analyte was added after extraction (Table 17.2). Use of a postextracted spiked sample corrects for matrix effects. Low, mid, and high QC levels need to be tested for recovery. Apart from the criteria that recovery should be consistent (independent of concentration), precise and reproducible for both the analyte and IS, there is no required minimum. Higher recovery is better, as even small changes in a low recovery assay can have great impact. Some laboratories do require a minimum by standard operating procedure (SOP).

The use of a postextraction spike works well for off-line extraction, but what about on-line methods such as turbulent flow [47]? When matrix is directly injected onto an on-line extraction with subsequent LC-MS analysis, an isolated extract is not obtained. In this case, the extraction recovery is presumed to be quantitative. To comply with the draft EMEA guidance, interbatch variability in response should be assessed by analyzing at least six batches of matrix in triplicate and achieving an overall CV within 15% [15].

Provided a close structural analog has been chosen as the IS, comparable recovery should be seen. Indeed, this should be one of the many defining criteria used in selecting an appropriate IS. Whenever possible, a stable label is preferred. Even still, it is important to ensure that the IS is properly equilibrated with the analyte in the matrix before beginning extraction. High affinity binding to matrix components can be difficult to disrupt. For instance, 2 mM of a competitive displacer was incubated for 2 h at 37°C to fully displace nanomolar levels of roxifiban from platelets [48]. Even the use of a stable label will not compensate for a procedure that improperly equilibrates IS before extraction.

Achieving consistent recovery from plasma or serum is less problematic than when one is working with blood, cells, tissues, or hard matrices such as bone. Partitioning between blood cells and plasma is important to measure for two reasons. First, it may determine the choice of matrix, blood, instead of plasma. Second, time-dependent partitioning is possible, so differences in the harvesting of plasma will impact PK. Similarly, slow release of analyte from tissue homogenates can complicate analyses. Proper introduction and equilibration of IS before extraction is needed to overcome differential recovery [49]. Recovery from study samples that have undergone different periods of equilibration should be tested. Slow equilibration of IS with analyte in the matrix may require a longer equilibration time, more vigorous mixing, or the addition of acid or a competitive displacer to overcome high affinity binding. Matrices such as bone require complete digestion before analysis [23]. Matrices that are more complex and difficult to homogenize will always present challenges to prepare standard and QC samples that mimic study samples. For complex cases, dosing radiolabeled drug and assessing recovery from study samples is an alternative approach.

Through-LC recovery, which for absorptive compounds can be greatly reduced at lower concentrations, is generally not tested or known. When low level adsorption is seen, the standard curve may have decreased sensitivity at lower levels than that seen from upper standards. Using high levels of an appropriate IS may compensate by masking active surfaces and exhibiting similar recovery at lower levels. It is more appropriate to select a better stationary or mobile phase that overcomes these low level losses. Peak tailing and varying background are often associated with reduced through-LC recovery at low concentrations. Both symptoms illustrate a propensity for high affinity binding to the injector or stationary phase. Including additives to the

mobile phase can overcome losses, but it must be done with care to avoid affecting MS response. The lack of understanding and correcting through-LC recovery problems can result in sensitivity changes during the run, poor apparent detection sensitivity, and variability in the chromatographic baseline. It is one reason why an extensive number of conditioning samples may be needed in an assay.

17.6 CARRYOVER

Carryover in an HPLC system is contamination by the preceding sample. It is generally predictable and quantifiable by examination of the method, allowing an accurate assessment of its impact. Method development should define the individual sources (e.g., liquid handler vs HPLC) so that its causes can be eliminated or reduced. Method validation should accurately measure carryover to define its expected contribution when samples are assayed. Relative carryover is measured by injecting the following sequence, blank-ULOQ-blank. An acceptable result is when the difference of the blanks is $\leq 20\%$ LLOQ. If carryover is more than 20% LLOQ, its acceptance should be justified and appropriate precautions used to minimize its impact.

A direct, yet inconvenient correction is to restrict the dynamic range of the assay. Precautions include the proper placement of samples to minimize changes in concentration and protection of samples with washout blanks. Even still, the influence of carryover is unknown when the preceding sample is above the limit of quantification (ALQ). It is not until the ALQ sample is reassayed that an assessment can be made. Concentration-dependent carryover can complicate this assessment. Saturation of non-specific binding sites causes concentration-dependent carryover and is most evident when a larger relative carryover is seen on subsequent injections (delayed washout). One can implement procedures that recognize high carryover within a run and *a priori* rules to reject individual samples or batches [50]. If not reduced and uncontrolled, one should be prepared to fail individual results or runs, intersperse blank samples, and state its potential impact on study results.

For these reasons, there is considerable commercial effort to reduce the absorptive nature of materials used in HPLC systems, particularly within autosamplers. Improvements include using more inert materials, avoiding contact with the syringe needles, and improving needle or valve washing. The present carryover criteria have all but eliminated the direct injection of matrix in bioanalysis, as this approach often results in the buildup of absorptive components [47].

Carryover forced a revision of earlier guidance that recommended randomizing samples. Randomization could result in low level (24 h) samples immediately following a high level (2 min IV) sample. In this example, the preferred sequence to minimize carryover would be to start assaying the latest time point samples and move to earlier ones.

17.7 DILUTIONS

Guidance does not mandate within study dilution QC samples to be used, provided dilutions are conducted with like matrix and tested within validation. The extent to which study samples are diluted must be tested during validation. Although allowed,

this approach does not check that dilutions were properly done during study sample analysis. A preferable approach is to include dilution QC samples whenever required by study samples.

When drug levels exceed the range of the highest dilution, additional dilution factors can be tested during sample analysis. If dilution is performed with an unlike matrix, QC samples must be diluted in the same manner as the study samples and analyzed with the diluted samples. All diluted QC samples should be created within the assay calibration range.

A few laboratories employ a procedure that measures the same sample at multiple dilutions within the same run. This procedure is more common when drug levels are unknown, high dilutions are needed, and/or curve ranges are limited. While this approach can provide more confidence in assay results by replicate demonstration of parallelism, *a priori* acceptance and reporting rules must be established.

17.8 STABILITY

Procedures must evaluate the stability of analytes during sample collection and handling. Conditions used in stability experiments should reflect situations encountered during actual sample handling, including the collection of plasma from blood. A common test is to process plasma or serum during blood collection as shown below:

- spike fresh blood with analyte(s) at low and high QC concentrations
- incubate at ice, room, and body temperatures (or warm blood to 37°C before room or ice processing)
- harvest plasma after 0, 30, 60, and 120 min of storage using a nonrefrigerated and/or refrigerated centrifuge
- transfer plasma to fresh tubes, and store frozen until analyzed together in a single set.

When analyzed, the compound is considered unstable when the measured concentration is less than 85.0% of the initial concentration. If performed at 37°C, this procedure can also be used to assess blood-to-plasma partitioning, as T0 plasma concentrations different from the nominal blood concentration reflect partitioning. As noted previously, knowing the blood-to-plasma ratio can define the assay matrix. For drugs that are highly partitioned into blood, analyte should be measured in blood and not plasma. Highly unstable compounds or those showing time-dependent blood-to-plasma partitioning will require special attention. Testing blood stability in all species is preferred since blood-to-plasma partitioning can be time and species dependent and enzyme levels vary across species. Blood should be freshly collected to avoid loss of enzyme activity.

Esterase activity in rodents is elevated but reduced in higher animals and man. For prodrug and acyl glucuronide assays, conditions that stabilize mouse or rat blood are generally sufficient for human, dog, or monkey studies. However, it is common to have difficulties if the procedure used to establish stability in humans is assumed to be sufficient for rodents. Likewise, oxidases and other enzymes can show similar species specificity. Thiols that form mixed disulfides can be challenging and require conditions to stabilize their breakdown back to parent drug. Owing to interspecies differences in

enzyme activity, it is preferred to determine stability in all test species at the beginning of method development.

Sensitivity to light is often known or easily tested, and hence, yellow lighting for sample processing and amber containers for storage are commonly required. Chemical and light stability assessments have generally been performed on new drug candidates, so this information should be available.

The effect of different anticoagulants on stability should also be considered. EDTA, a common anticoagulant, can be an effective phosphatase inhibitor. Phosphate ester prodrugs collected in EDTA plasma are stabilized. Heparin does not afford the same protection. Likewise, a drug that is a strong chelator may yield different apparent stability in EDTA than when using a nonchelating anticoagulant. Heparin can afford stability by binding drug while EDTA cannot. The stability in different anticoagulants should not be considered equivalent and must always be tested.

When measuring metabolites, it is essential to use an assay that is specific for the metabolite and to employ proper stabilization of any phase II conjugates or interconverting forms (e.g., lactone $\rightarrow$ acid). Phase II conjugates are common. Fortunately, their physiochemical properties often afford a relatively simple separation. The same is not true for phase I metabolites. It is common to have multiple hydroxylated forms, of which either one or many may maintain activity and be of interest for routine measurement. It is critical that all others (and their phase II conjugates) are separated from the metabolites being measured. During method development, it is helpful to obtain a source of known metabolites and spike them into the matrix. Preliminary stability tests using changes from initial response can guide decisions on what stabilization is needed.

The use of selective inhibitors is preferred since these can be added in limited quantities and can result in minimum change to the matrix. This assumes that the general enzymatic process is understood. For esters and lactones, esterase inhibitors such as diisopropyl fluorophosphates (DFP), phenylmethylsulfonylfluoride (PMSF), and 2-thenoyltrifluoroacetone (TTFA) can be effective. For phosphate esters, EDTA and Halts phosphatase inhibitor are commonly used. For oxidases, antioxidants such as ascorbic acid can be effective. Amides may have sensitivity to esterases, but they can also be hydrolyzed by fatty acid hydrolase or proteases. For peptides or proteins, a general protease inhibitor such as aprotinin is often needed. Since an understanding of its metabolic fate may still be evolving for new chemical entities, some screening of different classes of stabilizers may be needed [51].

Many peptides and proteins show high nonspecific binding, requiring the use of special sample collection tubes such as the NuncTM immunoassay tube. Adsorption losses may be misinterpreted as instability. To minimize losses, testing of containers should be considered. Adsorption losses are common in matrices such as urine and CSF and may be overcome using surfactants (CHAPS, Tween-20, Triton X-100), protein (BSA), or EDTA to minimize surface contact or using additives (organic, acid, or base) to enhance solubility. If samples were collected before a procedure is established, it may be possible to recover the absorbed compound from the collection tube. In other cases, drug is irreversibly bound and cannot be recovered. For urine, generally smaller aliquots, rather than the large primary collection vesicle, are stored. Laboratory tests should determine whether or not additives are needed at the collection site. It is a mistake to not test drug adsorption onto urine or CSF containers before a study is started. The procedure should account for any dilution on addition of a stabilizing

solution. Provided they can be properly aliquoted, concentrated solutions are preferred, as the dilution is limited. If a fixed amount is added to the container, one should test extremes in urine volumes. Although it is better to adjust the aliquot to the weight or volume of the urine collection, this is often impractical in the clinic [52].

Use of a low pH (3–4) acid or buffer (formic and citrate) affords optimum chemical stabilization of esters or lactones. Lowering the pH is also important to avoid migration or anomerization of acyl glucuronides [53]. Direct assays for acyl glucuronides must ensure specificity to the 1- β isomer, as one cannot assume that other migrated isomers yield the same MS response. It is therefore important that the 1- β anomer be resolved from all other forms [54]. One should always consider reducing the pH when collecting samples of an acid-containing drug to avoid the breakdown of any potential acyl glucuronides. However, there can be times when the drug itself is less stable under these conditions, so some preliminary stability assessment is warranted before defining sample collection conditions.

When labile ester-containing drugs are measured, both an esterase inhibitor and low pH may be needed. Using a high concentration of acid or buffer alone to denature all enzymes should be avoided. What stabilizes drug and metabolite may destabilize the matrix and complicate the assay. Stabilizers added at high concentration can result in difficulties because of inhomogeneous sampling and poor recovery from a viscous matrix. Hemolysis and gelling are far more common when adding stabilizers. Difficulties in sample collection at clinics may not be predicted from laboratory experimentation. Stabilizers may also cause interference, either directly as chemical interference or indirectly by suppressing ionization (matrix effects).

When adding stabilizers, it is important to correct for any dilution. Stabilizer added to blood within the Vacutainer™ will require an assessment of its partitioning into both blood and plasma. This is generally assumed to be, when corrected for hematocrit, equal. When preparing tubes, it is essential that vacuum is maintained and a regular assessment of the effectiveness of the stabilizer is performed. If plasma or serum harvesting can be done without the need to add stabilizer to blood, then a simpler dilution is possible. In practice, the use of a commercial tube that contains a solid stabilizer (e.g., sodium fluoride) is preferred, as this avoids dilution and preparation issues. While sodium fluoride may afford adequate esterase protection for some drugs in certain species, it may be inadequate for others. This is commonly the case in rodents, so special collection procedures may be needed for rodent studies, whereas a commercially available tube containing sodium fluoride may work for clinical studies.

Once proper means to measure drug levels and stabilize samples have been achieved in method development, validation can proceed. Validation will include appropriate assessments to determine stability. No sample can be reported without ensuring that stability determinations covered the period of sample storage and processing. Stressed (minimum low and high) QC levels must be within 15% of their nominal value. Assessments in the unaltered matrix of qualifying QC samples include

- long-term stability under storage conditions
- freeze–thaw cycles
- bench-top (room temperature and/or ice processing)
- processed sample or extract stability
- reinjection integrity.

Details on the stability procedures are given within the references and are not repeated here. Stability assessments can be an ongoing process as the studies progress. Most laboratories will require a minimum of three freeze–thaw cycles, six or more hours of bench-top stability and extract stability, and two weeks of long-term stability within the validation. If the sample extract is to be reanalyzed, successful reinjection of an acceptable run is required (reinjection integrity). This is different from an extract stability assessment, which is demonstrated using a fresh standard curve.

The stability of reference standards and IS stock solutions must also be demonstrated under storage and bench-top conditions. Secondary stock solutions not immediately used must also be tested. An apparent degradation of more than 10% is unacceptable. Mixtures of stock solutions can be more problematic, as common ion effects may limit solubility. Frozen stock solutions are to be avoided. Testing of different solvents and containers during method development is good insurance that stability assessments in validation will be successful.

17.9 SPECIFICITY AND SELECTIVITY

FDA guidance states that the specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix [9]. This assessment is made by ensuring that background response from six lots of blank matrix is $\leq 20\%$ of the response from the LLOQ sample and $\leq 5\%$ of the IS. Unless a nonselective channel is monitored or analysis at low levels is performed, LC-MS-MS chromatograms generally show few interfering peaks.

FDA guidance recognizes that for MS-based methods, testing six independent matrices for interference may not be definitive [9]. In the case of LC-MS assays, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised. Selectivity is defined within the guidance as “the ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present.” Guidance also requires that “each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).” The most definitive test is to measure the LLOQ concentration from six different lots of matrix to $\pm 20\%$ acceptance.

Laboratories need to decide whether a matrix lot that fails can be reanalyzed and how many additional lots should be tested. A reasonable rule would ensure that if more than 10% of tested lots fail, the analyst should consider redeveloping the method. Owing to the specific detection employed in MS, fewer lots of matrix are screened for interference and assessed for selectivity than when using less specific assays such as immunoassay. Accurate quantification may be affected by causes other than nonspecific detection, so measuring QC concentrations in at least six different lots of matrix is required to support assay selectivity.

An assay should be tested whenever clinical studies are performed, when there is the potential for concomitant medication, or when it is used in DDI studies. Over-the-counter (OTC) interference assessments include a mixture of commonly used medications at an appropriately high concentration. For prescribed medications or in DDI studies, tests of the other components are typically done at the anticipated $C_{\max}$ or at a higher value. The potentially interfering drugs and their significant metabolites should be added to the blank matrix and the assay tested to ensure that there is $\leq 20\%$

of LLOQ response and $\leq 5\%$ of IS response. This assessment tests specificity or the direct interference as noted from background response. Infrequently, comedications may cause an indirect effect if they are abundant, may coelute, and are preferentially ionized. Potential interferences in LC-MS are often predictable based on their molecular weight.

Guidance also states that “potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested.” Finding a source for these potential interferences can be a challenge. However, the analyst is expected to ensure that every attempt was made to secure and test them. Compounds are added at physiological concentrations to the matrix, fortified with analyte(s), and analyzed. Normal acceptance rules (four of six QC samples must be within 15% or 20% at LLOQ) apply.

The suitability of an assay to measure drug levels in special populations (age/gender, renal/hepatic impaired) should be considered before starting sample analysis. Differences in the clinical chemistry of special populations can impact assay performance. Elevated phospholipids in lipemic populations can cause matrix suppression not apparent in standards prepared from normal plasma. Hemolysis may be more prevalent or extensive, particularly when special processing is required or remote clinics are used. Accumulation of metabolites in slower clearing populations may result in a metabolite interference not previously seen in normal or healthy populations. It is therefore important to have an understanding of the anticipated metabolism before making this assessment. Assay levels may also need to be adjusted to better cover these populations.

Since biotransformation generally involves a change in the molecular weight of the drug, interference from metabolites when measuring parent drug levels is less frequent than when measuring metabolites. Phase II conjugates present concern when coeluting ether glucuronide fragments within the ion source or when acyl glucuronides degrade in-process back to parent drug. Either process results in overestimating parent drug exposure. More common is to have many isomeric or isobaric metabolites, all of which should be resolved from the metabolite being measured. For isomeric metabolites, this generally requires chromatographic separation [55]. For isobaric metabolites, a specific multiple reaction monitoring (MRM) fragmentation is more likely to be achieved. Analysts should take caution in assessing the specificity of a method to measure metabolites. Access to samples from a previous or pilot study helps and should always be considered as a precautionary step. Alternatively, metabolites from *in vitro* sources or animal *in vivo* studies can be spiked into the matrix to assess their potential for interference. Testing from a prior or pilot study is preferred since it affords the best assessment of analytical relevance. The spiking approach, however, should be considered for first-in-human studies.

17.10 CONTAMINATION

In contrast to carryover, contamination is unpredictable and may occur during any part of the study (dosing, sample collection, or analysis). We focus first on sample analysis and later illustrate means to assess study contamination. Contamination may occur in a

limited number of study, standard, or QC samples and to a varying extent. Therefore, assessing its impact can be difficult.

Contamination has become more problematic as instrument sensitivity has increased and samples are processed in closer proximity. For example, a tube-based, HPLC-UV assay that measures microgram per milliliter levels does not present the contamination challenge of a 384-well SPE (solid-phase extraction) LC-MS/MS assay that measures picogram per milliliter levels. Well-to-well or cross-contamination during sample preparation can be reduced by proper programming of the liquid handler method to avoid dripping or tip-to-tip contamination. Disposable pipette tips, although more expensive than fixed tips, can overcome contamination. If fixed tips are needed, appropriate wash steps should be performed. Placement can be considered to minimize its impact; however, isolating high level samples from neighboring wells is impractical.

Other general considerations include separating weighing rooms, where high level stock solutions are prepared, from the LC-MS laboratory. Likewise, bench areas should be covered with disposable paper and instrumentation regularly cleaned to avoid system contamination. Reagent contamination (matrix, solutions), at very low levels, can be difficult to recognize as analyte contamination versus chemical background. An easy test includes adding more MRM transitions so that several profiles are required to have the same relative abundance as analyte. By increasing assay specificity, the analyst can be assured that the problem is contamination and not simply chemical background. For chemical background, an additional transition may serve as the detection channel in a revised method.

If contamination during extraction is suspected, one may use cross-contamination markers to indicate well-to-well contamination during sample processing. Compounds are added in a checkerboard pattern and analyzed to note adjacent well contamination [56]. When used, the analyst must prove that the markers do not influence the assay. The impact must be judged by the bioanalyst using rules defined within an SOP. Options include rejecting the sample(s) or batch, raising the LLOQ, or tolerating the bias (contamination < experimental error).

The bioanalyst has the responsibility to resolve any questions regarding sample analysis. The study director has the responsibility to determine the overall cause(s) for contamination and assess its impact. When needed, the analytical method can also serve to trace contamination within the animal facility or from other sources. Confirmation by reanalysis can generally resolve analytical questions, provided the original sample was not contaminated at first sampling. Given that dosing often occurs at concentrations up to 1,000,000-fold greater than bioanalysis, even a small fraction of dose transferred into or about the tube can find its way inside the sample. For this reason, many laboratories take second samples to avoid random errors associated with sampling contamination.

Following issuance of the EMEA Guideline on Control Animals, more attention was paid to resolving contamination issues [21]. For all pivotal studies that include a toxicokinetic evaluation, control samples should be analyzed. In nonrodent studies, full profiles are required. In rodent studies, sufficient samples about the $T_{\max}$ are needed. When significant contamination is at a level that can impact the validity of the study, the sources of contamination should be investigated. This includes independent analytical and in-life investigations, which are reviewed and reconciled by the study director. It is the responsibility of the study director to assess the significance of impact (greater than no adverse effect level) on the study results. Options to remediate the problem in

future studies should be assessed and implemented. Findings should be stated in the written summaries and highlighted in the nonclinical overview.

When assessing contamination in control animals, the assay range should be considered. To minimize spurious signals being detected as contamination, the LLOQ should be set at an appropriate level. One approach for trough sampling targets an LLOQ that is a percentage of the concentration expected to occur at 24 h after the lowest dose. Interanimal variability must be considered to ensure that the lowest exposed animal within the low dose group can still be measured.

The objective is to determine whether control animals were exposed to drug. Therefore, control samples can be run on a separate plate to minimize the potential of analytical contamination. Contamination sentinels, or additional blanks, can also be used to indicate analytical contamination. An appropriate acceptance rule would require that all contamination blanks included in each analytical run do not yield measurable values. If blanks are below the limit of quantitation (BLQ) and toxicology control samples show quantifiable exposures, control samples can be reassayed in replicate to both confirm analysis and define a reportable value. Confirmation of animal exposure can be accomplished by monitoring for metabolites. For metabolites assayed with parent drug, their ratio should be different from standards or change over time to ensure the lack of sample contamination during analysis.

17.11 SYSTEM SUITABILITY AND RESPONSE CHANGES

Only qualified and properly maintained instruments should be used for drug analysis. To qualify instruments at time of use, a system suitability test may be used. System suitability samples test that the instrument is sufficiently sensitive, selective, and reproducible for the current analytical run. This generally includes replicate testing of the analyte for sensitivity and precision near the LLOQ before starting an analytical run. Similar tests following the run may be used to confirm instrument stability. A mixture of components can test multiple analytes for assay specificity, such as the resolution of critical pairs in a separation. System suitability tests do not replace the required run acceptance criteria. They can be effective in helping to decide whether sample extract can be simply reinjected or a new extraction and reanalysis are needed.

A means to judge individual results, based on the response of ISs, is often dependent on the assay. However, most laboratories have developed general rules that allow *a priori* decisions to reject sample results that show aberrant IS response. The rationale is that any significant change in observed response can reflect an unknown error in sample processing (double spike of IS solution) or an assay change (recovery or matrix effect, injected volume, or instrument response). While an assay may be rugged enough to compensate for an error such as a reduced injection volume, it is sometimes difficult to differentiate an apparent response change from a more fundamental problem. The criterion that only a significant (>50%) change in an individual IS response from the mean IS response is generally used to reject sample results.

Figure 17.1 illustrates an example in which conditioning of the instrument was insufficient before starting an analytical run. This example also illustrates how an individual subject profile may be different from others within the study or QC samples. Today, most laboratories use this type of assessment to help troubleshoot assay runs and make proper decisions on whether samples can be reinjected, reassayed, or not

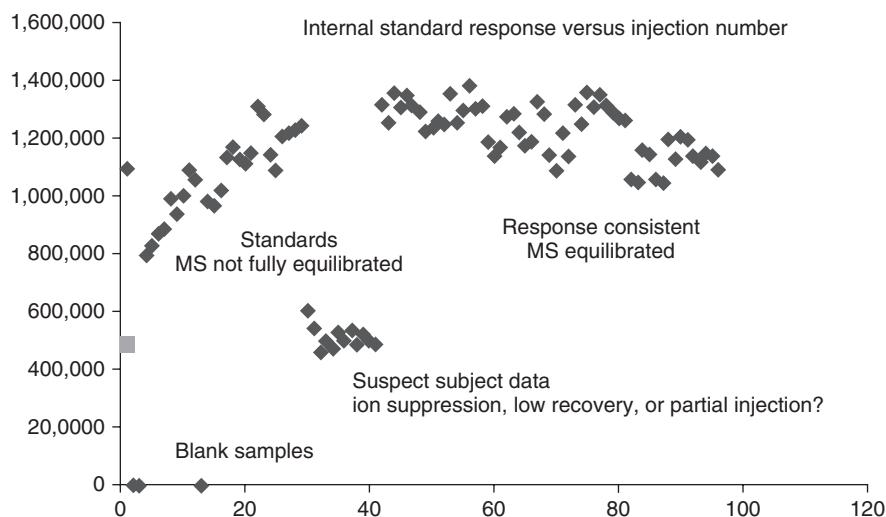


Figure 17.1 Plot of internal standard response versus injection number illustrates the need for proper conditioning (equilibration) of the LC-MS instrument before starting an analytical run. It also allows assessment of how individual subject data should be treated and when MS sensitivity changes.

reported. Some laboratories include other within-run monitoring such as phospholipid assessments to determine whether ion suppression occurred.

Figure 17.2 illustrates an example where a less potent derivatization reagent was uncovered by monitoring changes in d_3 - Δ^9 -tetrahydrocannabinol IS response. Aged QC samples showed a pronounced difference from fresh standard or QC samples, suggesting either poor IS recovery or less extensive derivatization. When another bottle of derivatization reagent was used with more vigorous vortexing, there was no difference in response between fresh and aged QC samples. The fortunate use of a stable label IS compensated for these changes, as indicated from standard and QC samples. Use of a stable label rather than a structural analog as IS is always preferable, particularly for more complex extractions and derivatizations. Provided the IS is well equilibrated with analyte in the matrix, a stable label will compensate for any subsequent changes. When using stable label ISs, having additional rejection criteria are less critical.

17.12 SAMPLE REANALYSIS

Samples may be reanalyzed for an assignable cause such as a technical error, poor chromatography, unacceptable IS response, or evidence of carryover. The original result is discarded, and the sample is reanalyzed in singlet. The acceptable reanalysis value is reported.

Samples may also be reanalyzed for anomalous results or for PK reasons. These analyses are performed in a manner similar to replicate confirmations of contamination. Typically, confirmatory reanalysis is performed in duplicate. Provided the two replicates agree, a comparison is made between their mean and the original result. Alternatively, the median of the three may be reported. If any one of the three results is below the

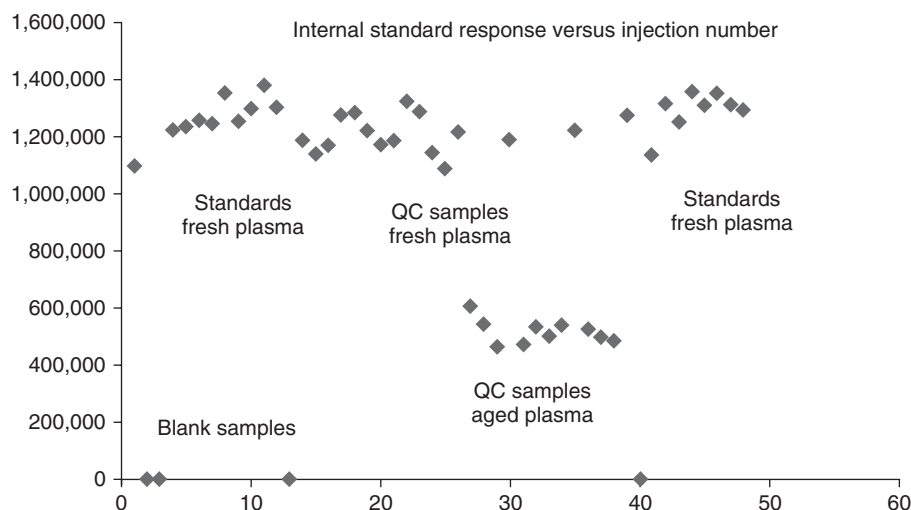


Figure 17.2 Plot of d_3 - Δ^9 -tetrahydrocannabinol IS response versus injection number for the analysis of Δ^9 -tetrahydrocannabinol in human plasma illustrates how the extent of derivatization was reduced in aged plasma when using a less potent solution. Fresh reagent and more vigorous vortexing returned IS response of aged to normal plasma (not shown).

LLOQ, the less of the two remaining results is usually reported. When two or more are below the LLOQ, the result is typically BLQ. If only sufficient volume remains for a single determination, a comparison is made between the two results. Either of the three events may occur: the original result is confirmed and reported (<15%), a mean value is reported (15–30%), or no value is reported (>30%). There are several acceptable means to judge replicate repeat analysis. A decision tree, established within an SOP, should define the procedure.

There are a number of situations where the performance of QC samples may not adequately mimic that of study samples from dosed subjects (incurred samples). Reasons for the difference can be considerable, including metabolites converting to parent drug, protein-binding differences in patient samples, recovery issues, sample inhomogeneity, matrix effects and interference from formulation, or drug-modulated differences in clinical chemistry. These factors can affect the reproducibility and accuracy of the concentration determined in incurred samples. Since accuracy is difficult to determine in a study sample, replicate analysis (interassay precision) is used as a surrogate for accuracy. Irreproducibility, therefore, exposes inaccuracies, which warrant further investigation. While the causes for irreproducibility are often characterized and minimized during method development, it is important to assure that the assay is under control when the method is applied [57]. For this reason, more recent studies have required the assessment of ISR. An underlying objective is to determine whether and why the variance noted from incurred samples differs from QC samples.

Within GLP toxicology studies, a proper evaluation of ISR and accuracy needs to be performed once on each species used in toxicology studies. Consistency of the animal's genetics and environment makes it unnecessary for additional incurred sample investigations to be performed once the initial assessment has been performed. The chance of incurred sample variability is far greater in humans. Therefore, randomly

selected subjects should include a minimum of 10% of total analyzed samples. For larger studies, 5% of total sample size is allowed. Samples must be analyzed within the period of stability [12,13].

In both GLP and clinical studies, the difference between the initial and reanalyzed values should be $\leq 20\%$ (small molecules) in at least 67% of reassayed samples. Unlike repeat analysis for anomalous results or for PK reasons, ISR is performed in singlet from selected samples about the $C_{\max}$ and in the elimination phase. It is recommended that the selected samples be representative of each study period and dose cohort. Initially, this was recommended to be referenced to the original result, assuming that it was more likely the correct value. The proper approach is to reference to the mean value:

$$\% \text{Variability} = \frac{\text{Repeat} - \text{Original}}{\text{Mean}} \times 100$$

Reference to the mean produces a consistent assessment, regardless of the order by which the two results were obtained [13]. For this reason, LIMS software, such as Watson, support either calculation.

When the criteria cannot be met, an investigation to understand the assignable cause of the irreproducibility is warranted. This will proceed from several probable causes to one assignable cause [58]. The simplest cause may be an assignable execution error, which may isolate the failure to that day's run. More complex reasons may implicate other reasons (Table 17.3). Once defined and corrected, a decision regarding reporting of data can be made. Study sample analysis cannot be accepted until the investigation is complete, documented, and appropriate follow-up actions are in place. In case of an unassignable cause, all ISR samples can be reanalyzed, along with additional samples, in two or more different batches. Results of all relevant batches should be evaluated to obtain interday ISR data. No assignable cause can require the redevelopment/validation and reanalysis of all subject samples.

TABLE 17.3 Assay Issues and Probable Causes for ISR Failures

Issue	Causes
Improper processing of study samples	Execution error (dilution, IS spiking) not seen in QC samples
Lack of rugged method	Assay not optimized and variance in study samples greater than that in QC samples
Interference from isomeric or isobaric metabolites	Metabolic specificity not fully tested
Metabolites $\rightarrow$ parent	Improper stabilization procedure
Rate of interconversion affected by the law of mass action	Interconverting forms not tested at same ratio as in study samples
Subject differences in protein binding	Extraction process not rugged
Differential recovery	Time-dependent recovery observed in study samples but not QC samples
Sample nonhomogeneity	Sampling error (improper thaw/vortex)
Endogenous interference and matrix effects	Error in defining matrix variability across populations because of insufficient lot screening

Investigation of ISR failures may implicate results, runs, studies, or assays. Understanding the cause will determine its impact and the direction taken. A unique sample may not have a reportable value. A unique population may present problems for the assay, failing study results and requiring revision/revalidation of a new assay. If the assay is implicated, it can invalidate results from other studies in which the assay was used. It is therefore critical to thoroughly test assays before use and ensure proper training of staff.

ISR results may be documented in the final bioanalytical or clinical study report and/or as an addendum to the method validation report. In selecting samples to be reanalyzed, a variety of concentrations, time points, and patient populations should be chosen, depending on the drug and its DMPK (drug metabolism and pharmacokinetic) properties. This can be a challenge when the analyst has limited information. First-in-human, proof-of-concept, special population, and bioequivalence studies are examples of studies that must include incurred sample concentration verification. Most perform ISR within all clinical studies. Study sample results obtained for establishing ISR may be used for comparison purposes and are generally not used in calculating reported sample concentrations.

To properly handle failed ISR runs, the laboratory must have an SOP on how to conduct and document investigations. The principal investigator should lead the analytical investigation and report their findings to the study director or the individual who monitors the overall conduct of the study, quality assurance, and management. Probable causes are reduced to definitive cause(s) through a series of hypothesis testing. The investigation report is issued with the study report in which the problem is described, reanalysis results are defined, and an assignable cause is identified. The identified cause serves to define preventative and corrective actions to avoid future errors.

Ruggedness testing during method development and validation can serve to reduce ISR failures. Robustness assessed by tracking assay performance across different analysts, laboratories, and studies can be invaluable in defining trends and causes for failures [59]. A critical question is to determine why there was an analytical error not uncovered by normal standard and QC acceptance criteria. Some assay-related causes of ISR failures are shown in Table 17.3. It is expected that ISR failures be fully investigated to an assignable cause and corrected, in a manner similar to assessing manufacturing failures when out-of-specification results are seen. It should be considered that imprecise assays may, on occasion, fail ISR in the same statistical manner in which one set of QC samples fail, while others pass acceptance criteria. The analyst should work to ensure that the assay can be executed more precisely before undertaking a larger ISR sampling.

17.13 MULTIPLE ANALYTE ASSAYS

It is becoming more common to expect that LC-MS assays perform multiple analyte assessments within a single run [27,48]. Cassette dosing and metabonomics are the extreme examples [60,61]. Within regulated bioanalysis, this is desired for antiviral indications where comedication drug therapy (highly active antiretroviral therapy (HAART)) is needed to overcome drug resistance. In many areas, the desire to measure parent drug and its metabolites drives multiplexed assays.

Simultaneously measuring multiple analytes increases the statistical probability of run failures. Results should not be rejected for the remaining analytes if one analyte fails. Concentrations from the first accepted run should be reported. If this analyte is repeated in simultaneous assays when analyzing for different analytes, it is not necessary to quantitate the already reported analytes. All results should be maintained and available for review.

17.13.1 Drug Metabolites

It is common to have metabolite exposures at significantly different levels than the parent drug, particularly in early clinical studies where metabolite levels are unknown. On repeat analysis of diluted ALQ parent samples, a second reportable value for the metabolite is often obtained. Source data from all acceptable runs, regardless of whether the concentrations were reported, should be retained.

MIST guidance requires one to demonstrate steady-state exposure in at least one toxicology species for all major (>10% of parent AUC) circulating human metabolites. Traditionally, this has been assessed by single dose ADME studies using radiolabeled drug. Studies such as these can be difficult and expensive to perform early in drug development. It is worth noting that the requirement to demonstrate exposure at steady state has made multiple tracer (100 nCi) dosing using AMS a viable option. Regulatory expectations are that these assessments will be completed after proof-of-concept trials but before launching larger clinical trials. This affords some time to assess steady-state exposures and nonradiolabeled approaches such as using tiered assays.

For extensively metabolized drug candidates, measuring large numbers of metabolites at steady state may be better achieved using tracer dose AMS studies. If one chooses to measure metabolites using LC-MS assays, tiered assays afford a means to make more informed decisions. In comparison to a traditional ADME study (50 μ Ci dose, liquid scintillation counting), multiple LC-MS assays defer synthesis of GMP (good manufacturing practice)-grade radiolabel, tissue distribution, dosimetry estimates, and the cost of a clinical ADME study. Savings by attrition of compounds that do not meet proof of concept is significant. In contrast to doing nothing until a late clinical ADME study is performed, the tiered assay approach avoids surprises and focuses resources on unanticipated, unique, or disproportionate metabolites.

Without reference standards, UV and MS responses are sometimes used as an estimate of parent drug and metabolite levels. UV response is often more consistent, particularly when its chromophore is unaffected by metabolism. MS ionization and MS/MS fragmentation have considerable variability for structurally similar (and dissimilar) metabolites. One should not assume that UV or MS response will be comparable. Assessment at high levels from *in vitro* sources can afford more security in these estimates. Relative response factors of the metabolite to parent drug are determined and used to calibrate MS response for lower level determinations. Comparisons of this type should be considered as first-tier assays.

Another approach compares response ratios across species. This assumes similar stability, recovery, matrix effects, and response proportionality in all species or minimizes them by dilution into human plasma. Interspecies differences in enzyme levels, protein binding, and phospholipid levels suggest that analyte stability, recovery, and matrix effects may vary across species. Mixing animal samples with an equal amount of human plasma compensates for these differences and has been shown to be a good procedure

by which simple response ratios afford safety margin estimates [62]. Response proportionality by testing study samples at multiple dilutions (parallelism) is also required. If studies were performed at different times and samples stored for different periods of time, long-term stability is still in question. Reanalysis of aged study samples may be used to support long-term stability. However, reference of stability to initial response rather than its nominal concentration may mask instability, as the breakdown product of one metabolite may fortify an unstable analyte. Ratio comparisons across species should be regarded as second-tier assays.

Third-tier assays generally employ calibrators that have been qualified with some limited assessment of stability. One approach previously mentioned uses metabolites from radiolabeled animal or human *in vitro* studies spiked into human plasma as standards for quantification [31]. To ensure accurate quantification, the radiolabeled profile must separate all metabolites from each other. Metabolite radioactivity is referenced to parent, which has been measured using LC-MS. Resulting curve ranges, therefore, reflect the original proportions within the spiked radiolabeled sample. Dilution in matrix affords the standard curve. The advantage of this approach is that steady-state assessments across species can be made quickly without the need to isolate or synthesize metabolites. Human metabolites that are disproportionate are readily noted. Those metabolites that are insignificant or for which there is adequate safety coverage may need little further investigation. It should be noted that similar approaches are possible using nonradiometric detection, provided response is specific only to metabolites and proportional to their concentration. Unfortunately, inductively coupled plasma-mass spectrometry (ICP-MS), chemical reaction interface for mass spectrometry (CRIMS) and N or S-chemiluminescence detection suffer from either high background in biological matrices or an inability to detect low background elements such as fluorine [63–65]. This approach, however, requires that the metabolite is observed in animals or in human *in vitro* sources.

A second, more time-consuming approach involves isolation of individual metabolites before NMR identification and quantification [29,30]. This approach is closer to the traditional method of isolating metabolites, synthesizing them, and assessing their purity. The difference is that the metabolite isolated from biological sources need not achieve a level of purity expected by synthesis. Only microgram levels are needed, and although the isolated metabolite should be the major component, it need not be high in purity. As NMR response is independent of structure, accurate quantification is achieved by direct measurement of a metabolite resonance using a parent peak for calibration. In contrast, synthetic reference standards are generally qualified by difference, subtracting impurity identified in their preparation.

Since cross-species comparisons reference human-to-animal exposure, the exact assessment of purity is not critical. That said, the direct determination is likely more accurate when the compound cannot be either synthesized or isolated in high purity. Limited supplies will make it difficult to characterize impurities, so a direct measurement will be more accurate. Likewise, the inclusion of a reference standard provides much greater accuracy than just measuring response. Furthermore, the direct measurement approach using NMR should provide both the confidence needed for structural integrity and purity assessment for its use in a regulatory assay.

In combination with strategies for biologically sourcing metabolites and qualifying them without a certificate of analysis, tiered assays can provide a reasonable estimate of steady-state exposures. Samples should be stored at -70°C and analyzed as soon as

received. Whether this is achieved using single or multiple assays is dependent on the extent and diversity of metabolism, required sensitivity and dynamic ranges, and risks one is willing to take by trying to achieve too much within a single assay. Analysts and development teams should consider whether a metabolite is worthy of GLP bioanalysis at its present stage of drug development or whether a tiered approach would be better.

Human metabolites for which there is abundant safety coverage are of less concern and support a tiered determination. The MIST guidance should not replace structure–reactivity assessments. Metabolites not expected to be active or reactive, such as sulfate or ether glucuronides, pose less concern [66]. Alternatively, low levels of highly reactive metabolites may not be seen in plasma, as they do not escape the liver in significant abundance. Accurate measurement of a trapped metabolite or both precursor and product metabolites in plasma may be required. Each metabolite should be considered on a case-as-case basis. Having an accurate assessment is critical when metabolism-mediated toxicity is observed [67].

Once the human metabolism is known, a solid decision can be made regarding what assay(s) would be effective in supporting clinical drug development. To allow proper assessment of PK–PD, parent and significant human metabolites should be measured to regulatory standards. It may be worthwhile to include abundant metabolites into this assay, particularly if they are active, potentially toxic (structural alerts), or implicated in drug clearance. Decisions regarding assays for toxicology studies in animals include safety concerns in humans.

17.13.2 Drug Interaction Studies

DDI is a major reason for toxicity, particularly when new drugs are used in larger, more diverse populations. New chemical entities often clear the body as metabolites or through transporters, both of which are subject to inhibition or induction by other drugs or their metabolites. In addition, the presence of genetic polymorphism and disease as well as age- or gender-related differences in enzyme or transporter function can serve to reduce the normal safety margin. The previous FDA Guidance on DDI studies has focused on *in vitro*–*in vivo* correlations, providing guidance on what studies are needed in clinical drug development [68,69]. More recent publications have addressed both CYP-mediated and transporter-mediated drug interactions [70,71].

When desired, it is possible to perform a more general screening of new drug candidates using a cocktail of marketed drugs that serve as cytochrome P450 probe substrates. As many as 10 analytes (parent and metabolite for 5 probe substrates) are measured in this coadministration study. Common substrates in DDI cocktails include warfarin (2C9), dextromethorphan (2D6), omeprazole (2C19), midazolam (3A4), and caffeine (1A2, NAT-2, XO). Other combinations have used debrisoquin (2D6), (*S*)-mephenytoin (2C19), chlorzoxazone (2E1), caffeine (1A2), and dapsone (3A4). Flurbiprofen has been added to cover 2C9. Other cocktails include mixtures of preferred probe substrates that assess clinical relevance to the drug under development [72,73]. This approach could find more utility, provided a common set of probe substrates and metabolites were used. Mixing of probe substrates and metabolites requires more extensive assay development and validation for each DDI study.

Measuring a metabolite that is specific to one biotransformation pathway provides a more powerful assessment of a drug interaction than just measuring a change in parent drug exposure. Unfortunately, the interaction of large and small molecules is

less predictable than small-molecule interactions. Small-molecule interactions mostly occur as a result of liver enzyme or transporter inhibition and are well predicted from *in vitro* studies. Large–small molecule interaction can be target-related and noted from enhanced or reduced pharmacological effect [74]. Regardless, most drug development programs now include an assessment of the effects of biologics on the systemic levels of small-molecule drugs.

17.14 ASSAY TRANSFERS AND CHANGES

There can be multiple opportunities to transfer assays. Each of these affords a chance to improve an assay. Assay transfer within a laboratory or across laboratories involves an assessment of its equivalence following the change. Depending on the nature of the change, this may involve either a partial or full validation. Transfers do not require cross-validation or conformance testing. However, testing at least QC samples from the other laboratories may expose a bias. Inclusion of incurred samples may expose issues not seen using just QC samples. Laboratories need to demonstrate ISR many times within clinical development, so requiring a similar test at assay transfer would be a wise investment. Cross-validation using incurred samples is only required when two different assays are used within the same study. However, at New Drug Application (NDA) filing, an assessment of exposures across populations is made, so assays are expected to give equivalent results in all studies.

At the discovery-to-GLP transfer, one should take every opportunity to learn from discovery studies. MS optimization should test ionization, instrument tuning, sensitivity, and assay range. Often, a generic extraction and fast gradient separation suitable for discovery needs revision to meet regulatory expectations. Automated screening of LLE (liquid–liquid extraction) or SPE procedures can test diverse conditions for high recovery and minimum matrix effects [75]. Mobile phase and LC column screening should test gradient or isocratic conditions using various mobile phases and columns. Attention to the separation of any interference, carryover, peak shape, and MS sensitivity should be made. MS sensitivity is affected by the separation for several reasons. ESI is concentration dependent, and mass flux to the source is impacted by peak shape and resolution. Efficient drying of ESI droplets is aided by reducing surface tension, so better response is achieved when using a higher percentage of organic in the mobile phase. Having the right pH or ionizing agent (e.g., ammonium) is also critical to ionization. For absorptive compounds, through-column recovery may be low. While calibrators may compensate for reduced LC recovery, the net effect is poorer sensitivity. It is also common to substitute a better, more abundant analog or preferably stable label IS at this phase of method development.

At the IND toxicology-to-clinical phase, assay sensitivity and a wide dynamic range are critical as one prepares for SAD (single ascending dose) and MAD studies in man. Achieving a high sensitivity assay may require larger volumes and the use of a more selective extraction or exploring alternative ionization or derivatization approaches. The addition of active metabolites to better assess PK–PD is a common change. At this time, it is unwise to include too many metabolites until samples from the top dose of the MAD study are profiled. Profiling and tiered assays can provide estimates as to whether these metabolites are unique or disproportionate and therefore require

long-term monitoring using a regulated assay. This approach will minimize changes to the clinical assay throughout drug development. As discussed previously, a human urine assay is generally needed.

When moving further to proof of concept, assays in other fluids or tissues may be needed to demonstrate penetration to the site of action or toxicity. Site-of-action questions will generally require analysis only in human and the pharmacology species. Toxicity questions will include human and the most sensitive safety species. In many cases, a representative human sample is unavailable. For human lung, bronchoalveolar lavage and macrophage exposure can serve as its surrogate. Some virology indications are supported by showing penetration into PBMCs. This is particularly important when drug is known to be actively transported or limited by intracellular metabolism. There is limited guidance on the extent of validation required for a penetration study. If limited to a single matrix and critical to explaining efficacy or toxicity, a full validation including stability assessments should be considered. For tissues, stability assessments may be limited to homogenates. This is generally sufficient for toxicology studies, as drug safety sites often can perform homogenization before freezing tissue samples.

Whenever assays are transferred across different companies, such as when a pharmaceutical company transfers an assay to a contract research organization, there is risk that any change could impact assay performance. The dilemma is whether one should allow changes to better optimize an assay or stay with the present assay. The transfer of knowledge and good communication are critical. Assessments of accuracy, precision, and failure rates provide metrics on its success. To confirm a lack of bias, the exchange and analysis of both QC and incurred samples can demonstrate equivalence before starting a new study. If additional tests are required beyond what is normally covered by the SOP, preparing and executing a validation plan is essential.

17.15 DOCUMENTATION

When preparing analytical and validation reports, a summary table of all analytical runs should list runs with run identification, dates of analysis, whether runs passed or failed, reasons for any failures, and, for analytical runs, any deviations from the validated method. QC data from validation runs that only failed to meet QC acceptance criteria with no assignable cause for failure should be included in the precision and accuracy estimation. Deviations from SOPs and assay procedures and significant unexpected events should be identified and their impact assessed. This includes any defined outliers [12]. For those performing large numbers of studies, having a LIMS is a critical component to ensure consistency in documentation and to manage the time associated with these tasks.

Source data documentation should include the conditions of use. For instance, stability determinations during method validation should record experimental conditions such as storage temperature and duration. Materials should be traceable to source and date of collection. Modification of calibration response and QC levels should be documented with sufficient detail to demonstrate that the changes were justified or followed established procedures. For chromatographic methods, source documentation should include original and reintegrated chromatograms for accepted runs, along with the reason for changing integration parameters across a run or for individual samples within a run. Electronic audit trails that record changes to integration parameters must be enabled [12].

A total of 5% of all chromatograms from randomly selected subjects, including QC samples and standards, must be submitted with an NDA and/or ANDA (Abbreviated New Drug Application). For bioequivalence studies, 20% of chromatograms from serially selected subjects are submitted.

Final reports for either validation or study sample analysis must include a complete account of the performance of the method. This includes a tabular listing of the actual QC results from all runs during method validation and accepted runs during study sample analysis. A table listing reassayed samples, reason for reanalysis, and values for the original, reassay, and final reportable result should be included in the final report.

Drug concentration data from the rejected runs need not be included in the final report; however, a brief description of the reasons and a tabular listing of rejected runs should be provided. Compliance has the added value that thorough documentation at the end of each study makes work easy when the common technical document (CTD) is assembled.

17.16 INSTRUMENT QUALIFICATION

Qualification of instrumentation and computer validation are beyond the scope of this chapter. Suffice it to say that the proper installation and operational and performance qualification of all instrumentation are required before performing regulated bioanalysis [76–79]. This responsibility is shared among the vendor, analysts, and informatics organizations. Testing and validation of functionality unique to your laboratory requirements will not have been performed by the vendor and therefore must be done. Design qualifications tend to be the domain of the vendor, although this aspect can be assessed during the vendor audit and at the time of instrument selection. User specifications and a validation plan should be developed. From vendor assessments, system functionality can be verified and any gaps identified. Consider customization when configuring and testing options. UAT should include a traceability matrix to regulations. Failures in executing UAT scripts are categorized as to their severity and decisions made for any procedural or software changes. Results and all reports must be thoroughly reviewed before the production release memo is issued. SOPs, postproduction, validation review, change management, disaster recovery, deviation, and exception documentation are required.

17.17 EXPLORATORY CLINICAL STUDIES

Many organizations have sufficient discovery or preclinical resources and confidence in animal studies that would predict success in the clinic. Demonstrating proper exposure at a therapeutic dose or target engagement in the clinic is expected for IND candidates. When unsure of success, eIND studies allow the assessment of new chemical entities that have undergone limited safety testing [80]. Subtherapeutic doses or microdoses afford the opportunity to determine ADME properties using AMS or target engagement using a positron emission tomography (PET) ligand [81]. A microdose is defined as <1/100th of the dose of a test substance calculated (based on animal data) to yield a pharmacologic effect of the test substance with a maximum dose of $\leq 100 \mu\text{g}$.

For some, the eIND presents a fast-to-fail decision. There is always the concern that DMPK properties at very low doses are not reflective of therapeutic doses because of dissolution rate-limited absorption or saturation of clearance [82]. Having clarity on the biopharmaceutics is therefore fundamental to understanding whether one or several new chemical entities are good candidates for microdosing. Not having a good *in vitro*–*in vivo* correlation and poor allometric scaling of the PK in animal species could be justification.

If microdose PK is the only desired endpoint, cold dosing of microgram quantities in humans and LC-MS measurements of picogram per milliliter concentrations in plasma are alternative means to estimate human exposures [83]. In a preclinical test, proportionality for several drugs in rats was considered as validation of a general methodology to support microdosing in humans [84]. Sprague-Dawley rats were given oral doses of 0.167, 1.67, 16.7, 167, or 1670 mg/kg. LC-MS provided sufficient sensitivity to study the PK of antipyrine, carbamazepine, atenolol, and digoxin, but not metoprolol. Dose proportionality was achieved between a microdose and up to 1000-fold higher doses for antipyrine, carbamazepine, and digoxin. State-of-the-art MS (API 5000) instrumentation was required. There was limited sample cleanup and no derivatization. The LLOQs ranged from 5 to 20 pg/mL. This illustrates the potential to quickly adapt existing LC-MS assays to an eIND study. Others have followed with examples of the potential of microdosing to obtain an early assessment of ADME properties [85].

High sample enrichment and derivatization may be needed to achieve microdosing objectives. For some small molecules and most peptides, immunoprecipitation can be used to enrich a larger volume of sample before analysis [86]. If the capture antibody from an enzyme-linked immunosorbent assay (ELISA) is used, the resulting LC-MS assay will share its epitope specificity. More details are presented later in the analysis of larger molecules. Molecularly imprinted polymers, sometimes referred to as *plastic antibodies*, are an alternate means to enrich samples [87]. They are more readily produced than antibodies but have considerable limitations. Derivatization generally employs means to make molecules more thermally stable or readily ionized in APCI or by attaching ionized surfactants in ESI [88,89]. In either approach, one should test derivatization conditions to ensure that they are robust and carried to completion. Limiting reagents or conditions can generate differences across samples.

17.18 LARGER MOLECULES

Peptides, small proteins, and oligonucleotides are representative of mid-size molecules (1–10 kDa) that can be directly measured using LC-MS to low nanomolar or sub-nanomolar levels. Van den Broek [90] has reviewed the use of LC-MS methodology to perform quantitative bioanalysis of peptides in biological fluids. Noted are the common requirements to add displacement reagents (similar peptides) or other proteins (serum albumin) to compete with binding sites and to add acid, solvents, or surfactants to overcome nonspecific binding. Immunodepletion of most abundant plasma proteins is also mentioned as a means to enrich samples. If time allows, developing an antibody to immunocapture the desired peptide would be preferred. In an example of derivatization, an octyl quaternary ammonium group was added to enhance ESI properties [89]. The addition of both a charged and hydrophobic group by derivatization aids

by enhancing its surface properties and ion yield. Reporting of both extraction and recovery through immunoprecipitation and derivatization from the low and high QC samples in the validation would be expected. Yields should be sufficiently high in both processes so as to avoid assay variability.

The analysis of insulin using LC-MS illustrates its value both as a biomarker and, when dosed, for its PK [91]. Radioimmunoassays (RIAs) and ELISA of insulin often are nonspecific across higher species, making it difficult to separate endogenous animal insulin from dosed human insulin in toxicology studies. Dosing of human insulin reduces endogenous animal insulin, so measuring a composite profile with a nonspecific RIA is problematic. Predose and insulin precursor C-peptide levels can be determined, but a more direct measurement with a specific assay affords one the chance to measure both drug levels and the effect.

While peptides may have picomolar potency and may require high sensitivity assays in clinical studies, toxicology studies may be supported using far simpler LC-MS assays. Hu and Kamberi [92] reported the analysis of apolipoprotein A-1 mimetic peptide D-4F in rabbit plasma from 0.02 to 40 µg/mL. When using a mixture of LC-MS and immunoassays in toxicology and clinical studies, one should have a clear understanding of assay differences to assess safety margins. The same can be said of discovery-to-preclinical bioanalysis when assessing the minimum effective dose. Assay bias should be assessed to ensure adequate safety margins and human dose selection.

Cross-validation of ligand-binding and LC-MS assays using both QC and study samples can help to illustrate bias. Although this is not required within the guidance, it should be considered when making assessments in large molecules. Larger molecules have tertiary and quaternary structures that may be critical to ligand-binding assays but unrecognized by LC-MS. If required for potency, immunoassay could be the better choice. Likewise, differences in specificity may be exposed when comparing assays. LC-MS requires extraction, so total drug is measured, whereas some immunoassays measure only free drug levels. Differences can be considerable.

Plotting study sample results for an immunoassay on one axis and LC-MS results on another should yield a correlation. There are no *a priori* acceptance criteria. The ideal correlation is a 45° line (slope = 1) that would indicate perfect agreement between the assays. The best fitting line will display the actual relationship. A comparison of precision between the assays can be done by computing the standard deviation of replicate results for each sample. Comparison of individual and grand means can be done using analysis of variance.

Therapeutic oligonucleotides have been under development for two decades [93]. Oligonucleotides are a special challenge and present their own stability and sensitivity problems. Hybridization-based ELISA methods generally afford the required speed and sensitivity for these potent drugs. Identification of individual metabolites, including those generated from 3'- or 5'-exo/endonucleases, is generally not possible using ELISA. While LC-MS may afford a more direct means to determine the metabolism of oligonucleotides, ionization efficiency in negative ESI is often limited and variable [94]. Matching chromatographic separations to MS detection is also a challenge. Reducing and controlling the formation of salt adducts through addition of bases such as triethylamine are critical to successful analysis. At best, low nanomolar concentrations have been measured [95]. Besides the parent drug, the 3'N-1, 5'N-1, 5'N-2, and 5'N-3 metabolites were also capable of being measured in rat plasma.

These types of separations require system suitability samples to be analyzed both before and after runs to ensure that separation of any critical pairs and MS sensitivity have been maintained. It is also important to understand the activity of any metabolite. Any multiplexed assay for oligonucleotides should be based on a strategy that is similar to small-molecule drug metabolites. Significant circulating active metabolites in humans should be included with the parent assay to assess PK–PD. Hopefully, this can be achieved within a single assay. It may be impossible to source others, so a tiered strategy may be needed. Particular attention to off-target toxicity is needed, as the similarity of oligonucleotide metabolites to parent drug may force one to consider regulatory assays for all metabolites.

Differences between present acceptance rules (15/20% for small vs 20/25% for large) have resulted in some confusion when mid-size molecules are measured. For instance, when oligonucleotides or peptides are measured using LC-MS, should acceptance criteria be tighter than when measured using immunoassay? As peptides and oligonucleotides are typically manufactured without heterogeneity, tighter standards may be applied. However, both an ELISA and an immunocapture LC-MS assay may share a reagent expressed with high variability (e.g., polyclonal antibody). A reasonable approach might be to target $\pm 15\%$ but to allow the acceptance criteria to be adjusted up to $\pm 20\%$, contingent on the accuracy and precision noted within the validation.

New approaches to deliver drugs, such as ADCs, are good examples of where the released peptide drug measured using LC-MS may be measured to the same rules as its parent biologic. In many cases, the parent ADC is measured using ELISA and results would be accepted with an accuracy and precision of 20% (25% at LLOQ). However, one could equally make the case that a small peptide measured by LC-MS as a protein surrogate should be determined to small-molecule rules (15% accuracy and precision, except 20% at LLOQ).

Moving to larger molecules (biologics) requires careful consideration of whether a highly specific LC-MS assay is useful. If ELISA measures all active components, a functional assay should be the primary measurement. What role does a peptide-as-protein surrogate assay then play in drug development? When high affinity antibodies are difficult to obtain, there may be good reason for LC-MS detection. Its higher specificity can compensate for a less selective capture reagent.

Proteins are not generally directly amenable to LC-MS bioanalysis. This is particularly true for proteins that have undergone extensive posttranslational glycosylation or to which polyethylene glycol (PEG) has been added. Proteins, following their isolation can be reduced in size using proteolysis and measured by LC-MS. Tenecteplase (MW 58,777 Da) has been determined in rat plasma in the range of 5–125 $\mu\text{g/mL}$ [96]. Plasma samples were reduced using dithiothreitol and reacted with iodoacetamide before overnight trypsin digestion. LC-MS analysis of three tryptic peptides served as surrogate for the protein drug. This relatively simple procedure afforded sufficient sensitivity to determine the PK in rat. While the LLOQ of the ELISA was much lower, this approach will be of increasing use to speed early drug development.

The LC-MS approach is time consuming but can provide detailed information of amino acid changes that are unrecognized by an immunoassay. For instance, one can interrogate whether a specific Asn residue has been hydrolyzed to Asp and measure its amount in plasma. This is a common degradation of peptide drugs, including insulin. Rearrangement can also occur during this process, which would require chromatographic separation. The LC-MS assay could provide complementary information on

impurities, degradants, and metabolites that cannot be determined using an immunoassay.

Enfuvirtide (MW 4492 Da) and its deamidated metabolite M-20 were determined in human plasma [97]. The assay range for parent drug was 10–2000 ng/mL, while that for M-20 was 10–500 ng/mL. A deuterated stable label was added as IS before protein precipitation. The resulting supernatant was directly analyzed using LC-MS. The same drug and its M-20 metabolite were measured in a similar assay [98]. Deuterated stable label IS was added before chymotrypsin digestion. LC-MS analysis of four smaller peptides was shown over the 100–10,000 ng/mL range. Since the peptide drug was of sufficiently low molecular weight, either a direct or indirect analysis under low resolution MS/MS conditions afforded an accurate measurement of the separated drug and M-20 metabolite. The easier method would avoid proteolysis; however, this assay was an order of magnitude less sensitive.

Blending of exposure data across studies and species from multiple LC-MS and ligand-binding assays will be of increasing importance in the drug development of peptides, oligonucleotides, and protein therapeutics. It will be critical to demonstrate adequate analytical figures of merit for stability, recovery, matrix effects, and specificity. A fundamental understanding of the differences in these assays through appropriate cross-validation must also be obtained.

17.19 DRIED BLOOD SPOTS

DBSs have been infrequently used for many years in the field of bioanalysis. Over the past four years, there has been considerable interest, largely driven by Glaxo Smith Kline, in the use of DBSs instead of plasma [99,100]. There are numerous advantages, particularly from the *in-life* portion of the study. For rodent studies, less blood (15 μ L) is required, which reduces the numbers of animals. Toxicology of satellite animal groups can be eliminated. Repetitive sampling from the same mouse also reduces interanimal variability in PK studies. In the clinic, labor-intensive plasma harvesting is eliminated. Needlestick bleeds replace indwelling catheters. Likewise, the need to store and ship samples at reduced temperatures is eliminated. The overall effect is to reduce errors at remote clinics, allowing higher quality from global studies.

The challenge becomes one for the bioanalyst. The analyte must be well recovered from filter paper without the introduction of matrix effects. As only a fraction of the total blood spot is used, the punch sampling must be sufficiently large to ensure the required LLOQ. Reemergence of DBSs has been aided by the improved performance of LC-MS systems. Owing to their sensitivity requirements, highly potent drugs may not be amenable to DBSs. However, there are a considerable number of drugs that require no greater than low nanogram per milliliter detection sensitivity, a target achievable for many DBS applications. High affinity enrichment and smaller-volume analysis including the use of capillary LC-MS may assist in obtaining additional detection sensitivity. Sampling across the blood spot must be consistent to ensure a reproducible sample since aliquoting a precisely measured volume at collection is not done. At present, automation is limited. There is considerable effort by automation companies to provide more innovative, off-the-shelf solutions in the near future.

As more investigators explore the potential of DBSs in clinical studies, there will undoubtedly be regulatory bioanalytical guidance that comes forth in the general area

of microsampling. Many are performing qualifying studies in which blood, plasma, and DBSs together are sampled and analyzed. When corrected for hematocrit and blood–plasma partitioning, the PK would be expected to be equivalent. This type of test is likely needed to bridge data for a new chemical entity that is moving through drug development.

17.20 CONCLUSIONS

The nature of regulated bioanalysis is ever changing. New methodology, technology, and novel drug candidates challenge past practices. Best policies from GMP and GCP (good clinical practice) are being incorporated into GLP. Most notable has been the use of ISR to confirm or refute original results and the use of out-of-specification procedures to investigate the causes of failed runs [101]. Although this has placed additional costs on the industry, it has greatly improved the quality of bioanalytical data. No longer does the first reportable result go unchallenged.

As a result of the Third Crystal City Bioanalytical Workshop, common procedures were adopted for validation and sample analysis of both large and small molecules, regardless of the analytical technology. These changes in regulated bioanalysis were greatly needed to assist in measuring more complex drug candidates. Products made by synthetic routes are integrated with those from biological sources, including modified peptides, proteins, and antibodies. LC-MS and ligand-binding assays share drug development roles, defining PK–PD and assessing drug safety. As such, it is likely that future guidance may also better integrate biomarker assays into regulated bioanalysis.

Global drug development has brought forward new cultures and perspectives into our science. Adaptive clinical trial design put more emphasis on bioanalysis to escalate doses and biomarker assays to define proof of concept. The next generation of regulated bioanalysis will see new challenges from evolving science and technology that will further shape work practices and standards.

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18 Leveraging Advances in HPLC and Sample Preparation to Maximize DMPK Data Quality

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18.1	Summary	1
18.2	Introduction	3
18.3	Regulatory guidance for the validation of quantitative chromatographic assays	4
18.4	Sample preparation considerations	4
18.5	A practical review of the principles of chromatography	10
18.6	Emerging technologies	14
18.7	Elevated column temperatures	19
18.8	Ion-pair reagents	21
18.9	Multiplexing LC-MS/MS	21
18.10	Metabolite interferences in quantitative assays	22
18.11	Conclusions	23
	References	23

18.1 SUMMARY

The reliable reporting of data from the quantitative analysis of drugs and their metabolites is the backbone of drug discovery and development; therefore, the bioanalytical methods that produce the data should be sufficiently accurate and reproducible. This chapter provides an overview of different technologies that offer opportunities to improve the data quality and efficiency of method development, method validation, and biological sample analysis.

The regulatory requirements of data quality are reviewed. Specifically, regulators require that bioanalytical methods be validated to ensure accurate and precise results. The quality of an analytical method is normally assessed by the performance of standards and quality control samples (QCs). Current regulatory guidance recommends an acceptance criterion of 15% for accuracy and precision of all standards and QCs, with the exception of the lower limit of quantitation (LLOQ), where the acceptance criterion is increased to a 20% deviation.

Technologies that improve sample cleanup are reviewed because data quality is the sum of effective sample preparation and appropriate chromatographic separation of endogenous matrix components. In other words, poor sample preparation cannot be overcome by high performance liquid chromatography (HPLC). Inadequate sample cleanup and chromatography often leads to matrix effects that are revealed by irreproducible results for some bioanalytical methods. Plasma phospholipids have been identified as playing a major role in causing matrix effects in liquid chromatography-mass spectrometry (LC-MS) bioanalysis. Therefore, in order to minimize the potential for matrix effects, the removal of phospholipids is an important component of any sample cleanup process. Moreover, the process of sample extraction is often a rate-limiting step in the workflow associated with quantitative bioanalysis.

Of the three commonly used extraction techniques for plasma or serum samples, protein precipitation ("dilute and shoot") is the least suited for assays that require low levels of quantitation because of its susceptibility to ion suppression and matrix effects from remaining endogenous components. Liquid-liquid extraction and solid-phase extraction (SPE) are techniques that offer much cleaner sample extracts that in turn serve to make the method more robust and scalable. SPE is particularly powerful in this regard because of its unique ability to utilize a variety of retention mechanisms. The most widely used SPE sorbent phases are those that are based on retention and elution mechanisms that involve polar and/or nonpolar interaction mechanisms. To fulfill the requirements of selectivity and retention, vendors now offer mixed-mode sorbents that afford hydrophobic and hydrophilic interactions in addition to ion exchange. With mixed-mode strong cation- or anion-exchange SPE, significant cleanup is achieved during the 100% organic wash step, where phospholipids bound by reversed-phase interactions are removed along with any other neutral hydrophobic interference.

To reduce time and effort, on-line sample preparation (direct sample injection), using either turbulent flow chromatography (TFC) columns or single-use SPE-packed cartridges, is a viable alternative to off-line sample preparation.

A practical review of chromatographic principles is provided. It is important to maintain chromatographic resolution as flow rates are increased or column particle sizes are decreased; otherwise, no improvement in productivity is realized. The long-term trend toward ever smaller column particle sizes is an effort to achieve the same performance as is possible with larger particles but in a shorter analysis time. The development and application of small particles (sub-2- μm particles) and fused-core (pelicular) high resolution columns is discussed.

Although increasing resolution by using smaller particles is a common approach to enabling faster separations, selectivity has the greatest effect on resolution. Selectivity is governed predominantly by analyte interactions with both the stationary and mobile phases. Of these, the stationary phase is the most important for chromatographic resolution. Selectivity differences may be brought about by changes in hydrogen bonding, dipole-dipole interactions, and/or ionic interactions with the stationary phase. The ionization state of an analyte resulting from the pH of the mobile phase may also alter the degree of interaction with the stationary phase. Column temperature is another easily adjustable parameter that can provide alternative selectivity leading to increased resolution and an overall reduction in analysis times. The conformational changes that occur with long-chain alkyl stationary phases as a function of temperature can produce selectivity differences among analytes.

Parallel or multiplexed LC-MS/MS systems that can analyze the eluent from several LC systems using a single mass spectrometer are a solution to support high throughput production of quality data. Multiplexed systems with ultrahigh performance capable chromatography systems are available. Included in some systems is the ability to alternate between on-line extraction methods and conventional LC methods.

The presence of unidentified metabolites can lead to inaccurate measurement of analyte concentrations because of ionization suppression, interference in peak integration, or, most problematic, in-source regeneration of the parent drug from phase II metabolites, as the metabolites are not present in the calibration standards and QCs. The importance of chromatographic separation in quantitative analysis should never be overlooked.

Minimizing the risk factors that can affect method reproducibility is an important aspect of developing a quality method. Developing a quality method requires that sufficient attention be given to the fundamentals of sample preparation and chromatography in the face of the never-ending drive for higher throughput.

18.2 INTRODUCTION

Analytical methods employed for the determination of drugs and metabolites in biological matrices such as urine, plasma, serum, and even tissue are essential throughout drug discovery and development. Over the last decade, the development of LC-MS technology has revolutionized bioanalysis. The combination of LC and MS/MS offers unparalleled sensitivity and specificity few other techniques can match [1–3]. Indeed, there was a period following the introduction of LC-MS/MS instrumentation that sample cleanup and high resolution chromatography were thought to be superfluous [4,5]. Fortunately, practitioners soon learned that adequate sample cleanup and chromatographic retention were needed to separate matrix interferences and biotransformed products away from the analyte to obtain better specificity, accuracy, and precision for drug determination in biological matrices [6–9]. Two review articles summarize the literature related to the development of fast LC-MS methods [10] and bioanalytical sample preparation [11].

Despite the advantages offered by the LC-MS/MS technology, the development of rugged and reproducible methods for bioanalysis remains a challenging endeavor. Indeed, there are reports that address the systematic troubleshooting of LC-MS/MS methods [12,13]. Therefore, the successful use of LC-MS/MS for bioanalysis requires an understanding of the mechanisms of various sample extraction processes and the underlying principles of both chromatography and mass spectrometry.

The reliable reporting of data from the quantitative analysis of drugs and their metabolites is the backbone of drug discovery and development. As the drug continues through development, the decisions become more critical; therefore, the bioanalytical methods that produce the data should be sufficiently accurate and reproducible. The regulatory agencies of different countries have their respective guidance documents describing the requirements for bioanalytical method validation and the application of these methods to routine drug analysis. For example, the FDA considers the quality of bioanalytical data generated during toxicology studies to be critical in determining the safety of a drug [14] and therefore requires that the bioanalytical methods be validated to ensure accurate and precise results [15–17]. Bioanalytical method validation is the

process that demonstrates that an analytical method used to quantify an analyte in a biological matrix is reliable, reproducible, and suitable for its intended purpose.

18.3 REGULATORY GUIDANCE FOR THE VALIDATION OF QUANTITATIVE CHROMATOGRAPHIC ASSAYS

The pharmaceutical industry is governed by enacted laws and regulations issued under those laws by regulatory agencies of many different countries. These regulatory agencies on occasion offer suggestions of best practices in the form of guidance documents that they will normally consider acceptable, if followed [17–20]. The FDA Guidance on Bioanalytical Methods Validation is one such guidance that describes best practices for the validation and subsequent application of bioanalytical methods [21]. Complementary guidance is available from the European Medicines Agency [22,23]. The fundamental parameters for method validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability [24–27]. The reader is directed to the chapter titled *Analytical Method Development and Validation in Accordance to the Regulatory Guidelines* for an in-depth discussion of the regulatory requirements associated with the application of bioanalytical methods.

The quality of an analytical method is normally assessed by the performance of standards and QCs. Current guidance recommends an acceptance criterion of 15% for accuracy and precision of all standards and QCs, with the exception of the LLOQ, where the acceptance criterion is increased to a 20% deviation. However, standards and QCs may not adequately reflect the composition of study samples from dosed subjects, that is, incurred samples. The limitation of using spiked control samples to assess “real” samples has been acknowledged for years, and there is general acceptance of the inherent risk of obtaining erroneous results. Confirmatory reanalysis of incurred samples is a recent best practice to determine the reproducibility of a method and ascertain the potential influence of metabolites, protein binding, sample homogeneity, and matrix effects otherwise not determined when using spiked standards and QCs [20,28,29]. Minimizing the risk factors that can affect method reproducibility is an important aspect of developing a quality method and is a primary focus of this chapter. Developing a quality method requires that sufficient attention be given to the fundamentals of sample preparation and chromatography in the face of the never-ending drive for higher throughput.

18.4 SAMPLE PREPARATION CONSIDERATIONS

As LC and MS technologies improve and analysis times reduce, the process of sample extraction becomes a rate-limiting step in the workflow associated with quantitative bioanalysis. For example, reducing overall cycle times is critical for clinical development when multisubject pharmacokinetic (PK) studies can generate a few thousand plasma and urine samples. Moreover, the development of drugs of ever increasing potency will continue to challenge the analytical chemist to lower the levels of quantitation (LLOQ). An LLOQ of 100 pg/mL is a common request for clinical development programs, and when this is coupled with the demand for lower sample volumes, the analytical chemist is well challenged.

Technologies for sample preparation have progressed in lock step with improvements in LC-MS/MS instrumentation. Robotic liquid handling systems with multiple probes for solvent and sample aspirating/dispensing in 96-well plate formats have been developed and commercialized to speed up sample preparation time [30–32]. Parallel processing of samples by protein precipitation or SPE can take anywhere from 10 min to an hour for a single 96-well plate.

The reader is directed to the chapter titled *Direct Biofluid Analysis Systems* for a more complete discussion of sample preparation.

18.4.1 Ion Suppression/Enhancement

A very common form of sample extraction for plasma or serum samples is acetonitrile precipitation of proteins followed by chromatographic separation using ballistic gradients on short analytical columns. Cycle times of <2 min are routine. However, it has become apparent that inadequate sample cleanup and chromatography often leads to, what is now termed, *matrix effects* that are revealed by irreproducible results for some bioanalytical methods [5,33]. Matrix effects cause either analyte ionization suppression or enhancement and occur when endogenous components coelute with the analyte [9,34–38]. The more common effect of ion suppression is observed primarily in electrospray ionization (ESI), where analyte ion signal is attenuated by competition from the ionization of bulk ions inside the solution droplets [39].

A number of techniques to visualize ion suppression have been proposed in the literature. The most widely adopted method for the qualitative assessment of matrix effects involves monitoring the MRM (multiple reaction monitoring) transition of a postcolumn-infused analyte while blank matrix is injected [36,40]. Ion suppression is revealed as dips in the constant background ion signal of the analyte. For the quantitative assessment of matrix effect, a different technique is used, wherein the response of the analyte spiked into extracted blank matrix is compared with that of the analyte spiked into matrix-free reconstitution solution [9,35,39,41,42].

Plasma phospholipids have been identified as playing a major role in causing matrix effects in LC-MS bioanalysis [43–45]. The two common structural classes of phospholipids are glycerophospholipids and sphingomyelins [46–48], and their concentrations in human plasma range between 1.6 and 3.0 mg/mL [49]. Although there is significant lipid variation within the population, because of diet and metabolic rate, phosphatidylcholine (PC) is the primary phospholipid circulating in plasma, accounting for about 60–70% of the total phospholipids [50–52]. As a zwitterion, PC can cause ion suppression in both positive and negative ESI modes because of its ability to ionize in both environments. Therefore, in order to minimize the potential for matrix effects, the removal of phospholipids is an important component of any sample cleanup process. The effectiveness of the cleanup can be monitored by using positive ESI selected reaction monitoring of m/z 184 $\rightarrow$ 184, with the precursor ion (m/z 184) generated in the source by collision-induced dissociation (CID) of phospholipids [53]. An alternative approach utilizes either positive ion neutral loss scans of 141 Da or a negative precursor ion scan of m/z 153 [54].

Considerable attention has been given to approaches for the removal of phospholipids during sample extraction. One report evaluated popular and commercially available ion exchange and mixed-mode SPE sorbents for their ability to remove

phospholipids. It was determined that reverse-phase retention mechanisms are detrimental in eliminating ion suppression caused by late-eluting phospholipids. If an analyte and its metabolites can be retained using an ion exchange mechanism alone, mixed-mode extraction phases should be avoided [55]. Moreover, it is recommended that elution of analytes be performed using aprotic solvents such as acetonitrile rather than using protic solvents such as methanol because of the increased solubility of phospholipids in the latter [56].

Sorbents containing lanthanide metal active surface elements were used successfully to remove phospholipids through a two-step cleanup process involving liquid–liquid extraction with methyl *tert*-butyl ether followed by SPE on a proprietary column [57–59]. Similarly, almost 99% of phospholipids were removed from serum by using cerium-modified columns [60]. More recently, a product (Sigma-Aldrich HybridSPE™) became available, which incorporates zirconia-coated particles. The product is available in a 96-well plate format and has demonstrated selective affinity toward phospholipids while remaining nonselective toward a range of basic, neutral, and acidic compounds [61]. More recently, Waters has introduced a sample preparation product called *Ostro*™ with a proprietary packing that selectively removes phospholipids while filtering precipitated proteins after protein precipitation.

It should not be forgotten that formulation excipients such as polyethylene glycol (PEG), sometimes administered in large quantities with the compound, can create ion suppression effects that are not present in control matrices [62–64]. Similarly, significant ion suppression was observed with a formulation containing polysorbate 80 (Tween 80®) [64,65].

Adequate sample cleanup along with sufficient chromatographic separation remain the primary tools to control matrix effects. Even then, it is important to adequately compensate for any alteration of ion signal by using an appropriate internal standard, most often a stable-isotope-labeled (SIL) analog of the drug [66]. An SIL internal standard is chemically identical to the analyte and is therefore considered to be essential in any quantitative assay that uses MS detection to avoid the influence of residual matrix effects on ionization efficiency [42,67]. Nevertheless, the use of an SIL internal standard does not always guarantee correction of matrix suppression [68]. The only way to avoid or minimize matrix effects is to develop a method that uses adequate sample cleanup and chromatographic resolution.

18.4.2 Orthogonal Approaches to Sample Preparation/Chromatography

Of the three commonly used extraction techniques, protein precipitation (“dilute and shoot”) is the least suited for routine plasma or serum assays with low LOQs (limits of quantitation) because of its susceptibility to ion suppression and matrix effects. For an alternative methodology, the reader is directed to the chapter titled *Analytical Method Development and Validation in Accordance to the Regulatory Guidelines* for a discussion on direct plasma analysis.

Liquid–liquid extraction and SPE are techniques that offer much cleaner sample extracts that in turn serve to make the method more robust and scalable. SPE is particularly powerful in this regard because of its unique ability to utilize a variety of retention mechanisms [69]. Moreover, the availability of SPE products in a 96-well format affords the opportunity for high throughput analysis using automated robotic liquid handlers with minimal operator intervention.

The most widely used SPE sorbent phases are those that are based on retention and elution mechanisms that involve polar and/or nonpolar interaction mechanisms. Specifically, sorbents that incorporate C₁₈ ligands are the most popular. However, the combination of polar and nonpolar retention mechanisms is also the least selective because nearly everything is retained from the biological matrices along with the analyte of interest. Although this retentiveness is sometimes an advantage in cases where metabolites and parent drug have very different polarities, in general, methods based on this type of interaction mechanism will then require more refined HPLC conditions to compensate for the lack of sample cleanup selectivity. On the other hand, a sorbent that utilizes an ion exchange interaction can be highly selective for molecules that contain functional groups capable of exhibiting either a positive or a negative charge under appropriate acidic or basic conditions [69]. The drawback with high selectivity is that, on occasion, metabolites with a significantly different pK_a from the parent drug will be difficult to isolate.

To fulfill the requirements of selectivity and retention, vendors now offer mixed-mode sorbents that afford hydrophobic and hydrophilic interactions in addition to ion exchange [55,70]. The advantage of incorporating both of these retention mechanisms is the potential to isolate structurally diverse analytes with adequate selectivity and high recovery [56,71]. Moreover, during mixed-mode strong cation- or anion-exchange SPE, significant cleanup is achieved during the 100% organic wash step, where phospholipids bound by reversed-phase interactions are removed along with any other neutral hydrophobic interference. With a reversed-phase only SPE, the final organic elution step simply releases the hydrophobic interferences along with the analyte of interest into the eluent.

Xue *et al.* [72] developed a simplified protein precipitation and mixed-mode cation-exchange SPE method for the extraction of a basic drug from human plasma. The concept was to directly transfer the protein precipitated supernatant onto an unconditioned Oasis[®] MCX μ Elution 96-well extraction plate. The simplified method offered equivalent performance and eliminated the time-consuming conditioning and rinsing steps.

18.4.3 Micro-Solid-Phase Extraction Tips

In order to eliminate the time-consuming steps of excess solvent evaporation followed by sample reconstitution, vendors have developed SPE products with smaller sorbent beds. Examples include the Waters Oasis μ Elution Plate and Omix[®] tips from Varian. SPE in pipette tips using monolithic sorbent technology is a particularly interesting development because it offers new automation possibilities not previously available [73,74]. Specifically, the elimination of a vacuum manifold enables true walkaway automation and the micro-SPE tip can perform exhaustive extractions simply by passing the sample back and forth over the sorbent bed. The micro-SPE tips have sufficient capacity to extract up to 100- μ L aliquots of plasma, and analytes can be eluted with as little as 60 μ L of organic solvent [75,76].

18.4.4 Turbulent Flow Extraction

To reduce time and effort, on-line sample preparation (direct sample injection) based on TFC offers an alternative and TFC on-line with LC-MS/MS has demonstrated its

utility and potential in the bioanalytical support of preclinical [77–80] and clinical studies [81–83].

Under conditions of high linear flow rates in columns packed with large, irregularly shaped particles ranging in size from 35 to 75 μm , the solvent flow profile will change from laminar to turbulent. Under the conditions of turbulent flow, low molecular weight molecules will diffuse faster into particle pores than larger molecular weight molecules. The practical implication is that biological samples can be injected directly onto a column and small molecules are retained at the column head while large macromolecules, such as proteins, and other unretained polar analytes, wash away. This is a highly effective technique to conduct on-line sample extraction [84–86].

Typical conditions for TFC involve flow rates above 4 mL/min with 10 mm i.d. columns and above 1 mL/min for 5 mm i.d. columns. For efficient extraction of analytes, the stationary and mobile phases selected need to provide a retention factor of at least 50 column volumes, preferably 100–200 column volumes [87–90].

Turbulent flow on-line extraction can be performed with two different configurations. For single-column methods [91], samples are loaded onto the column under turbulent flow conditions and then analyte elution occurs with a mobile phase of suitable strength under laminar or turbulent flow conditions. The peak capacity of the chromatographic separation can be low because the same solvent is used for both extraction and separation. Moreover, the high flow rates require splitting of the effluent before introduction into the ion source of a mass spectrometer.

The preferred configuration involves two columns; that is, samples are retained on a turbulent flow column, while proteinaceous material is diverted to waste and then the desired analyte(s) is eluted onto an analytical column (Figs. 18.1 and 18.2) [88]. The eluent is transferred through a tee to the analytical column into which the analytical

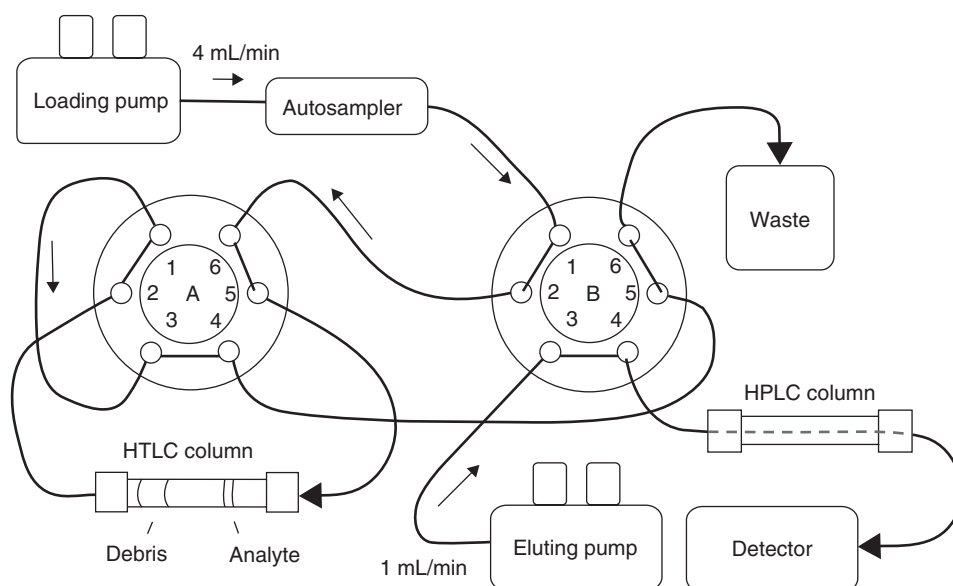


Figure 18.1 Turbulent flow extraction. Cohesive Technologies' "Quick Elute" program showing equilibration and loading steps.

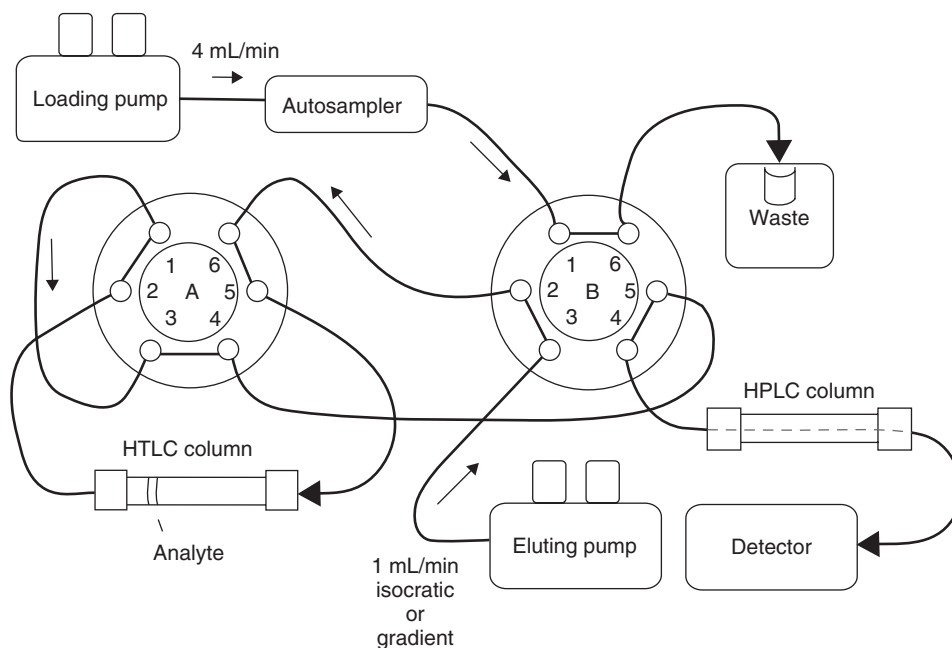


Figure 18.2 Turbulent flow extraction. Cohesive Technologies' "Quick Elute" program showing the elution step.

pump delivers a weak mobile phase at a higher flow rate relative to the extraction flow to focus analytes on the analytical column [92]. In general, the extraction flow rate in the mixing step needs to be minimized in order to maintain the desired focusing effect [93]. Once the sample is transferred, the compounds are eluted and separated with a gradient or step elution on the analytical column. Dual-column extraction normally provides better chromatographic separation and peak shape than single-column methods. It is worth noting that to increase the extraction column lifetime and avoid column and system clogging, samples should be diluted or even extracted before injection.

18.4.5 On-Line SPE

Another alternative to be considered for automated on-line SPE is the SymbiosisTM system from Spark Holland. The Symbiosis system evolved from earlier ProspektTM systems and consists of a RelianceTM autosampler, a dual high pressure dispenser (HPD) solvent delivery system with six-port valves, a gradient pump set, and dual automated SPE cartridge exchange (ACE) system. Significantly, the system uses single-use (i.e., disposable) SPE cartridges, thereby reducing the potential for column and/or system clogging. Samples do not normally require sample dilution before injection.

The automation features offered by the Symbiosis system have allowed for automated and unattended method development that has proven very useful for rapid method development of robust assays [94]. The authors were able to complete method development within 4 h in a fully automated mode.

The Symbiosis system has also been used to provide a fully integrated approach to bioanalysis [95–97]. The Vial to FileTM process involves placing study samples into

an autosampler and then all subsequent operations related to data generation occurs in a hands-free, 21CFR11 compliant environment. Custom-designed sample vials from Sarstedt that incorporate septum pierceable caps were used to provide cryogenic storage of unknowns and compatibility with the Symbiosis autosampler. The extraction protocol featured microliter pickup of internal standard concurrent with the sample aliquot.

A recent application of the Symbiosis system is on-line extraction of dried blood samples using sorbent sampling cartridges [98,99]. The technique requires the application of 5–10 μL of blood onto a fibrous material packed into a Symbiosis compatible cartridge. Blood samples absorbed on these cartridges are subsequently eluted on-line using the Symbiosis system for SPE and LC-MS/MS analysis. The continued development of this strategy could result in a significant decrease in the use of animals in drug development studies; specifically, with low blood volume requirements, the characterization of a drug candidate's PK could be performed using a single animal rather than with the current practice of composite PK from multiple animals. Indeed, determining PK from a single animal would most likely provide a better representation of the actual kinetics.

18.4.6 Supported Liquid Extraction

An efficient alternative to traditional liquid–liquid extraction for sample preparation is supported liquid extraction (SLE) offering aqueous sample volumes of up to 400 μL [100,101]. SLE is very efficient, often delivering higher analyte recoveries and cleaner extracts than an equivalent liquid liquid extraction (LLE) method. Moreover, SLE in a 96-well plate format is easily automated with no manual steps such as capping, mixing, centrifugation, and decapping required. SLE plates are most commonly packed with an optimized grade of diatomaceous earth to provide reproducible flow characteristics from well to well.

18.5 A PRACTICAL REVIEW OF THE PRINCIPLES OF CHROMATOGRAPHY

18.5.1 Resolution

The performance of a column can be measured in terms of the height equivalent to a theoretical plate (H), which can be calculated from the column length L and the column efficiency, or theoretical plate number, N :

$$H = \frac{L}{N}$$

The number of theoretical plates is calculated from the equation

$$N = 16 \left(\frac{t_R}{W} \right)^2$$

or

$$N = 5.54 \left(\frac{t_R}{W_{1/2}} \right)^2$$

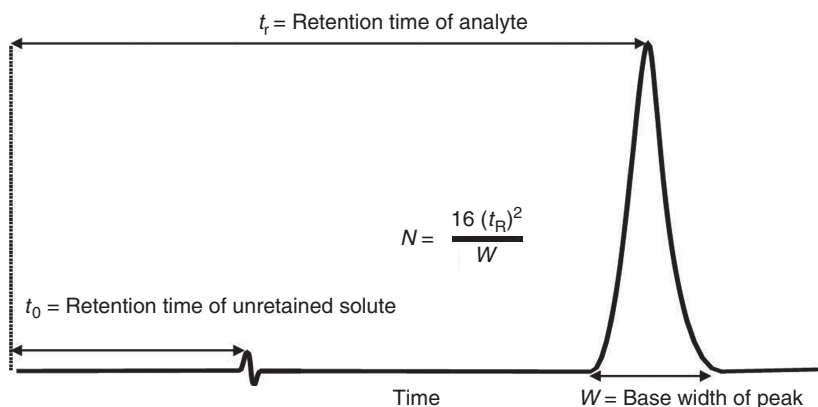


Figure 18.3 Column efficiency expressed as the number of theoretical plates (N).

where t_R is the analyte's retention time, W is the peak width, and $W_{1/2}$ is the peak width at half height (Fig. 18.3). A lower plate height indicates a more efficient column, and a minimum plate height of about twice the particle diameter is generally expected for an efficient column. The van Deemter equation describes H in terms of its dependence on the linear flow velocity (μ):

$$H = A + \frac{B}{\mu} + C\mu$$

where A , B , and C are the coefficients of eddy diffusion, longitudinal diffusion, and axial diffusion (resistance to mass transfer through and between mobile and stationary phases), respectively (Fig. 18.4). Peak (band) broadening results from an analyte diffusing through a column by following random paths with different lengths. The A term can be related to particle size, how well the column is packed, and the internal diameter of the column. An analyte diffuses from the center of the column out to the

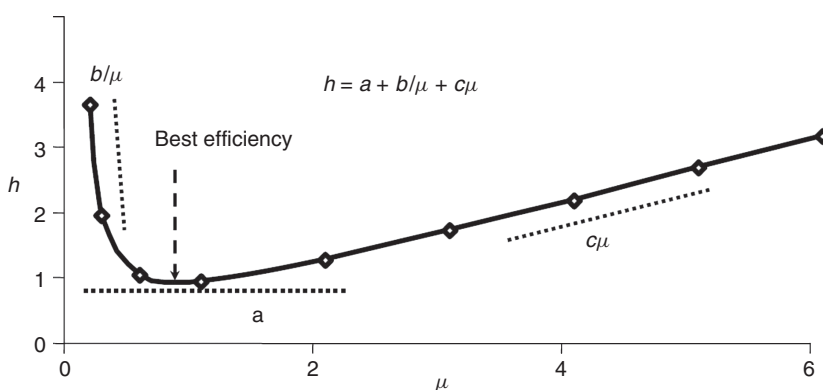


Figure 18.4 van Deemter plot for laminar flow chromatography. The van Deemter curve is a composite of three curves— a (eddy diffusion), b/μ (longitudinal diffusion), and $c\mu$ (axial diffusion, resistance to mass transfer through and between mobile and stationary phases).

edges, and as the velocity of the mobile phase increases, the analyte spends less time on the column, thereby decreasing the effects of longitudinal diffusion. Resistance to mass transfer (axial diffusion) is the partitioning that takes place between the solvent, column packing, and the analyte. If the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase, and the analyte band becomes broadened. In this case, the higher the velocity of the mobile phase, the broader the band.

Chromatographic separation of two analytes, also called *resolution* (R_S), is related to the number of plates in the column, selectivity of the chromatographic system, and the retention of each analyte:

$$R_S = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k'}{k' + 1} \right) \quad (18.1)$$

where N is peak efficiency, α is the selectivity, and k' is the retention (capacity) factor. To obtain high resolution, the three terms must be maximized. For example, increasing N by lengthening the column leads to an increase in retention time and increased band broadening, which is not desirable for high throughput bioanalysis. The capacity factor might be optimized by changing the column temperature (Section 18.7). Column selectivity is normally optimized by changing the mobile phase composition, column temperature, or stationary phase.

Resolution between two peaks in a chromatogram can be determined by

$$R_S = 2 \left[\frac{(t_R)_A - (t_R)_B}{(W_A - W_B)} \right] \quad (18.2)$$

where, t_R is the retention time of an analyte and W is the Gaussian curve width of the analyte. When resolution is unity, acceptable resolution is achieved, whereas baseline resolution between two peaks requires $R_S \geq 1.5$.

Productivity can be defined as being proportional to resolution per unit time. In other words, it is important to maintain resolution as flow rates are increased; otherwise, no improvement in productivity is realized. The long-term trend toward ever smaller column particle sizes is an effort to achieve the same performance as is possible with larger particles but in a shorter analysis time [102–105].

18.5.2 Optimizing Chromatographic Performance

18.5.2.1 Selectivity. Although increasing resolution by using smaller particles (i.e., increasing N) is a common approach to enabling faster separations, the selectivity term, α , has the greatest effect on resolution. Selectivity is governed predominantly by analyte interactions with both the stationary and mobile phases. Of these, the stationary phase is still the most important consideration for chromatographic resolution. Selectivity differences may be brought about by changes in hydrogen bonding, dipole–dipole interactions, and/or ionic interactions with the stationary phase. Nevertheless, hydrophobic (reverse phase) separations on C_{18} stationary phases remain the most popular choice in most part because of familiarity and availability.

Fortunately, column vendors continue to introduce unique stationary phases that offer the potential for dramatic improvements in selectivity. Examples include biphenyl

stationary phases that show excellent selectivity for aromatic or fused-ring compounds or pentafluorophenyl propyl stationary phases that are very selective and retentive for organohalogens or other compounds containing basic or electronegative functionalities.

18.5.2.2 pH. In reversed-phase chromatography, hydrophobic interactions are the primary mechanism of selectivity between the stationary phase and the analytes of interest. The ionization state of an analyte may alter the degree of interaction with the stationary phase. When a molecule is ionized, it is more likely to participate in hydrogen bonding. In a reversed-phase, aqueous-rich mobile phase, the solute will spend less time participating in hydrophobic interactions with the stationary phase and more time hydrogen bonding in the aqueous portion relative to the neutral species. The result is less retention. When there are significant concentrations of both ionized and neutral forms, the ionized form will have less hydrophobic interaction with the stationary phase while the neutral form will have more hydrophobic interaction. The result is broad or asymmetrical peaks. Moreover, an analyte in its ionized state may participate in unwanted ionic interactions with exposed silanol groups or metal contaminants present in the silica. These ionic interactions may lead to poor peak shape or irreproducibility.

It is possible to predict the ionization state of an analyte relative to the mobile phase pH from the analyte pK_a . The ionization state can then be controlled by simply altering the mobile phase pH. It should be noted that for nonionizable compounds, the retention characteristics remain relatively unchanged throughout the pH range, although the use of different buffers may influence their peak shape.

Partial dissociation of weakly acidic or basic analytes occurs when the pH range of the mobile phase is within two units (above or below) the pK_a of the molecule. Therefore, to avoid unpredictable secondary interactions, the mobile phase pH should be maintained at least two units above (bases) or below (acids) the pK_a of the molecule.

A general observation is that an acidic functionality will tend to have greater retention and efficiency at a pH below its pK_a value and less retention at a pH above its pK_a value. Basic compounds follow the opposite trend; that is, basic functionalities are often less retained at a pH below their pK_a values and demonstrate greater retention and better peak shape at a pH above their pK_a values.

18.5.2.3 Buffer Selection. Next to organic solvent, buffer selection is the most important variable in the development of HPLC method [106]. For example, when separating mixtures containing acids and/or bases by reversed-phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer to achieve reproducible results. Optimum buffering capacity occurs at a pH equal to the pK_a of the buffer. In general, adequate buffering capacity occurs only within ± 1 unit of their buffer's pK_a . Beyond that, buffering capacity will be inadequate. Table 18.1 lists some commonly used buffers for reversed-phase HPLC. Other criteria such as ionic strength, ion-pairing properties, and mass spectrometer compatibility should be considered before selecting any mobile phase modifier. The concentration of the mobile phase buffer usually has little effect on retention in reversed-phase HPLC, provided the buffer concentration is sufficient to control pH; buffer concentrations above 10 mM are usually effective. It should be noted that in situations where ion exchange interactions are occurring between basic analytes and acidic silanols on the surface of silica stationary phases, analyte retention can be affected by buffer concentration. Increasing buffer concentrations may then be used to suppress unwanted ion exchange interactions.

TABLE 18.1 Commonly Used Buffers for Reversed-Phase HPLC

Buffer	p <i>K</i> _a	Buffer Range
Phosphate	2.1	1.1–3.1
	7.2	6.2–8.2
	12.3	11.3–13.3
Formic acid ^a	3.8	2.8–4.8
Acetic acid ^a	4.8	3.8–5.8
Citrate	3.1	2.1–4.1
	4.7	3.7–5.7
	5.4	4.4–6.4
Tris	8.3	7.3–9.3
Triethylamine ^a	11.0	10.0–12.0
Pyrrolidine	11.3	10.3–12.3

^aVolatile buffers suitable for LC-MS/MS.

18.6 EMERGING TECHNOLOGIES

18.6.1 Hybrid Silica Particles

Silica particles possess excellent mechanical strength and efficiencies, but they have a limited range of pH stability. When mobile phases are used with pH < 2, the bonded phase is susceptible to hydrolysis [107], and at pH > 8, particle erosion can occur because of the dissolution of the base silica particle, which can result in a loss of column efficiency, an increase in column back pressure, and bed collapse of the silica packing material [108]. Chromatographic columns based on organic polymers, graphitic carbon, alumina, titania, and zirconia have been demonstrated to have little or no performance degradation when employed with high pH mobile phases (pH 8–14) at elevated temperatures (60–120°C) [109–111]. Unfortunately, the use of these nonsiliceous materials presents their own limitations. For example, organic polymers are generally less mechanically strong and exhibit significantly reduced efficiencies. Graphitic carbon and metal oxides show strong adsorption for many polar compounds resulting in poor peak shapes.

In an effort to maintain the predictable chromatographic performance afforded by silica-based materials, column vendors have turned their attention to the development of hybrid particles that offer high efficiencies and mechanical strength with extended pH stability.

In 1999, Waters launched the XTerra[®] family of HPLC columns featuring a first generation of hybrid particle technology. The hybrid particle was synthesized by replacing one out of every three silanols with a methyl group. The hydrophobicity introduced by the methyl group was distributed through the entire structure of the particle backbone, resulting in a rugged hybrid (inorganic/organic) particle. The presence of 33% fewer residual silanols (after endcapping and bonding) provided higher efficiency chromatography for basic compounds. In 2005, Waters introduced a second-generation hybrid material that utilized an ethylene bridged hybrid (BEH) structure [112]. Compared to the first-generation methyl hybrid particle of XTerra columns, the BEH particle offered vastly improved efficiency, mechanical strength,

and pH range. The BEH hybrid particle is one of the key enablers behind Waters Ultra Performance LC (UPLC™) technology.

Introduced by Phenomenex in 2004, the Gemini product line was the first media to use Two-In-One (TWIN™) technology. Phenomenex used an organosilica grafting process to incorporate highly stabilizing ethane cross-linking. The organic groups are evenly distributed into the grafted layers on the silica surface of a pure silica core. This provides extended pH stability, while maintaining the high efficiency and mechanical strength of a silica particle.

18.6.2 Chiral Columns

Substances that differ only in the spatial arrangement of their atoms are called *stereoisomers*. Certain stereoisomers that differ only in their capacity for rotating the plane of polarized light are termed *optically active* or *chiral*. Optically active isomers are called *enantiomers*. Enantiomers can differ in their potency and selectivity and may exhibit different PK. Indeed, toxicity can be linked to one of the enantiomers [113,114]. Studies of the differences in the pharmacological activities of stereoisomers of a chiral drug are necessary in the drug development process and also required by the regulatory bodies [115].

The separation of chiral drug candidates generally relies on the formation of transient diastereomeric complexes with the stationary phase, resulting in differing chromatographic properties of the enantiomers. Complexes can be formed by hydrogen bonding, ion pairing, metal-ion-to-ligand attraction, π -acid/ π -base interactions, or van der Waals attractions. Resolving enantiomers by forming inclusion complexes with cyclodextrin-based stationary phases is perhaps the most widespread approach in the field [116–118]. Of these, triacetate, tribenzoate, and triphenylcarbamate derivatives of cellulose and amylose derivatives are most common. Several reports, however, have demonstrated the utility of β -cyclodextrin as a chiral mobile phase additive for the separation of enantiomers using reversed-phase HPLC [119–122]. The use of chiral mobile phase additives (CMPAs) provides the flexibility to choose from a variety of achiral stationary phases including sub-2- μ m particles. Indeed, by combining the CMPAs with fused-core particles and ultrahigh performance liquid chromatography (UHPLC) instrumentation, fast/ultrafast enantiomeric separations are possible [123].

Efforts to increase the efficiency of chiral separations have led investigators to utilize supercritical fluid chromatography (SFC) because of the low viscosity, high diffusivity, and low surface tension of the eluent [124–127]. Super- and subcritical carbon dioxide modified with methanol or other solvents easily outperforms normal-phase HPLC because flow rates are typically five times faster with no loss of resolution. Moreover, SFC offers an environmentally friendly alternative to the conventional solvents used in HPLC. It should be noted that some basic compounds and, in particular, amines do not resolve well by using SFC [128]. The effect of the modifier on a chiral separation varies greatly with analyte, but it has been reported that 2-propanol, rather than the more common modified methanol, often leads to better selectivity [129].

Protein-based chiral columns offer an end user the ability to perform chiral separations in a reversed-phase environment where there is applicability to an extremely broad range of chiral compounds, including amines, acids, esters, sulfoxides, amides, and alcohols. Examples of proteins that have been immobilized onto 5- μ m spherical silica particles for chiral chromatography include human serum albumin, α 1-acid

glycoprotein, and the stable enzyme, cellobiohydrolase. The retention and enantioselectivity of these phases can be regulated by changes in pH, buffer concentration, and the nature and concentration of organic modifier.

18.6.3 Monolithic Columns

Modern monoliths emerged between the late 1980s and the mid-1990s to be used as columns for HPLC [130–132]. A monolith is a polymerized porous support structure that fills entirely the column volume without any interparticular voids typical of packed columns [133–135]. Moreover, a monolith is not packed into a column because they are normally prepared *in situ* by polymerization. Monolithic columns have proven to be a very good alternative to particle-packed columns for high efficiency separations in HPLC [136–139]. Because of their small-sized skeletons and wide through pores, these columns provide lower flow resistances and can be operated at high flow rates. Increases in efficiency, however, are moderate because there is greater contribution of a mobile-phase mass-transfer term to band broadening [140]. Also, solvent consumption is considerably higher than that in conventional packed columns and might be an issue for cost-conscious laboratories.

To reduce sample preparation and analysis time, the direct injection of biological samples or diluted supernatant resulting from protein precipitation onto an HPLC column is highly desirable [141,142]. However, the analysis of neat or diluted matrix samples is normally impractical because of the severe buildup of matrix components on HPLC columns made of particulate sorbents. Monolithic columns have proven to be more resilient to this type of approach because of their higher permeability along with the absence of traditional column frits. These differences allow biological matrix components such as proteins to easily pass through the column not affecting column lifetime. Nevertheless, it is always advisable to utilize a higher ratio of dilution in a “dilute and shoot” paradigm to ensure robust LC-MS methods with reduced potential for ionization matrix effects.

18.6.4 Hydrophilic Interaction Chromatography

Reverse-phase LC remains the most widely used technique in HPLC. Molecules are separated based on their hydrophobic interactions between the nonpolar stationary phase and the organic functionalities of typical analytes. However, the retention of polar analytes often requires a highly aqueous mobile phase to achieve retention. Highly aqueous systems sometimes lead to problems such as phase collapse (dewetting) [143,144], decreased MS ionization efficiency because of poor mobile phase desolvation, and yet may still not allow retention of very polar analytes. Some specialized phases were developed to allow the use of highly aqueous systems such as polar embedded phases, hydrophilically endcapped reversed-phase bonded silica, wide-pore low density bonded silica, short-chain phases, and other special designs [143].

Hydrophilic interaction chromatography (HILIC) is a mode of chromatography that can address these issues. HILIC requires mobile phases that are highly volatile (80% organic), are ideal for compound ionization by ESI-MS, and can retain highly polar analytes. The term *hydrophilic interaction chromatography* was first coined by Andrew Alpert to distinguish this technique from normal-phase chromatography [145]. HILIC is run on polar stationary phases such as silica [146–148], amino [149–151], diol

[152], polyhydroxyethylaspartamide, and cyclodextrin-based packing [145,153]. A high organic, low aqueous mobile phase is used to retain analytes with increasing orders of hydrophilicity. According to Alpert, retention is proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase [145]. On silica columns, the mechanism of HILIC involves various combinations of hydrophilic interaction, ion exchange, and reversed-phase retention by the siloxane on the silica surface [154]. This combination of retention mechanisms results in a unique selectivity that allows for polar retention [154–157].

Conventional SPE methods often contain an elution step that uses a high concentration of an organic solvent. This SPE eluent is not always compatible with reversed-phase analysis. A typical sequence would be to evaporate and then reconstitute into a solvent that more closely matches the initial mobile phase conditions [158]. In HILIC, the high organic eluent is ideal for direct injection, thus eliminating the need for lengthy evaporation and reconstitution steps and increasing throughput [146,154].

18.6.5 Sub-2- μ m Chromatography

18.6.5.1 The Promise of van Deemter. As early as the 1950s, van Deemter realized that peak efficiency could be improved by reducing particle size [159]. Since then, particle technology has continued to advance, whereby column vendors are now able to offer a variety of stationary phases with sub-2 μ m diameters and with the requisite mechanical strength [160–162]. The development of hybrid silica particles was a clear enabler for this technology.

From the resolution equation it can be seen that resolution is proportional to the square root of N . Because N is inversely proportional to particle size, as the column particle size is reduced, resolution increases by the square root of the difference. Similarly, with N being inversely proportional to the square of the peak width, as particle sizes decrease, peak widths become narrower; that is, there is an increase in concentration [163–165]. These improvements, though, come at a cost. Because the pressure required to pump mobile phase through a column is inversely proportional to the square of the particle diameter, the backpressures required for the use of these small-particle columns become quite high [165,166]. Indeed, pressures of up to 4000 bar were used with small-diameter columns packed with sub-2- μ m particles to achieve extraordinary separations [163].

In 2004, Waters Corporation introduced the ACQUITY UPLC that became the first commercially available system capable of pumping mobile phase at pressures up to 15,000 psi. Subsequent introductions of higher pressure capable systems came from Agilent Technologies (1200 RRLC series), Thermo Scientific (Accela 1250), Flux Instruments (Rheos Allegro), PerkinElmer (Flexar™ FX-15), and Dionex (UltiMate™ 3000 RSLC).

An important consideration that limits the use of even smaller particles and higher operating pressures is the occurrence of viscous heating. Viscous heating occurs from the dissipation of the frictional forces when liquid passes through the column bed. The generation of heat results in an increase in mobile phase temperature along the column axis [167,168]. This physical phenomenon produces band spreading, increasing peak width, and produces fronting and tailing. Indeed, the frictional heating of the

mobile phase is thought to be the primary reason for the underperformance of sub-2- μm columns when compared to 3.5- and 5- μm columns [169–172]. Therefore, the use of narrower bore columns with sub-2- μm particles is an important consideration to reduce the impact of radial temperature gradients and maximize chromatographic efficiency.

18.6.5.2 Optimizing Sub-2- μm Chromatography. The goal of any LC analysis is to optimize the resolution between critical pairs of analytes in the shortest time. Unit resolution may be acceptable for many applications, whereas increasing the resolution to 1.5 is considered appropriate for nearly all quantitative analyses. Optimizing selectivity by proper choice of stationary phase and chromatography conditions (e.g., organic modifier, pH, gradient) is far more powerful at improving resolution than increasing separation efficiency with smaller particles. Systematic evaluation of chromatographic conditions can be facilitated by using an automated LC method screening tool.

The transfer of an HPLC assay to UPLC is reasonably straightforward with the following recommendations [173,174]:

- By taking advantage of the high resolution potential of sub-2- μm columns, mobile phase solvent strength can be increased, thereby reducing run times.
- By maintaining optimum mobile phase linear velocities to ensure adequate peak resolution while operating at no more than 80% of the maximum rated pressure of the system. Flow rates are simply scaled in proportion to the difference in column dimensions in order to maintain the same linear velocity.
- Column re-equilibration times can be reduced as a result of low system dwell volumes.
- Injection volume should be appropriately adjusted for the column diameter to achieve good peak shapes. The smaller injection volumes are more than compensated by the increased peak height resulting from the use of sub-2- μm particles.
- By limiting sample volume injections to $\sim 50\%$ of the total loop volume. For systems that utilize air-gap sandwiching of the sample, reducing sample volume allows better utilization of the sample loop and higher injection precision.

18.6.5.3 Linear Velocity. Optimum linear velocity is inversely proportional to particle size, whereas the pressure required to operate at the optimum velocity is inversely proportional to the *cube* of the particle diameter. The pressure limits of the LC pump will therefore restrict the length of column if packed with sub-2- μm particles and operated at optimal flow velocity (~ 0.5 cm/s). Nevertheless, a significant advantage of using sub-2- μm particle columns is the ability to use higher linear velocities without detrimental effects on separation quality. It should be noted that maintaining a separation under gradient conditions when the mobile phase linear velocity is increased requires that the gradient times (t_G) also be adjusted accordingly:

$$t_{G2} = t_{G1} \times \frac{v_2}{v_1} \times \frac{F_1}{F_2} \quad (18.3)$$

where t_G is the gradient time, v is column volume, and F is flow rate.

18.6.6 Pelicular (Fused-Core) Silica

The recent introduction of fused-core silica particle technology has unlocked opportunities for developing higher throughput assays [175–177]. Examples include MAC-MOD Analytical Halo[®] and Phenomenex[®] Kinetex columns. Using sol–gel processing techniques that incorporate nanostructuring technology, a durable, homogeneous porous shell is grown onto a solid silica core. The solid silica cores range in diameters from 1.25 to 1.9 μm and are surrounded by 0.23–0.5 μm superficially porous shells for total particle sizes between 1.7 and 2.7 μm . The highly optimized growth process produces an extremely narrow particle size distribution and dramatically reduces two major sources of peak dispersion, namely, eddy diffusion and resistance to mass transfer. In practice, these fused-core silica particles have shown substantial improvement in chromatographic peak efficiencies over fully porous particles [178–180]. Specifically, the reduction in axial diffusion allows the use of higher flow rates without sacrificing chromatographic performance [181,182]. Compared to sub-2- μm particles, the fused-core particles offer similar efficiency and/or peak capacity at a fraction of the backpressure [183,184]. The adoption of fused-core silica particle technology has been rapid in the pharmaceutical industry for both early discovery [185,186] and late development programs [187].

18.7 ELEVATED COLUMN TEMPERATURES

18.7.1 Theory

Method developers often seek compromise rather than optimization. An often overlooked parameter is temperature that is an easily adjustable parameter and can provide alternative selectivity leading to increased resolution of critical isomer pairs along with an overall reduction in analysis times.

The benefits of using high temperature liquid chromatography (HTLC) were recognized in the 1960s when pioneers such as Giddings and Snyder demonstrated that increasing column temperature could lead to decreased separation times and altered selectivity [188,189]. Recent developments in technology and a better understanding on how temperature can enhance selectivity have created renewed interests in this area [190–192]. As early as 1988, Antia and Horvath [192] proposed in a theoretical study that up to a 100-fold increase in separation efficiency for HPLC can be achieved at significantly elevated column temperatures. A recent publication from Plumb *et al.* described an application utilizing a sub-2- μm HPLC column at elevated temperature to identify metabolites in urine samples. The authors reported a peak capacity of ~ 700 for a 10-min analysis and greater than ~ 1000 in 1 h in gradient elution mode [193]. A number of articles have reviewed the recent advances and applications of HTLC [194–197].

18.7.1.1 Van't Hoff Equation. Increasing temperature alters analyte retention by changing the free energy (enthalpy and entropy) between the analyte and the stationary phase [198,199]. The temperature-dependent free energy changes can be represented using a Van't Hoff plot (i.e., $\ln$ retention factor vs $1/\text{temperature}$). Van't Hoff plots are often linear, and the slope of the line can be used to estimate enthalpy, assuming the analyte retention is governed by only one retention mechanism. Several studies, however, have suggested that changes in temperature affect multiple retention mechanisms,

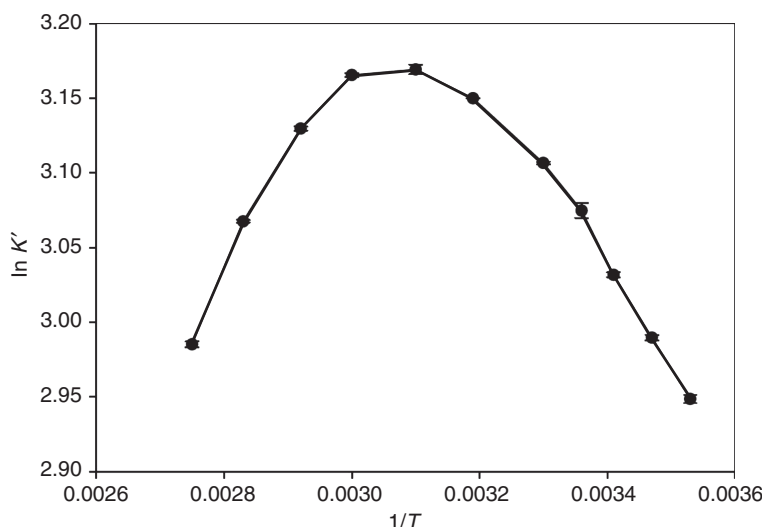


Figure 18.5 The effect of temperature on resolution. Retention factor of an analyte measured as a function of temperature and represented in a Van't Hoff plot.

including acid–base dissociation rate variations and varying adsorption/desorption kinetics in the stationary phase. The end result is nonlinear Van't Hoff plots, and the phenomenon is particularly evident for ionizable analytes (Fig. 18.5) [200–202].

In general, chromatographic peak efficiency increases as temperature increases, especially at higher flow rates [190]. On the other hand, selectivity can either increase or decrease depending on temperature, and with several other parameters also affecting selectivity (pH, mobile phase, stationary phase, etc.), it is very difficult if not impossible to predict how selectivity will change with temperature. The decrease in linear velocity and increase in the diffusion coefficient with temperature usually results in a decrease in k' (although not always). The decrease in k' would suggest a decrease in resolution with increasing temperature. With the effects of efficiency, selectivity, and retention factor combined, the end result is that resolution generally decreases with increasing temperature [191].

18.7.2 Reality

The conformational changes that occur with long-chain alkyl stationary phases as a function of temperature can produce selectivity differences among analytes. The promise of modulating selectivity by adjusting column temperature is certainly an appealing notion. Shen *et al.* [191] demonstrated enhanced resolution of diastereomers using elevated column temperatures of 100°C and an ion-pairing reagent. As a result of the increased column temperature, the authors achieved a twofold reduction in cycle time and a fivefold improvement in sensitivity.

Still others have employed temperature programming (thermal gradients) as an alternative to solvent composition gradients for the analysis of pharmaceutical compounds [203–206].

Given the difficulty in predicting the effect of temperature on selectivity, it is recommended that chromatography be evaluated over a range of temperatures. Automation

of the process by using chromatographic methods that slowly increment temperature overnight can reduce the overall development time. Concerns regarding analyte thermal stability and irreproducible chromatography because of column thermal instability remain, and they can be alleviated only by controlled investigations.

18.8 ION-PAIR REAGENTS

Volatile ion-pairing reagents such as perfluorinated carboxylic acids are useful because of their compatibility with both LC and MS [207–209]. Perfluorinated carboxylic acids such as trifluoroacetic acid (TFA), heptafluorobutanoic acid, and nonafluoroheptanoic acid have been used with LC-MS and were found to provide similar ion-pair behaviors when compared to the more conventional sodium heptane sulfonate additive. The use of perfluorinated carboxylic acids as ion-pairing reagents to extend the retention time of very polar analytes in reverse-phase chromatography has been reported. In one recent report, Gao *et al.* [209] used perfluorinated carboxylic acids as additives in the reconstitution solution of bioanalytical samples and discovered that the retention times of polar molecules, such as methadone, were significantly extended. The use of perfluorinated carboxylic acids to enhance diastereomer separations has also been reported [191].

Unfortunately, the use of TFA is known to cause ion suppression and must be dealt with if this acid is used in the mobile phase [210]. The use of surface-tension-lowering modifiers to modify the mobile phase and mitigate ion suppression is a common solution [211]. Postcolumn addition of acids and solvent carriers to aid ionization is known as the *TFA Fix* [212].

18.9 MULTIPLEXING LC-MS/MS

Automation of the pipetting and incubation steps of *in vitro* assays makes it possible for a single analyst to generate many more samples in an 8-h period than can be analyzed by current LC-MS/MS systems. Similar improvements in automation of sample extraction have given the analyst the ability to prepare large batches of *in vivo* samples for LC-MS/MS analysis. Once again, the LC-MS/MS interface is the bottleneck and other options are required. It is essential that whatever processes are established that maximize throughput, they must do so without compromising quality.

Parallel or multiplexed LC-MS/MS systems that can analyze the eluent from several LC systems using a single mass spectrometer are one solution to support the high throughput production of quality data.

Waters MUX technology uses indexed LC–MS interfaces from multiple LC systems running in parallel with MS sampling time split between the LC systems [213,214]. The MUX–electrospray interface incorporates either four or eight nebulization-assisted electrospray probe tips arranged radially about the sampling cone. The spray from each tip is successively admitted to the sample cone via a hole in a sampling rotor driven by a programmable stepper motor. The sampling rotor has a supply of heated dry nitrogen to facilitate desolvation of the analyte from the spray selected. An optical encoder on the shaft of the rotating assembly is used to ensure that each mass spectrum is correctly

assigned to the corresponding liquid stream. The inherent duty cycle of this approach, however, limits the MUX system for fast chromatography with multiple components.

The Cohesive Technologies multiplexing system consists of four fully independent HPLC systems fed by two injection syringes on a twin-arm HTS PAL autosampler. Samples are introduced into the atmospheric pressure ionization MS interface via a selection valve. Timing and triggering for injection, gradient start, and data collection are all synchronized by the Aria software. Each system operates independently, permitting multiple methods to run simultaneously, with the MS remaining idle for <4% of the time. With each system operating independently, it is possible to analyze standards, QCs, and study samples on the same hardware. The Aria system uses a modified Twin PAL autosampler from CTC. The two arms and four injectors allow the system to keep up with the required injection speed while maintaining thorough wash cycles for both the syringes and the injectors.

The latest iteration of the system combines the power of on-line extraction with the speed of UHPLC. The Transcend™ system uses Cohesive Technologies' patented TurboFlow® technology with ultrahigh performance capable chromatography systems. The system can alternate between on-line extraction methods and conventional LC methods in a multiplexed manner.

18.10 METABOLITE INTERFERENCES IN QUANTITATIVE ASSAYS

Drugs are xenobiotics to living organisms, which therefore biotransform them to less toxic, less active, and more hydrophilic forms to enhance their excretion. Metabolic pathways are categorized as either phase I or phase II reactions, and both classes of reaction may occur. In phase I reactions, enzymes (e.g., cytochrome P450) modify the parent compound by hydrolysis, oxidation, and/or reduction [215–217]. Phase II reactions conjugate polar groups to the parent or phase I modified compound. Glucuronidation is the main phase II reaction in mammals [218], with other pathways including sulfation, methylation, acetylation, and conjugation with amino acids or glutathione [219,220].

Metabolite identification is central to many of the activities in the discovery and development of drugs. However, quantitative analysis of parent drugs during the early stages of the pipeline often occurs without prior knowledge of the major metabolites of the target analyte. With a requirement for rapid, high throughput bioanalysis, it should not be surprising that a number of methods are developed, wherein the target analyte not only coelutes with endogenous components of biological fluids but may also coelute with biotransformed products of the drug. The presence of unidentified metabolites can lead to inaccurate measurement of the analyte because of ionization suppression, interference in peak integration, or, most problematic, in-source regeneration of the parent drug from phase II metabolites, as the metabolites are not present in the calibration standards and QCs [221]. Common examples of metabolites that undergo in-source regeneration are *N*-oxides [222,223], and both *O*- and *N*-glucuronides [224–227].

The importance of chromatographic separation in quantitative analysis using LC-MS/MS should never be overlooked. Without prior knowledge of the metabolic pathway of a target analyte, it would be best practice to screen a few unknown samples, if available, for metabolites before method validation. The goal of the screening would be to ensure that multiple analytes with similar structures and masses are free of cross

talk [228]. Endogenous isobaric interference from the sample matrix should also be considered [229].

Jemal *et al.* recommended a set of procedures using incurred samples to establish the validity of an LC-MS/MS method for analyzing samples collected from dosed subjects [230]. Specifically, the stability of incurred samples should be investigated before and after sample preparation and SRM (single reaction monitoring) transitions attributable to putative metabolites be scanned using both the same and chromatographically modified LC-MS/MS methods.

18.11 CONCLUSIONS

High throughput bioanalytical methods are essential to support the rapid discovery and development of drugs in the pharmaceutical industry. Nevertheless, the data being produced by these methods needs to be reliable in order to support decision-making processes, that is, the data should be sufficiently accurate and reproducible. Because LC-MS/MS offers high sensitivity and selectivity, rigorous chromatographic resolution and/or tedious sample extraction protocols may not be required; however, appropriate attention still needs to be paid to the quality of the methods to avoid issues with routine sample analysis. A number of sample preparation methodologies have now matured and automation is commonplace. Nevertheless, with chromatographic analysis, times of frequently <3 min, the bottleneck in sample analysis remains with sample preparation.

The importance of sample preparation stems from three major concerns—removing interferences from the biological sample matrices, concentrating analyte(s) of interest, and improving analytical system performance. The choice of sample preparation method should be dependent on the quality of the data required. It makes little sense to invest a month of development time on a method that affords pg/mL sensitivity with accuracy and precision of <15% each for a drug discovery screen that simply requires data that indicates one candidate has better bioavailability than 10 other candidates in a cassette-dosed animal model. On the other hand, it may be important to invest such time to develop and validate a method for a lead drug candidate undergoing human safety assessments when such data is subject to regulatory scrutiny.

The final method should be orthogonal to maximize selectivity and reduce ion suppression. If sample extraction utilized C₁₈ SPE, then the analyst should consider something other than C₁₈ chromatography, for example, a phenyl stationary phase. Alternatively, if strong cation-exchange SPE was selected for sample extraction, any reverse-phase LC method could be utilized. The method should be assessed for recovery, selectivity, precision, accuracy, and ruggedness. Formal validation may yet be required.

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19 High Throughput Quantitative Mass Spectrometry

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19.1	Introduction	1
19.2	Basic considerations for high throughput LC-MS/MS	4
19.3	Automation of sample preparation and batch processing	7
19.4	Sample preparation and pooling	7
19.5	Mass spectrometry method development—tuning and choice of mass analyzer	8
19.6	LC method considerations	11
19.7	Higher throughput serial LC-MS/MS	13
19.8	Multiplexed systems	14
19.9	Staggered parallel dual column with column reconditioning	16
19.10	Multicolumn parallel chromatography—single ionization source	16
19.11	Multiple column parallel chromatography with multiple ESI sources	18
19.12	Staggered parallel chromatography	19
19.13	Informatics	20
19.14	Non-LC-based high throughput mass spectrometry	21
19.15	Laser-based ionization methods	22
19.16	The future promise of ambient ionization methods	23
19.17	Summary	26
	References	27

19.1 INTRODUCTION

Studies from the late 1980s showed that 40% of the attrition of all drug candidates in clinical trials could be attributed to poor pharmacokinetic (PK) properties [1]. Currently, the primary mechanisms for clinical failure are lack of efficacy and toxicological issues while PK-related failures have been reduced to <10% [2]. This dramatic change

can be attributed to many pharmaceutical companies doing more *in vivo* PK studies and also establishing *in vitro* metabolism groups that were charged to develop and carry out a collection of *in vitro* assays to find compounds with appropriate ADME (absorption, distribution, metabolism, and excretion) properties. To respond to this daunting challenge, high throughput analytical techniques were desired to collect the data. As it turned out, quantitative liquid chromatography–mass spectrometry (LC-MS) fulfilled this need. Moreover, innovations in high throughput quantitative LC-MS have been driven by the ever-increasing need for industrialization of the bioanalytical process within the pharmaceutical industry.

The power of hyphenated chromatography/mass spectrometry techniques, that is, gas chromatography–mass spectrometry (GC-MS), was well established for the analysis of drug compounds and metabolites in the 1980s and into the early 1990s [3]. However, the GC-MS application range was limited to ~15% of the known chemical space since the compounds had to be volatile or made volatile with chemical derivatization [4]. Generally, a GC/MS method that required derivatization requires more extensive method development and sample preparation time than a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method [5]. It had long been known that mass spectrometry (MS) had the potential to supplant other liquid chromatography (LC) detection techniques. The coupling of liquid chromatography with ultraviolet detection (LC-UV) has limited detection sensitivity and was further confounded by specificity constraints, requiring longer run times to obtain sufficient chromatographic resolution. Fluorescence and electrochemical detection offer improvements in specificity and sensitivity of compound detection; these techniques were also coupled with drawbacks, such as the need for derivatization, intrinsic molecular properties, but most often, increased analytical cycle times to ensure analytical reproducibility, particularly in the case of electrochemical detection [6].

Before the advent of API (atmospheric pressure ionization), many other interfaces using various ionization sources were developed to couple LC with MS, including direct liquid introduction [7], moving belt [8,9], particle beam [10,11], thermospray [12,13], and continuous-flow fast atom bombardment (FAB) [14,15]. Intrinsic liabilities and robustness limitations precluded these interfaces from being used for high throughput quantitative analyses. For example, excessive flow splitting from an eluent flow rate of >1 mL/min to the low as 1–10 μ L/min inlet flow rate was required for continuous-flow FAB. Particle beam was more suited for environmental analysis of medium polarity compounds but, in general, exhibited limited sensitivity. Thermospray, a precursor and closely related LC-MS interface to the newer electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) technologies, was the most established and utilized technique for small molecule analysis due to its ability to accommodate higher LC flow rates [12]; however, thermospray was not a truly robust technique. Extended operational use resulted in excessive source fouling, blockages in the thermospray probe, and inconvenient manual intervention, which was not a trivial exercise due to the need for operation within a high vacuum. Thermospray interfaces required extensive tuning; exhibited degradation of thermally labile functional moieties, such as acyl glucuronides; confounding mass assignment; and when compared retrospectively to API, exhibited limited structural coverage.

Publications that outlined the utilization and application of LC-MS dramatically increased through the mid-1990s, primarily because of the invention of second-generation commercial APCI [16,17] and ESI sources [18–20], which could

accommodate higher LC flow rates [21]. Their rapid uptake and acceptance is clearly demonstrated through the explosion of abstracts in the MS community at the 1994 American Society Mass Spectrometry Conference. A total of 413 LC-MS presentations (35% of all presentations) were shown, ~81% featured ESI and ~9% featured APCI. These sources provided a robust, straightforward way to couple LC to a mass spectrometer [21].

In contrast to previous LC-MS interfaces, the API interface enabled facile coupling of LC-MS systems, demonstrated improvements in ionization efficiency, and demonstrated rapid interchange between ESI and APCI probes, as the technique operated outside of the high vacuum region of the mass spectrometer. Contamination from the analysis of minimally purified samples, historically responsible for interface fouling and leading to variance and degradation in mass spectrometer ion transmission performance, could be readily rectified, as the interfaces and ion optics were accessible to the operator for cleaning between runs.

Improved robustness resulted from a number of significant evolutions in interface and inlet (orifice) designs. The original interface technologies employed an on-axis ESI, such as the Branford Analytica and Finnigan MAT first-generation interfaces. The inclusion of a heated capillary ion transfer tube resulted in a degree of gas phase ion declustering and, together with differential pumping systems and multipole ion transmission technologies, provided an analytical footprint for acceptance of LC eluent flow rates of 1 mL/min for APCI and split flow of 200 μ L/min for ESI. The development and commercial launch of ESI interfaces incorporating off-axis [22] and, eventually, orthogonal [23] electrospray emitters relative to the mass spectrometer inlet (orifice), continued to dramatically increase the robustness of API interface. Also, instrument evolution of high purity curtain gas ion optics facilitated higher interface gas loads and eluent flow rates to be used [24]. Incremental improvements in differential pumping, partial pressure ion extraction technologies, interface pumping capacity, and orifice cross section resulted in improved instrument sensitivity. Moreover, these improved API interfaces enabled direct coupling of the LC eluent to the ionization source without flow splitting, reducing postcolumn resolution loss (band-broadening) inherent within early split flow API interfaces [25]. Interfaces could accommodate LC eluent flow rates up to 2 mL/min directly via APCI and up to 1.0 mL/min with pneumatically assisted ESI sources using heated N₂ gas (sometimes referred to as *ionspray*) [22]. It should be noted that the ability of API technologies to predominantly form a single, predictable precursor ion for small molecules, based on ionization mode (positive/negative), has enabled MS operation earlier in the drug discovery process. For example, open-access workflows have been developed to provide additional confidence assignment in chemical synthesis [26]. These features of API, leading to the moniker of “soft” ionization properties, have also enabled automated tuning capabilities for additional efficiency gains.

A striking example of the potential improved throughput using API was demonstrated for renin inhibitor quantitation from human serum using an isocratic LC-MS assay with an APCI source [27]. This assay described analyte peaks 3.4 min after injection, conservative numbers by today’s standards, but may be indicating a degree of empirical experimental design that was ahead of its time in terms of resolution of coextracted matrix constituents, together with the chromatographic resolution needed in selected ion monitoring experiments. More importantly, the method was able to detect and quantify down to levels of 50 pg/mL of the drug, demonstrating the capabilities

necessary to compete with the existing electrochemical and fluorescence detection techniques.

Before the advent of API ion sources, it was known that compound selectivity and thus sensitivity (signal-to-noise ratio, S/N) could be enhanced by performing tandem mass spectrometry (MS/MS) [28,29]. The most commonly used MS/MS experiment in quantitative efforts is the selected reaction monitoring (SRM) scan. The experiment is typically conducted on a triple quadrupole mass spectrometer using the following conditions. The first quadrupole (Q1) transmits a precursor ion (based on mass-to-charge ratio, m/z) of the compound of interest, usually a protonated or deprotonated ion, representative of the original analyte. The second quadrupole (Q2, often called the *collision cell*) is filled with neutral collision gas; a collision energy (accelerating voltage) is applied to induce energy transfer through collision and create a series of product ions through collisional activation of the precursor ion. Lastly, the third quadrupole (Q3) is set to transmit a specific, prominent product ion, before signal amplification. The SRM mode of analysis requires the analyte to transit through two distinct stages of mass-to-charge filtering, thereby increasing analyte specificity, while greatly diminishing the background noise. Performing a SRM scan event, together with an orthogonal separation modality, such as an LC separation, provides three distinct filtering mechanisms to increase specificity in measurement, namely, chromatographic retention time, protonated/deprotonated molecular ion m/z ratio, and product ion m/z ratio. The enhancement in selectivity, sensitivity, and operational utility of the modern triple quadrupole mass spectrometer represents one of the most powerful analytical techniques devised to date.

LC-MS/MS soon became the indisputable gold standard technique for quantitative analysis for *in vitro* and *in vivo* biological samples as is evident by the most commonly performed *in vitro* assays (Table 19.1). The increasing capabilities and utility of LC-MS/MS provided an enabling solution to performing *in vitro* assays on all lead compounds produced in early drug discovery. With this in mind, many researchers in the past two decades began to seek ways to improve quantitative LC-MS/MS throughput. This chapter presents the various methodologies used to obtain high throughput quantitative MS. A series of topics describes specific methodologies that have been demonstrated to directly improve the throughput of the quantitative LC-MS/MS experiment. These topics include automation, MS acquisition method setup, optimization of single and multiplexed LC systems and interfaces into the mass spectrometer, and data reduction. An additional topic covers some new and emerging non-LC-based quantitative MS techniques that show the promise of improving throughput. Although this chapter focuses on *in vitro* assays, many of the techniques described can and have been applied to *in vivo* sample analysis.

19.2 BASIC CONSIDERATIONS FOR HIGH THROUGHPUT LC-MS/MS

To obtain the full advantage of high throughput MS, the entire experimental process requires optimization and understanding of the timelines for each component in the overall process. A question of key consideration is “what is high throughput?” Frankly, the definition of high throughput is somewhat arbitrary; however, the time frames for independent components in the process are germane to defining an approximation to the answer. Perhaps the more telling parameter for high throughput is the cycle time

TABLE 19.1 *In Vitro* Metabolism Assays

Test	Technique(s)	Analyte Monitored	Sample Matrix	Throughput Bottleneck/Comments
CYP inhibition (drug–drug interactions)	LC/MS/MS	Specific probe molecules	Liver microsomes	Number of compounds tested; number of enzymes to be tested; LC run time
	Fluorescence	Fluorogenic probes	Recombinant proteins/buffers	None: relevancy of recombinant protein used, interference from fluorescent compounds
	Luminescence	Luminescent compounds	Recombinant proteins/buffers	Limited types of CYP isoforms
CYP 3A4 induction	Luminescence	PXRE-luciferase	Cells	Highly tuned specific assay
CYP isozyme profiling	LC/MS/MS	Drug and individual metabolites	Recombinant proteins or liver microsomes	Tuning of the mass spectrometer for individual compounds
Caco-2/efflux transport	LC/MS/MS	Drug compound	Buffers	Tuning of the mass spectrometer for individual compounds
PAMPA	96-well plate with UV detection	Drug compound	Buffer	Multiplexed detection, cost-effective—useful in early discovery phase
Metabolic stability	LC/MS/MS	Drug compound	Extract from hepatocytes	Tuning of the mass spectrometer for individual compounds

Abbreviations: PXRE, progesterone X receptor element; PAMPA, parallel artificial membrane permeability assay

for each event. Sample preparation includes off-line or on-line extraction/filtration techniques to reduce sample complexity and novel methodologies to pool samples. Sample preparation has evolved to become generic with/without zwitterionic analog or stable labeled internal standards. Incorporating 96-well plating automation has realized 10 s per sample [30] for preparation schemes, additional parallelization into 384-well plates and above has further reduced these steps to the 2- to 5-s time frame. This at least gives the process a throughput goal for optimization. Sample introduction into the LC-MS system, discounting the influence of carryover, has been realized in the 5- to 10-s time frame, although typically occurs in the 45- to 90-s cycle time frame in which carryover control is desirable, utilizing generic autosampler wash protocols [31–34]. Chromatographic peak widths in the subsecond regime are now readily achievable, utilizing gradient/recycle single-plex LC-MS systems with cycle times of 60 s down to <10 s [35]. Introduction of multiplexing LC systems facilitates eight samples in <60 s, together with incorporation of multianalyte per sample analysis, effectively realizes 1–2 s per analyte in targeted SRM mode and 1–2 s for full scan data [36]. Interfaces can now provide stable response in the 10- to 100-ms time regime; as such, these are now no longer not truly additive to the cycle time. As alluded to above, MS acquisition time frames are in the 1- to 2-s to subsecond regime as a function of the scan events required. It should be noted that the time frames to open and close files has been evaluated as a time-critical variable, and the 1–3 s required for this event have been eradicated from some processes in which a single file is acquired and deconvoluted postanalytically [37]. Lastly, data reduction and posting of data has been somewhat lagging. Functionally, 10 s per sample in review is an appropriate time frame for current workflows. This leaves us with a somewhat realistic argument to the proposed question; it appears that high throughput is 1–2 s per sample, irrespective of the number of independent analytes being assayed.

To achieve these throughput requirements, generic process systems are required. Method development is a key component in targeted assay measurement; however, in high throughput quantitative analysis of multiple chemically diverse compounds, process development is paramount. For quantitative LC-MS/MS, the goal is to produce a separation that resolves the analyte from background components. Unlike LC with a UV detector, the compound can coelute with many compounds because the tandem mass spectrometer will filter out the analyte precursor and product ions away from interfering species via mass discrimination. Generic LC separation, wherein desalting is functionally embedded in the separation, is the common and often only requirement for the LC separation. MS method development includes finding the appropriate precursor and product ion pair transitions for the analyte and maximizing the signal response by adjusting the collision cell pressure and voltage. Data reduction method development includes integration of the chromatographic peaks, assigning calibrators, quality controls, expected concentrations, and acceptability criteria, before posting data to a corporate database.

The key bottleneck for an assay depends on the type of analysis that is being performed (Table 19.1). For example, metabolic stability assays in which 96-well plates filled with unique compounds are analyzed, the critical bottleneck is optimizing the response of the mass spectrometer, that is, selecting the appropriate precursor and product ions and collision conditions for the SRM experiment, with saturation of response mitigated. In contrast, for a P450 inhibition assay in which the same analyte(s) are detected for each sample, the most important throughput constraint is sample analysis

time, predominantly linked to the time of the LC method, requiring development of a short, robust, and generically applicable LC method.

19.3 AUTOMATION OF SAMPLE PREPARATION AND BATCH PROCESSING

Automation of processes is an integral component of the high throughput quantitative ADME workflow, intrinsically linked to the type of *in vitro* assays and projected sample volumes. Detailed process review is necessary to assist infrastructure planning, such as sample tracking, software customization, and amount and types of automated sample preparation equipment, personnel (skill sets and number), and LC-MS/MS systems. Automation of processes requires significant investment capital and long developmental lead times. Ideally, the throughput of sample incubation/preparation should exceed the LC/MS/MS analytical throughput. As the analysis time is reduced, preanalytical systems will be required to provide increased numbers of samples to optimize the usage of the analytical equipment.

In a recent review of automation for *in vitro* analytical laboratories by Saunders [38], key examples of equipment used to automate sample incubation and preparation using sample-plate-based robotic systems were described. The review detailed the extent of automation required for sample volume and types of analyses performed. The type and extent of automation can range from very little (i.e., an autosampler in the LC-MS/MS system), which is well suited for a small start-up company or a university laboratory, to a fully automated system for a larger pharmaceutical or contract service laboratory. Fully automated systems control sample incubations, sample preparation, tuning of the mass spectrometer, automatic transfer of samples to the mass spectrometer, data acquisition, and data processing. These systems are custom built by the company and require extensive internal resources to establish communication and control all the diverse components within the analytical workflow. Examples of fully automated systems have been reported by Janiszewski *et al.* [39] and, more recently, by Laycock [40]. The majority of laboratories fall short of these modular and fully implemented automation solutions. In a typical laboratory, a multiuse multitip liquid handling platform is tasked with dispensing samples, performing off-line extractions, reaction quenching, and dispensing into new plates for subsequent LC-MS/MS analysis. As volume and sample needs increase, these “islands” of automation are readily scaled to match throughput requirements.

19.4 SAMPLE PREPARATION AND POOLING

Numerous and varied ways to perform sample preparation have been described in this volume and are detailed elsewhere (see chapter titled *Direct Biofluid Analysis Systems*) [41]. It cannot be overstated that sample preparation is crucial for the reliability and robustness of the assay using the current LC-MS/MS workflows. The clean-up process not only removes interfering analytes but also enables the filtration of particulates (i.e., microsomes) and reduction of proteins that eventually diminish the performance of the LC separation. Just as the instrumental parts of the analysis need to be optimized for high throughput, so does sample preparation. Off-line procedures such as solid-phase

extraction, sample dilution, and protein precipitation can be multiplexed and allow for plates to be transferred directly for LC-MS/MS analysis [42]. On-line sample clean-up methods have been demonstrated using restricted access media columns and turbulent flow columns and are summarized by Xu *et al.* [41].

Sample pooling is an effective strategy that is employed to improve throughput by reducing the number of analyses conducted. Multiple analytes can be combined [43] and run simultaneously through the biological system (biological cassetting). Smalley *et al.* [44] successfully demonstrated Caco-2 permeability analysis using a biological cassette of 10 samples. The method used on-line solid phase extraction (SPE) LC-MS/MS and achieved a standard curve range of 10–2500 nM with a run time of 3.5 min. Sample pooling involving post dosing sample combination (analytical cassetting) may also be performed after the biological assay is completed [45]. This strategy is generally preferred to eliminate any questions that arise from competition effects (i.e., inhibition) of compounds when presented simultaneously to the enzyme in question. Compounds that can be pooled together typically have different retention times, to minimize the chance of ion suppression effects resulting from coelution with other compounds within the pool.

Another potential approach to reduce the number of analyses, not widely deployed for ADME analyses, is the use of subsetting compounds based on chemical properties. Using *in silico* methods, specified compounds with diverse chemical motifs are selected for potency screening from a limited number of well plates. Statistical models are used to predict compounds that have similar chemical properties to the training set hits, to “cherry pick” from the compound collection for additional screening [46]. The authors noted that the results from the subsetting approach compared favorably with data obtained from a traditional screening approach and led to a reduced sample analysis burden of ~75%.

Another pooling approach is to determine the effect the compound has on multiple enzymes in a single analysis using a cocktail of probe substrates. This workflow was demonstrated by Kim *et al.* [47] for the analysis of inhibitory potential of nine CYPs by measuring the metabolized products formed from microsome incubations containing six- and three-probe compounds, respectively. The incubations were pooled together before analysis. The LC/MS/MS run time was 6.5 min for each pooled sample. The methodology provided only a modest improvement in analytical throughput because of the slow LC analysis conditions when compared to what can be achieved using the RapidFire™ (see below). However, this assay did effectively increase the coverage of CYP enzymes studied and somewhat minimized material usage.

19.5 MASS SPECTROMETRY METHOD DEVELOPMENT—TUNING AND CHOICE OF MASS ANALYZER

For experiments that perform a single or a limited number of samples for each analyte (i.e., metabolic stability, Caco-2), mass spectrometer method development becomes a critical factor in throughput enhancement. Practically, sample is infused or loop injected into the mass spectrometer for selection of precursor and product ions to be monitored on a triple quadrupole instrument. Additionally, interface gas flows, polarity, and ion optics setting are tuned to optimize transmission of the precursor ion. Subsequently, the collision cell accelerating voltage is adjusted to identify the most prominent product ion, before fine-tuning of collision cell conditions to optimize

the response of the SRM transition. Computer-driven programs have been developed to automatically perform this task [37,48–52]. These programs perform a series of multiple loop injection experiments to optimize these previously described variables and can significantly reduce the development time requirements. Success with these technologies does come at a price. When loop injection is performed, the signal profile changes quickly (i.e., peaks can become as narrow as or narrower than a LC peak), thus the analyte signal is changing very quickly as a function of time. This can result in limited time for algorithms to find optimal settings for each parameter. Reduction in the flow rate to allow increased residence time of the flow injected analyte bolus is often used to extend the amount of time a steady signal is delivered to the instrument. This process overcomes constraints within the selection of SRM transitions; however, tuning of the ion source parameters may be compromised since optimization of some of ion source voltages can be dependent on eluent flow rate. Ideally, obtaining a steady flow of sample at the same flow rate used for the chromatography method is required. Conversely, performing these procedures manually consumes a significant amount of time (4–10 min per sample); thus, a 96-well plate with eight discrete analytes would require 32–80 min to complete tuning of the instrument. Moreover, choosing the appropriate analyte(s) serving as tuning compound is critical since carryover issues can occur, which for sticky compounds could require extensive time (hours) to flush the compound out of the instrumentation setup. In many cases, instrument tuning time is greater than the overall analytical time frame for quantitative LC-MS/MS analysis of the samples themselves.

Alternate approaches to relieve this bottleneck have been described by Kieltyka *et al.* [53] utilizing an autosampler to infuse samples into the MS system. Besides delivering sample to the source, the system using QuickQuan 2.0 was able to tune the mass spectrometer at a rate of 2.5 min per analyte. For a plate consisting of 48 discrete compounds, tuning was completed within just <2 h. These investigators reported that using this tuning procedure and a dual chromatography system for analysis, they were able to obtain a throughput of 1300 compound assays with 500 discrete compounds per instrument per month with a 95% success rate. Figure 19.1 summarizes the informatics workflow for this automated tuning process.

In larger laboratories with common vendor-supplied triple quadrupole mass spectrometers, databases have been developed to store and recall analyte tuning parameter files, reducing the time requirements for subsequent method development for the same analyte [40]. The realization of this protocol owes much to the reproducibility of MS systems manufacturing. For successful implementation, instruments using shared tuning parameters must meet a specific performance requirement, using a standard compound analyzed using fixed ionization source and collision cell conditions, to ensure that each instrument is performing optimally.

A strategy to avoid optimizing the SRM transition is to use another type of mass analyzer. For example, a single quadrupole in full scan [54] or single ion monitoring mode [55] has been utilized for quantitative ADME experiments. As discussed previously, this leads to compromised selectivity, decreased sensitivity, and concordantly, increased sample consumption. Ion trap mass spectrometers have also been utilized, whereby the ion trap is operated in product ion MS/MS mode. Quantitation is established by extracting the analyte-specific product ion(s) produced from a given precursor ion and is performed during postacquisition analysis from the total ion chromatogram. Typically, the mass window for these mass chromatograms is approximately ± 0.5 Da

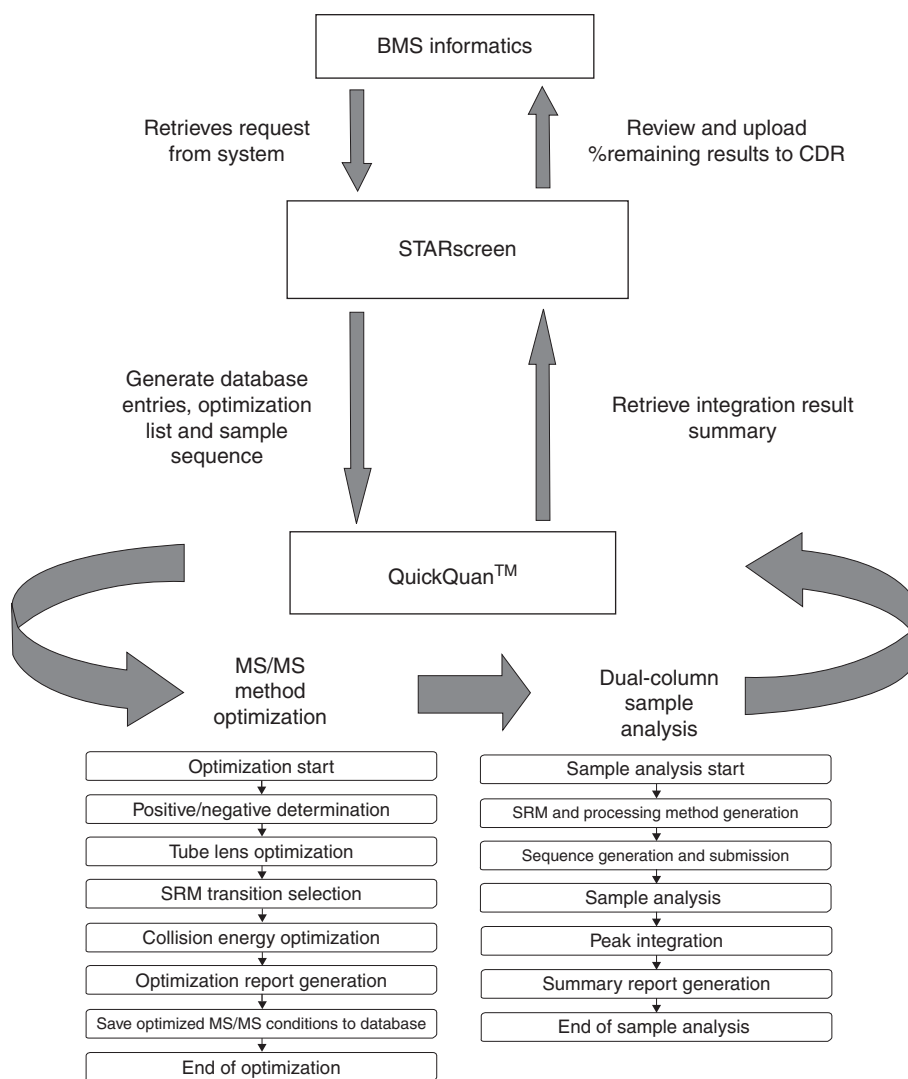


Figure 19.1 The integrated informatics workflow employed by Kieltyka *et al.* [53]. A customized software tool, STARscreen, provides corporate gate-keeping function and tasking lists for QuickQuan 2.0, including all relevant import list details and final data release/upload. The automated analyte MS optimization process is executed via QuickQuan 2.0 using infusion syringe driven optimization, allowing appropriate time to generate high quality MS acquisition methods. Batch import and process method assignment is controlled automatically through QuickQuan 2.0, including user-specified generic methods. Staggered dual-column analysis is executed for accelerated throughput, of particular note in the process; the import list generated by STARscreen incorporates the analytical staggering process automatically, using alternating samples from two discreet plates. Manual data review and data release processes follow initial autointegration.

wide, a function of the unit mass operation of the ion traps used. A key efficiency advantage to this approach is the use of “universal” collision conditions, circumventing tuning for specific SRM transitions [56]. Typically, this platform is 10 times less sensitive than the current triple quadrupole MS-based platform [53].

Another application involves the use of an orthogonal acceleration time-of-flight (TOF) mass spectrometer [57,58]. The TOF detects all ions within a given mass range (i.e., it cannot be fixed to monitor for a specific mass) and can be more selective than the quadrupole based on its higher mass resolution capability. Moreover, current TOF mass analyzers have a very fast duty cycle—20 Hz or faster, which improves its detection capabilities for sampling subsecond wide chromatographic peaks that elute in an ultrahigh performance liquid chromatography (UHPLC) separation. Initial experiments performed with a TOF with a resolving power of 5000 showed ~5–25 times less sensitivity when compared to the triple quadrupole together with limited dynamic range [57]. Using narrow mass width extracted ion chromatograms of 0.1 Da for quantitation, linearity and sensitivity (signal-to-noise ratio) approached that produced by a triple quadrupole system. However, at this resolving power, chemical noise is not always fully resolved from the analyte, so higher resolving power would be of further utility. There is growing interest in revisiting the TOF experiment now that TOF/Q-TOF (quadrupole–time-of-flight) instruments with resolving powers exceeding 20,000 and better sensitivity are commercially available. These technologies permit the use of even narrower mass width ion-extraction chromatograms (0.01–0.005 Da), to produce an extracted ion mass chromatogram for the molecular ion with minimal contributions from chemical noise. Since data are acquired over a wide mass range without *a priori* mass spectrometer tuning, acquired data can be processed to find masses of expected analytes and metabolites, especially when accurate mass measurements are performed [59]. This approach has been termed as *quantitative/qualitative (Quan/Qual) workflow* [60] and has been applied routinely for metabolic stability assays. Moreover, when a Q-TOF instrument is utilized, data-dependent MS/MS experiments can be triggered to provide additional structural information [58]. Even higher resolving powers 100,000 full width at half maximum (FWHM) in a 1-s scan cycle can be achieved using the Exactive™, a benchtop orbitrap mass spectrometer [61]. Reports have shown this system supporting metabolic Quan/Qual stability experiments [60].

19.6 LC METHOD CONSIDERATIONS

The orthogonal nature of LC-MS/MS and relative differences in cycle times between these techniques has realized the greatest diversity and efforts in enhancement of throughput. The mass spectrometer, when set in SRM mode, is capable of detecting analyte response every 10–100 ms, whereas the LC system typically requires minutes to deliver the sample to the mass spectrometer. Thus, sample analysis throughput improvements are primarily dependent on optimized sample delivery to the mass spectrometer. This can be accomplished by reducing LC separation time or multiplexing (multiple LC separations to a single MS system) to align the throughput scales of the systems. The main function of the LC separation in a SRM experiment is to separate analyte from species that cause ion suppression, such as nonvolatile salts, proteins, or phospholipids.

In early experiments, isocratic high performance liquid chromatography (HPLC) methods were common and achieved run times of <3 min per injection [6,27]. However, isocratic methods require redevelopment of the LC method conditions each time a different analyte is selected for analysis. Moreover, isocratic methods suffer from column contamination from retained lipids and proteins.

A more practical approach involves the inclusion of a binary gradient HPLC method, the goal being to provide appropriate LC separation quality across the majority of chemical space, without individual analyte reoptimization, minimizing ion suppression, and column contamination. An important consideration to achieve high throughput LC separations is reduction of system volume for gradient mixing experiments. System volume is composed of a delay volume in pumping systems, solvent mixing, extra-column tubing, LC column volume (at least 60% of the LC column volume unpacked), and postcolumn interface volume. Pump dead volume results in a gradient delivery delay to the column and also lengthens column reequilibration time. Manufacturers have reduced extra-column volume significantly with the development of UHPLC systems. Improvements in column packing materials have resulted in reduced variability from residual silanol activity. Better reproducibility of silica particle cross section has resulted in a reduction in tortuosity, the A term in the van Deemter equation. Manufacturing processes have also improved column packing reproducibility. Finally, gradient separations result in chromatographic peaks with reduced peak tailing, leading to improved detection limits.

A generic gradient LC separation will include a 3- to 5- μm , C18-functionalized silica particle packed into a column of dimension 2.1 mm in diameter by 30–50 mm in length. Flow rates are typically delivered in the 0.5–1.0 mL/min range. Generic reverse-phase linear gradient separations start at a low organic concentration, typically, acetonitrile at 0–5%, 95–100% aqueous 1 mM ammonium formate, and 0.1% formic acid, and elute at a sufficient organic concentration to remove proteins and some lipids (95–100% acetonitrile). The solvent flow rate and slope (gradient time), along with the stationary phase of the column, are principle drivers in determining the speed of the LC-MS/MS analysis and the chromatographic resolution achieved. Generic LC conditions become more tailored and refined if insufficient compound detection occurs or if the compound will be continuously analyzed for many studies. During process refinement, different variations of C18 columns or stationary phases (i.e., C8, cyano, phenylhexyl, etc.) may be investigated to obtain increased throughput and improved selectivity.

Recent advances in LC column technologies have resulted in further choices for high throughput LC-MS. Good results have been demonstrated with the use of monolithic columns [62,63], where the intrinsic porosity ($\sim 15\%$ > standard columns) provides for separations at significantly higher flow rates (up to 5 mL/min). With preinterface solvent flow splitting, chromatographic separation of bupropion and its metabolites was achieved in 23 s [64]. Similarly, fused-core columns have become available that provide higher resolution separations than conventional spherical semiporous 3- μm particles, with reduced separation timescales. Fused-core LC columns enable higher flow rates (>1 mL/min) without producing a pressure drop that exceeds the 400-b pressure limits of standard HPLC systems. These benefits do come at a price. Gradient reconditioning before the next assay is required, which is essentially dead time in an assay. Alterations in the workflow, such as multiplexed LC analysis (see below), are necessary for realization of the true chromatographic efficiency gains.

The evolution of UHPLC has provided an alternate methodology to accelerate LC cycle time [65]. With UHPLC, better chromatographic separations can be achieved in a shorter timescale than that with conventional HPLC columns, since columns with smaller (sub-2 μm) particle size particles realize improved theoretical plate heights at the same linear velocity, when compared to columns with 3- to 5- μm particle sized particles. Moreover, UHPLC pumping systems have been designed with reduced system volume ($\sim 60\ \mu\text{L}$) and higher operating pressure regimens, up to 1800 b. Cycle time reductions of two- to threefold have been demonstrated by multiple laboratories [66–68], with a concomitant increase in sensitivity through chromatographic peak compression.

Sample throughput is impaired by autosampler cycle time constraints. For a highly optimized LC method ($<60\ \text{s}$), a significant portion of the injection-to-injection cycle time is invested in a number of autosampler tasks. Typically, the autosampler receives a signal from either the mass spectrometer or the data system that the previous analysis is complete and starts a cycle. The autosampler aspirates the next sample in the queue, transports the sample or an injection needle to enable injection, and injects the sample. Following injection, the autosampler is tasked with preparing the system for the subsequent injection (i.e., washing the sampling valve). As LC throughput increases, the cycle time of the autosampler becomes the dominant factor in efficiency enhancement as preinjection delays of up to 20 s can occur. Technologies such as “inject ahead” have been developed to reduce this problem [69]. Inject ahead enables the autosampler to wash the injection device and inject the next sample during the analysis of the previous sample.

19.7 HIGHER THROUGHPUT SERIAL LC-MS/MS

Solid-phase extraction provides less chromatographic resolution than that of LC; however, sufficient resolution can be obtained to desalt the analyte and reduce the levels of proteins, salts, and detergents that can suppress the analyte response in a rapid timescale MS analysis. Inman *et al.* [70] described an off-line SPE method that enabled 24 s per sample MS analysis times for metabolic stability samples. The method used SPE cards in a 96-well plate format. Samples were processed using a SPEExpress system, composed of a Harvex liquid handler with an array of 96 needles, and transferred into the SPE elution zones on the card-containing Empore C18 media. After that, the analytes were washed with aqueous mobile phase to remove buffer salts. The sample plates were transferred into the Elutrix unit and eluted from the SPE resin into the mass spectrometer with a C18 guard column coupled in between to enhance peak shape. The Elutrix unit had the capacity to hold 35×96 -well plates for unattended analyses. Good correlation between traditional LC-MS/MS and SPE-MS (solid-phase extraction–mass spectrometry) were observed ($R^2 > 0.92$) for *in vitro* clearance samples from human and dog microsomes. This chapter described the impact of limited chromatographic resolution and reduced recoveries for highly polar analytes, which affected the results obtained by the SPE method.

An innovative on-line SPE instrument, RapidFire, has been introduced by Biocius (formerly BioTrove). Originally designed as system for high throughput biochemical screening [71,72], the system has recently been applied to *in vitro* screening of CYP inhibition [73], Caco-2 (Özbal 2009, (Fig. 19.2) Personal communication), and

metabolic stability [74] assays. The RapidFire system utilizes three isocratic pumps to deliver a low organic strength mobile phase and two eluents with high organic strength mobile phases delivered at flow rates of 1–1.5 mL/min. Columns similar to those described by Janiszewski *et al.* [51] but with smaller bed volumes are utilized. The system consists of four valves (three high speed–low dead volume switching valves and a four-port valve), an injection needle that utilizes vacuum aspiration of the sample to an injection loop, a set of wash stations, a three-dimensional translatable well plate stage, and a well plate stacker. An injection cycle consists of three distinct steps: load, desalt, and analysis. The valve timings are set so that a single sample can be analyzed every 6–10 s. Recent CYP450 inhibition assay studies screened for inhibition of 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4 isoforms [73]. Good correlation was obtained for data acquired on pooled samples analyzed by RapidFire, MS/MS and a 2-min LC-MS/MS method. Lim *et al.* [73] reported that a 96-well plate was analyzed in 15 min by the RapidFire method, while the LC-MS/MS method required 4 h. A drawback of the reported data was significant carryover, between 1% and 4.1%.

Multiple column functionalities are available for highly polar compound extraction (hydrophobic interaction liquid chromatography (HILIC) extraction media), overcoming a number of the analytical constraints observed by Inman. When coupled with quadrupole TOF system, it is plausible to obtain both quantitative and qualitative information on consecutive injections of the same sample through postacquisition data mining.

19.8 MULTIPLEXED SYSTEMS

Currently, the most efficient LC-MS systems have maximized MS data collection rate and minimized the time spent detecting noise. These designs have moved away from the serially delivered single LC-MS/MS platform, requiring sacrifices in LC resolving power, while retaining the inherent lag time of LC reconditioning and autosampler processes. When a single analyte is being analyzed in a very fast UHPLC run, the peak width is approximately one-thirtieth (2 s wide/60 s gradient) the time of the separation. Many techniques have been devised to increase the number of analyte signals detected during a single MS acquisition. Multiplexing the number of chromatography columns attached to a single mass spectrometer is a commonly used option. There are three major families of multiplexed experiments: staggered parallel dual-column analysis with off-line column regeneration, true parallel chromatographic separations (MUX), and staggered parallel.

Figure 19.2 (a) An example of data generated using the RapidFire system for metabolic liability analysis, as describe by Luippold *et al.* [74]. Single data files are acquired, following similar preanalytical principles as described in Fig. 19.1, with 8 s per sample acquisition rates. Analyte (top) and internal standard (bottom) are automatically integrated using QuicCalc, area responses are displayed in a tabular format on for rapid data review and release. Of particular note, the process assays the latest time points first (likely the lowest concentration) to minimize errors associated with carryover effects in quantitation. (b) Studies comparing the RIAS (rapid and integrated analysis system, utilizing the RapidFire) and a validated LC-MS/MS assay (10-fold lower throughput). Reference compounds were assayed in duplicate across three separate days using both processes; calculated half-lives ($t_{1/2}$) between platforms indicate excellent correlation. (See color insert.)



19.9 STAGGERED PARALLEL DUAL COLUMN WITH COLUMN RECONDITIONING

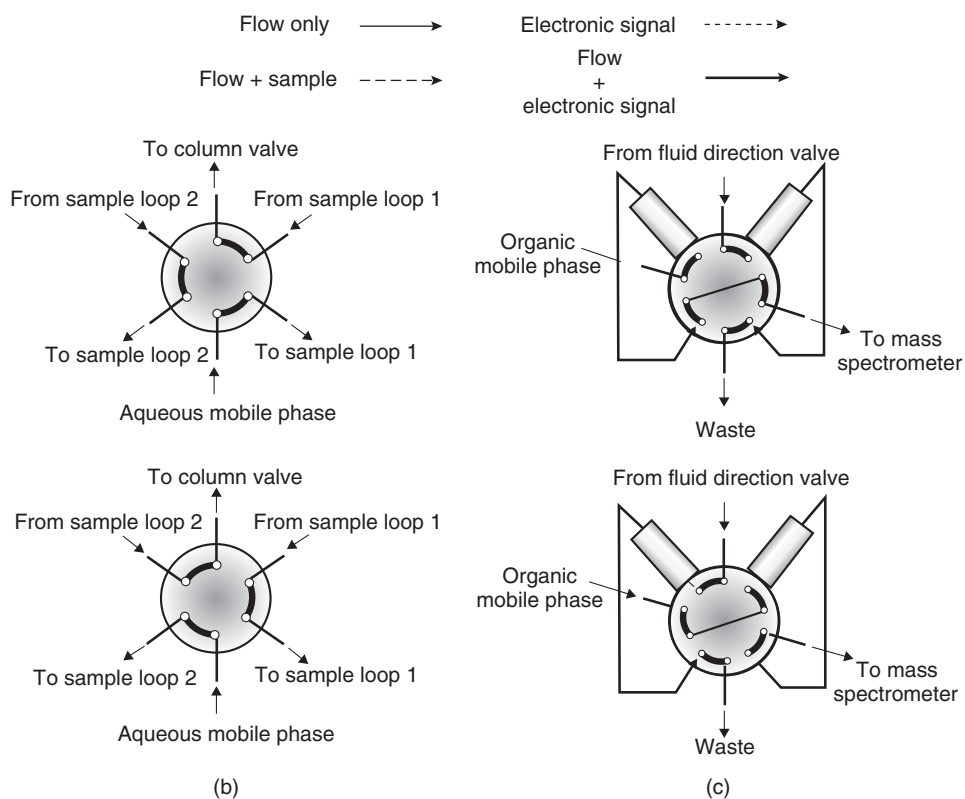
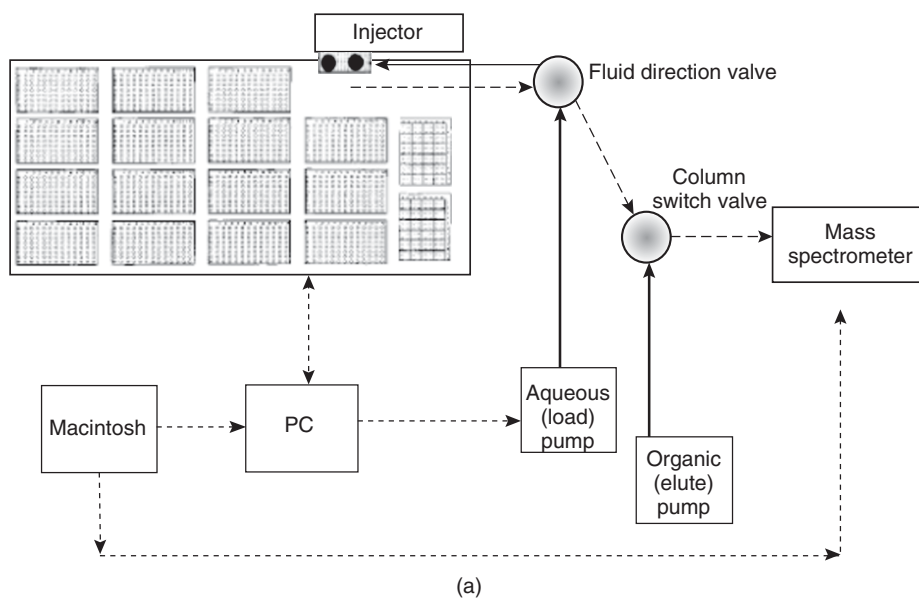
A simple method to overcome the gradient reconditioning time involves a dual-column analytical separation with a secondary pump for off-line column reconditioning. The analytical setup is composed of two 6-port valves, a gradient mixing LC pump, and an isocratic or gradient conditioning pump. This setup enables parallel processing of LC columns to be effected. When an analytical separation is being delivered to the mass spectrometer from the gradient LC pump, the second column undergoes reconditioning with the starting conditions for the gradient separation, isocratically delivered by a secondary LC pump. Subsequently, valve switching occurs to index the reconditioned column in-line to the autosampler and detector for the next assay while reconditioning the previously used LC column. Enhancements such as inject ahead autosampler operation may be employed to further improve throughput. The dual column configuration has been successfully deployed to support a variety of *in vitro* assays.

An example of this analytical setup was described by Janiszewsk *et al.* [51], using an OptiLynx 1 mm $\times$ 15 mm, 40- μ m pellicular pore C18 columns. The system consisted of a dual-arm autosampler with a dual chromatography system to provide column cycling and peak stacking. Two isocratic pumps delivered low and high organic strength mobile phase to the columns, which were set on opposing arms of a 10-port switching valve. The system timing delivered high organic strength mobile phase 18 s after injection onto column 1 to elute the analytes to the mass spectrometer. At 24 s after injection, aqueous flow was diverted through the autosampler and the second sample was injected onto the second column. During this time, the first injection needle was washed before subsequent injections and the process was repeated. The system was utilized for a variety of ADME studies including metabolic stability and Caco-2 assays and was reported to have a capacity to run over 2000 samples per instrument per day (Fig. 19.3).

19.10 MULTICOLUMN PARALLEL CHROMATOGRAPHY—SINGLE IONIZATION SOURCE

Xu *et al.* [55] demonstrated the utility of an eight-way “true” parallel LC-MS system for the analysis of metabolic stability samples. The system consisted of an LC pump, Gilson 215 eight-tip autosampler, an AB/MDS-Sciex API 165 mass spectrometer, two Valco valve manifolds, eight 10 mm $\times$ 1 mm i.d., 3- μ m particle sized HQC18 PEEK microbore columns, and a Valco switching valve to divert the solvent front to waste.

Figure 19.3 (a) The dual-column high capacity LC-MS configuration, as described by Janiszewski *et al.* [51]. The system schematic indicates the positioning of custom dual injection system and the 17-plate deck of the Gilson 215 multiprobe autosampler, together with in-line selector valves for dual-column/dual-pump operation, all operated under computer and pump signal control. (b) The specific valving configurations for the aqueous solvent flow (sample collection from the dual injection loops, LC column reconditioning, and gradient starting conditions). (c) The valve orientation to provide sample loading and subsequent elution to the mass spectrometer for dual-column analysis.



Using an accelerated (ballistic) gradient separation, of ~ 0.8 min per cycle, the system was capable of running 240 samples per hour. To enable analyte focusing during injection, sample preparation utilized trichloroacetic acid instead of acetonitrile. The group reported that on average, 65–70% of samples were successfully analyzed by the system. Three major reasons for failure included poor compound solubility, weak ion signal, or experimental pipetting/injection errors.

19.11 MULTIPLE COLUMN PARALLEL CHROMATOGRAPHY WITH MULTIPLE ESI SOURCES

A variety of multiple electrospray systems have been studied for many different MS applications [75–78] and were later investigated for the use in quantitative efforts [79–81]. Multiple spray sources can be termed as *nonindexed* or *indexed spray sources*. For nonindexed spray sources, spray is continually sampled by the mass spectrometer from all sources. Indexed sources enable only one channel to be sampled by the mass spectrometer at a time. For quantitative efforts, nonisobaric species must be analyzed from each channel (e.g., mass spectrometrically distinct compounds) when a nonindexed source is utilized.

A simple dual-spray system was described by Hiller *et al.* [81] using a Sciex 3000 triple quadrupole MS system. A Turbospray source with two electrospray and heater units was developed. The dual sprayers were coupled to two separate LC systems each of which had column regeneration capabilities. The autosamplers were synchronized to simultaneously inject sample so that data could be recorded in a single data file to generate a cycle time per compound of 30 s. Utilization of the dual-probe approach generated a twofold improvement in throughput with data quality that was deemed adequate by the authors for discovery-based quantitative applications.

The most widely used multiple sprayer source was the multiplexed electrospray interface, MUX, introduced by Waters in 1999 [75]. The device consists of API interface that composed of either four or eight sprayers. A rotating valve with a machined slot is located between the fixed electrospray probe tips and the skimmer cone of the instrument. The rotating valve serially selects an electrospray probe to sample for a finite period (50–200 ms), while excluding the majority of eluent sampling from the other electrospray emitters. The rotating valve indexes to the next sprayer (50 ms) and so forth to realize a sampling duty cycle of 400–1000 ms for a four-channel system and 800–2000 ms for an eight-channel system. The rotating valve was designed to reduce the gas load on the vacuum system at the interface and eliminate any ion suppression effects caused by the other sprayers. Independent LC columns were connected to each sprayer tip and solvent delivery was provided by either a single LC pump, split across all columns and sprayers, or column-/sprayer-independent LC pumps. Data for each column were encoded into individual channels and into a single data file. With such a system, the same or different analytes could be analyzed on all channels.

Morrison *et al.* [82] and others have demonstrated that the system does suffer from reduced sensitivity related to the duty cycle constraints. Lower flow rates or flow splitting was required for optimal performance to further reduce the load of liquid introduced to the API region. Cross talk between spray channels have also been a body of concern with this method, although selection of the appropriate assay type and

sampling strategy negated this bias [82], as was demonstrated by Fung *et al.* [49] in utilizing the MUX interface to multiplex their Caco-2 permeability assay.

The dual-channel MUX interface is more commonly implemented now and has been successfully employed on TOF and Q-TOF instruments to enable the introduction of reference masses to obtain accurate mass measurements [77,78]. Utilization of a lock mass enables on-the-fly correction of the mass-scale and improves mass axis stability. When coupled with higher resolution measurements, improved mass axis stability enables the use of narrower mass widths (i.e., increased specificity) when extracting the signal for an analyte from the mass chromatogram, leading to improved sensitivity and specificity.

An alternative dual- or four-spray-indexed interface was described by Schneider *et al.* [80] In this interface, an electrospray probe was paired with an ion lens located near the tip of the probe. A voltage was applied to synchronously enable or disable the spray from reaching the entrance lens to the mass spectrometer. The signal emitted from a probe could be fully turned-off or -on rapidly, within 50 ms of the change in the lens voltage.

In terms of throughput, multiple sprayers increase throughput as a function of the number of sprayers introduced into the system. After their initial introduction, these systems have lost favor because of the reduction in sensitivity and increased complexity in terms of MS hardware when compared to other multiplexed systems.

19.12 STAGGERED PARALLEL CHROMATOGRAPHY

The use of staggered parallel technologies has ameliorated a number of the issues associated with the parallel systems described above. The concept of staggered parallel chromatography is shown schematically in Fig 19.4. Sample is injected onto column 1, using an independent LC pump, and most often, acquired to an independent data file. After a fixed time (Δt_{inj}), another sample is injected onto column 2. The same steps are repeated for columns 3 and 4 in a four-channel multiplexing system. Before elution of analytes from column 1, eluent streams (analytes) are delivered into the mass spectrometer by means of a selector valve. Following a predefined acquisition window, column 1 eluent is diverted to waste and column 2 is selected for elution to the mass spectrometer. The same steps are repeated for columns 3 and 4, and the cycle repeats again. The advantage of such a system is that it enables multiple columns to be coupled to a mass spectrometer fitted with a conventional ESI/APCI source for class-specific analysis. The staggered parallel strategy maximizes the time spent by the mass spectrometer detecting analytes, which elute in 3- to 10-s peak widths and minimizes the time spent detecting background and chemical noise. With a minimal increase in run time compared to a single LC system, over a threefold improvement in sample throughput is typically achieved.

The concept was originally described by Van Pelt *et al.* [83] using a single LC with pressure regulation to control the flow to the individual columns and calibrated loops to delay to the delivery of mobile phase to the columns. The concept was refined by using three [84] or four [85] columns with multiple LC pumps and a multiport autosampler for *in vitro* Caco-2 screens. With the four-column system, it was reported that 600 samples could be analyzed during an overnight period (~16 h). These technologies have now been commercialized into a dual-CTC arm,

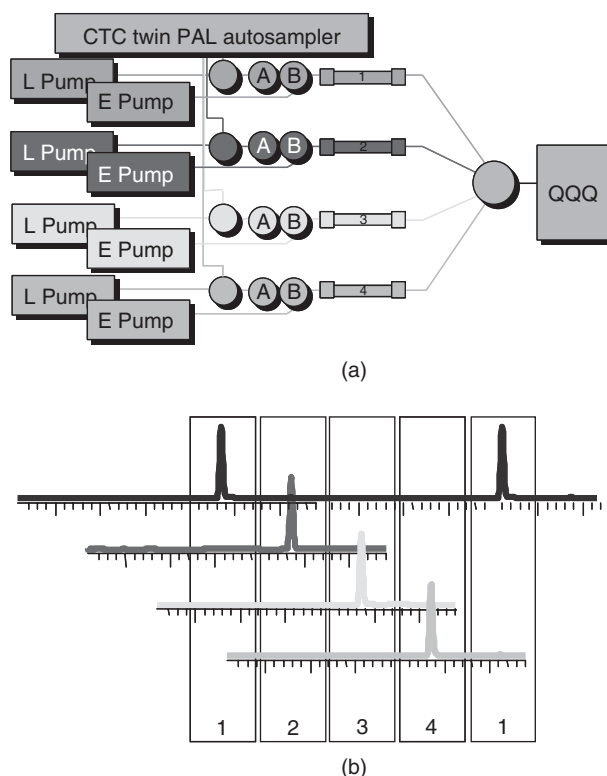


Figure 19.4 (a) The four- or eight-channel LC-MS configuration, as commercialized by Thermo Fisher. The system schematic indicates eight LC pump systems, facilitating either four-channel operation with column switching or eight-channel operation as single dimension LC separations. A dual-arm CTC autosampler with two- to eight-injection valves provides sample aspiration and injection. Dual-switching valves (A/B) enable column switching selection for analysis and a selection valve gates the appropriate LC eluent stream to the triple quadrupole mass spectrometer (QQQ). (b) Sample delivery is staggered in time such that the appropriate eluent stream windows (shaded and numbered) are serially selected for MS detection with an interacquisition delay for independent acquisition file open/closure events. (See color insert.)

two- to eight-pump, and one- to eight-channel staggered parallel system, as shown in Fig. 19.4. Where the same assay is run across multiple channels, there has been concern raised to intercolumn retention time variability. Variability can be reduced by purchasing columns with the same lot of packing material. Briem *et al.* [85] reported that 2000 injections could be made on the columns without notable increase in backpressure or change in chromatographic properties.

19.13 INFORMATICS

As the number of data points generated increases, so does the burden on data processing and reporting. This requires increased efficiency and robustness in the procedures used in method development (discussed above), sample tracking, data analysis, data processing, and posting, and reviewing [39].

Data processing mainly consists of peak integration, generation of calibration functions from fitting standards, and converting sample signal response into a representative concentration and has almost exclusively been developed inside each company by customizing vendor-provided software with the use of visual basic scripting. Most MS vendors have fully automated peak integration routines. However, automated procedures can fail to pick non-Gaussian peaks, especially those at low S/N ratios. These results require manual review and integration, which for large data sets is very time consuming. For single-shot data, the data can be directly posted into the corporate database for the compound tested. For dosing experiments, response curves are plotted and fitted to find the IC_{50} values, which are subsequently reported to the database. Finally, after data posting is completed, the data are reviewed to determine what compounds have favorable or unfavorable *in vitro* ADME properties. Using defined search criteria, the results can be automatically filtered to provide this information. An excellent example of the interconnectivity and rational design of automation, auto-tuning, multiplexed measurement, library searching and final results posting was recently shown by Laycock [40]. Proprietary data processing systems have been developed and described, accommodating many of the principles described so far, namely, single data file acquisition and rapid manual data review processes, to achieve manual data review and release of 96-well plate data in 5 min [86]. Ascent™ is a commercial solution to instrument-independent peak processing and automatic data release, which has been developed by Indigo Biosystems. Software algorithms provide a numerous array of chromatographic, analytical troubleshooting, and quality scoring solutions to remove the majority of manual peak review. While these technologies have been predominantly employed within the high throughput clinical diagnostic laboratory, the analytical efficiency needs are similar, and it is anticipated that the quantitative *in vitro* setting will embrace this approach [87,88].

19.14 NON-LC-BASED HIGH THROUGHPUT MASS SPECTROMETRY

A continual theme in the previous sections involved efficiency gains through the sampling capabilities of LC-MS/MS. This has been predominantly achieved by sample pooling, reducing the autosampler and LC cycle times, or multiplexing the number of LC systems connected to the mass spectrometer. An alternate approach is to eradicate the LC dimension entirely. The gains would be simplification of the equipment burden and removal of the cycle time limitation imposed by the autosampler/LC hardware. The obvious concerns are reliability, reproducibility, and robustness of these approaches, particularly in light of the inherent reduction in matrix effects observed when an appropriate LC separation is utilized. *In vitro* ADME samples are less complex than plasma-based samples and may be more amenable to such approaches. A newer suite of technologies have been proposed, including MALDI or the recently introduced ambient ionization techniques including desorption electrospray ionization (DESI), direct analysis in real-time (DART), or laser diode thermal desorption–atmospheric pressure chemical ionization (LDTD-APCI). Each of these techniques proposes the potential for no or minimal up-front sample preparation before analysis and depends on product ion MS/MS or single ion reaction monitoring to provide an effective method filtering out the response of contaminant species.

19.15 LASER-BASED IONIZATION METHODS

One alternative that was commercialized under the product name FlashQuant consisted of a MALDI ionization source coupled to a triple quadrupole instrument (MALDI-QQQ) [89–92]. Samples were spotted onto MALDI target plates with matrix and were analyzed by rastering the MALDI plate through the laser beam. The fastest sample analysis rates ever reported were achieved by this equipment at 1.2 s per sample (Fig. 19.5) to enable analysis from 384 wells in <7.7 min [93].

Since a triple quadrupole was utilized, SRM transitions were used to provide compound specificity and removed the difficulties of detecting small molecules in the presence of interfering ions. This technique did not resolve ion suppression effects

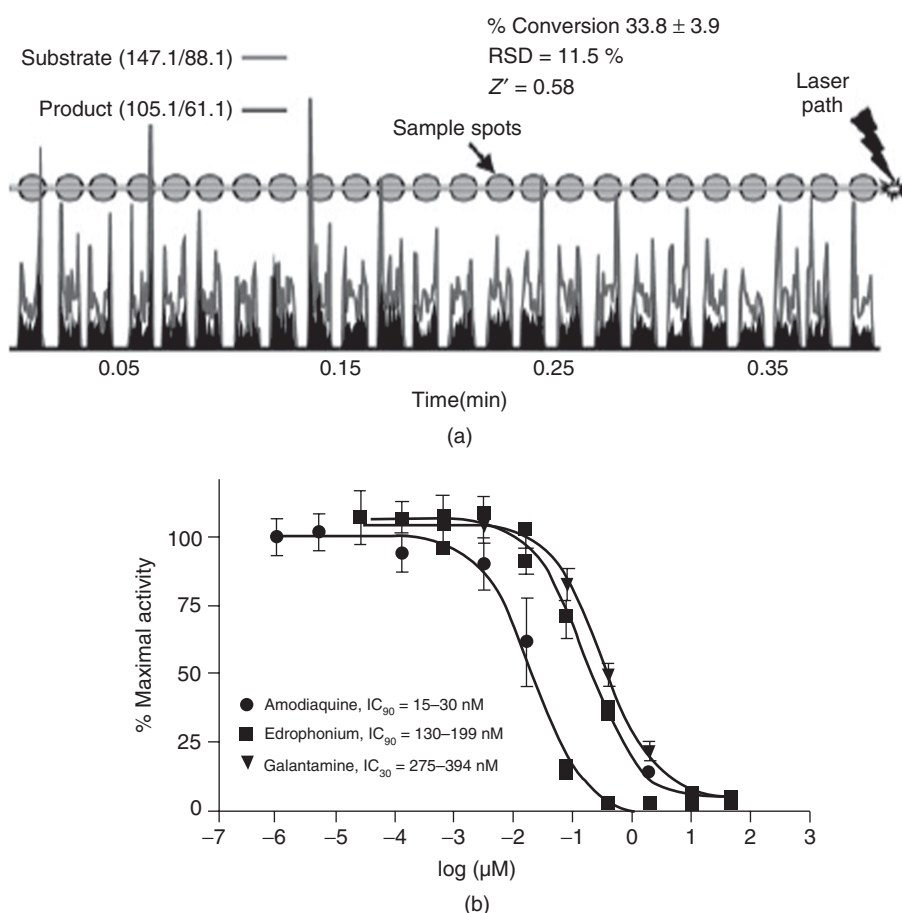


Figure 19.5 (a)MALDI-QQQ readout of the substrate acetylcholinesterase (light gray) and enzyme product, choline (black) used to probe AChE inhibitors as described by Rathore *et al.* [93]. Rastering rate of the laser beam was set at 9 mm/s, identical to the spot width of a 384-well plate, realizing a sampling rate of 1.2 s per sample, accounting for plate stage movement. Reproducibility (%RSD) of measurement of substrate and product was <12%. (b) Ten-point concentration-dependant inhibition assays for AChE against known inhibitors, results are plotted from triplicate analysis with mean and $\pm$ standard deviations shown.

from the presence of salts, proteins, and other molecules, which will generally reduce sensitivity. For less complex biochemical systems, no sample clean-up was typically required [93]. However, for more complex sample mixtures such as microsomes or plasma, an off-line liquid–liquid extraction or SPE clean-up step was typically required, limiting the overall process sample throughput. The effect of ionization suppression effects was illustrated by Kovarik *et al.* [92]. Using protein precipitation of plasma and 4 times washing *in situ* on the MALDI plate, acceptable accuracy and sensitivity of 1 ng/mL was obtained for propranolol. Addition of an off-line LC separation using a 1-mm LC column with fraction collection before spotting improved analytical sensitivity to 50 pg/mL.

The throughput of MALDI-QQQ clearly demonstrates the capability to acquire data representative of high throughput, even at the single analyte per spot rate; however, new bottlenecks such as spotting compound and matrix to MALDI plates would be added to the workflow, which require additional automation and degrade the overall process efficiency. Further, the original FlashQuant system lacked a plate-stacking capability, thus removing the capacity for sustained, unattended automated analysis.

Another laser-based methodology that has recently been introduced for high throughput quantitative MS is LDTD-APCI [94]. In this ionization source, 2–10 μ L of sample are spotted into a 96-well plate made of a polypropylene with stainless steel well walls. The plate sits on an x – y translatable stage, and the sample is moved to align with the source assembly. Once in position, a plunger from the source is placed into the well to seal off the sample well. An infrared (IR) laser thermally desorbs analyte into the vapor phase; vaporized sample is swept through a transfer tube by a carrier gas (air), to a corona discharge needle for ionization, before sampling by a mass spectrometer.

Wu *et al.* monitored competitive inhibition for the CYP isozymes 1A2, 2C9, 2D6, and 3A4 using human liver microsomes. For the metabolic probe molecules monitored in this study, acetaminophen, 4'-hydroxydiclofenac, dextrophan, and 6 β -hydroxytestosterone, the signal obtained for 5 μ M of each analyte in 0.25 mg/mL human liver microsome data were similar and, in some cases, better than data generated by flow-injection with APCI or ESI. When operated in fully automated mode, a 96-well plate was analyzed in 18 min for a sample throughput of 11.3 s per sample.

19.16 THE FUTURE PROMISE OF AMBIENT IONIZATION METHODS

During the past five years, many new ionization techniques have been introduced in the field of MS, which enable direct analysis of samples with minimal or no sample clean-up. These ionization techniques have been classified as ambient ionization techniques. The fundamental principles and applications for ambient ionization are described in more detail in this volume in the chapter titled *A Perspective on the Evolving Field of Ambient Ionization Mass Spectrometry*. An inherent advantage of these techniques is the generation of ions in an “open-source” before transmission into the mass spectrometer; thus, samples can be introduced from alternate technologies to the mass spectrometer (i.e., from glass slides or capillaries, thin-layer chromatography (TLC) plates, or paper). The major constraint for analyzing a batch of samples is physically introducing the samples (on a sampling device) to the mass spectrometer.

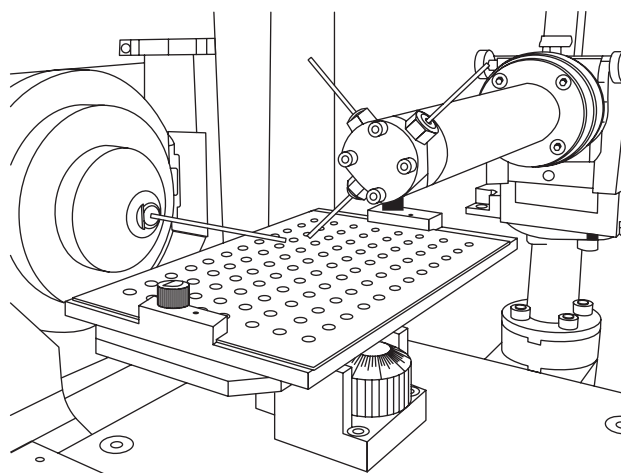
A second potential advantage these techniques offer is eliminating time spent developing a specific separation method along with concomitant reduction in SRM method development time.

DESI and DART have been utilized to provide quantitative information at increased throughputs from biological samples. Initial reports demonstrated many utilities of the DESI source for the detection of specific analytes from a variety of surfaces [95]. The high throughput capabilities were demonstrated by moving pharmaceutical tablets using a conveyor belt at a rate as fast as 0.33 s per sample [96]. For liquid samples, a DESI source with a 3D stage (automated in two dimensions) has been developed and interfaced to a linear ion trap mass spectrometer by Manicke *et al.* [97] (Fig. 19.6). They demonstrated the ability to analyze samples containing either propranolol (PRN) or carbamazepine (CBZ) at a sampling rate of 2.5 s per sample for analytes in 10% urine or porcine brain total lipid extract from a 96-sample array plate for with a total analysis time of ~ 3 min per plate. Moreover, good precision data were obtained for the chosen analytes: relative standard deviation (RSD), 10% from urine and $<5\%$ from brain lipid extract with deuterium-labeled internal standards. Full scan MS mode enabled a limit of detection of 10 fmol for PRN from a neat solution and increased to 400 and 200 fmol for 10% urine and brain lipid extract samples, respectively. As expected, the biological extracts increased the observed chemical noise. Notably, these detection limits could have been markedly improved with the use of MS/MS scan on the ion trap or a triple quadrupole operated in SRM mode.

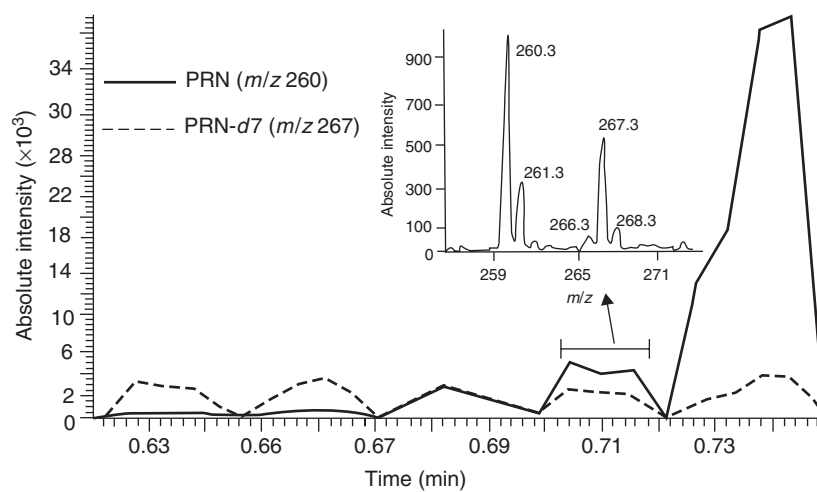
One key attribute of DESI is the low protein ionization efficiency, compared to small molecules and peptides, resulting in the potential for very little sample preparation before analysis. Additional ionization modes may be explored, such as desorption atmospheric chemical ionization (DAPCI) [98]. An automated DESI source coupled to a tandem mass spectrometer would be highly applicable for a full suite of *in vitro* ADME techniques, once automated plate handling coupled to the source and automated spotting from 96- and 384-well plates were implemented. For example, for metabolic stability, samples from various time points could be analyzed quantitatively and qualitatively using the same sample spots if Q-TOF, linear ion orbitrap, or quadrupole ion trap mass spectrometers were utilized. Using many of the same principles discussed above (sample pooling and using multiple probe molecules), CYP inhibition assays could be performed at the fastest cycle times ever recorded.

The DART ionization source developed by Cody *et al.* [99,100] has also been shown to perform well in a variety of qualitative and semiquantitative analyses. Liquid

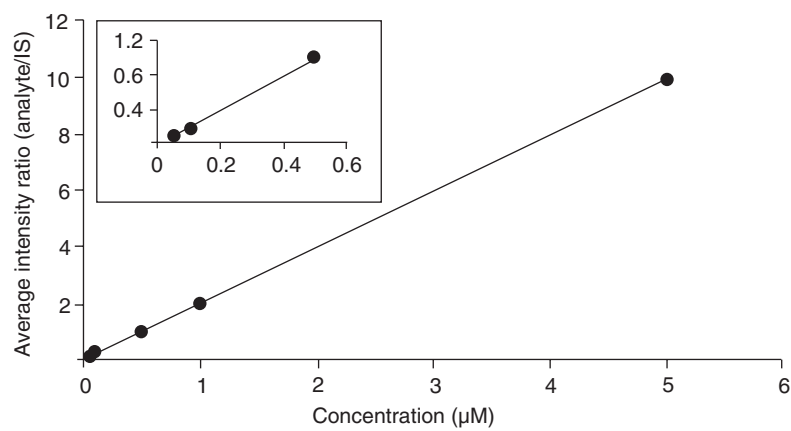
Figure 19.6 (a) A schematic diagram of the automated 2D stage DESI sampling system, as described by Manicke *et al.* [97]. The “96-well” layout is composed of a glass slide and raised printed hydrophobic ink containing polytetrafluoroethylene (PTFE) in the geometrical arrangement of a standard 96-well plate. The custom built X–Y moving table aligns the appropriate spot to the DESI sampling probe for analysis, following positional and incident angle alignment of the DESI tip. (b) The sampling rate of the system of 1.5 s per sample from neat solutions, composed of 0.5-s interwell movement and 1 s of spot analysis. Propranolol (PRN) (solid line) and propranolol-*d*7 (PRN-*d*7) internal standard (dashed) chromatograms are shown from an expanded region of the 96-well plate analysis. (c) The representative calibration curves (intensity ratio vs concentration for propranolol) generated from neat solutions of propranolol and stable labeled internal standard ($N = 5$ replicates per concentration level). Error bars indicate reproducibility (%RSD) of 5–10% for the lowest standard and $<5\%$ at other concentrations.



(a)



(b)



(c)

samples are introduced into the ionization source region using a glass TLC micro-capillary tube, solvent is evaporated, and ions are generated by chemical ionization processes. Cycle times in the <5 s per sample regime have been shown, from the time of sample introduction until complete evolution of the analyte from the tube. With these principles in mind, Yu *et al.* [101] developed a DART source interfaced with an automated sampling system to enable analysis of drugs from plasma samples using a triple quadrupole mass spectrometer. A critical modification to enhance the sensitivity of the DART source was an additional pumping interface between the exit of the DART source and the entrance of the ABI 4000 mass spectrometer. This reduced the gas load of He produced by the DART source to enable sufficient pumping by the mass spectrometer and contained analyte enriched carrier gas to the API interface. The second improvement involved automating the procedure for transferring sample from a plate to the ionization region using a HTC-PAL autosampler with a glass dip tube instead of a syringe for sampling.

Matrix effects varied for each analyte. For example, prapracaine gave a response of 5.4% of the neat solution response when sampled from plasma. Linearity was achievable for the compounds studied, and the sensitivity was within the range that was usable for bioanalytical studies, that is, verapamil was detectable at a level of 0.1 ng/mL. The overall sensitivity was 2–10 times lower than that achieved by electrospray for the compounds surveyed. The cycle time for each sample was at ~0.8 min per sample. The sampling rate was dictated by the speed of the HTC-PAL for fetching and placing samples into the source. This time could be further reduced using a multiplexed HTC-PAL system in which the next sample could be dipped while the current sample is analyzed.

The authors noted that DART ionization is unable to maintain intact glucuronide conjugates during the ionization process, thereby yielding higher apparent amounts of the parent molecule. Moreover, the system is limited to analyzing molecules with a molecular weight (MW) of <1000 Da.

Recently, IonSense has announced a 3D sampling stage for the DART source, the SVP 3+D scanner at the 2010 Pittcon Conference (Musselman 2010, Personal communication). It is likely that cycle times for this implementation of DART will compare favorably with DESI and FlashQuant.

The application of laser-based and ambient ionization techniques can truly increase the throughput achieved by quantitative MS. By eliminating the LC, the complexity of the analytical system is reduced. These techniques show great capability to reduce sample analysis times from minutes down to seconds. However, for these techniques to be successfully implemented, mechanisms to automatically handle plate loading and removal from the source region must be made available along with the ability to store and access a large number of plates. Moreover, new advancements in data handling and processing will also need to be developed.

19.17 SUMMARY

The realization of high throughput quantitative MS has required a significant internal investment of resources for the development of customized workflows and human interfaces. In many instances, the techniques being employed have achieved the 1–2 s per analyte goal of “high throughput,” most notably in minimizing the dead time associated

with each discreet event and removing manual processes. A number of inefficiencies still remain within the process, most notably the LC separation time domain and post-analytical data review. The nascent potential of ambient ionization methods may play a significant role in eliminating these steps to match the analytical throughput of the mass spectrometer. Recently developed instrument-independent quantitative peak processing tools may also further align these throughput efficiencies.

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20 The Role of Biomarkers in Drug Discovery and Development: Enabling PK/PD

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20.1	Summary	1
20.2	Introduction	2
20.3	Definition of biomarkers and the roles they play in drug discovery and development	3
20.4	Pharmacokinetic/pharmacodynamic-model-based drug discovery and development	6
20.5	PK/PD enabling biomarkers	7
20.6	Assay and data quality considerations for PK/PD biomarkers	13
20.7	Conclusions/future challenges	17
	Acknowledgements	20
	References	20

20.1 SUMMARY

The ultimate goal of drug discovery and development is to find the right drug and to administer it at the right dose with the right frequency to the right patient. Biomarkers can be an invaluable tool to meet this goal by confirming the efficacy and safety of drug candidates, enabling the understanding of their mechanism of action (MOA), and determining the efficacious dose and dosing regimen by establishing the correlation between the pharmacokinetics (PK) and pharmacodynamics (PD) of the drugs. They can also aid the selection of a patient population that will respond to treatment and benefit the most from their therapeutic effects, thus creating the path for personalized medicine. Biomarkers are objectively measured indicators of pathogenic processes of disease development or responses to therapeutic intervention. This chapter provides a brief description of the key concepts related to the use of biomarkers in pharmaceutical drug discovery and development. The role of target, mechanism, outcome,

and surrogate biomarkers in the early stages of drug discovery, starting with target identification, nonclinical model development, through clinical translation and proof of concept (POC) applications are described. A brief section on PK/PD-model-based drug development and translational research illustrates how technological advances enabling more accurate and robust assessment of drug concentrations in target tissues and systemic circulation together with PD response data lead to more accurate human dose projections and more optimal clinical trial designs. Considerations for the characteristics of optimal PK/PD enabling biomarkers are presented, including study design and sampling considerations and an overview of the most commonly used methodologies for the development of quantitative biomarker assays. Since assay development and validation for biomarkers can take significant time and is expensive, the importance of biomarker validation using fit-for-purpose assays is discussed. The challenges and solutions to various aspects of the fit-for-purpose validation are described in the context of the drug development stage requirements. Throughout the chapter, historical perspectives and representative examples for the challenges, solutions, and applications of biomarkers are provided.

20.2 INTRODUCTION

Biomarkers have been used for over 100 years in medical practice and have been playing a key role in drug discovery and development for over half a century. The National Institute of Health Biomarker Definitions Working Group defined biomarkers as various biochemical, physiological, imaging, and behavioral characteristics that are objectively measured as indicators of normal or pathologic processes or in response to therapeutic intervention [1]. Perhaps, the two most well-known examples of biomarkers are blood glucose for insulin-dependent diabetes and low density lipoprotein cholesterol (LDL-C) for hypercholesterolemia and cardiovascular disease. Their history illustrates that the discovery, validation, and application of biomarkers in medical therapy and drug discovery require interdisciplinary research between biology, medicine, and drug development and can take several years to decades. The earliest records of diabetes can be found in Egyptian papyrus records in 1552 BC, mentioning frequent urination as the symptom, and from the eleventh century, diabetes was diagnosed by tasting the urine of subjects as its sweet taste was connected to the disease. However, it was not until the nineteenth century that the first chemical tests to measure sugar in the urine were developed. Furthermore, it took over 100 years of medical and biology research to discover and link insulin with type 1 diabetes, with the first successful treatment of a depancreatized dog with insulin occurring in 1921. The first home tests for urinary glucose became available in the 1960s, and Clemens' patented blood glucose meter in 1971, enabled the easier monitoring and medication management of diabetics [2]. Closer monitoring of the glucose biomarker levels combined with adaptive therapy, applying more frequent doses and self-adjustments according to individual activity and eating patterns, have significantly delayed the onset and progression of long-term complications in diabetic patients, thus illustrating the importance and utility of the biomarker in managing clinical outcomes for diabetes [3]. As for the history of LDL-C, the concept of using biomarkers in the prevention and treatment of cardiovascular diseases can be traced back to the Framingham study [4]. The investigators initiated this study in 1949 to "seek a single essential cause to produce cardiovascular disease."

It was soon realized that complex and multifactorial interactions lead to the pathogenesis of atherosclerotic cardiovascular disease. However, it was through this study that the quantitatively measured clinical parameters of total cholesterol and LDL-C as traditional risk factors for coronary heart disease were identified. Subsequent pharmaceutical research to identify drugs that inhibited cholesterol synthesis led to the discovery of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that we now know as “statins.” Statins were approved in 1987 to lower total cholesterol and LDL-C, and in 1994, statins were shown to reduce cardiovascular events [5].

As illustrated by the above examples, there are a number of important roles that biomarkers play in clinical applications including diagnosis, monitoring disease progression or reversal, and patient selection for clinical trial stratification. In recent years, modern drug discovery has recognized the importance of biomarkers in the earlier stages of drug discovery and development. Their utilization is often required in compound selection strategies for nonclinical development using the efficacy and/or safety biomarker profile of drug candidates, as well as for the proof of medical hypothesis by linking drug effect to the biological target using relevant and validated target and mechanism biomarkers [6].

20.3 DEFINITION OF BIOMARKERS AND THE ROLES THEY PLAY IN DRUG DISCOVERY AND DEVELOPMENT

20.3.1 Target, Mechanism, Outcome, and Surrogate Biomarkers for Drug Discovery and Development

The drug discovery and development process consists of several stages. It starts with the identification of viable targets and continues with the screening and selection of potent drug candidates against the target. This is followed by testing these potential candidates to confirm their nonclinical *in vivo* efficacy and safety prior to proving their safety and efficacy in humans and ultimate benefit to the patients. In the candidate screening and selection process, it is important to demonstrate if a drug candidate is truly interacting with the target by directly or indirectly preventing it from performing its biological function, and if this interaction will result in modifying the disease. *Target biomarkers* provide feedback on direct inhibition of the target enzyme. *Mechanism biomarkers* provide information on the target inhibition, downstream from the target, in the disease pathway. *Outcome biomarkers* are indicative of the clinical outcome of the disease and are sometimes referred to as *surrogate biomarkers* since they are indicative of how the patient feels and functions. These concepts and definitions are illustrated by the examination of the well-known cholesterol synthesis pathway shown in Fig. 20.1. The first step in this pathway is the conversion of acetyl-coenzyme A to mevalonic acid (MVA) by HMG-CoA reductase. The target enzyme is HMG-CoA with the hypothesis that its inhibition will result in reducing blood cholesterol. MVA is the product of the first step in the enzymatic synthesis and thus is the target biomarker for this pathway. The other intermediates in this pathway are potential mechanistic biomarkers, while LDL-C is the outcome biomarker. On the basis of the results of the Framingham study, LDL-C has been validated as an outcome biomarker for cardiovascular disease. Since the connection between coronary heart disease and LDL-C is scientifically and medically well established, it is also accepted as a validated surrogate biomarker for

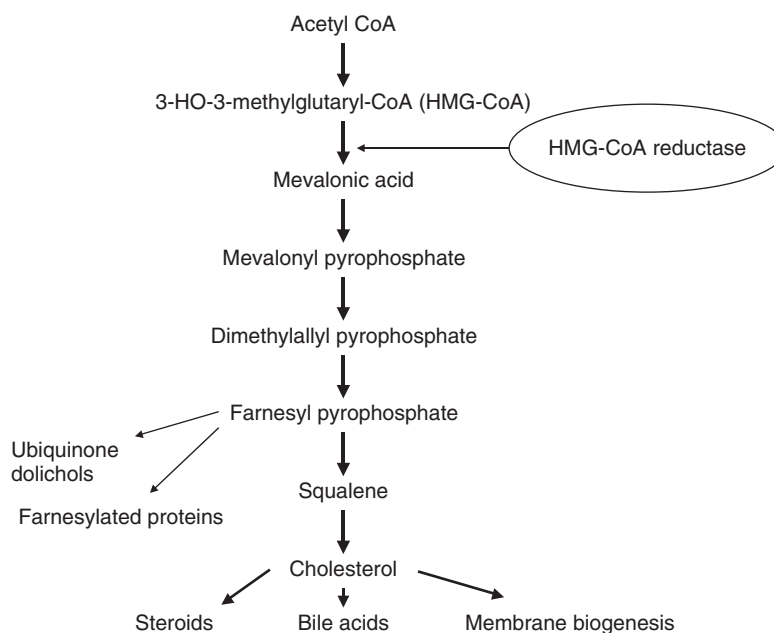


Figure 20.1 Schematic of the cholesterol biosynthetic pathway. Mevalonic acid is the target biomarker for the conversion of HMG-CoA by HMG-CoA reductase [19].

this disease. Validated target, mechanism, and outcome biomarkers are invaluable in drug discovery and development as they can lead to earlier validation or disqualification of a new target, provide feedback on drug design and selection, and save significant time and cost. For instance, by demonstrating that a drug candidate “hits” the target via inhibiting the formation of the target biomarker, but results in no modulation of the outcome biomarker that has been linked to the disease state, a target can be proved nonviable. Similarly, if target modulation was not achieved *in vivo* then this information can be provided to the medicinal chemist to design and synthesize more potent candidates. Another benefit is time and cost savings. For instance, confirming modulation of cholesterol synthesis by measuring the steady state concentration of systemic LDL-C can take four to six weeks, while potent inhibitors of HMG-CoA show modulation of the target biomarker, MVA, less than 24 h post dose in the blood of patients, thus enabling the evaluation of potential novel compounds much faster and at lower cost. As illustrated in Fig. 20.2, acute changes in plasma mevalonate levels have been demonstrated to correspond to the potency of statins. In addition, since the target organ for statins is the liver, plasma drug concentrations can be difficult to measure, so the plasma biomarker modulation facilitates the assessment of target inhibition. Therefore, plasma mevalonate can be used in succinct nonclinical PK/PD modeling studies to select an efficacious dose and is translatable from nonclinical models to humans.

20.3.2 Disease Biomarkers and Diagnostic Markers

As described above, outcome or surrogate biomarkers that are well understood and validated for a given disease state can be utilized to diagnose or confirm a disease. It is

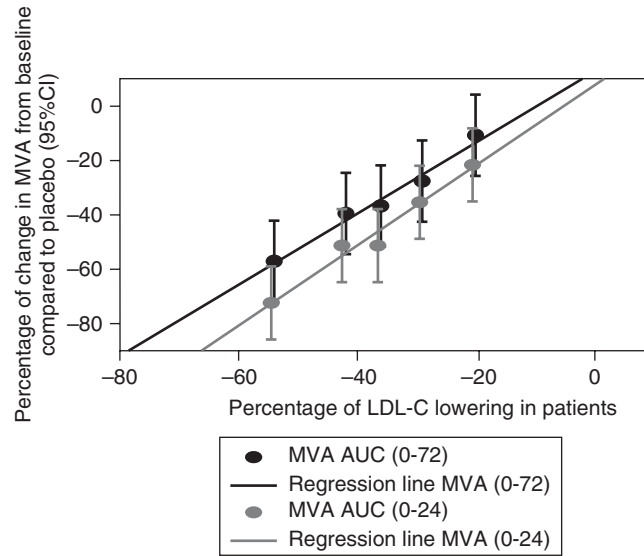


Figure 20.2 A relationship exists between the change in mevalonic acid (MVA) area under the curve (AUC) following a single dose of statins in healthy subjects and steady state changes in LDL-C in patients.

important to note that the normal range of some biomarkers can have significant inter-subject variability. Therefore, it is not the actual biomarker levels, but changes to these levels, that are diagnostic of a developing or advancing disease state. For instance, until recently, research on one of the most widely used tumor biomarkers, prostate-specific antigen (PSA) for prostate cancer, suggested that the rate of increase in PSA (the PSA velocity) was a more specific marker for prostate cancer than the absolute blood levels [7]. Thus, it was recommended that baseline levels were established for subjects in the higher risk populations and monitored for changes with a given frequency (i.e., approximately two years). The rate of PSA increase is also believed to have a value in prostate cancer prognosis. For instance, men with prostate cancer whose PSA level increased by more than 2 ng/mL in a year before the diagnosis have a higher risk of death from prostate cancer despite surgery [8]. Most recent PSA diagnostic tests approved by the Food and Drug Administration (FDA) between 2000 and 2010 are actually used to determine the ratio of free PSA to that of total PSA, including the fraction that is bound to serum proteins. In men with prostate cancer, the ratio of free to total PSA is decreased, and the risk of men acquiring cancer increases if the free to total PSA ratio is less than 0.19–0.25 [9].

20.3.3 Biomarkers for Patient Selection and Trial Stratification

A great promise of modern drug development is personalized medicine that enables the physician to select the treatment most beneficial to the patients to cure, or slow down the disease with the least amount of risks from unwanted side effects. Biomarkers, often codeveloped and validated as a diagnostic, with a given therapeutic can enable the selection of the patients who are most likely to respond to the treatment, thus obtaining

the most benefit. The development of companion diagnostics became possible because of the increased understanding of the role that particular genes play in the disease pathology.

Examples of this for cancer are Herceptin[®] that is approved for the treatment of human epidermal growth factor receptor 2 (HER2) overexpressing breast cancer tumors, as well as Vectibix[®] and Erbitux[®] that are approved for V-Ki-ras2 Kirsten Rat Sarcoma viral oncogene homolog (KRAS) expressing colorectal cancer. The cytochrome P450 genotyping test approved in 2003 by the FDA using gene chip arrays can be used to determine if a patient has mutations in their CYP4502D6 gene that can affect their ability to metabolize certain drugs, which can lead to harmful drug–drug interactions or inappropriate dosing and treatment. Human gene expression analysis already plays and will continue to play an increasing role in the identification and validation of new disease targets and the development of individualized medicine with more effective and safer treatment options [10–12].

20.3.4 Pharmacodynamic Biomarkers

Biomarkers are also a means to assess PD response. Their use in understanding effect and exposure relationships is essential for PK/PD-model-based drug development enabling better predictions of efficacious dose and regimen [13–16]. Target and mechanism biomarkers that are directly linked to a target's activity or its MOA through a given biochemical pathway or outcome can potentially be used as PD markers. Their full utilization requires translatability between nonclinical models and human, validation of the animal models for nonclinical *in vivo* efficacy screening, as well as confirming the MOA in POC clinical studies. Blood concentrations of drug as a result of therapeutic dosing have long been assessed to determine PK parameters; thus, they can be considered a biomarker of dose response. On the other hand, in some cases, the blood concentrations of drug cannot be measured because of exposure levels below the limits of detection of the technology used or rapid conversion of the parent drug to metabolites. In these instances, PD biomarkers are often recommended to help to establish tolerated doses and dosing regimen.

20.4 PHARMACOKINETIC/PHARMACODYNAMIC-MODEL-BASED DRUG DISCOVERY AND DEVELOPMENT

20.4.1 PK/PD-Model-Based Drug Discovery and Development

The goal of PK/PD modeling is to link the drug exposure to the pharmacologic response and thus determine the optimal dose and dosing regimen to attain and maintain an efficacious concentration for successful treatment. In recent years, significant advances have been made in this area due to the development of more robust bioanalytical methods to determine PK parameters by measuring circulating drug concentrations systemically and in target organ tissues, as well as measuring biochemical PD biomarkers. An example of this is the use of physiologically based pharmacokinetic (PBPK) models to characterize multivariate systems [13]. PBPK modeling is a mathematical modeling method that is used for predicting the absorption, distribution, metabolism, and excretion (ADME) of a compound *in vivo*. PBPK models are also usually multicompartiment models in which the compartments correspond to predefined organs

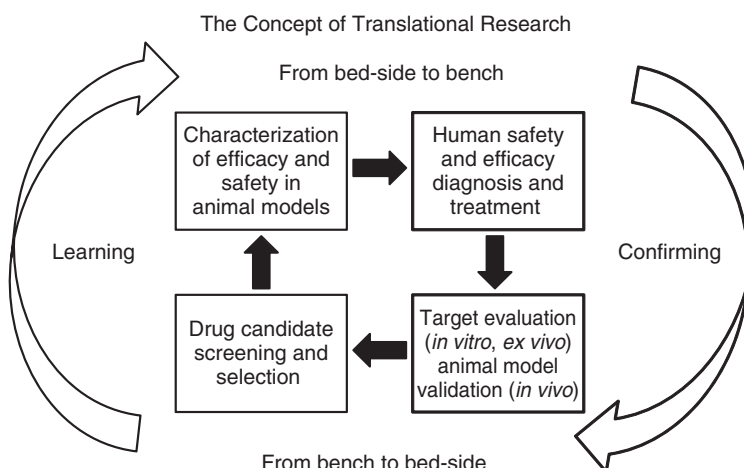


Figure 20.3 Translational research process illustrating the bidirectional and iterative integration of nonclinical basic research (learning) and its application after clinical translation (confirming).

or tissues in the body, and the interconnections correspond to blood or lymph flows. Measurement of drug concentrations systemically (in plasma or serum) and in the target organs, as well as biomarkers that correspond to the PD response in these same compartments, enables the development of more robust PBPK and PBPK/PD models resulting in better predictions of optimal dose.

20.4.2 Translational Research

Translational research is a recent interdisciplinary approach that focuses on successfully advancing fundamental discoveries from the discovery to clinical setting, and is often interpreted to include a bidirectional component where clinical findings are integrated back into the nonclinical space [17–19]. More broadly, this bidirectional component can be interpreted to include a ‘learning and confirming’ model of iteratively increasing and applying knowledge from nonclinical to clinical testing [20]. The basic components of this translational research process are illustrated in Fig. 20.3. Owing to the sequential process of drug discovery and development from *in vitro* and *in vivo* nonclinical models to human, it is paramount that the translatability of nonclinical knowledge to clinical relevance, and vice versa, is well understood and integrated in a successful and more efficient drug development process.

20.5 PK/PD ENABLING BIOMARKERS

As defined previously, PD is the science of drug action on an organism. It may be studied at many organizational levels (molecular, cellular, tissue/organ, and whole body) and at all stages of the drug development process. In addition, by enabling the quantitative integration of information across different species and throughout the clinical phases of development, PD measures can be represented by a number of

TABLE 20.1 Characteristics of Optimal PD Biomarkers

Biomarker Attribute	Desired Characteristic for PD Modeling
Mechanism of action	The biomarker's connection to the mechanism of action and disease state is well understood and correlated
Translatability	Biomarker is expressed in nonclinical models and clinical subjects and is correlated with the mechanism of action
Biological variability is well understood	Intra- and intersubject variability is well understood, the range of biological variability is low relative to modulation with disease state and drug effect
Accessibility in biological matrix	Sample collection is noninvasive, small sample size preferred
Measurability	Objective, quantitative techniques can be applied

different biomarker classes and utilize a wide range of laboratory and analytical techniques. Therefore, as an integral part of PK/PD modeling, the selection of effective PD biomarkers has become an essential strategy to optimize drug development resources.

20.5.1 Characteristics of Optimal PD Markers for PK/PD

When considering a PD biomarker for conducting modeling and simulation in pharmaceutical development, there are several attributes of the biomarker that would be regarded as ideal for this purpose (Table 20.1). Among these traits are its linkage to the drug/target MOA and linkage to the disease state, translatability between nonclinical models and the clinic, biological variability and modulation, and accessibility for sampling and measurement. For example, plasma and urine concentrations of MVA have been shown to correlate with the biosynthetic rate and long-term changes of cholesterol levels in nonclinical models and humans [21–24]. Therefore, determining the concentration of MVA in these accessible (peripheral) biological fluids can be used as a mechanistic biomarker of HMG-CoA reductase inhibition localized in a sequestered cellular target space (e.g., liver) and can thus provide a quantitative link between target and outcome (cholesterol level). In addition, information that has been collected on PD biomarkers that have been successfully translated to the clinic can be fed back to discovery efforts to further optimize drug candidates and nonclinical models [25]. The biomarker should also reflect the underlying biology by demonstrating a measurable impact on the biological mechanism or pathway that confirms a drug target has been hit and is having a correlating effect on the disease outcome [26]. In this regard, biomarkers that are more proximal to the drug target, rather than distal clinical end points further down the disease pathway, may be more desirable because they could minimize the influence of potential intervening variables between the target and outcome.

An ideal biomarker for effective PD modeling purposes should not be subject to a large amount of “noise.” Biological variability, such as marked circadian and diurnal rhythms, food effects, induced stress, or various disease states, may mask changes in biochemistry that are caused by a mechanistically based drug intervention [27]. The biomarker should also have an adequate modulation window between treated and non-treated subjects, dosed groups, or healthy versus diseased, so that a change can be

quantified relative to inherent accuracy and precision errors of the employed analytical methodology. It is also beneficial if the biomarker can be quantified with objective versus subjective techniques. For example, clinical trials for pigmentary disorders involve measurement of melanin. Melanin is a highly insoluble pigment of varying composition that is not particularly amenable to commonly used quantitative methods. Therefore, instruments that measure the ratio of reflected light to incidental light from skin, such as reflectance spectrometers and tristimulus colorimeters, are increasingly being used by dermatologists for the quantitative measurement of melanin [28]. While this approach can provide noninvasive information for the melanin content of skin *in vivo*, interobserver bias or characteristics of a particular instrument's aperture limit the accuracy and reproducibility of these measurements. Therefore, a biomarker that can be developed, which reflects the skin melanin content in absolute quantitative terms, such as pyrrole-2,3,5-tricarboxylic acid (PTCA), could be a more desirable methodology. Once melanin is oxidized to its unique monomers, such as PTCA (Fig. 20.4), melanin content can then be accurately determined by validated analytical technology such as liquid chromatography tandem mass spectrometry (LC-MS/MS). These more objective measurements of melanin concentration in skin are less susceptible to interobserver or instrumental bias and can provide more reliable PK/PD modeling. Another important consideration is to understand how the concentration of a particular biomarker can be influenced by the external influences (such as diet), endogenous biosynthesis rates, and interacting biochemical pathways. Static (as a single time point) and kinetic (as multiple time point or time course) measurements can provide more insight into the turnover and synthesis rates [29]. For instance, it is well known that carbohydrate and fat intake can affect the plasma lipid profiles. Measuring the lipid concentration at a single time point (static concentration) provides a snapshot in time, but it does not reveal the origin of the lipid (endogenous vs dietary). Stable isotope tracers combined with static and dynamic (metabolomic and fluxomic) measurements can provide a more complete assessment of the biomarker modulation [29,30].

There are also practical considerations when considering an optimal PD biomarker for PK/PD. A biomarker that can be obtained in a noninvasive manner from a matrix that is easily accessible and is relatively abundant has several advantages, such as being more cost effective because of ease of sampling, the use of fewer study subjects, and greater clinical study compliance [31]. Biomarkers requiring a small sample size for their quantification also facilitate the ability to collect more time points from the same subject. This advantage is compounded if several relevant biomarkers can be multiplexed into a single quantitative analytical method. Similar to xenobiotic analysis, it is also important to assess if the biomarker has issues that could complicate sample handling, such as stability or induced changes post collection, which may interfere with accurate endogenous measurements. Finally, in a resource-constrained environment, quantitative method development for the analysis of biomarkers tends to be tool driven. Therefore, when a biomarker must be available in time for the intended use in a program, some classes of biomarkers may be prohibitively difficult to quantify accurately because of their molecular composition and the available technology.

20.5.2 Fit-for-Purpose PK/PD Markers

Developing and applying PD biomarkers for mechanistic PK/PD modeling and simulation imparts value and a quantitative basis for translational research throughout the

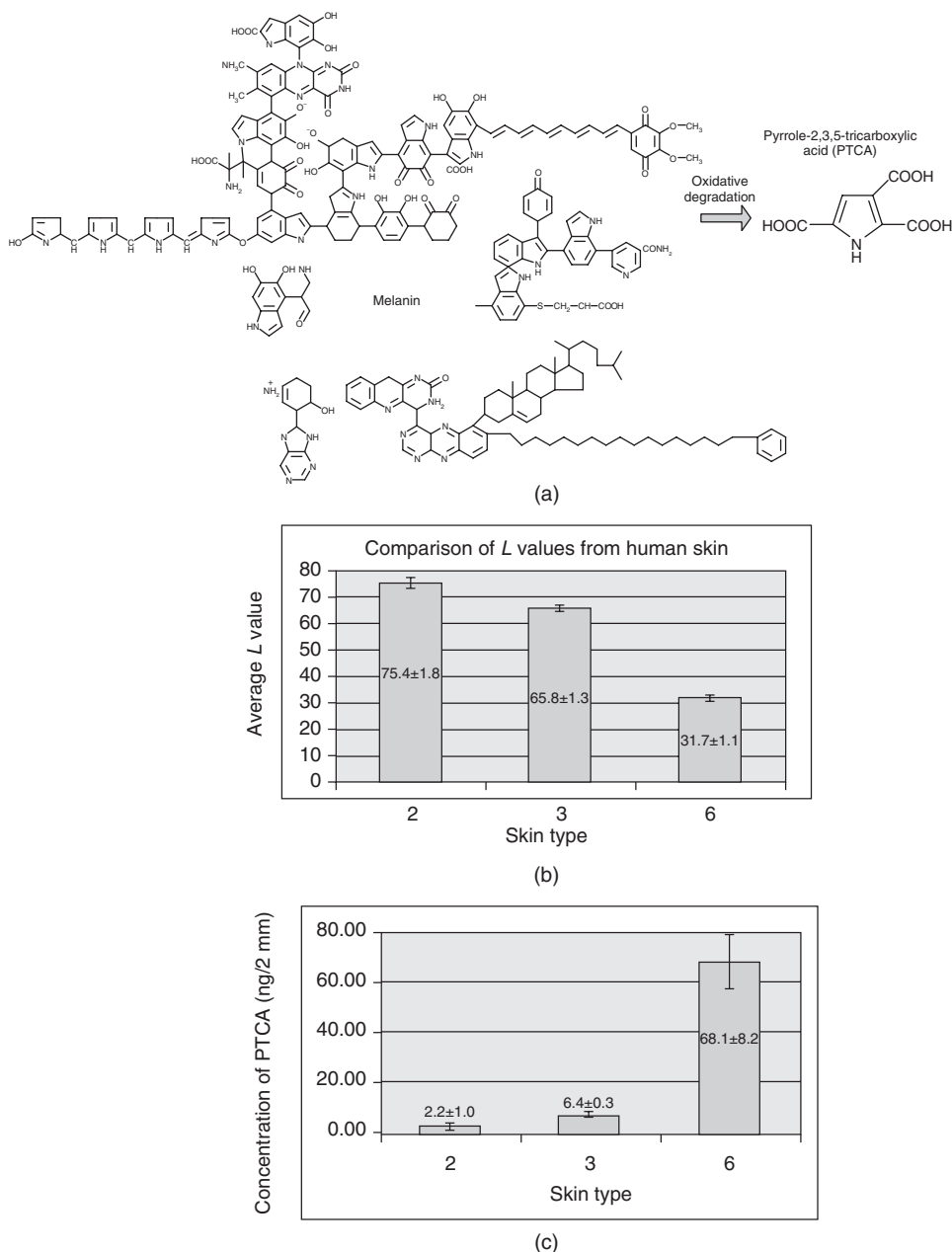


Figure 20.4 (a) Melanin can be oxidatively degraded to its unique monomers which are more amenable to common quantitative analytical methodology such as LC-MS/MS. (b) Noninvasive chromametric measurement. Melanin can be measured noninvasively using light reflecting technology to provide subjective pigment measurements, with lighter skin color reflecting more light and thus giving a larger calculated *L* value (arbitrary value obtained from chromametric measurement). (c) Comparison of PTCA concentrations obtained from different skin types. However, more objective and quantitative levels of melanin can be determined using LC-MS/MS following its conversion to PTCA monomers in 2-mm skin punch biopsies. *Source:* Part (b) and (c) graphic courtesy of Kim Wade.

drug development pipeline. The intent of this approach is to provide informed decision making in early drug development that can be applied at later stages to reduce cost through smaller study sizes or duration. Therefore, it is important to develop biomarkers, as well as the analytical tools for measuring them, that are fit for the purpose of answering the PD question that is being posed.

The desired characteristics of fit-for-purpose biomarkers depend on their intended use and the stage of drug development. For example, biomarkers selected to validate a therapeutic target or mechanism may not be appropriate for accurately scaling dose predictions across species or to form the basis of clinical trial simulations. Once the degree of confidence in mechanism has been established for candidate drugs that have an effect on this target, then the utility of the biomarker at different stages in the drug development process can be considered. If monitoring the biomarker provides a high linkage between target/mechanism and predicting dose/outcome then this could potentially be used for later definitive nonclinical or clinical PK/PD study analyses using a validated assay. For example, androgen levels within prostate tumor cells may be used as target or mechanistic biomarkers for therapies, such as Abiraterone, developed to block intracellular CYP17 enzymes and thus combat castration-resistant prostate cancer [32]. These steroid determinations may be highly useful early on in drug development to demonstrate proof of mechanism and validate the target in xenograph or *in vitro* systems. However, because of the invasiveness of sample collection, analytical challenges in their measurement, and narrow modulation window, other biomarkers such as PSA may be needed for later stage PK/PD studies and patient treatment decision making.

20.5.3 Utility of Quantitative and Qualitative Biomarker Assays

At the cellular and whole animal level, drugs can act on many biochemical pathways and in many target tissues. These actions could lead to primary responses which, in turn, may induce secondary responses, that can either enhance or diminish the primary response. Systems biology is an emerging discipline that evaluates how individual components of a complex biological system dynamically interact within the system [33]. When given a pharmacological treatment that is aimed at single components of a pathophysiological process, systems biology can provide a collective understanding of how the whole system is affected. If effectively integrated in early drug discovery and hypothesis driven, systems biology could offer an understanding of the MOA of new molecular entities, allow the modeling of different pathways individually, and estimate their overall contribution to the net effect.

Therefore, when doing studies to understand systems biology and PD hypotheses related to early validation of a disease target, more multiplexed or qualitative assays could be more appropriate than a highly accurate and precise quantitative method developed for a single biomarker. The application of “polyomic” technology, such as genomics, proteomics, lipidomics, and metabolomics, offers the potential to generate more successful drug candidates because each technology offers more extensive and complementary approaches to the underlying biological and pathological processes being examined [34]. While producing large quantities of biological data, there is already evidence that these tools can be used to detect specific changes that predict disease and drug response [35–38]. Often without reference standards, or with the goal of doing a pattern comparison between a panel of biomarkers from diseased versus healthy or treated subjects, it may be adequate for scientists to use relative quantitative

or semiquantitative assays [27]. An understanding of the basic attributes of the individual technologies, for example, sensitivity and signal-to-noise, is required to determine their applicability. For example, microarray tools that provide a comprehensive survey of the cellular transcriptional landscape and identify gene expression patterns can predict or monitor drug responses. Advances in mass spectrometry (MS) have allowed scientists to monitor protein expression changes and correlate them to disease progression. Similarly, many imaging modalities, such as magnetic resonance imaging (MRI), have been successful for qualitative biomarker analysis.

20.5.4 Study Design Considerations for Biomarkers

The biomarker strategy should be integrated with the PK/PD strategy of a program to understand confidently the relationship between dose and the time course of biomarker response. Drug exposure and biomarker response relationships *in vitro* and in nonclinical species must be understood in order to enable PK/PD modelers to evaluate the effect of time on response (e.g., modulation window, delayed biomarker responses or responses lasting longer than exposure) and will have impact on the protocol design of the studies. Therefore, the utility of the results from a PD study can only be maximized when the study design considerations for biomarkers are adequately considered.

For an ideal PD biomarker, the signal should be quantitative and reflect activity of the compound in the tissue of interest, which is relevant to the indication. The biomarker response should also be evaluated over a wide range of doses and exposures in single and/or multiple dose studies and include sampling times that provide an understanding of the relationship between time of dosing (i.e., exposure) to biomarker response. Similarly, the relationship between biomarker response and efficacy must be understood to establish the degree of biomarker signal associated with the desired outcome. This will also establish the dynamic range, or modulation window, required for the biomarker assay. In addition, whether intermittent high levels of pharmacological activity or constant levels of pharmacological activity are preferred for efficacy must be correlated to the biomarker disposition. This may come from nonclinical models, or from studies that compare the biomarker signal in normal subjects with various levels of disease activity [25]. Some direct pharmacological effects may not be rapidly reversible or may have irreversible downstream effects on the disease. For example, repeated dosing can result in toleration, sensitization, or various reflexive feedback mechanisms. Therefore, doses selected should result in clearly separated levels of pharmacological activity based on biomarker data and exposure–response modeling [38].

There are a number of other factors, both intrinsic and extrinsic, that may affect biomarker calculations that should be considered when designing PK/PD studies [27,39,40]. For example, animal species and biological sample size (i.e., animal size) or availability may limit the amount of biological sample that may be collected from each subject and may increase the number of subjects needed for each study or necessitate the use of highly sensitive, low sample volume assays. The utilization of dried blood spot sampling for biomarkers can certainly facilitate the collection and storage of test samples [41]. Multiple mechanistic biomarkers of interest, or biomarker and drug measurements, may need to be multiplexed into a single assay method to adequately cover peak/trough analyte levels. In addition, in order to account for intra- and intersubject variability in drug effect at each time point, it is important to characterize the entire biomarker sampling interval with and without drug on board.

Variation in biomarker levels from time to time, be it from reproducible rhythms (circadian/diurnal), food effects, disease state, and so on, make it critical that the placebo biomarker response profile is understood so that changes in the biomarker during drug intervention are not attributed to drug activity when they are really just biological variability. It may be necessary to draw predose samples from each subject if significant intersubject biomarker variability exists at the beginning of a study. Finally, proper sample collection and handling strategies, beyond those assessed as part of an analytical method validation, must be evaluated to determine their effect on biomarker variability. Matrix (e.g., plasma or serum), type of blood anticoagulant, immediate sample freezing or processing, platelet inactivation, and tissue or cell lysis protocol are some of the types of variables that need to be considered for biomarkers and implemented in a PK/PD study design.

20.6 ASSAY AND DATA QUALITY CONSIDERATIONS FOR PK/PD BIOMARKERS

As previously described, a biomarker can be a biochemical, physiological, imaging, or behavioral characteristic that is objectively measured as an indicator of a normal or pathologic process or a response to therapeutic intervention. In the context of this chapter, the primary focus is on biochemical markers. Biochemical markers can be characterized by a known chemical formula and structure and can belong to various compound classes, such as steroids, lipids, phospholipids, carbohydrates, nucleotides, peptides, proteins, and so on. The two primary methodologies for the identification of biochemical biomarkers are metabolomics and proteomics. Each of these comprises a comprehensive set of study designs and analytical technologies often in comparative studies between normal versus diseased and treated versus untreated study groups to identify differentially expressed biomarkers. Metabolomics primarily focuses on nonprotein, nonpeptide biomarkers and utilizes various sample extraction protocols followed by liquid chromatography (LC) and/or gas chromatography (GC) separation technologies combined with MS and/or nuclear magnetic resonance (NMR) detection. Proteomics, on the other hand, focuses on the identification of peptide and protein biomarkers using separation methods amenable to resolving larger molecular entities, including 2D gel and capillary electrophoresis, reverse phase high performance liquid chromatography (RP-HPLC), most often followed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or electrospray ionization (ESI) LC-MS/MS detection. Once the biochemical biomarkers are identified, assays can be developed for their quantification. The two most often used technology platforms for this in body fluids and tissue extracts are immunoanalytical, or ligand-binding, assays and more recently, LC-MS/MS with the combination of immunocapture sample enrichment, or IMM-LC-MS/MS. This section provides information on the factors that determine which platform to utilize for biomarker quantification and discusses the key challenges of biomarker assay validation using these technologies.

20.6.1 Technologies for Biomarker Measurements

A variety of factors influence which technology platform should be used to develop a biomarker assay. These factors include technical feasibility, fitness for

purpose/validatability, translatability between nonclinical and clinical settings, throughput, availability at contract research organizations (CROs) to support larger scale clinical studies, and time required versus time available for method development.

Technical feasibility considerations should assess the selectivity and sensitivity of the method to accurately and precisely quantify the biomarker in the biological matrix relative to the biological variability and expected modulation of the biomarker. Biomarker concentrations with a significant modulation window between normal and diseased states and low intra- and intersubject variability can be differentiated using a method with greater variability, while highly variable biomarkers with a narrow difference between normal versus disease states have limited chance to be differentiated unless the method variability is minimized. Figure 20.5 illustrates this point for results obtained from a nonclinical study where animals were administered a statin drug and plasma mevalonate levels were monitored by LC-MS/MS. The modulation window between the low and high dose groups is a combination of the difference between the individual subject variability and the established assay accuracy.

Enzyme-linked immunosorbent assay (ELISA) and LC-MS/MS assay have inherently different selectivity and specificity based on the analytical principles they employ. The selectivity of a method is the measure of its ability to determine a particular analyte in the presence of other interfering components in the matrix. A method that is perfectly selective for an analyte is specific [42,43]. The inherent selectivity of immunoanalytical or ligand-binding assays, such as ELISA, is based on the selectivity of the antibody–antigen interaction. Antibodies are heterogeneous in their antigen specificity, which is attributed to the polyclonal B cell response that leads to their formation. This known heterogeneity, combined with variable affinity to the antigens, can cause considerable variability in the specificity and sensitivity of immunoassays.

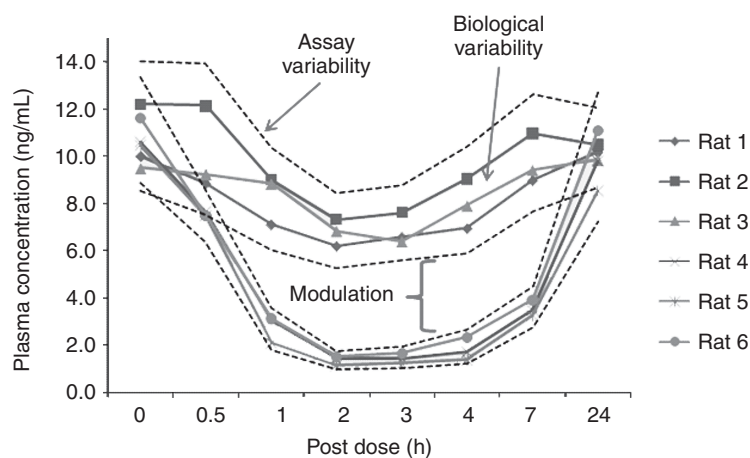


Figure 20.5 Rat plasma mevalonic acid concentrations measured by LC-MS/MS following oral administration of a statin (rats 1–3: 10 mg/kg; rats 4–6: 100 mg/kg). Variability in a biomarker measurement comes from both technical and biological sources. Assay variability must be minimized when developing a method in order to accurately determine the modulation window of a biomarker in different states (i.e., healthy vs disease, treated vs untreated). The established overall accuracy error of the assay was 15% (represented by the dotted lines).

The three main contributors to the selectivity of an LC-MS/MS method are (i) sample preparation, including directed extraction of the analyte from interfering components; (ii) LC separation; and (iii) selected reaction monitoring (SRM) detection based on the precursor and product ion mass-to-charge (m/z) values. Thus, LC-MS/MS assays tend to be more specific than immunoassays. On the other hand, LC-MS/MS assays are limited to analytes with molecular weights below ~ 10 kDa, the m/z range detected by contemporary instrumentation. Most commonly used platforms are triple quadrupole mass analyzers with m/z ranges < 4000 . Using an ESI source that is capable of producing multiply charged ions can potentially bring small proteins within the useful m/z range of a triple quadrupole mass spectrometer. Other mass analyzers, such as time-of-flight (TOF) instruments, have a higher m/z range. However, they are often combined with ion sources, such as matrix-assisted laser desorption/ionization (MALDI), that are not amenable for quantitative measurements. Gas chromatography-mass spectrometry (GC/MS) can also be used for quantification of low molecular weight, highly volatile compounds.

A fundamental challenge in developing and validating robust quantitative biomarker assays, in contrast with assays for drug measurements, is the lack of relevant biological matrix that is devoid of the analyte(s) of interest. This not only requires an assessment of assay selectivity but also necessitates a strategy for preparing assay calibrators. Often a substitute calibrator matrix cannot be identified because of interfering components or because of inadequate analyte recovery compared to the biological matrix of interest. An alternative approach to remedy this issue that has been successfully applied to MS-based methods is the use of a stable-isotope-labeled isoform of the biomarker compound as the calibrator [44]. The incorporation of C^{13} , N^{15} , $2H$, or other heavy elements into the synthetic biomarker allows it to maintain similar recovery, chromatographic, and ionization properties as the endogenous form but provides the shift in mass to be resolved on the mass spectrometer.

In terms of sensitivity, ligand-binding ELISAs routinely reach 1–0 pg/mL sensitivity, especially using electrochemiluminescence (ECL) or fluorometric detection. LC-MS/MS assays have similar sensitivity in the 1–10 pg/mL and low nanogram per milliliter range depending on analyte responses and matrix effects. More recently, immunoaffinity enrichment using analyte-specific antibodies has been coupled with LC-MS/MS analysis to increase the sensitivity [45]. There are also significant differences in the precision and accuracy of the various platforms. Immunoassays usually have greater variability because of their simple sample preparation procedures that often use straight, or minimally, diluted plasma or serum, and have inherent specificity and potential cross-reactivity issues. Commonly, for validated ELISA assays, $\pm 20\%$ accuracy and precision with $< 30\%$ total error is acceptable. LC-MS/MS assays, on the other hand, use more elaborate sample preparation procedures and utilize an internal standard to correct for process and analysis variability and, therefore, are usually validated within $\pm 15\%$ accuracy and precision criteria. The main characteristics of various assay technologies that can be used for biomarker quantification are shown in Table 20.2. Besides the technical considerations listed above, some logistical consideration such as typical development time lines are also included.

20.6.2 Fit-for-Purpose Assay Validation Considerations for PK/PD Studies

While biomarkers are increasingly used in internal decision making within pharmaceutical companies, there is a focused interest in their utilization for regulatory evaluation

TABLE 20.2 Comparison of Assay Characteristics for Commonly Used Quantitative Biomarker Assay Technologies

Assay Characteristic	Ligand binding/Immunoassay	LC-MS/MS
Selectivity	Wide range of analytes Favored method for large MW proteins On the basis of antibody–antigen interaction Can measure multiple analytes nonselectively that cross-react with capture/detection antibody Can be multiplexed	Applied for <10 kDa MW polar/ionizable analytes On the basis of HPLC separation, molecular weight and fragment ion m/z values in selected reaction monitoring (SRM) detection Typically measures one specific analyte/SRM channel at a given retention time, can be multiplexed
Sensitivity	in picogram per milliliter	in picogram per milliliter to nanogram per milliliter
Precision (%CV)	<20	<15
Accuracy (%RE)	<20	<15
Throughput	Sample preparation in automated parallel processing, incubations: few hours to overnight/96-well plate Reading time: <5 min/plate	Sample preparation in automated parallel processing in <30–120 min/96-well plate LC-MS/MS analysis time: 2–15 min/sample
Sample preparation	Usually none, direct analysis of serum and plasma	Extraction, addition of stable-isotope-labeled internal standard
Reagent needs	Antibody reagents are needed; Labeling of reagents are required (source of variability)	Usually no specific reagents are needed; Stable-isotope-labeled internal standards may need to be synthesized
Equipment and sample analysis cost	Equipment cost is <\$100, 000 (~\$60/sample)	Equipment cost is <\$500, 000 (~\$100/sample)

Abbreviations: MW, molecular weight; CV, Coefficient of Variation; RE, Relative Error.

of new drug candidates. The FDA Critical Path Initiative outlines the framework and evidence needed to qualify biomarkers for regulatory drug evaluation purposes and defines some of the critical biomarker needs in various disease areas [19]. This need for high confidence in biomarker data used in scientific or regulatory decision making emphasizes the need for high quality, reproducible, and reliable assay measurements that are translatable between nonclinical and clinical applications and validated for their intended purpose. In a recent conference, the American Association of Pharmaceutical Sciences (AAPS) Clinical Ligand Assay Society Biomarkers Workshop addressed the key challenges in biomarker research and summarized validation recommendations for immunoanalytical ligand-binding biomarker assays in a summary report [46]. Follow-up publications continued to discuss the need for iterative, fit-for-purpose approaches to biomarker method development and validation keeping in mind the intended use of the data, as well as the regulatory requirements associated with that use [43,47].

The fit-for-purpose concept advocates the progressive rigor employed for biomarker assays from initial characterization and qualification of assays to fully validated methods. Some of the key concepts related to this are described in the sections below and summarized in Table 20.3.

20.6.3 Assay Validation Versus Biomarker Validation

Regardless of which purpose the biomarker is used for, its successful utilization in scientific decision making requires its validation for the intended purpose. This fit-for-purpose validation consists of two critical steps: (i) the technical validation of the analytical method used to quantitatively measure the biomarker and (ii) the biological validation of the biomarker, confirming its linkage to the relevant biological and pharmacological hypothesis that is being tested. These two validation steps often occur concurrently, since a reliable analytical assay needs to be developed first to be able to test the biomarker's linkage to pharmacology in an animal model or human. Once the biomarker linkage to biology is tested and modulation of the biomarker between normal versus disease state or response to a therapeutic intervention has been shown, the analytical method requirements can be finalized and the method validated. Depending on the drug discovery program, multiple potential biomarkers in multiple animal models and species may be under investigation until a decision-making biomarker is selected. The development of multiple biomarker assays in multiple species and matrices and their testing in relevant disease models and clinical populations is a resource-intensive process with respect to both cost and time. Therefore, carefully designed and robust strategies for the development and fit-for-purpose validation of translatable biomarkers between nonclinical and clinical applications need to be formulated in the form of a biomarker research and operating plan [17,18]. The existence of this research plan containing input from nonclinical and clinical biology, pharmacology, PK, analytical, and PK/PD scientists can assure timely availability of validated biomarkers and assays to support nonclinical and clinical decision making.

20.6.4 Technology/Methodology Translation Considerations for PD Markers

The translatability of methods from the nonclinical to clinical application, including sample collection and preparation, is an important consideration for any biomarker—including PD markers. While it is not uncommon and often helpful to determine biomarker levels in the target tissues at the site of action in nonclinical models, the clinical applicability of these is limited because of the invasiveness of sample collection. In these cases, it is helpful to characterize the correlation between the tissue concentrations and circulating biomarker concentration in plasma, serum, or secreted concentrations in urine to determine if sampling from a less invasively obtained biological matrix can replace the tissue sample in the clinical setting. Other aspects for clinical translation are availability of the technology at CROs and throughput and validation requirements.

20.7 CONCLUSIONS/FUTURE CHALLENGES

The identification, analysis, and application of biomarkers in the development of therapeutics have come a long way over the past decades. The information provided by

TABLE 20.3 Characteristics of Fit-for-Purpose Ligand-Binding and LC-MS/MS Biomarker assays for Applications in Drug Development

	Drug Development Stage and Purpose of Biomarker Assay		
	Discovery	Nonclinical Development	Clinical Development
Assay Characteristic	• Identification of biomarker candidates • Use biomarkers for target validation • Evaluate biomarker response in animal model	• Validation of the biomarker's linkage to the disease state and/or mechanism of action • Characterization of the efficacy and safety in animal model • Drug candidate selection	Validation of the biomarker in human, for purposes of • Clinical trial go/no go decisions (mechanism, compound efficacy) • Clinical trial dose range determination (PK/PD) • Clinical trial design (length, size of population, powering of studies) • Compound differentiation • Disease diagnosis and prediction • Surrogate end points • Treatment decisions
Reference standard	Consistent—source determined	Stability as reference material and in biological matrix	Well-characterized (identity, purity) stability established, inventory/supply established
Assay dynamic range, LLOQ and ULOQ	Estimation based on endogenous levels and expected modulation	Verification based on incurred samples in animal studies, and in human, if feasible, if human assay is needed Establishment of LLOQ and ULOQ in multiple validation runs	Verification based on incurred samples Establishment of LLOQ and ULOQ in multiple validation runs
Selectivity and specificity	Determination based on reagent supplier or literature data, assess matrix effects, and potential interferences Determination of standard curve matrix (or surrogate matrix)	Verification of selectivity, specificity, and matrix effect in method validation runs	Verification of selectivity, specificity, and matrix effect in method validation runs

Precision and accuracy	Establishment of expectations for required accuracy and precision based on biomarker variability and expected modulation window	Determination of accuracy and precision in method validation runs (multiple runs for interassay values)	Determination of accuracy and precision in method validation runs (multiple runs for interassay values)
Recovery	Determination of recovery from biological matrix, establishment of expectations for desired sensitivity and robustness	Validation of recovery in spiked quality control samples and incurred samples	Validation of recovery in spiked quality control samples and incurred samples
Dilution linearity/parallelism	Verification if exist in incurred and spiked samples	Validation in spiked and incurred samples	Validation in spiked and incurred samples
Sample collection, processing, and storage stability	Establishment of feasibility, evaluation of stability at bench-top and in process	Establishment of bench-top and short-term stability, freeze/thaw, if used	Establishment of freeze–thaw and long-term stability

biomarkers to elucidate the drug target, biochemical mechanism, or disease outcome can be used nonclinically to predict efficacy and safety in humans. Therefore, biomarkers provide the cornerstone for PK/PD modeling and translational research. For proper design of PK/PD studies, biomarkers and the analytical methods used to measure them, require the degree of validation necessary to confidently answer the question the experiment was designed for. Technology also is advancing to the point where not only robust analytical methods for a PD biomarker can be established but mutianalyte and multiparameter assays (“-omics,” arrays, etc.) can also be developed to understand the PD system as a whole. Similarly, while some pharmaceuticals modulate well-established pathways and biomarkers, and can be applied to a population generally, such as statins, others require biomarkers to enable the selection, or stratification, of certain patient populations to demonstrate efficacy. While this systems pharmacology approach promises to lead to the design of better drugs with the right dose in the right patient, the amount of data generated and the associations that can be revealed are daunting. Therefore, bioinformatics will be an essential and integral part of future drug development to process and interpret large amounts of data. As biomarkers at the metabolome, genome, and proteome levels continue to be discovered, validated, and employed both for new drug development and in improving the utility of existing therapeutics, the next decades will no doubt bring more effective and safer therapies to patients.

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21 Bioanalytics for Human Microdosing

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21.1	Summary	1
21.2	Introduction	2
21.3	Biological accelerator mass spectrometry in microdosing	4
21.4	Liquid chromatography-mass spectrometry in microdosing	16
21.5	Positron emission tomography in microdosing	20
21.6	Conclusion	22
	Acknowledgments	24
	References	24

21.1 SUMMARY

Phase 0 human microdosing trials are an approach that can obtain early human data in the drug development process and improve overall efficiency. A microdose of a small molecule is defined to be one-hundredth of the anticipated pharmacological dose or 100 μg , whichever is lower, or <30 nmol of a protein product. The microdose is designed to have cellular effects but not systemic or therapeutic effects and is intended to get basic pharmacokinetic (PK) data in a limited number of volunteers. Early human data can eliminate compounds with poor PK parameters early in the development process before major investments are made in later clinical trials. Better early information in phase 0 improves the planning process and pipeline efficiency, reducing the likelihood of needing to extend or repeat later clinical trials. The European Agency for the Evaluation of Medical Products (EMA) and the US Food and Drug Administration (FDA) support streamlining the drug approval process as long as

safety is not compromised and have issued guidelines on how phase 0 trials are to be conducted.

The low doses used in phase 0 trials present detection and quantitation challenges to conventional analytical instruments, especially when test compounds possess low bioavailability, low systemic distribution, or high potency. Accelerator mass spectrometry (AMS), liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and positron emission tomography (PET) are bioanalytical techniques used for analysis in phase 0 studies. Each technique possesses strengths and weaknesses. LC-MS/MS is most common and least expensive. It is the easiest to use and can provide structural information on metabolites. It has limited sensitivity, however, and requires analytical standards for all metabolites. AMS has the best sensitivity and quantitative precision of the techniques. AMS quantitates a carbon-14 label but provides no chemical information, enabling detection of unknown or unexpected metabolites that must be identified with other techniques. AMS systems are rare and expensive, but off-site analysis is possible. PET provides unparalleled distribution information and real-time images of whether test compounds accumulate at targets. The positron emitters (carbon-11 or fluorine-18) used in PET have short radioactive half-lives and require an on-site cyclotron for production in addition to a PET scanner. PET is limited to short-term PK measurements and cannot distinguish metabolites from parent compound. Selection of a technique depends on the aims of the study and properties of the test compound. In many cases, combining the distribution information from PET with more quantitative measurements of AMS and LC-MS/MS provides the best picture of the behavior of a candidate compound.

21.2 INTRODUCTION

The current operational paradigm of drug development is time consuming and inefficient. Despite the great advances in biotechnology, computational power and models, analytical techniques, and genomics over the past 20 years, the rate of new compounds reaching the market has not increased. Furthermore, the pipeline lasts 10–20 years and costs of development are estimated at \$800 million to \$1800 million per registered drug [1–3]. As much as 40% of this cost is incurred in phase I trials that fail [4]. Up to 75% of this cost flows into compounds that never reach the market [5]. New approaches to drug development are needed to improve the efficiency of the development pipeline. Getting new compounds into humans earlier should reduce costs by identifying drugs destined to fail because of poor PK profiles before larger investments are made and by improving the planning of trials of those compounds moving ahead. Better informed planning should reduce the frequency of repeated or extended trials and potentially get new drugs to market more quickly. Phase 0 human microdosing trials are an approach that can obtain early human data [5].

A microdose is defined to be one-hundredth of the anticipated pharmacological dose or 100 µg, whichever is lower [6–8]. In the case of protein products, a microdose is limited to 30 nmol [7]. The microdose is designed to have cellular effects but not systemic effects and is intended to get basic PK data in a limited number of healthy volunteers. Phase 0 cancer clinical trials include patients with cancer, but doses of test compounds have no therapeutic effect [9–12]. Patients receive standard of care therapies during the studies.

Regulatory agencies in Europe and the United States support streamlining the drug approval process if safety is not compromised. The EMEA issued its Guideline on Microdosing in 2003 followed by a position paper on supporting nonclinical safety studies needed for microdosing [6]. The FDA followed with its Exploratory and Investigational New Drug Guideline in 2006 [7]. The guideline developed by the FDA Center for Drug Evaluation and Research (CDER) (2006) aimed to clarify the approaches, chemistry, manufacturing, and controls to be considered when planning exploratory studies in humans (also called *phase 0* or *preclinical trials*) [7]. It did not issue new regulations but rather interpreted existing recommendations on drug development. The FDA document describes three types of phase 0 studies: (i) microdose studies evaluating PK or imaging, (ii) studies evaluating pharmacologically relevant doses, and (iii) studies evaluating mechanism of action related to efficacy [7,13]. Multiple compounds can be administered in phase 0 trials allowing evaluation of several related compounds [13–15].

The aim of microdosing studies is to get drugs into humans earlier in the development process so that compounds destined to fail due to poor PK will be dropped before further investment is made [9–17]. The guideline delays full-scale good manufacturing practice (GMP) and some preclinical toxicity studies since microdosing uses very small amounts of material. There is no relaxation of good clinical laboratory practice (GCLP) when conducting phase 0 studies and full phase I studies must still be completed for compounds continuing in development [9,18]. Because very small amounts of test compound are used, quantitation challenges exist for measuring samples provided. In many cases, conventional LC-MS/MS cannot achieve the required sensitivity and either AMS or PET must be used to analyze the samples [14,18–23].

Beyond determining whether to continue development of a compound, early human PK and absorption, distribution, metabolism, and excretion (ADME) data provide insight for later human trials. By conducting phase 0 studies with intravenous (IV) and oral dosing, researchers can quickly assess bioavailability and address formulation before embarking on extensive trials [24]. The starting point and dose range for phase I can be selected with more confidence with early human data. Better early information in phase 0 improves the planning process and pipeline efficiency, reducing the likelihood of needing to extend or repeat later trials. In this case, an individual compound can get to market more quickly by minimizing delays, as it moves through the pipeline. Metabolite safety can also be addressed earlier with smaller doses of labeled drug [25].

Microdosing establishes PK of the subpharmacological dose. Lappin *et al.* [14,26] suggest that microdosing can predict pharmacological PK behavior. Although microdosing is not a perfect predictor of pharmacological PK, performing studies in the target species is often superior to allometry [17,27]. The collective experience of phase 0 trials is not yet sufficiently mature to predict when response will be linear across the entire dose range. It depends on whether receptors become saturated, reversible binding occurs, or first-pass metabolism produces an active metabolite [28]. A microdose is sufficiently small so that threshold, linear, or superlinear behavior cannot be distinguished.

Microdose levels of compounds labeled with short-lived radioisotopes appropriate for PET have been utilized for decades to determine tissue distribution. Isotopes appropriate for PET are produced with cyclotrons and then incorporated into tracer compounds. The chemistry must be done quickly because the isotopes decay quickly

and are very radioactive. The rapid decay of these compounds designed for PET works well for imaging of tissue distribution studies but is often unsuitable for PK studies.

The best way to measure the PK of these small doses is with an unambiguous tracer that can clearly be seen in small biological samples without knowledge of its metabolites. An atomic label that can be detected regardless of its chemical form is ideal. The radioisotope carbon-14 (^{14}C) with its low natural abundance ($^{14}\text{C}/\text{C} \sim 1$ part per trillion) is a fine candidate if the radiological hazards can be minimized. AMS is a rare isotope ratio mass spectrometry technique in which the rare isotope is a long-lived radioisotope such as ^{14}C . The technique is five to six orders of magnitude more sensitive than decay counting because it counts individual ^{14}C atoms in milligram-sized samples rather than waiting for their decay. The typical quantity of ^{14}C used in human metabolism studies is 200–40,000 Bq [29–32], comparable to the amount of ^{14}C in standard man (3700 Bq in 70-kg human) [33]. The small amount of isotope delivers lifetime radiation doses in the order of a couple microsievert, comparable to that received in a commercial airline flight across the United States. The level of radioactivity is so low that generally all samples (blood, urine, feces, and saliva) are nonradioactive, greatly simplifying waste disposal issues.

The strength of AMS is quantitative tracing of specifically labeled compounds. Metabolism studies are also attractive because the elevated ^{14}C provides an unambiguous signal that is clearly quantified. AMS does not provide chemical identification. That must be done by chromatography or other techniques before the tracer molecules are converted to a chemical form suitable for atom counting (either CO_2 or graphite).

Phase 0 trials require the high sensitivity to trace the small amounts of target compounds. Bioanalytical techniques suited for measuring experimental compounds are described below. All three techniques, AMS, LC/MS, and PET, have strengths and weaknesses. Selection of a technique depends on the aims of the study and properties of the test compound. In many cases, combining the distribution information from PET with more quantitative measurements of AMS and LC/MS provides the best picture of the behavior of a candidate.

21.3 BIOLOGICAL ACCELERATOR MASS SPECTROMETRY IN MICRODOSING

21.3.1 Overview of Accelerator Mass Spectrometry

AMS is an extremely sensitive analytical method used for the measurement of rare, long-lived isotopes. This technique was initially applied in isotope dating in the fields of archeology and earth science. More recently, AMS has been applied to the realm of experimental biology, including the areas of pharmacology and toxicology [34]. ^{14}C is the most common radioisotope used with AMS in biological studies, but ^3H [35], ^{41}Ca [36], and ^{10}Be [37] have also been used in biological AMS studies. The traditional analytical method of liquid scintillation counting (LSC) used in biological studies relies on nuclear decay events to quantify radioactive material. In contrast, AMS counts atoms of a rare isotope independent of decay by measuring the mass ratio of the radioisotope of interest relative to a stable isotope of the element. AMS is the most sensitive technique available for measuring ^{14}C , with the ability to quantify labeled material over six orders of magnitude down to the attomole (10^{-18}) level [34,38–40]. Owing to this

exquisite sensitivity, biological experiments using AMS produce negligible radiological exposure to the experimental subjects and do not perturb the normal biology of the system under consideration. The low level of radioactivity used in AMS experiments has enabled the use of human subjects in a number of biomedical tracer studies that would not be possible using other methodologies [34,39].

Studies in the biological sciences utilizing AMS include investigation of tissue turnover rates [41–43]; determination of PK parameters, including ADME, of drugs [14,21,26,44–46], toxicants [29,31], and nutrients [30,32]; measurement of the binding of drugs and reactive metabolites to proteins and DNA [46–48]; as well as risk assessment [49,50]. AMS is especially useful in microdosing studies because of its sensitivity that allows for the accurate measurement of extremely low levels of compound of interest. Measuring disposition and metabolism along with the molecular targets of investigational compounds in phase 0 studies can be done with AMS. Obtaining these data earlier in the drug development pipeline will help determine what compounds warrant further development. Those with unfavorable properties could be eliminated at an earlier stage before more is invested in a drug that has no chance of success. Microdosing experiments have made increasing use of AMS in recent years, introducing investigational compounds into humans earlier than is possible with more traditional techniques [14,26,51]. AMS is also being investigated for microdosing experiments with a growing area in pharmaceutical agents and biopharmaceuticals, which include large molecules such as polypeptides and proteins or macromolecules such as DNA segments [52,53].

21.3.2 Capabilities and Limitations of Accelerator Mass Spectrometry

Long-lived radioisotopes with low natural abundance are best suited for AMS measurement. Low natural abundance is important because the compound containing the radiolabel must be distinguishable from background levels, yet still be a low dose. The stable isotope ^{13}C does not fit in this category because it is naturally present in relatively high abundance, comprising approximately 1% of naturally occurring carbon.

AMS is unlike other forms of mass spectrometry in that it does not provide any structural information because the spectrometer is designed specifically for quantitation of the isotopes of a single element. Therefore, samples for AMS must be properly and adequately defined before analysis in order to ensure that meaningful results are obtained. The actual AMS measurement of a sample can be obtained in a few minutes; however, the sample preparation is a lengthier process and limits the throughput of AMS for ^{14}C analysis on most AMS instruments. AMS samples must be converted to a form that is easily conductive in the ion source and which does not allow for isotopic fractionation. Solid graphite is currently used on a conductive core such as cobalt or iron powder for ^{14}C analysis in most AMS instruments [54,55]. This proper sample definition presents a limitation of the technique, where a single HPLC trace may contain up to one hundred samples for AMS by graphitization [56]. The length of sample preparation and graphitization means several days pass between the time the samples are collected and the time that they are measured by AMS.

When sample material is plentiful and radiological dose is not an important consideration, other techniques such as LSC, isotope ratio mass spectrometry using ^{13}C , ^{13}C -NMR, or in some cases, fluorescent or chemical tagging of the compound or

molecule of interest may be more suitable than AMS. The level of sensitivity, importance of maintaining a low radiological dose, number and complexity of samples to be measured, and the cost of analysis are factors that should be considered in determining the appropriateness of using AMS for a biological study. The sensitive, quantitative, and precise nature of AMS makes it the most appropriate measurement technique for compounds with low bioavailability, low systemic distribution, or high potency. Experiments that require a compound in which only limited amounts can be produced or the tracer must be measured for extended periods of time are also well suited for AMS.

21.3.3 ^{14}C Measurement by Accelerator Mass Spectrometry

The majority of biological AMS experiments use ^{14}C because carbon is the basis of most biological materials and pharmaceutical agents. Recent developments have allowed for sample measurement in the gas phase; however, most AMS instruments measure samples in the solid state, which minimizes experiment-to-experiment carryover of signal in the instrument [57–59]. The solid form for measuring biological containing carbon by AMS is graphite. Samples containing 0.5–1 mg of carbon are optimal for graphitization and AMS, although samples containing 0.3–5 mg of carbon may be successfully measured [54]. In microdose applications, samples containing smaller quantities of carbon can be measured by the addition of a known quantity of carrier carbon that is ^{14}C deficient. The graphitization process consists of combustion of the biological sample to CO_2 , cryogenic isolation of CO_2 , and reduction to graphite in the presence of a cobalt or iron catalyst. The graphitization procedure has been described in greater detail elsewhere [54,55,60].

A schematic of a typical AMS instrument can be seen in Fig. 21.1. A cesium sputter ion source is used to generate negative carbon ions from the graphite sample. The ion beam containing those negative carbon atoms and other molecular isobars is mass selected following passage through a magnet and then introduced to the entrance of a tandem electrostatic accelerator. The ion beam accelerates toward and then collides with either a diffuse argon gas or a thin carbon foil that destroys interfering molecular isobars in velocity-dependent collisions and also removes one or more electrons from the atoms. The resultant positive ion beam is then further accelerated, followed by momentum and energy analysis. The rare ion (^{14}C) is counted using standard particle detection instrumentation. A change to the ion beam energy just after the ion source allows for the transmission of mass-13 ions through the first magnet and tandem accelerator with quantification of the $^{13}\text{C}^+$ ion current using an off-axis Faraday cup. This “bouncing” of the energy of the ion beam occurs several times a second allowing for the near simultaneous measurement of the $^{14}\text{C}/^{13}\text{C}$ isotope ratio of a sample. Absolute quantification of the $^{14}\text{C}/\text{C}$ isotope ratio in a sample comes from comparing the measured ratio of the sample to the measured ratio of similarly prepared standards.

Using the methodologies described, in a typical biological AMS study, a ^{14}C -labeled compound of interest can be routinely detected and quantified with 1–3% precision at levels ranging from ~ 10 pmol ^{14}C to 1 attomol (1×10^{-18} mol) of ^{14}C in samples containing 1 mg of total carbon in 5–10 min [38,54]. This measurement of ^{14}C by AMS can be compared to LSC that requires a gram-sized sample and a measurement time >48 h to obtain a precision of $\leq 1\%$. AMS is 1000 times more sensitive than LSC [61–63]. AMS experiments in humans have used ^{14}C label ranges from 185 Bq/person to 1.85 MBq/person, depending on the duration and type of study being performed

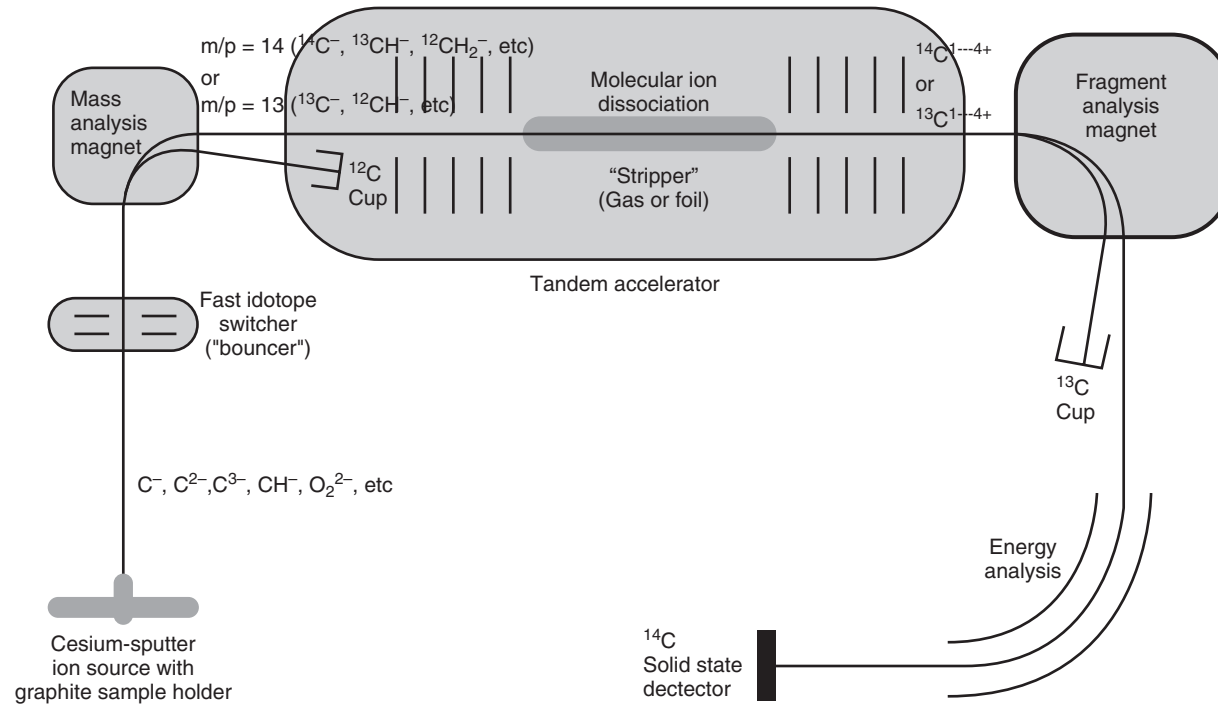


Figure 21.1 Schematic representation of the components of an accelerator mass spectrometer used to measure ^{14}C .

TABLE 21.1 Basic Performance Characteristics for the Biological AMS Instrument at Lawrence Livermore National Laboratory

Instrumental Performance	Approximate Value
Specificity (background signal with no ^{14}C)	Two parts per 10^{15}
Stability (loss of ^{14}C during sample preparation)	CV < 3%
Linear range of measurement	10^{-2} to 10^3 Modern
Instrument carryover between samples	None
Reproducibility	<5% error
Precision	2.5% (varies based on counting)
Lower limit of quantitation	5 amol ^{14}C
Upper limit of quantitation	100 fmol ^{14}C
Signal recovery	98%

[31,38]. The biological AMS instrument at Lawrence Livermore National Laboratory has measured over 70,000 samples during its operation over a period of nearly 10 years [64]. Important performance characteristics of this instrument are listed in Table 21.1.

21.3.4 Applications of Accelerator Mass Spectrometry in Drug Development

21.3.4.1 Basic Principles. Several basic principles can be applied to the unique experiments conducted with biological AMS to ensure that results are valid and informative. The investigator must understand what they are measuring by AMS. This understanding can be achieved by considering these three fundamental requirements for a successful AMS study:

1. absence of contamination in the sample,
2. appropriate amount of labeled compound, and
3. adequate sample definition.

Failure to satisfy one or more of these requirements of the sample can be very costly in time and resources and may entirely negate the value of the AMS experiment.

The absence of contaminating sources of ^{14}C is necessary to ensure that the measured ^{14}C is entirely due to the presence of the labeled compound being examined. Contamination can result in laboratory background levels as low as 5–10 μBq . The amount of labeled compound used in an experiment must result in a sample for AMS that falls within the range of 5 amol to 100 fmol ^{14}C in a milligram-sized sample. Samples with more than 100 fmol ^{14}C may generate a signal that exceeds the linear range of the counting electronics of the detector. Conversely, a sample that contains <5 amol ^{14}C will not produce adequate signal above background to be measured. Finally, the sample must be appropriately characterized by other methods such as HPLC before AMS in order to ensure that the correct and specific material of interest is measured because an AMS measurement provides no structural information. Chemical purity and radiopurity of the starting material should also be well characterized, typically using HPLC/MS and decay counting.

21.3.4.2 Work Area and Chemistry Requirements. The work area for conducting biological AMS experiments and performing sample preparation procedures must be clean and free of sources of ^{14}C -radiolabeled material as well as sources of extraneous

carbon. For AMS sample preparation, a laboratory that has no history of ^{14}C material use should be selected, as even microbecquerel quantities of ^{14}C can contaminate experimental samples [65,66]. The area for preparation of the radiolabeled compound must be separate from the dosing and sample collection areas. Samples to be sent for AMS measurement must also be stored separate from labeled material to prevent the possibility of contamination. The areas should be checked for ^{14}C background before beginning AMS experiments and must be monitored regularly for contamination. LSC is not sufficiently sensitive to detect contamination at low levels that can interfere with correct AMS measurement. Surveys for radioactive contamination should be taken and sent for AMS analysis in any laboratory in which samples are routinely prepared for AMS. The surveying procedure is described in greater detail elsewhere [65].

Chemicals to be used in experiments leading to the biological sample for AMS and the actual AMS sample preparation must be free of interfering radioisotope and should be sampled to determine the background ^{14}C levels by LSC and by AMS. Sodium-containing reagents should be avoided, as sodium can cause the quartz tubes used for graphitization to fail during the sample combustion step, resulting in loss of the sample. In many cases, it is possible to substitute potassium for sodium in reagents.

The major sources of potential errors encountered in AMS are the addition of carbon or ^{14}C from sources that remain unaccounted. This can be a result of contaminated solvents, glassware, laboratory equipment, airborne contamination, or even other researchers. The use of carbon-containing solvents for extractions or separations should be carefully considered in the determination of the total carbon present in the sample. Miscalculations in dosing or dosing of material that has an activity higher or lower than expected can result in excessive or inadequate signal intensity from the AMS sample.

21.3.4.3 Dosing and Collection of Labeled Material. The amount of ^{14}C -labeled compound necessary for adequate recovery and detection of signal by AMS can be approximated using several calculations. It is always advisable to perform initial experiments to optimize dosing, recovery, and detection parameters before beginning the full-scale AMS study. Performing these calculations and initial experiments will greatly increase the probability of obtaining meaningful results in the AMS study.

In estimating the doses of labeled compound that should be administered to achieve a desired degree of labeling in the collected sample, it is helpful to know the approximate bioavailability and volume of distribution of the compound under consideration. Although these parameters are unknown at the start of a phase 0 study, they can be estimated within one to two orders of magnitude for AMS signal-to-noise estimation. The type and quantity of tissue or other material (e.g., urine and feces) to be sampled should also be considered in the dosing calculation. For an experiment with an injected or highly bioavailable compound in which plasma is collected and the labeled compound is measured, the predicted optimal dosing level can be calculated as follows:

$$\text{Dose (in Bq)} = M_{\text{Target}} \times 226 \mu\text{Bq/mg C} \times V_{\text{Distribution}} \times C_{\text{plasma}}$$

where

M_{Target} = target fraction Modern in the collected sample, where
 $1 < M_{\text{Target}} < 100$

$V_{\text{Distribution}}$ = apparent volume of distribution for the compound (L)

C_{plasma} = carbon content of plasma (mg C/ μL).

The dose typically ranges from 300 Bq to 1.8 MBq. In the case of a compound with low bioavailability, the dose must increase according to the following equation:

$$\text{Dose (in Bq)} = M_{\text{Target}} \times 226 \mu\text{Bq/mg C} \times V_{\text{Distribution}} \times C_{\text{plasma}} \times (1/F)$$

where

F = bioavailability of the compound.

Predicted optimal doses can also be calculated for time points after administration of the labeled compound. In this case, the calculation is made based on the target fraction Modern for the final collection time point:

$$\text{Dose (in Bq)} = M_{\text{Final}} \times 226 \mu\text{Bq/mg C} \times V_{\text{Distribution}} \times C_{\text{plasma}} \times (1/F) \times e^N$$

where

N = number of elimination half-lives at the time of final sample collection.

These equations can be used for other materials (e.g., urine) by substituting the carbon content and tissue or fluid volume, as appropriate. When the target dose has been calculated in becquerel, the quantity of labeled compound (e.g., nmol) can be calculated based on the specific activity of the compound (nCi/nmol):

$$\text{Quantity(nmol)} = \text{dose} \times \text{specific activity}$$

The quantity of compound administered is typically in the range of 10–100 nmol.

For novel compounds, where little is known about bioavailability, volume of distribution, and elimination half-life, it may be necessary to perform initial range-finding experiments starting with low doses and working upward before conducting full-scale experiments. All samples with unknown levels of radioactivity should be quantified by LSC before graphitization and AMS analysis. Any sample that can be measured by LSC contains too much label to be measured by AMS.

Sample collection should be carried out using disposable materials, and care should be taken to avoid contamination or mixing of samples during collection, handling, and storage. Owing to the possibility of introducing contamination, separate sets of pipettes should be used for dosing experiments and for preparing samples for AMS analysis. If pipettes are used for dosing, they should not be used for general laboratory work or for preparing samples for AMS submission. Positive displacement pipettes and filter tips should be used at all times in all stages of experimental work and sample preparation to avoid the possibility of label carryover from one sample to the next. It is wise to always use new, disposable laboratory vessels and pipettes for all materials used in AMS experiments. This minimizes the potential for spread of contamination. For all experiments, control samples (predose or undosed) must be generated in order to provide a ^{14}C baseline for each experiment. Samples should be collected individually and sealed.

21.3.4.4 Sample Definition and Preparation. During the process of converting the sample material to graphite for AMS measurement, all chemical information contained in the original sample is lost. Thus, it is vitally important that the sample be adequately characterized before AMS analysis. This characterization will be specific for each type of sample and may in many cases be as simple as collecting and preparing a selected tissue or fluid (plasma, whole blood, urine, etc.). More complicated experiments may require additional separation and characterization techniques, including mass spectrometry, HPLC, flow cytometry, ELISA, isoelectric focusing, immunoblotting, or other analytical techniques, as needed to adequately separate and confirm the identity of the parent compound and metabolites.

It is essential to track all additions of carbon-containing compounds during the biological experiments and during sample preparation in order to produce meaningful data by AMS, since both total carbon and ^{14}C are measured to generate an isotope ratio. With some samples, it may be impossible to achieve the desired carbon amount from the experimental preparation. In this case, it may be necessary to add “carrier carbon” to these ultralow carbon mass samples. The primary source of carrier carbon now in use is tributyrin (glycerol tributanoate). This substance has the desirable characteristics of nonvolatility (bp $>300^\circ\text{C}$), high carbon content, and low ^{14}C content. A typical addition of carrier carbon occurs by adding 1 or $2\mu\text{l}$ of tributyrin in capillary tubes to the biological sample. It is always advisable to analyze each new type of sample for carbon content before graphitization to ensure that a proper carbon inventory is maintained.

21.3.4.5 Data Analysis and Interpretation. AMS reports result in Modern, which is a defined unit of $^{14}\text{C}/\text{C}$ isotope concentration equal to

- $1.180 \times 10^{-12} \text{ }^{14}\text{C}/\text{C}$,
- 13.56 mdpm/mg C,
- 226 $\mu\text{Bq}/\text{mg C}$, and
- 97.89 amol/mg C.

Conversion of this number to a meaningful result requires careful inventory of the carbon sources in the sample. For the simplest case in which a sample contains sufficient carbon as not to require the addition of carrier carbon, this conversion is facile. In many experiments, it is necessary to add carrier to make up the total amount of carbon necessary for the graphitization process to occur. In these experiments, the fraction Modern and the carbon mass of both the sample and the carrier must be considered in the calculation. Correctly accounting for all sources of carbon in the analyzed sample is essential, as a low estimate of carbon will artificially increase the apparent quantity of tracer, and an overestimate of carbon will deflate the apparent amount of tracer present.

The measured ratio is a composite value that includes the total amount of ^{14}C and the total carbon present in the measured sample. It is necessary to know the quantity of carbon and ^{14}C in the sample tissue, as well as the amount of carbon due to the tracer, in order to solve for the ^{14}C due to the tracer. If the only sources of carbon in the measured sample are the tracer and the tissue, determining the ^{14}C due to the tracer proceeds as follows:

$$R_{\text{Measured}} = \frac{{}^{14}\text{C}_{\text{Measured}}}{\text{C}_{\text{Measured}}} = \frac{{}^{14}\text{C}_{\text{tracer}} + {}^{14}\text{C}_{\text{tissue}}}{\text{C}_{\text{tracer}} + \text{C}_{\text{tissue}}} \quad (21.1)$$

In most cases, the carbon due to the tissue is much greater than the carbon due to the tracer, so that the tracer carbon can be neglected in the calculation:

$$R_{\text{Measured}} = \frac{{}^{14}\text{C}_{\text{tracer}} + {}^{14}\text{C}_{\text{tissue}}}{\text{C}_{\text{tissue}}} \quad (21.2)$$

Because the measured fraction Modern is a composite of the contribution of the tissue and the contribution of the tracer, rearranging to solve for the tracer ${}^{14}\text{C}$ gives

$${}^{14}\text{C}_{\text{tracer}} = \text{C}_{\text{tissue}} \times (R_{\text{measured}} - R_{\text{tissue}}) \quad (21.3)$$

where

$$R_{\text{tissue}} = \frac{{}^{14}\text{C}_{\text{tissue}}}{\text{C}_{\text{tissue}}} \quad (21.4)$$

If carrier carbon is also present, the general equation is as follows:

$$R_{\text{Measured}} = \frac{{}^{14}\text{C}_{\text{tracer}} + {}^{14}\text{C}_{\text{tissue}} + {}^{14}\text{C}_{\text{carrier}}}{\text{C}_{\text{tracer}} + \text{C}_{\text{tissue}} + \text{C}_{\text{carrier}}} \quad (21.5)$$

In this case, the carrier carbon is usually much greater than the carbon due to the tissue or the tracer, allowing these quantities to be neglected in the calculation:

$$R_{\text{Measured}} = \frac{{}^{14}\text{C}_{\text{tracer}} + {}^{14}\text{C}_{\text{tissue}} + {}^{14}\text{C}_{\text{carrier}}}{\text{C}_{\text{carrier}}} \quad (21.6)$$

which becomes

$${}^{14}\text{C}_{\text{tracer}} = \text{C}_{\text{carrier}} \times [R_{\text{measured}} - (R_{\text{tissue}} + R_{\text{carrier}})] \quad (21.7)$$

21.3.5 Specific Applications of Accelerator Mass Spectrometry

AMS excels at detecting very low levels of labeled compounds of interest and has been applied to a variety of types of experiments applicable to drug development. The common feature of these experiments is the ability to trace the fate of a labeled compound in a biological system. This can include determination of PK parameters, biotransformation of the labeled compound, formation of covalent adducts with cellular macromolecules, drug–ligand interactions, and tissue or subcellular localization of the compound of interest. Such experiments can provide valuable information about the pharmacological and toxicological characteristics of investigational compounds. AMS may likewise be used to investigate metabolism of endogenous, naturally occurring compounds within a biological system without perturbing the normal physiology of the system. The types of experiments listed below are by no means exhaustive but are intended to give the reader a sense of the potential value of AMS in the drug development process.

21.3.5.1 Pharmacokinetics. Several studies have compared the PK profiles of drugs using standard therapeutic doses and microdoses [14,21,26,31,45,51,67,68]. In these microdose experiments, a subtherapeutic level of a ^{14}C -labeled investigational compound is administered to animals or human volunteers, and the plasma levels of the compound are measured over time to determine the PK parameters of the compound. These experiments are essentially the same as traditional “dose and measure” PK experiments, but the investigational compound can be administered and measured at much lower levels than are possible using standard techniques. This allows for human studies to be performed without the risk of harmful effects that could occur with the administration of higher doses. While the utility of such microdosing experiments as a replacement for standard PK techniques remains a matter of discussion, several reports indicate that microdosing may be useful as a screening tool for drug candidates [14,21,26,67,69]. Microdosing may be particularly useful when a drug candidate presents with conflicting *in vitro* and animal PK data [67]. An understanding of the PK parameters of a drug candidate may disqualify the compound for further investigation based on a poor PK profile or may provide guidelines for predicting optimal doses within the therapeutic range. The plasma concentrations following oral and IV therapeutic (50 mg) and microdoses (100 μg) of sumatriptan are shown in Fig. 21.2 [26]. Despite the doses being different by a factor of 500, the dose normalized curves are very similar with the maximum plasma concentration occurring at about an hour. The biological half-life, volume of distribution, and clearance were all similar also [26]. The oral microdose had better bioavailability (20%) than the therapeutic dose (7.6%) [26].

In planning and conducting PK studies using AMS as a measurement technique, it is essential that an appropriate dose of the labeled material be administered to the test subject in order to allow accurate measurement at the selected time points. Assuming that the quantity of drug to be administered is significantly below the level required to elicit a pharmacological or toxic effect, dosing can be calculated based on the level of ^{14}C required to detect an adequate signal in the collected sample. If higher levels of drug are to be administered, the dose should be calculated based on the therapeutic dose

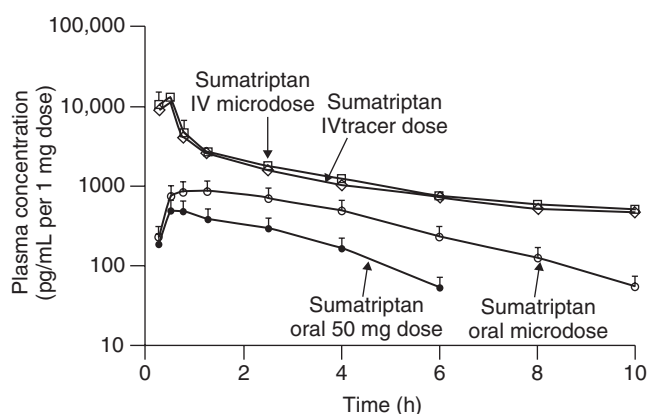


Figure 21.2 Semilog plots of mean plasma sumatriptan concentration–time profiles following a single oral dose of 100 μg ($\circ$), a 30-min IV infusion of 100 μg ($\square$), a single oral dose of 50 mg ($\blacklozenge$), and a 30-min IV infusion of 100 μg with a simultaneous oral dose of 50 mg ($\diamond$). Data are dose normalized to a 1-mg dose and error bars are ± 1 standard deviation. Source: (a) p. 144 from Lappin *et al.* [26].

range in addition to the radiological dose. The general steps involved in performing a PK study using AMS are as follows:

- Determine the specific activity of the ^{14}C -labeled compound of interest to be used in the PK study.
- Determine the mode(s) of administration of the compound of interest (injection, oral, dermal, subcutaneous, etc.). If appropriate, estimate the bioavailability of the compound.
- Determine the anticipated length of time the compound should be followed, the number of samples to be collected (blood and urine) and the sampling interval.
- Determine the minimum target fraction Modern to be achieved in the sample (blood and urine) at the final time point. In most cases, this level should be at least 10% above 1 Modern in order to allow appropriate resolution.
- Calculate the initial dose based on the estimated number of biological half-lives of the compound to produce the desired ending fraction Modern. Determine the total radiological exposure to be achieved using the calculated initial dose, assuming that the subject will absorb all radioactive decay energy. Consult with a health physicist to ensure that this level of exposure is acceptable based on current regulations and requirements. For purposes of assessing the radiological burden, a human can be assumed to have a normal ^{14}C level of 1 Modern, which for a 70-kg person corresponds to an energy deposit of 110 nJ/h, and assuming that the radiological dose is uniformly distributed throughout the body, this corresponds to 1.6 nSv/h [54].
- Use animal dosimetry data to initially estimate a compound's bioavailability and biological half-life, calculate the initial dose based on the target highest amount of label to be present in the sample, and proceed to calculate the total radiological exposure.
- The initial samples collected should be measured by LSC to ensure that the calculations were correct and that the level of dosing was correct for AMS measurement.

21.3.5.2 Biotransformation and Conjugate Formation. Identification of metabolites of a drug candidate or other molecule of interest can be greatly facilitated by AMS. Tracing of a ^{14}C label allows unequivocal identification of compounds originating from the ^{14}C -labeled parent compound, including metabolites and degradation products, even if the structures of these compounds are not previously known. This type of experiment may be performed by collecting blood and urine and separating components by HPLC or other separation techniques and identifying the parent compound as well as any metabolite or conjugate by measuring ^{14}C in the HPLC fractions.

Appropriate separation techniques are essential for the identification of metabolites. Although the presence of label introduced as the parent compound unambiguously demonstrates the source of the label, it is important to ensure that the collected material is reasonably pure to allow further characterization of novel metabolites. In many instances, the metabolites of a compound of interest may be unknown or incompletely characterized. A typical metabolism study consists of the following components:

- Characterize chemical purity, radiopurity, and specific activity of the ^{14}C -labeled compound of interest.

- Administer the labeled material and collect samples (blood, urine, tissue, etc.).
- Perform mass balance to account for all labeled material.
- Separate metabolites by standard techniques (HPLC, electrophoresis, etc.).
- Remove any solvents used in separations processes and analyze the sample fractions by AMS, adding carrier as necessary. In the case of HPLC fractions, the amount of carbon present in each sample is usually negligible compared to the amount of carrier carbon.
- Analysis occurs by comparison of cochromatography with authentic standards of detector peaks on the HPLC (e.g., UV) with AMS results to identify fractions containing the parent compound, its metabolites, and conjugates.

Glucuronide conjugation occurs with many drugs and it can be the single largest metabolite as shown in Fig. 21.3 [45]. Following a microdose of ^{14}C -acetaminophen, parent compound and major metabolites were separated by LC and fractions were analyzed by AMS [39]. In many studies, unexpected metabolites or conjugates are formed and labeled peaks elute at times different than the available standards [29,56]. Careful analysis of the elution spectrum can help narrow the possible metabolites. The ^{14}C signal clearly distinguishes the peak as deriving from the test compound and not an endogenous compound that happened to elute.

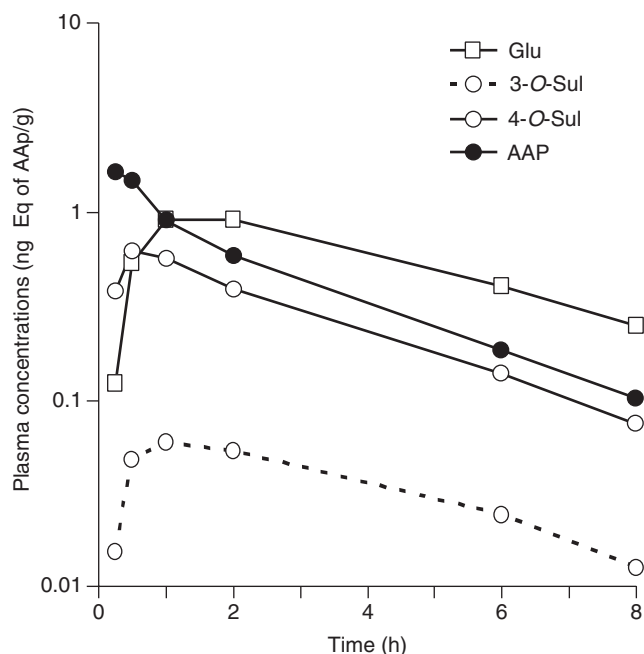


Figure 21.3 Time profiles of acetaminophen (AAP) and its metabolites in the pooled plasma of six subjects after oral administration of ^{14}C -AAP. Pooled plasma specimens collected at 0.25, 0.5, 1, 2, and 8 h were subjected to LC-AMS analysis. 3-*O*-Sul, AAP-3-hydroxysulfate; 4-*O*-Sul, AAP-4-hydroxysulfate; AAP, acetaminophen; Glu, AAP-glucuronide; LC-AMS, liquid chromatography–accelerator mass spectrometry. *Source*: p. 221 from Bauer *et al.* [85].

21.3.5.3 Protein and DNA Adduct Measurement for Determination of Reactive Metabolites. Incorporation of label into protein or DNA is a useful indicator of adduct formation or noncovalent binding of the moiety of interest. This approach has been used very successfully in the evaluation of known or potential carcinogens that exhibit DNA-binding activity [47,48,70,71]. This type of experiment may be particularly valuable in assessing the potential toxicity, mutagenicity, or carcinogenicity of a compound of interest. For example, AMS was used to detect DNA adducts in the colon after humans were exposed to a dietary-relevant dose of the cooked food carcinogen PhIP [47]. It may also be important to determine the quantitative fate of compounds that can form reactive intermediates capable of binding to cellular macromolecules, for example, compounds that can form immunogenic haptens or glutathione conjugates. Experiments of this type can also be designed to distinguish covalent and noncovalent interactions. As in the types of experiments described in previous sections, it is absolutely essential that the sample be thoroughly characterized and as pure as is reasonably achievable to ensure that results are not confounded by the presence of contaminating materials. These experiments include the following parameters:

- Determine the tissue to be sampled.
- Calculate the dose of compound to administer and the quantity of tissue to collect.
- Administer the dose and collect sample material.
- Separate and quantify the biomolecule of interest (protein and DNA).
- Perform AMS measurement on the sample.

21.3.5.4 Drug–Ligand Binding and Subcellular Localization. Tracing a radiolabel compound into tissues, cells, and even subcellular compartments is possible using AMS [69,72,73]. These experiments may be of great importance in determining the site of action and specific molecular target(s) of a drug or other compound of interest. Identifying the site of action or the molecular target of a drug candidate is very important in characterizing its biological activity and elucidating its mechanism of action. Such experiments could be a valuable screening tool to help in the selection of lead compounds for development as investigational drugs or to indicate that a compound has undesirable binding or localization characteristics early in the drug development process. As with other types of experiments, the sample material must be thoroughly characterized before AMS analysis. The steps involved in these types of experiments are similar to those described in the previous section but may include cellular fractionation in addition to isolation of target biomolecules.

21.4 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY IN MICRODOSING

21.4.1 Overview of Liquid Chromatography-Mass Spectrometry

In addition to AMS, liquid chromatography-mass spectrometry (LC-MS) is also used to analyze samples in microdosing studies. Each method provides advantages but also has limitations. The previous section of this chapter described in detail the use of

AMS for microdosing experiments. This section describes the use of LC-MS/MS in microdosing studies, highlighting its advantages and disadvantages compared to AMS.

All microdosing experiments require analytical methods capable of identifying and quantifying the analyte(s) of interest in biological samples at low levels. In some cases, it may only be necessary to measure the administered (parent) compound. More often, the parent compound forms one or more metabolites that must be characterized and measured. Metabolites of interest may be formed at low concentrations relative to the parent compound. This can present significant analytical challenges in microdosing studies, because the parent compound is administered at low doses that may already be near the lower limit of quantitation (LLOQ) for most instruments. Thus, a method that is suitable for measurement of a parent compound in a microdosing study may not provide adequate sensitivity to measure low abundance metabolites, particularly those metabolites with uncharacterized structures [74]. AMS provides unrivaled sensitivity for measurement of ^{14}C -labeled compounds in biological samples and can detect labeled metabolites in an LC elution with *a priori* knowledge, but it provides only quantitation. Samples measured by AMS must be structurally characterized using other analytical methods. In contrast, LC-MS can identify compounds of interest, and can quantify material, but lacks the sensitivity of AMS. The specific questions that need to be answered, and the level of sensitivity required to answer those questions, must guide the researcher's choice of analytical methods to be used in microdosing studies.

21.4.2 Advantages and Limitations of Liquid Chromatography-Mass Spectrometry

Some general considerations can be applied in selecting the appropriate analytical methods for use in microdosing experiments. These considerations include types of instrumentation available and the capabilities of each instrument, the level of sensitivity needed, analytical methods that must be developed to enable microdosing studies, dosing considerations, and cost of performing microdosing studies. It should be noted that many studies have used both LC-MS and AMS to obtain the desired information.

21.4.2.1 Instrumentation. Many research facilities have LC-MS equipment, while very few have AMS instruments in-house. Both types of instrumentation require skilled and knowledgeable operators, and each instrument requires a specific type of sample preparation. AMS instruments measure samples in the form of graphite or CO_2 gas, while LC-MS instruments measure samples that are injected into the instrument in liquid form. Methods for preanalysis sample cleanup and processing will depend on the needs of the particular experiment being performed. In addition to providing quantitative information, LC-MS allows molecular characterization of parent drug and metabolites. Unlike LC-MS, AMS requires the use of a ^{14}C -labeled test compound and gives results in the form of an isotope ratio. AMS provides quantitation and requires that analytes be separated and characterized by other means before AMS analysis. AMS measures analytes that contain the ^{14}C label irrespective of their chemical structures, which can result in measurement of both parent compound and metabolites if adequate separation of these compounds is performed before AMS analysis. LC-MS can distinguish between a parent compound and its known metabolites with different m/z ratios but may not be sufficiently sensitive to detect low abundance metabolites.

Through chromatographic retention time and MS/MS, an observed m/z can be compared to a reference standard of the parent. LC-MS cannot resolve endogenous isobaric interferences, however, so discovering and identifying unexpected metabolites is very difficult. In detecting metabolites by LC-MS, it is helpful for the researcher to know what types of metabolites are likely to occur in order to optimize instrument parameters for the measurement of each metabolite.

21.4.2.2 Sensitivity. The typical analyte concentration range for LC-MS instruments is in the order of nanograms per milliliter sample [74–76], although some instruments can measure analytes in the picogram to femtogram per milliliter range [76,77]. In contrast, AMS instruments provide the highest level of sensitivity currently available and can measure from analytes in the range of femtograms to attograms per milliliter [74,75,78]. While AMS allows greater sensitivity than any other available methods, it is also extremely sensitive to minute quantities of contaminating material, necessitating great care in performing experiments and handling samples. Depending on the design of the study, the utility of AMS may be limited by the fact that all sources of ^{14}C label are measured, so that both the parent compound and the metabolites are measured [77]. Thus, caution must be exercised in interpreting the results of those microdosing studies in which parent compound and metabolites are not adequately separated and characterized. This problem can often be circumvented by the use of HPLC separation of the parent drug and its metabolites, followed by AMS analysis of each collected fraction. However, collection of HPLC fractions can be problematic due to the possibility of peak overlap [77].

21.4.2.3 Analytical Method Development and Sample Preparation. Both AMS and LC-MS methods typically require methods for extracting the test compound or its metabolites from a biological matrix such as plasma or urine. AMS has the advantage of being able to detect and quantify the ^{14}C -labeled test compound in the biological matrix without extraction if quantitation is all that is desired. AMS analysis does not require the use of internal standards or the generation of standard curves for the analyte(s). LC-MS requires the development and validation of an analytical method with a known linear range and lower limit of detection for each analyte and makes use of internal standards for analyte quantitation. MS can be linked directly to chromatography, unlike AMS, thereby mitigating concerns about loss of material as could occur in pre-AMS chromatography and fraction collection. Both AMS and LC-MS may require considerable sample processing before analysis. For AMS analysis, the possibility of contaminating samples with ^{14}C from other sources must be minimized.

21.4.2.4 Dosing Considerations. While both LC-MS and AMS microdosing studies involve the administration of small quantities of a substance to test substance, the test compound in AMS experiments contains a ^{14}C label, which presents extra challenges to ensure that the label is in a structurally stable position. The additional sensitivity provided by the use of a radioisotope-labeled test compound can enable detection of low abundance metabolites, as discussed above, and can allow more frequent sampling than would be possible if LC-MS detection was used. The ability to measure very low levels of analyte by AMS can also enable experiments of greater duration than is possible with LC-MS-based experiments. Conversely, the potentially long washout time for ^{14}C -labeled test compounds may limit the use of multiple dosing experiments or crossover studies.

21.4.2.5 Cost. In addition to the direct sample analysis costs, the time required to prepare test material, design and perform experiments, and complete analyses affects the total cost of microdosing studies. In-house equipment can speed up experiments and give results in a shorter time frame than is typically possible with AMS experiments. The high sensitivity that can be achieved by AMS requires a ^{14}C -labeled test compound, which increases the cost and time necessary to complete microdosing experiments. Additionally, AMS analysis following HPLC separation and fraction collection is limited by the relatively low throughput of this technique. AMS-based microdosing experiments are inherently more expensive than LC-MS-based experiments, but AMS can provide significant advantages that offset the additional costs. In some cases, AMS is the only analytical method available that has the sensitivity to provide the needed information.

21.4.3 Examples of Liquid Chromatography-Mass Spectrometry Microdosing

The ability to simultaneously identify and quantify a circulating drug or metabolite has made LC-MS microdosing an attractive alternative to AMS-based microdosing experiments. While not all drugs are suitable for LC-MS microdosing studies, several proof-of-principle experiments have been performed to demonstrate that LC-MS microdosing can be a viable method. The continuous improvements in instrument sensitivity made in recent years have enabled detection and quantitation of compounds at levels that were not previously possible. While quantitation by mass spectrometry is inherently problematic, it may be adequate in many cases to answer the critical questions in microdosing studies.

Ni *et al.* [68] investigated the utility of microdosing using LC-MS/MS by measuring rat plasma and urine metabolites using the drugs atorvastatin, ofloxacin, omeprazole, and tamoxifen with administered doses ranging from $1.67\text{ }\mu\text{g/kg}$ (microdose) to $5000\text{ }\mu\text{g/kg}$ (standard dose). Their analytical instrumentation included a 5500 QTRAP SYSTEM (AB Sciex, Concord, ON, Canada), with chromatographic separation on either a Shimadzu Prominence instrument (Columbia, MD) or an Agilent 1200 system (Palo Alto, CA). Quantitation was obtained using Analyst software (version 1.5, AB Sciex, Concord, ON, Canada), and metabolites were identified using LightSight software, version 2.2 (AB SCIEX). In the microdosing experiments, the researchers were able to characterize the hydroxylation metabolite of omeprazole, the hydration metabolite of tamoxifen, and the glucuronide metabolite of ofloxacin, while additional metabolites were identified at higher doses. These researchers reported that low dose PK parameters were not necessarily predictive of PK parameters at higher doses in the rodent model [74]. Similar experiments using additional drugs provided further support for the use of LC-MS/MS for microdosing experiments [79].

Yamane *et al.* [80] used LC-MS/MS to measure nicarbidine in humans and developed a method capable of measuring in the linear range of $1\text{--}500\text{ pg/mL}$ for a microdose and up to $0.2\text{--}100\text{ ng/mL}$ plasma for a clinical dose. This experiment used a microdose of $100\text{ }\mu\text{g}$ per patient and a clinical dose of 20 mg . They used mass spectrometry to identify metabolites following the microdose and compared these results with the metabolites identified using the clinical dose [80]. Yamazaki *et al.* [81] performed microdose studies in eight humans with $100\text{ }\mu\text{g}$ of fexofenadine and found that microdose PK measured using LC-MS predicted the PK of a 60-mg therapeutic dose satisfactorily.

Balani *et al.* [75] demonstrated that useful PK data could be obtained for the drugs fluconazole and tolbutamide using a microdose of 1 $\mu\text{g/kg}$ in rats and compared the linearity of PK with oral doses of 0.002, 0.01, 0.1, and 1 mg/kg of tolbutamide or 0.005, 0.05, and 5 mg/kg of fluconazole. Using LC-MS analysis, they reported 0.1 nM as the LLOQ for fluconazole and 1 nM as the LLOQ for tolbutamide. On the basis of adequate detection of test compounds by LC-MS and linear PK between microdoses and higher doses for both drugs, it was suggested that LC-MS/MS could provide adequate sensitivity for some human microdosing studies [75].

21.4.4 Summary

The decision to use LC-MS/MS or AMS must be informed by knowledge of the advantages and limitations of each method and primarily by the questions the microdosing study need to address. For further guidance in designing microdosing experiments, the interested reader is referred to the excellent review by Ings [77]. As the development of mass spectrometers with improved sensitivity continues, LC-MS is likely to find additional applications in microdosing experiments, but AMS is expected to remain the method of choice for many microdosing applications requiring high sensitivity.

21.5 POSITRON EMISSION TOMOGRAPHY IN MICRODOSING

21.5.1 Overview of Positron Emission Tomography

PET is widely used in medical imaging applications. In PET, a radioisotope that decays by positron emission is used to determine the location of the isotope in the test subject. Positrons react with electrons in tissue to annihilate each other and release two 511-keV photons at 180° from each other. An array of photon detectors located around the subject measure the simultaneous photons, and computers are used to reconstruct the position of the decay within the tissue with spatial resolution of $\sim 4\text{ mm}$ [22,82]. In microdosing applications, PET can be used to monitor distribution and clearance of a test compound in real time.

Positron-emitting radioisotopes need to be produced with a cyclotron and are short lived. The common positron-emitting isotopes for microdosing studies are ^{11}C (radioactive half-life $T_{1/2} = 20\text{ min}$) and ^{18}F ($T_{1/2} = 110\text{ min}$). ^{11}C is most useful since nearly all drugs contain carbon. Because of the short radioactive half-lives, cyclotrons must be located at medical facilities. After production of ^{11}C , it is typically exchanged with ^{12}C in a drug molecule in high activity radiochemistry facility also on-site to produce specific compounds that can be distributed and traced. The short radioactive half-lives require that the chemistry is rapid, and significant shielding is needed to protect chemists synthesizing the compounds. Finally, a PET scanner must be available on-site to measure the photons produced by positron–electron annihilation. After seven radioactive half-lives, an isotope decays to about 0.7% of its initial activity and is usually too weak for useful tracing. For ^{11}C , this is only 2.5 h, limiting PET analyses to initial distribution and rapidly clearing compounds. Since PET depends on the radioactive decay for measurement, it delivers a low to moderate radiation dose to test subjects. PET studies generally describe a normalized tissue deposition of a compound using standardized uptake values (SUVs). SUVs normalize the accumulated compound

in a tissue as measured by PET to the body weight of the subject and the injected dose [83]. Traditional PK parameters can be measured shortly after administration of the labeled tracer before its activity drops below quantitation limits.

21.5.2 Examples of Positron Emission Tomography Microdosing Studies

The advantage of PET over AMS and LC-MS is real-time visualization of distribution and concentration of the test compound in all tissues. PET can provide some PK information, but the rapid decay of the radioisotopes can only assess rapidly cleared compounds and metabolites. In general, PET is ill suited for metabolite analysis because of the time required for analysis and the low levels of compound in the small samples analyzed. The development of robust ultra performance liquid chromatography (UPLC) systems in the past few years with much shorter elution than traditional HPLC system may make PK of positron-emitting isotopes more practical. PET does not provide precise quantitation like AMS or structural information like LC-MS, it tells you if the test compound is concentrating in a particular tissue. The *in vivo* visualization is particularly useful when targeting a specific tissue such as CNS or diseased tissue such as cancer [84].

21.5.2.1 Tracing Across the Blood–Brain Barrier. PET has been used on several occasions to monitor the transport of a compound across the blood–brain barrier (BBB). A microdose study was conducted with the ^{11}C -labeled antiamyloid drug (ST1859) in healthy and patients afflicted with Alzheimer’s disease (AD) [85]. The test compound crossed the BBB relatively quickly and slows to washout in both control and AD groups, good qualities for a compound targeted at amyloid plaques. The study was not designed for detecting differences in the control and AD groups, but it appeared as though differences were present (Fig. 21.4). The AD group had an earlier maximum concentration in most brain regions and higher radioactivity in the brain than the control, suggesting differences in the BBB of the two groups [85].

A comparison of therapeutic dose (80 mg) and microdose (0.05 mg) of verapamil labeled with both ^{11}C and ^{14}C for PET and AMS analyses was conducted to investigate linearity [21]. PET measured similar rate constants across the BBB and distributed volumes for each dose [21]. ^{14}C concentration–time profiles in plasma were also similar for each dose [21]. A much earlier study with [^{11}C]raclopride saw similar cerebellum to blood concentrations with a microdose (1–2 μg) and a pharmacological dose (200–400 μg) [86].

21.5.2.2 Targeting Cancer. The development of new cancer drugs depends on identifying markers of the cancer and concentrating the drug at a tumor site compared to systemic circulation. PET is ideally suited for assessing drug distribution *in vivo* and determining if a cancer drug does indeed target the tumor preferentially compared to healthy tissue. PET microdosing offers the potential to obtain PK parameters in individual patients before commencing a therapeutic dose regimen or a noninvasive way to assess response or stage of a tumor with specific markers [84,87].

Although not strictly a microdose application, small doses of 16α - ^{18}F -fluoro-17 β -estradiol (FES) have been used to assess estrogen receptor (ER) status [88]. ER status of breast cancer can be assessed *in vivo* because FES is a ligand for ER [88]. Additionally, ^{11}C -colchicine has been used in preclinical PET studies to image multidrug resistance

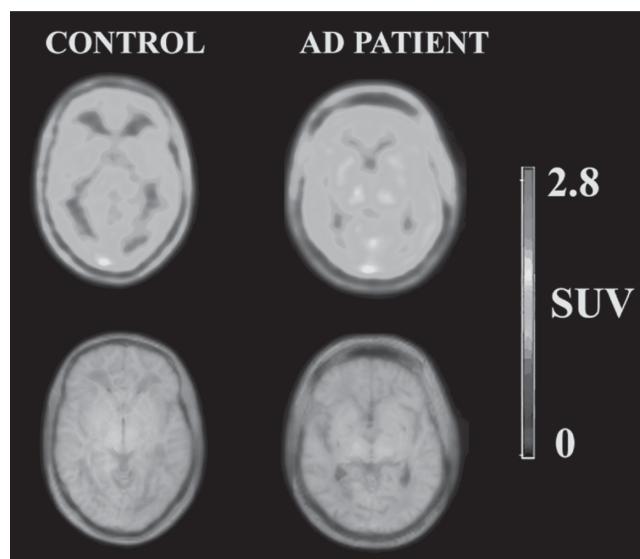


Figure 21.4 Transaxial magnetic resonance–coregistered positron emission tomography (PET) summation images recorded from 20 to 90 min after intravenous injection of [^{11}C]ST1859 into 1 control subject (HV 1) (left) and 1 AD patient (patient 9) (right). The lower row shows the same PET images with the transparency of the superimposed magnetic resonance scan set to 50%. The radioactivity concentration is normalized for injected radioactivity per body weight and expressed as the SUV. *Source:* p. 829 from Tozuka *et al.* [45]. (See color insert.)

and identify tumors that will not respond to some chemotherapy agents [89]. ^{18}F -Fluoride has also been used for bone imaging [90].

An early pre-phase I study that used one-thousandth of the phase I starting dose of ^{11}C -N-[2-(dimethylamino)ethyl]acridine-4-carboxamide showed tissue distribution, tumor concentration, and early time point PK data were the first microdose PET study [91]. Combining PET with microdialysis to assess unbound drug in extracellular spaces has been done and may become more common in the future [92].

21.5.3 Summary

PET provides unparalleled *in vivo* distribution information with very low chemical doses. The short radioactive half-lives of positron emitters prevent acquisition of PK information in many cases.

21.6 CONCLUSION

Each bioanalytical technique employed in microdosing studies has its strengths and weaknesses (Table 21.2). LC-MS/MS is the least expensive and most widely available of the techniques. In most cases, if LC-MS/MS has the sensitivity to measure the test compound accurately, it is the preferred method. LC-MS/MS requires *a priori* knowledge of metabolites, however, and cannot definitively distinguish test compound from other molecules. AMS is most quantitative of the techniques. It is expensive to use and relatively few facilities are in operation. PET is at best semiquantitative, but

TABLE 21.2 Comparison of Bioanalytical Methods Suitable for Microdosing Studies

Attribute	AMS	LC-MS/MS	PET
Sensitivity limit (g)	10^{-18}	10^{-12}	10^{-14}
Linear range	Five to six orders of magnitude	Three to four orders of magnitude	Three to four orders of magnitude
Sample matrix measured	Plasma PK only	Plasma PK only	Plasma and tissue PK
Tissues sampled	Blood, urine, skin, saliva, exhaled CO ₂ , cerebrospinal fluid, feces, biopsy when available	Blood, urine, skin, saliva, cerebrospinal fluid, feces, biopsy when available	Tissues imaged, no sampling required
Radiation dose	Very low, generally microsievert	None	Low to moderate, millisievert
Radiolabel required	Long-lived β -emitter, ¹⁴ C	None	Short-lived β + emitter, ¹¹ C or ¹⁸ F
Chemical separation for metabolite analysis	Chemical separation of drug and metabolites possible. Metabolites quantifiable without analytical standards	Chemical separation of drug and metabolites possible. Structural information of metabolites possible. Metabolite quantification requires analytical standards	No chemical separation of tissue signal
Drug administration	Intravenous or oral usually, dermal and inhalation possible	Intravenous or oral usually, dermal and inhalation possible	Intravenous usually, oral or inhalation possible
Sampling periods	Limited by plasma clearance, minutes to months	Limited by plasma clearance and sensitivity, minutes to days	Limited by short half-life of positron emitters, up to 1.5 h
Costs	High cost, limited availability	Low cost, greater availability	High cost, limited availability
Outsource	Analysis can be contracted	Analysis can be contracted	Analysis must be done in-house

in vivo imaging allows analyses of distribution and accumulation of test compounds at target sites. Combining PET with LC-MS/MS or AMS provides the most information in a microdose study.

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22 Bioactivation and Reactive Metabolite Assays

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22.1 Introduction: formation of reactive metabolite and drug-induced adverse effects	1
22.2 Detection of peptide and protein adducts	4
22.3 Semiquantitative determination of bioactivation	19
22.4 A generic protocol for <i>in vitro</i> trapping study	21
22.5 Data interpretation and utilization	21
Acknowledgment	24
References	24

22.1 INTRODUCTION: FORMATION OF REACTIVE METABOLITE AND DRUG-INDUCED ADVERSE EFFECTS

Drug molecules are typically subject to biotransformation, that is, metabolism, once administered. Drug metabolism usually serves as a defense/detoxification mechanism, converting a drug molecule to hydrophilic metabolites, followed by removal of the latter from the circulation through urinary and/or biliary (via fecal) excretion [1,2]. However, drug metabolism sometimes becomes a process of “metabolic activation” or “bioactivation,” generating chemically reactive species such as α,β -unsaturated carbonyls, epoxides, isocyanates and isothiocyanates, quinones, quinone imines, and quinone methides. These metabolites are electrophiles capable of modifying proteins or nucleic acids, forming covalent chemical bonds with amino acid (e.g., cysteine, tyrosine, and lysine) residues or nucleoside base (e.g., adenine and guanine) components (Fig. 22.1a). The underlying mechanisms of these chemical reactions are often nucleophilic addition and substitution in nature. Structurally altered proteins and nucleic acids could lead to perturbed cellular functions, and consequently, drug-treatment-related AEs. Drug-metabolizing enzymes that catalyze the formation of reactive metabolites include cytochrome P450s (P450s), peroxidases, amine oxidase, and flavin-containing monooxygenase. Because these enzymes are proteins, they are sometimes prone to structural modification by reactive metabolites formed within their respective active sites, resulting in the so-called suicidal enzyme inactivation. For P450s, enzyme inactivation could also occur through heme alkylation or heme-iron

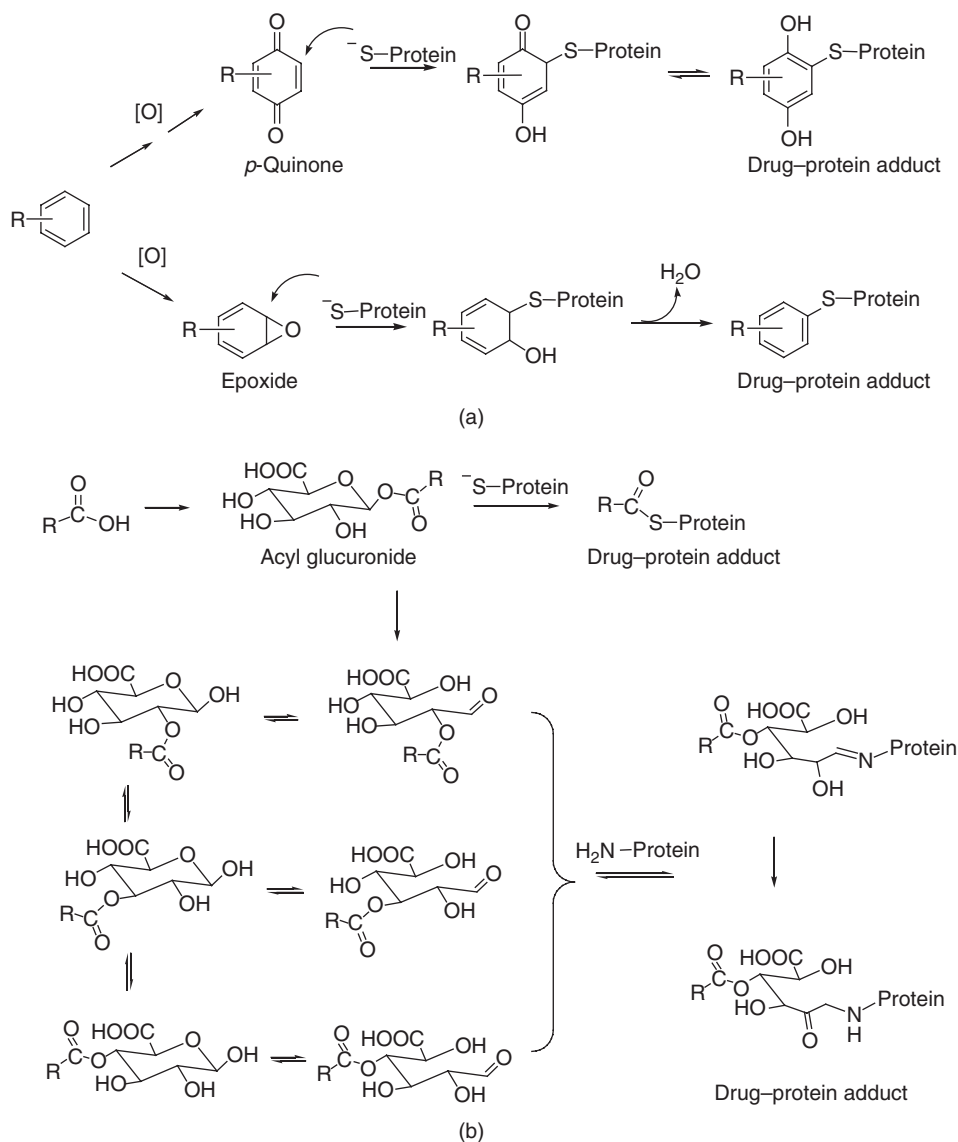


Figure 22.1 (a,b) Schematic representation of bioactivation and subsequent modification of proteins.

chelation. A covalently modified drug-metabolizing enzyme has the potential to cause drug treatment-related AEs not only by invoking a direct cytotoxic effect but also via drug-drug interactions due to diminished enzyme activity.

Parent drugs and their metabolites containing a carboxylic acid, alcohol, phenol, or amino functional group may undergo glucuronidation, sulfation, or acetylation. These biotransformation reactions occur in the presence of UDP-glucuronosyltransferases (UGT), sulfotransferases (SULTs), and *N*-acetyltransferases, respectively. Acyl glucuronides have been regarded as reactive species, in that they may acylate proteins through two different pathways, one of which is via displacement of the glucuronic

acid moiety by a cysteine, tyrosine, or lysine residue. The other mechanism involves migration of the aglycone at the pyranose ring, followed by scission of the hemiacetal ether bond to afford an aldehyde that reacts with a lysine residue to form an imine species, further intramolecular rearrangement of which yields a stable 1-amino-2-keto protein adduct (Fig. 22.1b). Acetylation and sulfation also mediate the formation of reactive metabolites, with acetate or sulfuric acid serving as a leaving group. The resulting cation species are capable of electrophilic addition to proteins or nucleic acids.

Drug molecules and metabolic products are eliminated from the circulation via biliary and/or urinary excretion, both of which in many cases are facilitated by active transporters such as bile salt export pump (BSEP), multidrug resistance-associated proteins (MRPs), and multidrug resistance proteins (MDRs). For those transporter substrates that are chemically reactive (e.g., acyl glucuronides or reversible GSH adducts), they could react directly with the transporter proteins or modify proteins/nucleic acids localized in a distal tissue compartment following migration assisted by transporters. Because active transporters serve to regulate the disposition of both xenobiotic (e.g., food, drug, and environmental chemicals) and endogenous substances (e.g., bile acids, cholesterol, and lipids), covalent modification of transporter proteins has the potential to disrupt transporter functions and produce drug treatment-related AEs resulting from excessive accumulation of cytotoxins.

Although circumstantial in nature, bioactivation appears particularly relevant to drug-induced hepatotoxicity on the basis that drug metabolism usually takes place in liver and reactive metabolites could readily alkylate/acylate hepatic proteins upon their formation *in situ*. This proposed scenario of liver injury is consistent with the observation that ~50% of clinical acute liver failure cases are likely drug treatment related [3]. In this context, mitochondria may either be a target or serve as a mediator for hepatotoxins, as the organelle not only produces ATP and other essentials to support cell survival but also regulates the programmed cell death process, namely, apoptosis. Insult from reactive metabolites would therefore start with structural modification of mitochondrial proteins and/or mitochondrial DNA (mtDNA), triggering pathogenesis such as inhibition of fatty acid oxidation, perturbation of cellular calcium homeostasis, oxidative stress, formation of peroxynitrite that induces membrane permeability transition, and collapse of mitochondrial membrane potential [4,5]. An alternative mechanism of drug-induced hepatotoxicity involves immune responses invoked by the formation of drug-protein adducts, which activate antigen-presenting Kupffer and Ito cells and produce T cells and antibodies that are cytotoxic to hepatocytes [6,7]. This type of liver injury usually exhibits a delayed onset of clinical symptoms on initial exposure to the offending drug. However, the AEs are to occur rapidly in response to rechallenge with either the implicated agent or a drug whose metabolism produces a structurally similar reactive species. Such a characteristic appears to represent a hallmark of immune-mediated AEs resulting from drug treatment. Injured hepatocytes undergo apoptosis if mitochondria remain functional and generate ATP. Necrotic cell death takes place when the damage to mitochondria is overwhelming and the organelles cease providing energy.

In addition to covalent modification of proteins and/or nucleic acids, reactive metabolites can form thioether or thioester with the cysteine residue of GSH (Fig. 22.2), a tripeptide consisting of L- γ -glutamyl-L-cysteinyl-glycine (Glu-Cys-Gly).

4 BIOACTIVATION AND REACTIVE METABOLITE ASSAYS

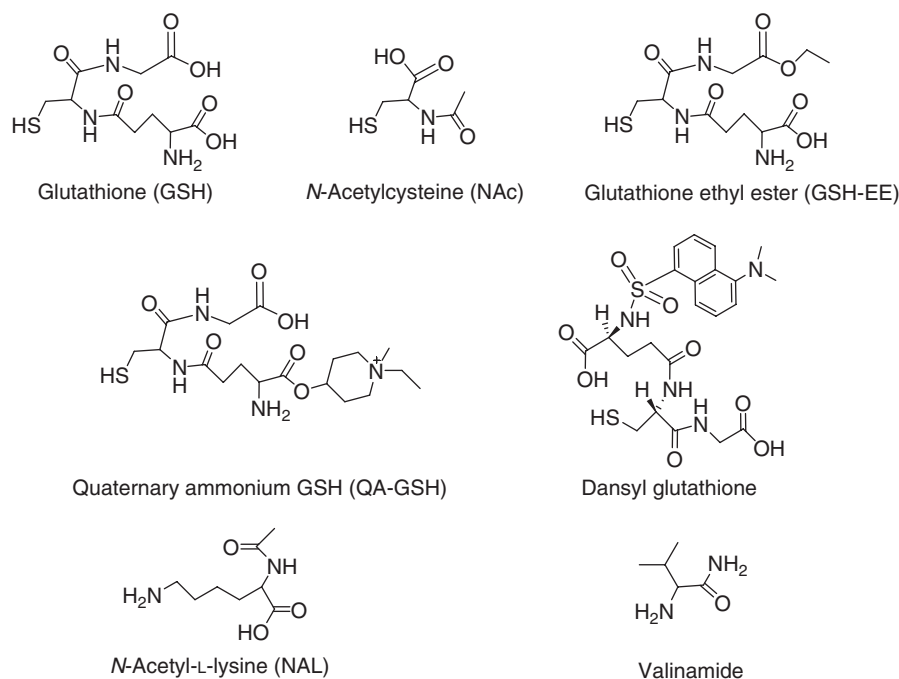


Figure 22.2 Structures of GSH and amino acid derivatives-related trapping agents.

This conjugation reaction may or may not require catalysis by glutathione-*S*-transferases (GSTs), depending on the chemical reactivity of the metabolites. The resulting GSH adducts are subject to hydrolysis of the tripeptide moiety, catalyzed by γ -glutamyltranspeptidase and dipeptidase. These hydrolytic reactions lead to the formation of cysteinylglycine and cysteine adducts, with the latter undergoing *N*-acetylation to afford the corresponding mercapturic acids [*N*-acetylcysteine (NAC) adducts]. Mercapturic acids usually are eliminated via urinary excretion. Although conjugation reactions with GSH generally represent detoxification, the thiol adducts sometimes serve as carriers for reactive metabolites, causing cellular damages in tissue compartments distal from the site where drug metabolism takes place. Possible scenarios include (i) retro-Michael reactions regenerating reactive species from which the GSH adducts are originated, (ii) further bioactivation of cysteine adducts via the β -lyase pathway, and (iii) exacerbation of tissue oxidative stress due to participation of thiol adducts in cellular redox cycling [8,9]. From a metabolite profiling perspective, identification of stable thiol adducts formed via the mercapturic acid pathway represents a bioanalytical option for the detection of reactive metabolites with short degradation half-lives in biological systems (see below).

22.2 DETECTION OF PEPTIDE AND PROTEIN ADDUCTS

Reactive species formed during drug metabolism processes usually are short lived, with their fate partitioning between reaction with biological molecules and further degradation to stable products. This has presented a technical challenge for direct detection

of reactive metabolites even with state-of-the-art instrumentation. An alternative is to identify derivatives resulting from reactions of the metabolites with the so-called trapping agents (Fig. 22.2). Such an approach has proved valuable during investigation of bioactivation mechanisms and identification of potential biological targets susceptible to covalent structural modification. In drug discovery and development settings, the data are utilized to construct an SAR aiding medicinal chemistry's effort to design and synthesize molecules with a low propensity to bioactivation.

22.2.1 Detection of GSH-Related Thiol Derivatives

Many reactive metabolites are electrophiles prone to react with nucleophilic amino acid residues of proteins, leading to the formation of covalently bound drug–protein adducts. One such amino acid is cysteine in its reduced form. Although the outcome can be very informative, analysis of protein adducts is relatively complex and resource consuming. In order to simplify bioanalytical procedures while not losing essential structural information, studies of bioactivation are often carried out via identification of the stable products resulting from nucleophilic “trapping” of electrophilic metabolites. For example, experiments are routinely performed to search for GSH adducts. The peptide GSH is an important endogenous substance, and one of its cellular functions is to scavenge reactive species (see above). GSH conjugation reactions also occur in incubations with liver microsomal or hepatocyte preparations when the thiol peptide is present. Detection of GSH adducts by MS is relatively straightforward due in part to the fact that MH^+ ions of those adducts produce characteristic fragments resulting from the neutral loss of 129 Da upon collision-induced dissociation (CID) [10]. A conventional approach is to analyze biological samples obtained from either *in vivo* or *in vitro* using the full scan functionality of an ion trap or triple quadrupole MS [11]. Initial identification of potential adducts is based on searching MH^+ ions corresponding to the combined molecular weights of GSH and the putative metabolites. Any molecular ion meeting the criteria is subject to CID, and further analysis focuses on the characteristic fragment ions such as those derived from the neutral losses of pyroglutamate (129 Da) and cysteine (75 Da). The probability of success for a full MS scan-based probe of GSH adducts is highly dependent on the experience of investigators when measured by the number of “false negative results.” An alternative is to use a neutral loss scan of 129 Da for detecting GSH adducts with triple quadrupole MS operated at positive ionization mode. Subsequently, the MH^+ ions identified are subject to CID, generating product ion spectra for structural analysis. One of the major limitations related to the neutral loss scan of 129 Da is its low sensitivity and selectivity; the former results from the inherited deficiency of full scan mode, while the latter is mainly due to interference by the biological matrix. This may be improved by scanning for those precursor ions $(M-H)^-$ that produce a specific fragment at m/z 272 with a triple quadrupole MS [12]. The ion of m/z 272 represents a diagnostic fragment of GSH adducts under the negative ionization mode, corresponding to the cleavage of the carbon–sulfur bond at the peptide moiety. Both the aforementioned approaches are relatively low throughput with application of “low” resolution MS (see below).

In order to improve sensitivity and selectivity, multiple reaction monitoring (MRM) has been used as an alternative to full scan for triggering the acquisition of enhanced product ion (EPI) spectra on a triple quadrupole linear ion trap MS, such as API4000 Q-Trap [13]. Thus, it was demonstrated that up to 114 MRM scans can be constructed

to monitor mass transitions resulting from the neutral losses of 129 and/or 307 Da, with the MH^+ ions proposed to target-specific GSH adducts whose structures are derived from a database of known bioactivation reactions including those observed for compounds with similar substructures. Detection of the desired signals by the MRM survey scan triggers a data-dependent acquisition of EPI, from which the structures of GSH adducts are deduced. As such, investigators should be able to obtain product ion spectra of targeted GSH adducts, and the sensitivity of detection could be as high as ~ 180 -fold better than the full-scan-based neutral loss survey scan. However, this approach would only enable identifying the GSH adducts “predicted” by a database based on which MRM survey scans are constructed. In other words, GSH adducts formed via “novel” bioactivation mechanisms are likely to escape from the detection.

Several derivatives of GSH have been synthesized for trapping reactive metabolites. One such agent is GSH ethyl ester (GSH-EE, Fig. 22.2) used *in vitro* to improve the sensitivity of detection [14]. The experiment was performed on a microbore liquid-chromatography-micro-electrospray-ionization tandem MS instrument. Detection of GSH-EE adducts was based on selected reaction monitoring (SRM) for simultaneously monitoring multiple MH^+ to $[MH-129]^+$ transitions, where $[MH-129]^+$ ions correspond to the neutral loss of pyroglutamate from the GSH-EE moiety. It appears that the sensitivity of detection was ~ 10 -fold better for GSH-EE adducts relative to GSH adducts. In a separate study, GSH and GSH-EE were added together into liver microsomal incubations in an attempt to increase the probability and certainty of identifying reactive metabolites by LC-MS analysis [15]. In this case, paired MH^+ ions with their mass difference of 28 Da were clearly indicative of GSH and GSH-EE adducts. Another GSH derivative synthesized for trapping purpose incorporated a quaternary 4-hydroxy-*N*-methylethyl piperidinium (QA-GSH, Fig. 22.2) [16]. Similar to GSH, QA-GSH “traps” electrophilic reactive metabolites, but detection of the resulting adducts by LC-MS was based on M^+ and $(M+H)^{2+}$ ions, as the agent already contains a fixed positive charge. Formation of QA-GSH adducts *in vitro* was semiquantifiable when the so-called QA-GSH standards were added during the sample processing and employed as internal standards for calibration purposes. Thus, the M^+ and $(M+H)^{2+}$ ions were monitored using either a multiple single reaction monitoring (mSRM) battery or a precursor ion scan of m/z 144, with the latter corresponding to the 4-hydroxy-*N*-methylethyl piperidinium ion liberated from QA-GSH adducts. Both GSH-EE and QA-GSH belong to a class of “artificial” trapping agents, and their trapping capability is dictated by chemical reactivity. For comparison, formation of GSH adducts in liver microsomal incubations sometimes can be enhanced via adding GST or hepatic cytosolic fraction [17]. A larger quantity of the metabolite formed should obviously improve the probability of detection. The experimental setup also better mimics the *in vivo* biological environment since GST is present in abundance in hepatocytes. Semiquantification is achievable in principle by employing a synthetic GSH adduct as an internal standard during LC-MS analysis.

The fragmentation of GSH adducts often is dominated by cleavages of the peptide bonds, and, as a result, the product ion spectra sometimes do not offer sufficient clues for structural analysis of drug portion of the adducts. Certain types of GSH adducts are chemically unstable, further complicating bioanalysis. An alternative trapping agent is NAc (Fig. 22.2) with a molecular weight of 163 Da. Unlike the corresponding GSH adducts, NAc adducts lack a peptide moiety and consequently their fragmentations are likely associated with drug portion of the molecules, hence more structural information

on the trapped reactive species and better understanding of the mechanism underlying bioactivation. In addition, NAc adducts can be detected *in vivo*, formation of which takes place via the mercapturic acid pathway following GSH conjugation reactions with reactive metabolites (see above). The conventional approach described above for detection of GSH adducts has also been employed for analysis of NAc adducts, such that identification of potential adducts focuses initially on searching MH^+ ions corresponding to the sum of 161 Da plus the molecular weights of putative reactive metabolites, followed by MS/MS analysis of the MH^+ ions in question. Similar to GSH adducts, NAc adducts produce fragments corresponding to the neutral loss of 129 Da, although the fragmentation is due to the cleavage of the thioether bond on the cysteine side of the molecule. On that basis, a neutral loss scan of 129 Da can be used for screening NAc adducts. In a recent report, NAc adducts formed either *in vitro* or *in vivo* were detected using a quadrupole linear ion trap MS with the polarity switching technique [18]. In the study, neutral loss scans of 129 Da, based on either full scan or MRM, triggered the acquisition of parent ion spectra and fragment-rich EPI spectra in a single LC-MS run for structural assignment. This method employs a generic data-acquisition protocol for detection of various NAc adducts and may therefore be suitable for fast screening of samples derived from *in vitro* and *in vivo* experiments. However, one of the limitations in such an approach is that it would only be able to identify those NAc adducts “predicted” by investigators. In addition, the neutral loss scan of 129 Da appears to exhibit lower sensitivity for detection of NAc adducts than for GSH adducts due likely to the fact that the two fragmentations are associated with cleavage of the chemical bonds with different strength. The thioether bond of an NAc adduct is much stronger than the peptide bond of a GSH adduct.

A recent development in instrumentation is related to the data acquisition with alternating low/high collision energy on high resolution MS (HRMS) such as a hybrid quadrupole time-of-flight (Q-TOF) MS. This improvement of methodology, in general, has increased not only bioanalytical throughput but also the sensitivity of detection for drug metabolites, including trapped reactive species [19,20]. The experimental setup starts with scans of ions at a low level of collision energy, followed by ramping collision energy to different levels in order to trigger CID. The first scan function at low collision energy detects the intact molecular ions, whereas the second scan at higher levels of collision energy generates MS^E spectra, where “E” represents collision energy. The MS^E spectra are equivalent to the ones obtained from nonselective tandem MS/MS scans, containing fragmentation information for the ions registered in the low collision energy scan [20]. However, MS^E spectra acquired by Q-TOF MS are not specific, as they lump together all fragments of the molecular ions that reach the detector at a given time. Such deficiency complicates the data interpretation but has been somewhat compensated by coupling HRMS with UPLC, which separates efficiently the analytes from each other as well as from the biological matrix [21]. Accurate mass-based postacquisition data processing software also aids interpretation of the MS^E spectra [22,23]. Consequently, a single sample injection onto UPLC-HRMS usually is sufficient to obtain both MS and MS^E spectra of the metabolites of interest. In this regard, detection of a diclofenac–GSH adduct may serve to demonstrate the utility of HRMS. Initial scans at low collision energy under the positive ionization mode of a sample from human liver microsomal incubations registered an MH^+ ion at m/z 583.1267, which corresponds to the exact mass of [diclofenac + GSH + O – Cl – H] (Fig. 22.3a). Subsequent automated switch to a scan at high collision energy

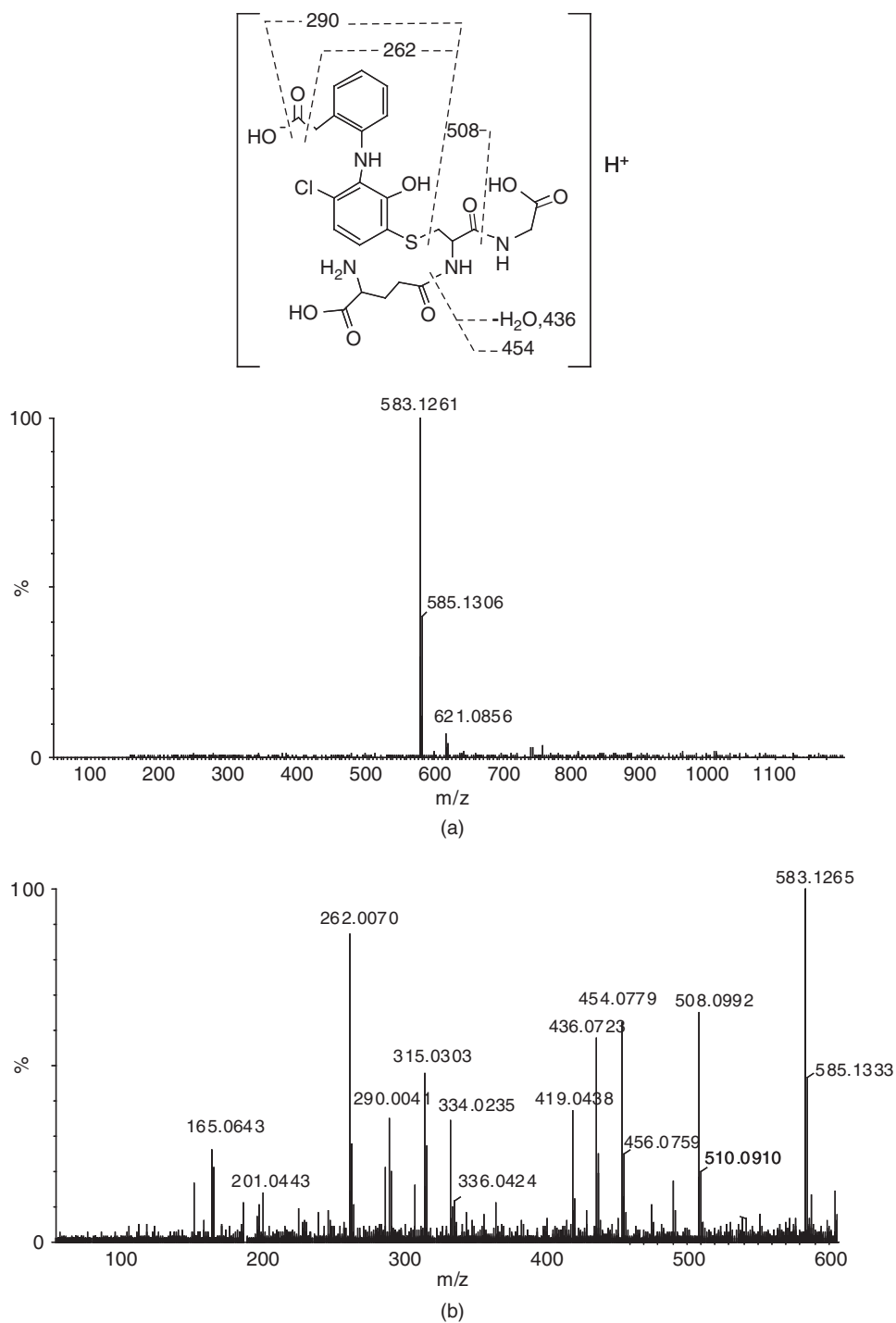


Figure 22.3 Detection by HRMS of a GSH adduct derived from diclofenac bioactivation.

produced an MS^E spectrum (Fig. 22.3b), followed by data processing of the spectrum by Metabolynx (Waters) for assignment of the fragment ions. The result supports the idea that diclofenac undergoes bioactivation to form reactive quinone imine or epoxide species, which are trapped by GSH conjugation reactions [24,25]. Similar to their counterpart with low mass resolution, HRMS also is equipped for searching GSH adducts via neutral loss scans [26]. In this case, consecutive pairs of low and high ionization energy spectra are generated for MH⁺ ions satisfying the neutral loss of 129.0426 Da within a window of ± 20 mDa. Once the instrument registers an MH⁺ ion with the specified neutral loss, it switches to MS/MS mode to collect an MS^E spectrum, providing sufficient information for subsequent structural analysis using data-processing software. The entire exercise often requires only a single sample injection, significantly enhancing the bioanalytical throughput. In principle, such an approach should be applicable for detection of not only GSH adducts but also drug metabolites with known characteristic neutral losses.

22.2.2 Detection of Amino acid- or Peptide-Related Adducts

Although GSH and NAc are the commonly used trapping agents, their utility to some extent is limited by their ability to form conjugates with reactive metabolites primarily via the sulfhydryl functional group. Some reactive species, such as quinones or quinone imines, may prefer to react with amines depending on their chemical reactivity, whereas certain thiol adducts could escape from detection by LC-MS analysis because they remain highly unstable and undergo degradation or rearrangement either in biological systems or during sample preparation [27,28]. In this context, individual amino acids or small synthetic peptides containing representative amino acid residues may serve as alternatives to GSH and NAc for trapping reactive metabolites formed *in vitro*.

It was therefore demonstrated that hydroxymethylvinyl ketone (HMVK) reacts readily with three model amino acids, namely, L-valinamide, *N*-acetyl-L-lysine (NAL), and NAc (Fig. 22.4) [29]. HMVK is a putative metabolite of 1,3-butadiene, which has been classified as a human carcinogen. Exposure to butadiene usually is associated with occupation and/or cigarette smoke. In the study, formation of adducts between HMVK and the model amino acids was monitored by HPLC with UV detection, while structural assignment was based on NMR analysis of purified products. Several HMVK-amino-acid adducts were identified, including those resulting from mono- and di-Michael addition(s) of the electrophile to the nucleophilic acceptors (Fig. 22.4). Among the amino acids tested, the reactive potential was in the order of NAc > valinamide > NAL, implying a free cysteine residue is a preferred site of modification. The detection of multiple different types of adducts has reinforced the idea that a variety of proteins are potential biological targets for HMVK *in vivo*. Similarly, cysteine, lysine, histidine, tyrosine, and serine were shown in a separate study to be able to trap quinone methides of different electrophilicity [30]. With HPLC-UV detection, three quinone methides, which were 4-methylene-2,5-cyclohexadienone derivatives, were observed to react rapidly with cysteine to form thioether derivatives with little or no competition from hydration in aqueous solutions. The α -amino groups of lysine, histidine, tyrosine, and serine were the primary sites of modification by the quinone methides, although alkylation also occurred with the side chain amino groups of lysine and histidine. The order of reactivity was cysteine thiol > N-terminal amine > *N* ϵ -lysine $\sim$ *N* Im-histidine, but this pattern of reactivity did not hold for peptides or proteins. For the

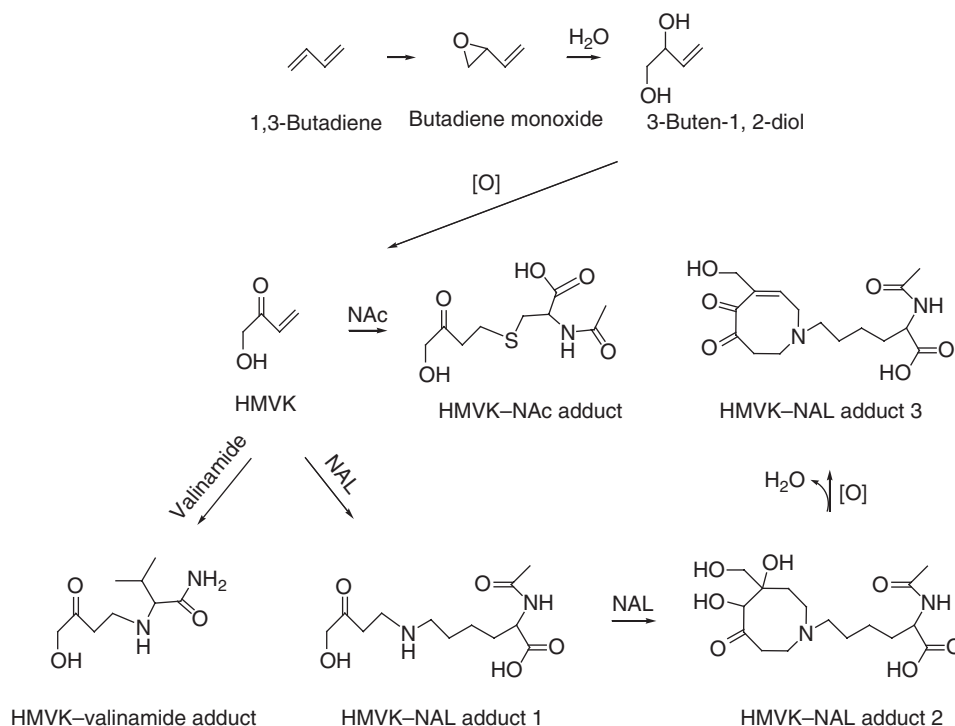


Figure 22.4 Structures of *in vitro* trapped adducts of HMVK with valinamide, NAL, and NAc.

synthetic tripeptide Gly-His-Lys and human hemoglobin, their primary sites of modification by the quinone methides were $N\alpha$ -glycine and $N\epsilon$ -lysine, respectively, based on LC-MS/MS analysis of the samples prepared with and without treatment by a protease.

Peptides may therefore represent better trapping agents when the focus of investigation is on the identity of modified amino acid residues. A generic experimental setup may include incubations of test compounds with human liver microsomes or recombinant P450s in the presence of a trapping peptide, followed by sample workup and LC-MS/MS analysis. For example, reduced oxytocin was employed as a model biological target of alkylation by the 2,4-diene derivative of valproic acid, an anti-convulsant agent that causes severe but rare liver injury [17]. Initial LC-MS scans registered an MH^+ ion of m/z 1491, consistent with a nucleophilic addition of two diene molecules to a single reduced oxytocin molecule. Subsequent CID of the MH^+ ion led to the assignment that the alkylation occurred at Cys-1 and Cys-6, suggesting that the sulfhydryl residues of proteins are vulnerable to attack following valproic acid bioactivation. Recent efforts include design and synthesis of trapping peptides that incorporate various nucleophilic amino acid residues in an attempt to capture different types of reactive species in a single experiment [31]. One such trapping agent was the 11-amino-acid peptide ECGHDRKAHYK containing not only cysteine but also other potential targets for nucleophilic addition/substitution, such as lysine and histidine. In incubation of a drug in question with the peptide in a bioactivation system, samples were partially purified and drug-peptide adducts retained on a cation-exchange chip

surface were analyzed by a surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) MS. Identification of those adducts was based on parent ion scans for MH^+ corresponding to a combined mass of the trapping agent plus putative metabolites. Although application of an SELDI-TOF MS eliminated the requirement for separation by LC of the analytes, the instrument, in general, exhibits a relatively poor precision in comparison with conventional LC-MS and is incapable of generating fragmentation data to aid structural elucidation of the observed adducts as it lacks a CID function. Such an experimental setup was driven primarily by industrial research, characteristics of which include a rapid turnaround time to answer a much simplified question about bioactivation, that is, whether reactive metabolites are produced from a given test compound. Peptide-based trapping agents have the advantages of mimicking biological protein targets better than individual amino acids and providing information on the sites of modification. However, similar to GSH adducts, drug-peptide adducts usually offer insufficient or no information at all for the drug side of trapped products even if they are subjected to fragmentation. This can be very unsatisfactory for those whose research focus is on an SAR with respect to bioactivation. In this context, data from trapping experiments with GSH, NAc, and various peptides are likely complementary to each other.

22.2.3 Detection of Protein Adducts

Formation of protein adducts is at the center of a working hypothesis relating bioactivation to drug-induced AEs (see above). Identification of modified proteins should therefore represent a step forward to better understanding of the pathogenesis of tissue injury. When the modified protein is a drug-metabolizing enzyme, studies of the corresponding protein adduct not only bear toxicological significance but also shed light on interactions between the enzyme and its substrate, hence the relevant topography of enzyme active site. Similar to any analyte in question, detection of protein adducts usually begins with separation or partial purification of those adducts from biological matrix, and the techniques may fall into two major categories, one of which is one- and two-dimensional gel electrophoresis. An alternative is chromatographic separation, represented by HPLC and size exclusion, ion exchange, and affinity chromatographies. Popular means of detection includes immunochemical and MS analyses.

Immunochemical detection coupled with electrophoretic separation essentially constitutes the so-called Western blotting (see *Bioanalytics for Human Microdosing*), capable of identifying multiple modified proteins including the antigen(s) that may be responsible for causing pharmacotherapy-related hypersensitivity. Polyclonal antibodies are usually produced by immunization of laboratory animals, such as mice or rabbits, with a carrier protein conjugated to a drug or metabolite in question. Antibodies raised are present in serum, termed *antisera*, and can be used as is or subject to purification before application. Detection of protein adducts resulting from drug treatment is therefore based on recognition by the antisera or purified antibodies of the adducts separated by one- and two-dimensional gel electrophoresis. A classic example involves identification of acetaminophen (APAP)-related protein adducts, in which polyclonal antisera was obtained from rabbits immunized with APAP linked to keyhole limpet hemocyanin (KLH) via NAc [32]. After characterized for its specificity toward APAP-protein adducts by a competitive enzyme-linked immunosorbent assay (ELISA), the antisera was utilized for analysis of the serum samples collected from

patients administered with APAP. The results revealed that the subjects with increased serum transaminase levels had APAP–protein adducts in their circulations, whereas those without signs of liver injury did not exhibit immunochemically detectable signals for protein adducts. A reversed sequence of the aforementioned detection procedure is viable to determine if patients are “immunized” by drug treatment. For example, sera from patients who experienced tienilic acid-induced hepatitis recognized CYP2C9 protein but not other members in the CYP2C family on immunoblotting, suggesting the presence of anti-CYP2C9 antibodies in these patients [33]. Additional experiments with mutants of CYP2C9 identified three regions on the enzyme protein that form a binding pocket interacting with the antibodies. Similarly, antisera against proteins modified by halothane was present in patients who developed drug-treatment-related hepatotoxicity [34]. Detection of antibodies that recognize either native or modified proteins is of mechanistic importance, as it implicates an activated immune system in the pathogenesis of idiosyncratic drug toxicity (see above). In this regard, a patient treated by halothane several years earlier experienced desflurane-induced hepatotoxicity [35]. Subsequent analysis of serum samples from the patient by ELISA revealed that the sera exhibited reactivity toward liver microsomal proteins from rats treated with halothane. This led to the hypothesis that the patient was sensitized by the early halothane therapy and consequently became susceptible to desflurane treatment on the basis that metabolism of both halothane and desflurane generates putative trifluoroacetic chloride, a reactive species capable of binding covalently to proteins and, presumably, activating the immune system [35].

More recently, chromatographic separation coupled with MS detection has become a popular method for analysis of protein adducts. Advantages of an LC-MS/MS-based approach reside with not only sensitivity and accuracy but also a capacity of generating data for structural elucidation. In this context, LC-MS/MS analysis, together with the use of various types of proteases, often provides information on molecular weight of protein adducts and the amino acid residues modified. In one instance, such a method was used for detection of APAP–albumin adducts in patients administered with the analgesic [36]. An earlier experiment established that *N*-acetyl-*p*-benzoquinone imine (NAPQI) formed during APAP bioactivation reacts with the only Cys residue of serum albumin, and digestion of the protein adduct by pronase E gives rise to an NAPQI-Cys-Pro-Phe (NAPQI-CPF) adduct. Thus, serum specimens from three patients overdosed with APAP were analyzed by LC-MS/MS, following treatment of the samples with protease E. Serum from healthy volunteers served as controls. The tripeptide adduct was observed only in those who were ingested APAP, and its level was ~10-fold higher in a patient with severe hepatotoxicity. From the same samples, the LC-MS/MS assay also was able to pick up signals for the NAc and Cys adducts of NAPQI, providing additional data in a relatively straightforward analytical process that enables better understanding of the fate of NAPQI [36]. With respect to covalently modified drug-metabolizing enzyme, studies of the resulting protein adduct aid structural elucidation of the enzyme active site, complementary to the data collected by other techniques. For example, grapefruit juice intake can cause significant increases in systemic exposures of CYP3A substrate drugs such as cyclosporine and midazolam. Two major components in grapefruit juice, bergamottin (BG) and 6', 7'-dihydroxybergamottin (DHBG), were identified as irreversible inhibitors (inactivators) of CYP3A, possibly via modification of the enzyme apoprotein. Subsequent effort was focused on studies of the protein adducts formed during enzyme inactivation [37,38]. Recombinant or purified

enzymes were used in order to simplify bioanalysis. In one study, DHBG was incubated with purified CYP3A4, and the samples were injected sequentially onto two different HPLC columns for chromatographic separation of analytes from the matrix before MS analysis. Two protein adducts were detected, one of which exhibited the molecular ion corresponding to the intact CYP3A4 plus 386.8 Da, suggesting covalent binding of an oxygenated DHBG derivative to the enzyme apoprotein. The second adduct appeared to be a “bis-adduct,” with its molecular weight equal to the apoprotein modified by two oxygenated DHBG derivatives [37]. Similarly, a protein adduct detected in a separate study by LC-MS showed an increase of mass by 387 Da from the native enzyme when BG was incubated with purified CYP3A5, implying BG was converted to DHBG followed by protein alkylation [38]. A proposed mechanism has therefore attributed the loss of CYP3A activity in grapefruit juice drinkers to oxidation of the furan moiety of DHBG to an epoxide species that alkylates the enzyme apoprotein and consequently inactivates the enzyme. Detection of the bis-adduct resulting from interactions of DHBG with CYP3A4 implicates a large enzyme active site capable of accommodating more than one substrate molecule. Additional information concerning amino acid residues critical to enzyme activity may rely on studies of the peptides derived from digestion by proteases of the drug-protein adduct. One example involves raloxifene, which causes irreversible inhibition of CYP3A4. The loss of enzyme activity was attributed to alkylation by raloxifene of CYP3A4 apoprotein, as the inactivated enzyme maintains an intact heme-Fe moiety that produces spectrally detectable P450 similar to controls [39]. Further analysis by LC-MS/MS of inactivated CYP3A4 following digestion by protease K and trypsin, respectively, revealed that the Cys-239 and Tyr-75 residues were targets of alkylation by a reactive species, designated as extended quinone or diquinone methide, formed during raloxifene metabolism [40,41]. These data collectively suggest that Cys-239 and Tyr-75 are important to CYP3A4 activity.

22.2.4 Detection of DNA Adducts

DNA consists of deoxyguanylate, deoxyadenylate, deoxythymidylate, and deoxycytidylate, while purines and pyrimidines, including adenine, guanine, cytosine, uracil, and thymine, are the base units for constructing DNA molecules. Electrophilic reactive species formed during drug metabolism may react with the nucleophilic sites of purine and/or pyrimidine base units, causing covalent modification of, and lesions to, the nucleic acids. In response to the insult, cells activate repair enzymes or polymerases to identify and correct the damage in order to maintain normal cellular function. The affected cells undergo programmed cell death or mutation when damages overwhelm the repair mechanism, resulting in tissue injury or triggering cancer development. From a chemistry perspective, the nucleobase units of DNA molecules are “hard” nucleophiles that are susceptible to the attack by “hard” electrophiles (see below) such as epoxides, aldehydes, ketones, and quinones.

In this context, acetaldehyde (Fig. 22.5a), classified as a possible human carcinogen, is present in various foodstuffs and is an oxidative metabolite formed following alcohol consumption or cigarette smoking [42]. In an attempt to analyze DNA adducts resulting from exposures to acetaldehyde, calf thymus DNA was incubated with the suspected carcinogen, in the presence or absence of NaBH_3CN , and the resulting samples were hydrolyzed with aqueous HCl to release adenine- and guanine-related products for

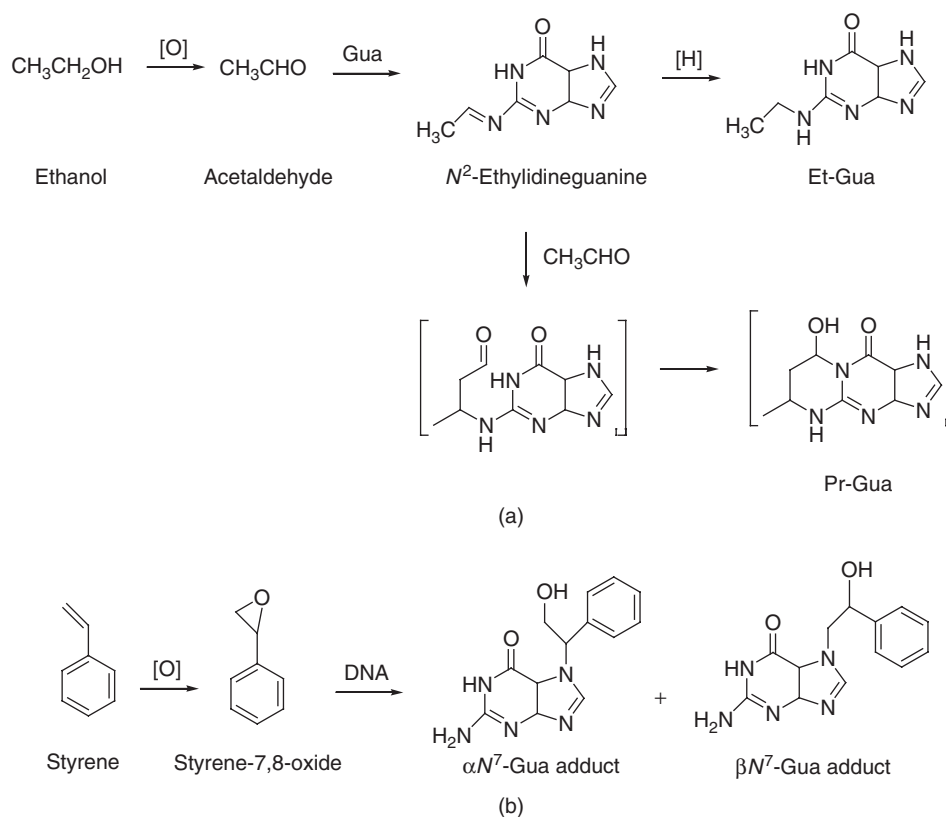


Figure 22.5 *In vitro* reactions of acetaldehyde with calf thymus DNA (a) and styrene-7,8-oxide with salmon testis DNA (b).

analysis by LC-MS/MS with selective ion monitoring (SIM). N^2 -Ethylguanine (Et-Gua) or 1, N^2 -propanoguanine (Pr-Gua) was the major adduct, depending on whether incubations contained or lacked NaBH_3CN . Thus, the underlying mechanism likely involves the reaction of acetaldehyde with the DNA molecule, resulting in the formation of an unstable Schiff's base N^2 -ethylidineguanine adduct, chemical reduction of which by NaBH_3CN (followed by hydrolysis of the DNA adduct) produces Et-Gua. In the absence of the reducing agent, N^2 -ethylidineguanine undergoes further reaction with an additional molecule of acetaldehyde to give Pr-Gua (Fig. 22.5a). When acetaldehyde was incubated with cultured HL-60 cells, both Pr-Gua and Et-Gua were detected by LC-MS/MS, suggesting that alkylation of DNA by the aldehyde occurs in intact cells [42]. Covalent DNA modification also took place following incubations of calf thymus DNA with 5'-acetoxy- N' -nitrosomnicotine (5'-acetoxy-NNN), a precursor to the reactive species 5'-hydroxy-NNN resulting from tobacco smoking [43]. The reaction mixtures were subject to enzymatic hydrolysis by phosphodiesterase and alkaline phosphatase, and the adducts identified by LC-MS/MS with selected reaction monitoring (SRM) of $(\text{MH}^+ \rightarrow [\text{MH}-116]^+)$ mass transitions were pyridyloxobutyl-dGuo (POB-dGuo), POB-dCyt, and POB-Thd. The $[\text{MH}-116]^+$ fragment formed upon CID corresponds to the loss of the deoxyribose moiety from the nucleobase adduct MH^+ ion. Similar to aldehydes, epoxides, such as styrene-7,8-oxide, also are potential

mutagens and/or carcinogens. In incubations of styrene-7,8-oxide with salmon testis DNA, several adenine and guanine adducts were characterized by LC-MS/MS and UV detection following hydrolysis of modified DNA in aqueous HCl [44]. It appeared that alkylation at the N^7 of guanine accounted for 93% of total DNA modification (Fig. 22.5b), followed by 4% at the N^3 of adenine and the remaining 3% distributed among N^2 of guanine, C^1 of adenine, and N^6 of adenine. The N^7 of guanine has been perceived as a high reactivity center because of its nucleophilicity and accessibility, which is followed by the N^3 of adenine with similar nucleophilicity but slightly more steric hindrance [45]. Other positions on the nucleobase units can also become a main target of alkylation, depending on a specific electrophilic reactive species, as in the case of the reaction between acetaldehyde and DNA, where modification occurred primarily at the N^2 of guanine.

Detection of DNA adducts formed *in vivo* is likely most relevant to biological and pathological processes, and numerous studies have been conducted in laboratory animals. For example, acrylamide (AA, Fig. 22.6a) is a chemical with various applications

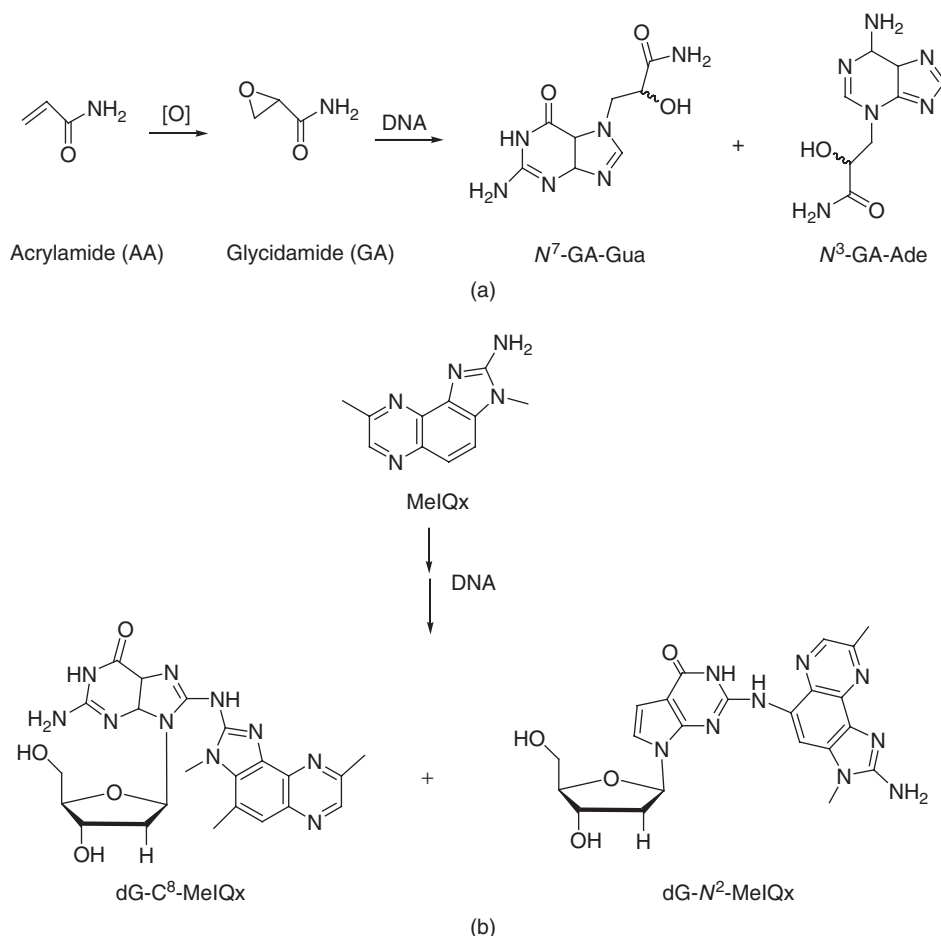


Figure 22.6 Formation of DNA adducts in mice dosed with acrylamide (a) and in rats dosed with MeIQx (b).

in industry, and its human exposures are associated with occupation as well as cigarette smoking and ingestion of fried starchy foods. AA is suspected to undergo bioactivation, getting converted to glycidamide (GA), which, being an epoxide, alkylates DNA [46]. Following a single dose from 1 to 50 mg/kg of AA to mice by intraperitoneal injection, liver, lung, and kidney were collected at 6 h for DNA extraction [47]. The resulting samples were subject to neutral thermal hydrolysis and partial purification by a size exclusion column. Two adducts, namely, N^7 -GA-Gua and N^3 -GA-Ade (Fig. 22.6a), were quantified by LC-MS/MS based on calibration curves generated with synthetic standards. The results revealed that formation of both adducts exhibited a dose-dependent response, with the level of N^7 -GA-Gua being ~ 100 -fold higher than that of N^3 -GA-Ade in rat tissues examined. Another example involves 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, Fig. 22.6b), a structural representative of more than 20 heterocyclic aromatic amines formed during meat cooking. MeIQx is a rodent carcinogen and is considered as an agent of potential cancer risk to humans. Thus, MeIQx- C^8 -dG and MeIQx- N^2 -dG (Fig. 22.6b) were detected and quantified in liver samples from rats treated with MeIQx, based on LC-MS/MS SRM of $(MH^+ \rightarrow [MH-116]^+)$ mass transitions [48]. The observed higher level of MeIQx- N^2 -dG relative to that of MeIQx- C^8 -dG implies that the former adduct is likely an important contributor to MeIQx-induced genotoxicity in rodents. In a separate study, the neutral loss of deoxyribose was employed to trigger data-dependent acquisitions of the MS^3 product ion spectra [constant neutral loss (CNL)- MS^3 scan] for putative adducts [49]. In addition to MeIQx- N^2 -dG and MeIQx- C^8 -dG, an adenine adduct was identified via the CNL- MS^3 scan of liver samples from rats dosed with MeIQx. The structure of the adenine adduct was assigned as MeIQx- N^6 -dA according to the product ion spectrum. Most significantly, DNA adducts were also detected in humans, one of which is N^2 -ethylidene-dG derived from reactions of acetaldehyde with DNA (see above). In this case, DNA isolated from human lung and liver samples was subjected to hydrolysis in the presence of $NaBH_3CN$, converting chemically unstable N^2 -ethylidene-dG to the corresponding quantifiable N^2 -ethyl-dG [50]. Quantification of N^2 -ethyl-dG was based on LC-MS/MS SRM of $(MH^+ \rightarrow [MH-116]^+)$, using synthetic $[^{15}N]N$ -ethyl-dG as a stable isotope labeled internal standard. The results revealed an average of 0.1 adducts per million nucleotides in human livers and a similar level (0.13 per million nucleotides) in human lungs. There was no apparent difference between cigarette smokers and nonsmokers with respect to the level of N^2 -ethyl-dG in their lungs, although the comparison was confounded by a small number of subjects investigated ($n = 4$) and a significant period of hospitalization without cigarette smoking before the samples were collected [50]. Nevertheless, the ability to detect and quantify DNA adducts in patients is clearly very important toxicologically.

22.2.5 Detection of Adducts Trapped by Nonbiological Agents

Although GSH and NAc often serve as trapping agents, some compounds known to form reactive species causing covalent protein binding *in vivo* do not produce trapped adducts, which may be attributed to the instability of the adducts or the nature of reactive metabolites. In the latter instance, electrophiles and nucleophiles are generally grouped as “soft” or “hard” according to their chemical properties. Soft nucleophiles are large in size with diffuse charge, such as the thiol group of GSH, and react readily with soft electrophiles, for example, α,β -unsaturated keto group. Hard electrophiles

are small with intense charge and tend to react with hard nucleophiles. As a result, soft nucleophilic trapping agents may not be able to effectively trap hard electrophilic metabolites and vice versa, leading to a false assurance that bioactivation is absent. Therefore, there is a need to explore nonbiological chemicals as alternatives for trapping different types of reactive species.

Cyanide ion is one of the most commonly used supplemental agents for trapping reactive metabolites. It is a hard nucleophile and traps certain hard electrophiles (e.g., iminium ion) with which GSH and NAc may not react. Another advantage is that the resulting cyanide adducts are generally stable and readily identifiable by MS because of the facile loss of 27 Da (HCN) upon CID. For example, metabolism of 1-benzylpyrrolidine in rabbit liver microsomes leads to the formation of a pyrrolidinium ion, identification of which was based on detection by GC-MS of mono- and bis-cyano adducts (Fig. 22.7) when incubations were performed in the presence of sodium cyanide [51]. Formation of the pyrrolidinium was attributed to the observed covalent protein binding by 1-benzylpyrrolidine. Metabolism of [^3H]phencyclidine in rabbit liver microsomes, while generating an iminium species, also was associated with covalent protein binding [52]. The degree of covalent protein binding was reduced by 75% in incubations containing 0.1 mM sodium cyanide, suggesting that the reactive iminium ion is responsible for phencyclidine-related protein modification. It should be noted that sodium cyanide at high concentrations may inhibit drug-metabolizing enzymes, leading potentially to decreases in the formation of corresponding cyanide adduct [51]. Several studies revealed that an optimal concentration of sodium cyanide was 1 mM or less when used with rabbit or human liver microsomes [52,53].

In order to facilitate LC-MS/MS detection, a mixture of $\text{Na}^{13}\text{C}^{15}\text{N}$ with NaCN (1:1) has been exploited for trapping reactive metabolites [54]. The isotopic patterns of the corresponding cyanide adducts are characteristic and easily recognizable during LC-MS analysis. This approach not only is useful with nominal MS application but also enhances HRMS detection of adducts trapped by cyanide. In a recent report, cyanide adducts were detected by UPLC coupled with TOF MS in 8 of 12 tested compounds following incubations with human liver microsomes in the presence of 1 mM of a mixture of KCN and $\text{K}^{13}\text{C}^{15}\text{N}$ (1:1) [55]. The exercise further explored the use of the so-called mass defect filter for identification of the trapped adducts during data mining processes, and its success highlighted that a combination of HRMS and a stable-isotope-mixed CN trapping agent could significantly increase the throughput for screening reactive metabolites formed in an *in vitro* system.

Methoxyamine is a hard nucleophile prone to react with hard electrophilic aldehyde to form the corresponding oxime adducts [56]. DBOPN ([1*S*, 5*R*]-3-cyano-1-(3-furyl)-6-{6-[3-(3 *R*-hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridine-2-yl-

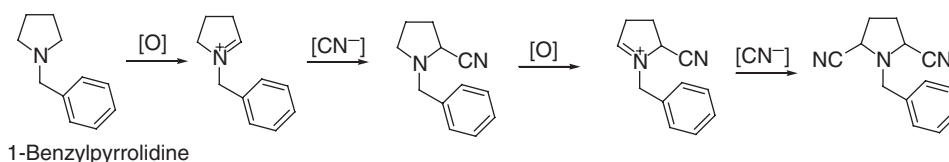


Figure 22.7 Formation of mono- and bis-cyano adducts of 1-benzylpyrrolidine.



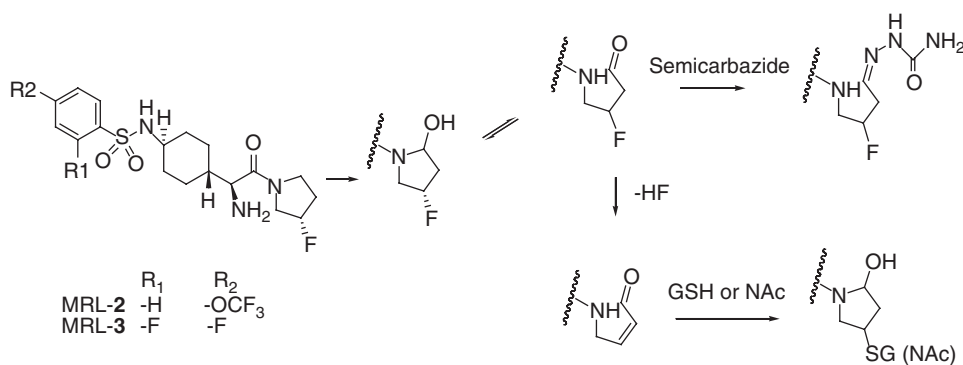


Figure 22.9 Formation of adducts of MRL-2 and MRL-3 with semicarbazide or GSH/NAc.

led to identification of five adducts by LC-MS/MS and NMR, four of which were derived from the mono- and bis-addition of methoxyamine to the oxidized furan moiety of DBOPN (Fig. 22.8a). Similar oxime adducts were also detected after incubating DBOPN with liver microsomes from dog, monkey, and human in the presence of hydroxylamine. Another example involves MRL-1, a furanopyridine derivative with a potential for anti-HIV treatment (Fig. 22.8b) [59]. MRL-1 caused covalent protein binding to rat, dog, monkey, and human liver microsomes in an NADPH-dependent manner, implying bioactivation and formation of reactive species. In liver microsomal incubations containing methoxyamine, metabolism of MRL-1 produced a ring-opened dihydrofurandiol tautomer that was trapped in the form of an *O*-methyloxime derivative (Fig. 22.8b). These two case studies clearly indicate that the furan ring represents a structural alert for bioactivation with the potential to form reactive epoxide and aldehyde derivatives in biological systems.

Semicarbazide is another small nucleophilic molecule capable of reacting with an aldehyde or ketone functional group to afford the corresponding stable semicarbazones. For example, two dipeptidyl peptidase-IV (DPP-IV) inhibitor analogs, MRL-2 and MRL-3 (Fig. 22.9), were subject to bioactivation in liver microsomes [60]. Covalent protein binding of the ³H-labeled MRL-2 and MRL-3 in rat liver microsomes was NADPH-dependent, suggesting P450-mediated bioactivation. Subsequent trapping experiments with GSH, NAc, and semicarbazide led to the identification of several adducts by LC-MS/MS analysis. A possible mechanism underlying the observed bioactivation involves hydroxylation at the C⁵ of the pyrrolidine moiety, with the resulting β -fluoroaldehyde partitioning between reaction with semicarbazide and elimination of HF. The latter process affords an α,β -unsaturated carbonyl that is trapped by conjugation reactions with GSH or NAc (Fig. 22.9). This case study highlights the value of different trapping agents in mechanistic studies of bioactivation.

22.3 SEMIQUANTITATIVE DETERMINATION OF BIOACTIVATION

Bioactivation of test compounds may involve phase I (e.g., epoxidation) and/or phase II reactions (e.g., acyl glucuronidation). If metabolism of the compounds in question is mainly through phase I reaction, incubations with liver microsomes are likely appropriate for evaluation of bioactivation. In cases where compounds undergo both

phase I and II metabolism, hepatocytes should be a better choice of *in vitro* systems for assessing bioactivation potentials. In general, radiolabeled tracers are required for quantifying covalent protein binding. ^{14}C -Labeled compounds are preferred from a chemical and metabolic stability perspective. On the other hand, ^3H tracers are often a suitable alternative to the corresponding ^{14}C tracers because the former are relatively easy to synthesize and possess higher specific radioactivity. However, the ^3H label may be lost if oxygenation of the test compound occurs directly at the carbon center where the ^3H label is attached. This can usually be tracked with appearance of $^3\text{H}_2\text{O}$ during sample analysis, and the data should be incorporated into the assessment of covalent protein binding. To quantify covalent protein binding is a resource-consuming exercise and has been a subject of other review articles [11,54].

Semiquantitative approaches employing radiolabeled trapping agents may become an attractive alternative to covalent protein binding analysis from a resource-saving perspective. For example, bioactivation of test compounds may be determined following trapping experiment using [^{35}S]cysteine and [^{14}C]sodium cyanide [61]. Four drugs, namely, clozapine, diclofenac, R-(+)-pulegone, and troglitazone, known to cause hepatotoxicity, were incubated in human liver microsomes in the presence of [^{35}S]cysteine or [^{14}C]sodium cyanide. The radioactive peak areas of the trapped [^{35}S]cysteine adducts exhibited a reasonable correlation with the extent of covalent protein binding determined via a conventional exhaustive wash method using radiolabeled test compounds. To include both [^{35}S]cysteine and [^{14}C]sodium cyanide in the trapping experiments enabled simultaneously trapping both soft and hard electrophilic reactive metabolites and therefore improved the accuracy of “predicting” the extent of covalent protein binding. In a separate study, a high throughput trapping assay incorporated the use of potassium [^{14}C]cyanide to assess the degree of bioactivation of unlabeled compounds in question [62]. These compounds were incubated with liver microsomes in the presence of [^{14}C]cyanide, and the resulting cyanide adducts were concentrated, while excess unreacted tracer was removed via solid-phase extraction. The cyanide adducts then were eluted from the solid-phase extraction columns and subject to liquid scintillation counting. The extent of bioactivation potential was estimated based on the amount of radiolabeled cyanide adducts derived from the test compounds. Such an approach can be useful for ranking compounds within the same structural class during drug discovery.

Dansyl glutathione (dGSH, Fig. 22.2) is a fluorescent-labeled GSH derivative, with a fluorescence tag attached to the GSH moiety [63]. Its advantage resides with the fact that while reactive species trapped by dGSH are characterized by LC-MS/MS analysis, the amount of dGSH adducts formed is quantifiable via the fluorescent detection. A different fluorescence-based assay was demonstrated to employ a GSH-embedded 96-well plate for high throughput screen of reactive species formed *in vitro* [64]. The 96-well plates are initially coated with poly(2-hydroxyethylmethacrylate) (pHEMA) polymeric membrane, followed by attachment of glutathione disulfate (GSSG) to the surface of pHEMA. The immobilized GSSG is reduced to GSH by treatment with DL-dithiothreitol (DTT) before use. Incubations of test compounds with liver microsomes are performed in the DTT-treated 96-well plate, and the resulting reactive metabolites are trapped via their reactions with GSH coated on the plate. Remaining unreacted GSH are “destroyed” by adding *N*-(iodoacetyl-amino-ethyl)-1-naphthylamine-5-sulfonic acid (1,5-I-AEDANS) at the end of each incubation, and the resulting GSH-1,5-AEDANS is quantified by a fluorescence measurement. The extent of GSH adducts formed in the

incubation is derived from a reciprocal relationship relative to the amount of GSH-1,5-AEDANS. This method provides a rapid semiquantitative assessment of bioactivation *in vitro* via P450-catalyzed pathway, while its limitations are associated with the preparation of GSSG-immobilized 96-well plates and low sensitivity of detection for the reactive species tested (e.g., 500 nM for NAPQI).

22.4 A GENERIC PROTOCOL FOR *IN VITRO* TRAPPING STUDY

Liver microsomal preparations are suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM), MgCl_2 (0.1 mM) with the final protein concentration of 1 mg protein/mL. A trapping agent dissolved in the phosphate buffer is added into the suspension with a final concentration of 5 mM for GSH or NAc, 1 mM for KCN/ $\text{K}^{13}\text{C}^{15}\text{N}$, or 1 mM for a DNA base. A test compound dissolved in methanol also is added to the suspension to give a final concentration of 10–50 μM . Incubations then are performed in the presence of 1.2 mM of NADPH at 37°C for 30–60 min. Reactions are terminated by adding 3 $\times$ of acetonitrile, and the mixture is sonicated for 5 min and centrifuged at 20,800g at 4°C for 10 min. The resulting supernatant is collected, while the protein pellet is extracted twice with 1 mL of aqueous methanol (75%, v/v). The extracts and the supernatant are combined and evaporated to dryness under nitrogen at room temperature. The residues are suspended in 300 μL of aqueous acetonitrile (66%, v/v), an aliquot (75 μL) of which is subjected to LC-MS/MS analysis [65]. Further details can be found in experimental protocols published previously [10,11].

22.5 DATA INTERPRETATION AND UTILIZATION

Identification of reactive metabolites and their protein/nucleic acid adducts has been a focus of academic research in the area of toxicology and will likely be a part of future investigations exploring the pathogenic process leading to clinical onset of toxicity. A clear mechanistic understanding should significantly improve the detection and subsequent treatment of various tissue injuries resulting from pharmacotherapy. Although a rare example, APAP–protein adducts quantified in the circulation have been shown to serve as a possible biomarker for hepatotoxicity in patients suspected of drug overdose [66]. An accurate and timely diagnosis in this case is critical, as treatment with NAc is most effective if the antidote is given within ~ 16 h of APAP administration. On the other hand, identification of GSH adducts and related metabolites formed via the mercapturic acid pathway may be of toxicological importance in addition to simply “trapping” reactive metabolites for bioanalysis. A number of thiol adducts are implicated as culprits in drug-induced AEs. For example, terbinafine metabolism generates 7,7-dimethylhept-2-ene-4-ynal, an allylic aldehyde that reacts readily with GSH via 1,6-Michael addition [67]. The resulting GSH adduct remains an electrophilic α,β -unsaturated carbonyl and also undergoes a retro-Michael reaction to convert back to the allylic aldehyde. These reactive species are suspected of covalently modifying MRP2 protein, leading to a compromised transporter activity and consequently drug-induced cholestasis. Alternatively, GSH adducts can undergo metabolism to form the corresponding cysteine adducts, which may be subject to bioactivation mediated by

renal β -lyase. The resulting reactive species are capable of modifying kidney proteins, causing nephrotoxicity such as that illustrated in patients exposed to haloalkenes [68].

From a pharmaceutical research perspective, one of the primary purposes of measuring bioactivation is to guide medicinal chemistry effort in drug discovery. There are numerous successful examples in terms of minimizing bioactivation during lead optimization processes [11]. Many of the scenarios reported usually involve the identification by LC-MS/MS of GSH adducts, followed by chemical intervention via incorporation of an electronegative functionality, such as F, CN, or heterocyclic moiety, into the structural series in order to reduce bioactivation potential. A few case studies also suggest that selection of a “meaningful” trapping agent should rely on the knowledge of specific toxicity. For example, MRL-4 (Fig. 22.10a) is a potent cGMP-dependent protein kinase inhibitor for prevention and/or treatment of coccidiosis, but the compound promoted mutation in Ames tests when assays were performed in the presence of rat liver S9 fraction. This observation indicates that mutagenic effect shown by MRL-4 was dependent on its bioactivation. Further studies of MRL-4 metabolism in incubations with rat liver S9 containing guanine or cyanide led to the identification by LC-MS/MS of two possible reactive species (Fig. 22.10a). The first was likely an iminium ion capable of reacting with both the trapping agents to afford *N*-cyanomethyl

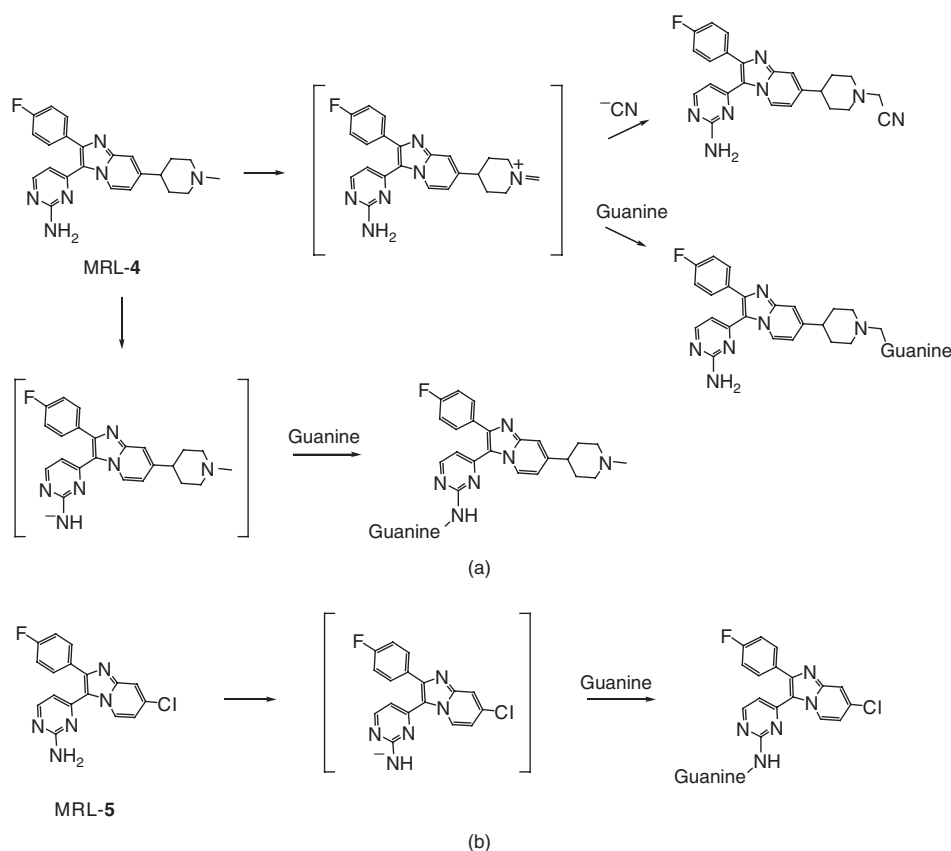


Figure 22.10 Bioactivation of MRL-4 and MRL-5 and formation of CN and guanine adducts.

and *N*-guaninemethyl piperidine derivatives, respectively. The second reactive metabolite presumably was a nitrenium ion, formation of which could begin with oxidation of the aminopyrimidine moiety to yield an *N*-hydroxyarylamine, followed by dehydration or a loss of sulfate/glucuronic acid if sulfation/glucuronidation of the hydroxyamine took place. The putative nitrenium ion reacted only with guanine, producing a guanine adduct. Subsequently, MRL-5 (Fig. 22.10b) was synthesized, which retained the arylamine functionality but replaced the piperidine moiety with chlorine. The compound, when evaluated for mutagenic and bioactivation potential, was Ames positive and formed a guanine adduct in incubations with rat liver S9. Collectively, these data support the idea that bioactivation is responsible for the mutagenic effect of MRL-4 and related arylamine analogs, whereas guanine adducts in this case serve as a better indicator for the formation of “toxicity-relevant” reactive species. Further lead optimization efforts eliminated arylamine from the structural class and consequently produced compounds that were Ames negative.

This example highlights the importance of minimizing bioactivation, while at the same time emphasizes the significance of data interpretation in relation to the knowledge of toxicity in question. Metabolites with different chemical reactivity are likely to target different biological molecules, some of which are biologically/toxicologically important, but others may simply serve as “scavengers” of reactive species. Such a scenario represents a dilemma for decision making in drug discovery, where data interpretation in many cases is prospective rather than retrospective. A core issue is the level of confidence with which one could claim a drug candidate would or would not cause harm to the patient according to a metabolism/bioactivation data set. Although this is a complex question, it appears that daily dose load is an important factor in determining the risk level of a compound, presumably due to saturable detoxification pathways. An empirical analysis suggests that drugs with a daily dose at 10 mg or less are associated with low incidence of drug-induced AEs [69]. Several recent studies attempted to test the hypothesis on whether reactive metabolites are the culprits responsible for clinical drug toxicity. In one study, 37 compounds were divided into 3 classes according to their safety status, including safe drugs (no warnings), warnings for toxicities, and black box warning/withdrawn from the market [70]. Bioactivation was measured by covalent protein binding quantified in incubations with human liver microsomes. The result suggests that majority of the compounds (30 out of 37) evaluated could be placed into their designated safety categories when daily dose was factored into the data analysis. A separate investigation of 50 drug products also was able to discriminate “toxic” from “safe” agents, using a criterion that normalized the extent of GSH adducts formed in human liver microsomal incubations to the daily doses prescribed [71]. Additional aspects deserving careful consideration during the selection of drug candidates include therapeutic areas and patient populations. A decision then can be made to balance the benefit and risk in order to satisfy unmet medical needs.

Last but not least, it should be noted that bioactivation represents only one of the several possible mechanisms underlying drug-induced AEs. Compounds can be intrinsically cytotoxic or can form metabolites that are cytotoxins but not chemically reactive. One such example is ximelagatran, a direct thrombin inhibitor prescribed for anticoagulation. The drug was removed from the market following clinical observations that ximelagatran treatment was associated with a significant elevation of liver enzymes and thus a high risk of severe liver injury [72]. Subsequent attempts to connect the

clinical AE and ximelagatran metabolism failed to identify GSH adducts or detect covalent protein binding in incubations with human liver microsomes or hepatocytes [73]. A probable cause of ximelagatran-induced hepatotoxicity remains obscure, although some evidence implies that the observed liver enzyme increases on drug treatment is immune mediated and dependent on specific genotypes of the major histocompatibility complex [74]. The parent drug also directly affected the plasma membrane fluidity and lipid composition in human hepatocyte incubations [75]. Similarly, troglitazone could serve to demonstrate that sometimes there are multiple mechanistic explanations for a pharmacotherapy-related AE in question. The drug was an agonist of the peroxisome-proliferator-activated receptor in the thiazolidinedione structural class for treatment of diabetes. Troglitazone was withdrawn from the market following numerous clinical reports of severe liver injury characterized by hepatocyte necrosis and bile duct proliferation. Metabolism of the drug leads to the formation of several reactive species, most of which are derived from P450-catalyzed oxidation of the chromane and thiazolidinedione moieties [76,77]. While these metabolites in principle could initiate pathogenesis of liver injury, the parent drug itself exhibited cytotoxicity to HepG2 cells expressing limited P450 activity [78]. Troglitazone also was subject to extensive sulfation in patients; the resulting sulfate derivative was the most abundant circulating metabolite with its plasma concentrations several-fold higher than that of parent drug [79,80]. Both troglitazone and the sulfate metabolite exhibited strong inhibition of BSEP *in vitro*, and their IC₅₀ values and clinical exposures were within a similar concentration range. Because BSEP is a hepatic canalicular transporter regulating bile flow and removal of bile acids from liver, a compromised transporter function on drug treatment could lead to an accumulation of toxic bile acids in hepatocytes and subsequent cholestatic liver injury in susceptible patients. On that basis, inhibition of BSEP by troglitazone and its chemically inactive metabolite represents an alternative to bioactivation as a hypothesis to explain the drug-induced hepatotoxicity. Collectively, the above case studies suggest that clinical AEs, including liver injury, do not necessarily follow the path of forming reactive metabolites from the offending drug. With respect to pharmaceutical research, a balanced approach appears preferable such that the effort of minimizing bioactivation potential does not result in a delay of pivotal preclinical safety studies. It is conceivable that further clarification of the relationship between bioactivation and pharmacotherapy-related AEs should affect greatly the strategy of drug discovery and development, leading eventually to improved drug safety.

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Abstract

A number of small-molecule drugs that cause severe adverse effects (AEs) undergo metabolism to form reactive species capable of covalently modifying proteins and/or nucleic acids. This has led to a working hypothesis that eliminating compounds susceptible to bioactivation in drug discovery should eventually produce safer medicines. A technical challenge associated with this strategy is to identify reactive metabolites that are often short lived either in biological systems or during sample preparation. Current bioanalytical options include capture of reactive metabolites with various trapping agents, such as glutathione (GSH) derivatives, synthetic peptides, cyanide, and nucleobase derivatives. These agents “trap” different types of reactive species, presenting the first hint on the mechanism of bioactivation. *In vitro* incubations usually are carried out in the presence of an appropriate trapping agent, with liver microsomes or hepatocytes serving as the source of enzymes responsible for bioactivation. The resulting samples are subjected to chromatographic separations and mass spectrometry (MS)-based detection of the trapped stable products. Similar procedures are applicable to specimens from preclinical species and humans for identification of GSH-related adducts formed *in vivo* via the mercapturic acid pathway. Sample analyses by triple quadrupole or ion trap mass spectrometers follow primarily a stepwise manner from neutral loss and/or parent ion scans to multiple-stage mass fragmentations in order to acquire sufficient data for metabolite structural assignment. Recent developments in instrumentation have centered on coupling ultraperformance liquid chromatography (UPLC) and high resolution MS; this combination is able to achieve a superior separation of analytes and acquisition of accurate mass spectra, hence a significant increase in sample throughput and improvement of metabolite detection. Use of accurate mass-based data-processing software further simplifies data interpretation, enabling quick structural assignment of the trapped products from which reactive species and bioactivation mechanisms are deduced. From a bioanalytical perspective, these experiments often require minimal sample preparation and method development, with a throughput usually satisfying the need of medicinal chemistry to construct a structure–activity relationship (SAR) with respect to the formation of reactive metabolites. The database accumulated, together with preclinical and clinical safety information, should also lead to better understanding of drug treatment-related adverse effects (AEs).

Keywords

bioactivation; bioanalysis; drug metabolism; DNA adduct; glutathione adduct; mass spectrometry; protein adduct; reactive metabolite; toxicity

23 Plasma Protein Binding Methods in Drug Discovery and Development: Bioanalysis

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23.1 Summary	1
23.2 Introduction	2
23.3 Traditional methods	4
23.4 Emerging techniques	9
23.5 Impact of high (undeterminable) protein binding on compound developability	14
23.6 Conclusions	15
References	15

23.1 SUMMARY

This chapter provides an overview of both conventional and unconventional methods to determine plasma protein binding. Plasma protein binding is an important ADME parameter required to understand drug efficacy and possibly toxicity. Plasma protein binding refers to the noncovalent interaction of drugs with plasma proteins such as albumin and α -acid glycoprotein (α -AGP). It may contribute to a wide variety of ADME phenomena such as drug–drug interaction potential and nonlinear, stereoselective, or interindividual pharmacokinetics variability. Therefore, the potential exists to identify and differentiate drug candidates based on plasma protein-binding values. Experiments using plasma protein binding advance our understanding of ADME properties to aid in candidate selection and development by determination of the unbound drug blood concentrations as well as (potentially) drug concentration at the site of action.

Recent progress in automation as well as increased routine performance of the analytical instrumentation has enabled a greater number of compounds to be assayed earlier in discovery, often at a high enough quality level such that repeat determinations later in development are not needed. Significant effort has been invested to search for high throughput screening methods that reliably and accurately determine protein binding in early drug discovery. The development of 96-well formats for equilibrium dialysis, ultrafiltration, and ultracentrifugation, combined with liquid handling automation have

transformed a formerly tedious, labor-intensive assay into a process, which can now be swiftly conducted. In addition, new approaches, referred to as *emerging techniques*, are also under investigation, including chromatographic methods such as the human serum albumin (HSA) column and spectroscopic methods such as surface plasma resonance, solid-phase microextraction, Transil membrane screening, and capillary electrophoresis (CE) methods.

Significant challenges still remain unsolved, particularly in cases of high protein binding with low exposure levels that fall below the normal limit of quantitation (LOQ). This chapter describes the impact of high (>99%), undeterminable protein binding on compound developability as well as possible solutions including plasma dilution techniques.

23.2 INTRODUCTION

The use of *in vitro* ADME (absorption, distribution, metabolism, and excretion) tools offers the exciting prospect of optimizing a drug candidate's efficacy while potentially reducing costly attrition during drug development. Over the last two decades, enormous investment and interest have blossomed in high throughput *in vitro* ADME screens, such as permeability, metabolic stability, drug–drug interaction potential, and transporter affinity. From a hierarchical perspective, *in vitro* methods of plasma protein binding for drug candidates have been less preferred and less frequently performed than other ADME screens. However, as the value of plasma-protein-binding data has become better understood, and, as more tools have been developed to conduct the experiment in a higher throughput manner, the conduct of plasma-protein-binding assays has increased in the early discovery or lead optimization space.

Plasma protein binding refers to the noncovalent interaction of drugs with plasma proteins such as albumin and α_1 -acid glycoprotein (α -AGP). The premise that only unbound or free drug is available to exert a pharmacological effect is known as the *free drug hypothesis* [1,2]. Although there is controversy in the literature, unbound drug levels are generally regarded to be the principal determinant of tissue distribution, cell entry, receptor interactions, and availability for elimination, thus impacting the pharmacokinetic/pharmacodynamic (PK/PD) relationship or off-target adverse reactions or toxicities. Plasma protein binding may contribute to a wide variety of ADME phenomena such as drug–drug interaction potential and nonlinear, stereoselective, or interindividual pharmacokinetics variability. Therefore, the potential exists to identify and differentiate drug candidates based on plasma-protein-binding values [3,4]. Plasma-protein-binding experiments advance our understanding of ADME properties to aid in candidate selection and development by determination of the unbound drug blood concentrations as well as (potentially) drug concentration at the site of action.

In 2002, the clinical relevance of plasma protein binding was challenged by Benet and Hoener [5], who through careful study demonstrated that protein binding is relevant primarily for IV administered drugs with a high extraction ratio and oral drugs with a high extraction ratio and a nonhepatic clearance mechanism. Examination of data for 456 currently marketed drugs showed that protein binding influenced exposure for 25, which fell into the two categories described above. If the therapeutic index is considered, protein binding influences even fewer compounds. Benet and Hoener's work might lead some to conclude that the protein-binding experiments are not worth

the time and effort they consume. However, the clear need for protein binding in the discovery and preclinical development stages to conduct allometric scaling and understand species-difference behavior is well understood. In rare cases, differences between animal and human plasma-protein-binding values may lead to significant differences in observed clearance, with severe negative outcomes. Protein-binding behavior of a drug candidate may not be in and of itself decision making, but has a profound influence on a variety of *in vivo* and *in vitro* properties during ADME and pharmacology experiments. Thus, its determination early in drug discovery can provide significant input into understanding tissue distribution and pharmacological effect.

Before the last decade, a significant stumbling block of plasma-protein-binding methods has been lack of miniaturization and automation. Plasma protein binding has been traditionally performed by equilibrium dialysis during drug development using ^3H - or ^{14}C -labeled compounds. The timing of these experiments is largely determined by the effort and expense to obtain radiolabeled material. The traditional methods consume a significant amount of material, are very labor intensive and not easily automated, but are considered sufficiently rigorous to permit data inclusion in regulatory submissions.

Significant effort has been invested to search for high throughput screening methods that reliably and accurately determine protein binding in early drug discovery. The development of 96-well formats for equilibrium dialysis and the coupling of this with liquid handling automation have transformed a formerly tedious, labor-intensive assay into a process that can now be swiftly conducted. However, as 96-well techniques are validated, the need for the final low throughput “definitive” radiometric experiment is becoming less and less compelling. In some situations, experimental results derived

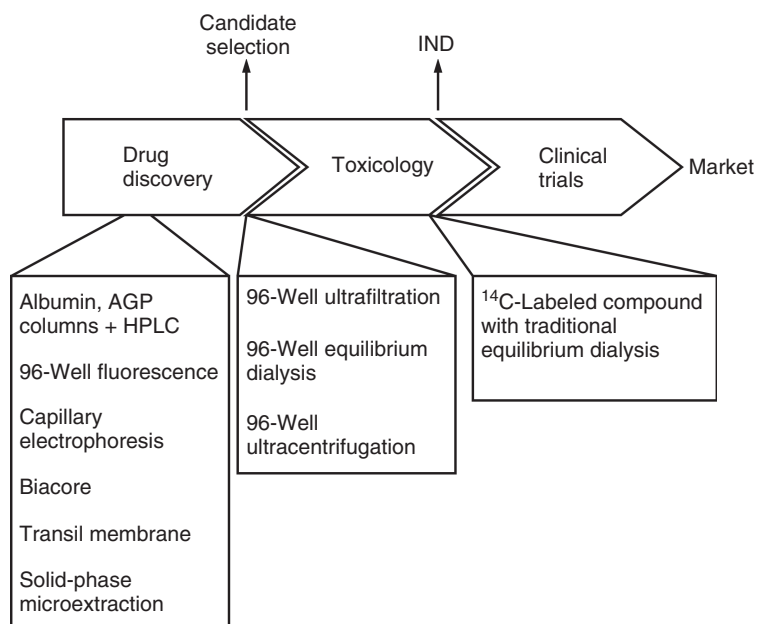


Figure 23.1 Overview of plasma-protein-binding methods during drug discovery and development.

from LC/MS/MS analysis may be used in regulatory submissions. In addition, new approaches referred to as *emerging techniques* are also under investigation and they will be described later.

An overview of normal timings of the techniques, which will be discussed are shown in Fig. 23.1. Early in drug discovery, rapid screening techniques that allow binning into low, medium, and high binding categories may be utilized. As a potential drug candidate progresses forward in the drug discovery process, and more of it is synthesized, more definitive approaches such as equilibrium dialysis are applied. Once a drug candidate is deemed to have a high likelihood of success, traditional equilibrium dialysis using radiometric detection may be performed. However, the approaches used across the pharmaceutical industry continue to evolve. With significant recent improvements in the high throughput automated devices used to conduct the incubations as well as the analytical instrumentation, the need for the “definitive” radiometric experiment is declining. Some companies may also choose not to invest resources in the conduct of binning experiments if higher quality data can be obtained with appropriate turnaround time from the 96-well equilibrium dialysis, ultrafiltration, or ultracentrifugation experiments. The maturation of plasma-protein-binding assays continues to be both an interesting and dynamic process.

23.3 TRADITIONAL METHODS

Plasma protein binding has been conducted for several decades as part of normal compound progression from animal experiments into the clinic. The original manually conducted experiments relied on simple approaches utilizing common laboratory equipments such as the centrifuge. An overview of the experimental process for “traditional” techniques [6], based on their popularity and longevity of conduct, that is,

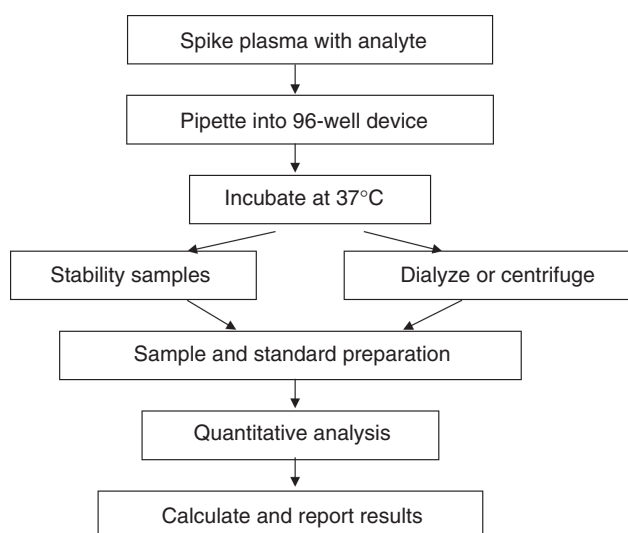


Figure 23.2 Process flow for traditional methods of plasma protein binding: ultrafiltration, ultracentrifugation, and equilibrium dialysis.

ultrafiltration, equilibrium dialysis, and ultracentrifugation is shown in Fig. 23.2. In addition, a summary of the advantages and issues, as well as, comparison with more innovative or higher throughput approaches described later in this chapter, is shown in Table 23.1.

When embarking on a protein-binding assay, several variables should be considered in the experimental design. The choice of which preclinical species to examine should be dictated by the purpose of the protein-binding experiments, such as PK calculations, PK/PD relationship, or exposure margin calculations for safety assessment studies. Plasma protein binding should be evaluated over an anticipated therapeutically and/or toxicologically relevant concentration range for the compound of interest. Additionally, these methods can be used to investigate individual binding proteins (i.e., HSA, α -AGP, preclinical species' albumin). Plasma harvested using EDTA as an anticoagulant is recommended. Concerns have been raised that the anticoagulant heparin may interfere with protein binding, and it should thus be avoided. During preparation of initial analyte-spiked plasma, minimal organic content (<2% by volume) should be maintained to minimize interference with protein binding. The stability of the compound in all species and matrices, including buffer solutions, should be assessed over the time period used for equilibration. This is accomplished by incubating an aliquot of the matrix standard and spiked buffer at the lowest concentration under the same conditions as the equilibrium dialysis apparatus. Samples taken at time zero and at the end of experiment should be assessed to determine percentage of analyte remaining.

23.3.1 Ultrafiltration

Ultrafiltration experiments [7–10] have been conducted in 96-well format for over a decade, thanks to the commercial availability of the filtration plates. In this experiment, an aliquot of analyte-spiked plasma is pipetted into an individual well of a single-use 96-well device. The bottom of this well is composed of a filtration membrane with a 10,000 Da molecular weight cutoff. The entire device is then centrifuged, during which time the unbound analyte solvated in plasma water and other low molecular weight components, will migrate through the filter to a collection well on the opposite site of the filter. Bound analyte will remain with the plasma proteins on the top half of the device, which is then discarded. The analyte-containing filtrate samples are then quantitatively assayed, in addition to unfiltered plasma spiked with analyte, once the centrifugation cycle is finished. The analyte percentage bound is then calculated using the following formula:

$$\left[\% \text{ Unbound} = \frac{\text{Drug concentration in ultrafiltrate post centrifugation}}{\text{Drug concentration in plasma pre centrifugation}} \times 100 \right]$$

Ultrafiltration is a relatively quick, inexpensive, and simple experiment to conduct but does suffer serious problems and inaccurate values due to compound adsorption to the top half of the filtration device. This can lead to serious overestimation of protein binding.

23.3.2 Equilibrium Dialysis

Equilibrium dialysis is generally regarded as the gold standard method for obtaining plasma-protein-binding information [11–16]. In addition to improvements in

TABLE 23.1 Comparison of *In Vitro* Methods of Plasma Protein Binding

Techniques	Advantages	Issues
<i>Traditional Techniques</i>		
96-Well ultrafiltration	% Binding Commercially available plates Automated	Nonspecific binding Must correct for volume shifts Not true equilibrium experiment
96-Well equilibrium dialysis	% Binding Automated “Gold standard” Flexible	May require long equilibration times Long-term plasma stability of analyte at 37°C may be problematic Devices not widely available
96-Well ultracentrifugation	% Binding Automated	Requires ultracentrifuge or long centrifugation times May generate errors because of lipoprotein interactions in supernatant
<i>Screening Techniques</i>		
Chromatographic methods (Human Serum Albumin, α -AGP columns)	Easy setup Low cost Relative low sample consumption Minimal hands-on preparation or intervention	Binning/relative ranking not absolute percentage binding
96-Well Fluorescence	Rapid Automated Easy setup K_d	Monitoring binding only to tryptophan residue of Human Serum Albumin and α -AGP May miss other binding interactions
Biosensor (Biacore)	Semiautomated % Binding and K_d	Higher time commitment and cost for setup More sample may be required Skilled operator required Solubility issues may be encountered
TRANSIL™ Membrane	Rapid Automated Sensitive % Binding and K_d	Individual proteins or blend of key proteins Not physiological conditions
Capillary electrophoresis	% Binding and K_d Wide dynamic range of K_d Online determination—no sample preparation required	Poor sensitivity Poor reproducibility Compound adsorption to capillary walls Highly acidic compounds problematic
Solid-phase microextraction	% Binding No sample preparation 96-well format	Not commercialized May observe sensitivity limitations and binding saturation for highly bound compounds

throughput via 96-well formats (see below), recent work at Astra Zeneca has shown that compound pooling may be utilized to increase the number of compounds assayed in a single experiment to as many as 10 [15]. On the basis of the high protein content in plasma (30–50 mg/mL HSA, 0.75 mg/mL α -AGP) relative to analyte concentration (typically 10–1000 ng/mL), compound pooling offers a simple but powerful mechanism to quickly obtain screening information. However, if multiple compounds bind specifically to α -AGP, pooling may introduce erroneous results compared to single-compound incubations.

Three different 48- or 96-well commercial equilibrium dialysis devices have been introduced since 2005. In the HT-Dialysis apparatus, eight dialysis membranes are vertically mounted between Teflon spacers that comprise the individual wells [14]. Owing to the vertical design, 96-well liquid handling devices can be used for automated buffer and plasma transfers. The device itself is reusable, with the membrane serving as the primary device consumable. The 96-well Dispo-Equilibrium Dialyzer proposed by Kariv *et al.* [17] utilize a single horizontally mounted dialysis membrane contained in a single-use unit. Use of liquid handling automation for sample handling is still feasible with the horizontal design, albeit with user intervention. The 48-sample Pierce Rapid Equilibrium Dialyzer (RED) system [16] offers a faster equilibrium time (1–2 h) because of a large surface area/volume ratio.

In a typical equilibrium dialysis experiment, blank phosphate buffer solution is added to one side of the dialysis membrane in each well and an equal volume of analyte-spiked plasma to the other side. The equilibrium dialysis device should be incubated at 37°C in a CO₂ atmosphere or with a strong phosphate buffer [17–19] to minimize plasma pH shifts over the experimental time course. Work done by Kochansky *et al.* has shown that pH may increase to 8.7 over 22 h incubation under ambient conditions [18]. This pH shift may alter the protonation state of the analyte thus affecting plasma protein affinity. Once the incubation is complete, individual aliquots are taken from both the plasma and buffer sides and quantitatively assayed by either LC-MS/MS or radiometry.

The analyte free fraction (f_u) is calculated using the following equation. This equation assumes that error added because of a Donnan equilibrium volume shift [20,21] is within the error of the assay method, and therefore negligible. The volume shift may be observed with long equilibrium times such as 24 h; however, compounds or devices that allow a rapid equilibration time of 4 h are unlikely to result in a significant shift.

$$f_u = \frac{C_{\text{buffer}}}{C_{\text{plasma}}}$$

where C_{buffer} is the unbound compound concentration in buffer after dialysis and C_{plasma} is the postdialysis plasma concentration.

The percent of drug unbound (free) and bound to protein are calculated as follows:

$$\% \text{ Free} = f_u \times 100$$

$$\% \text{ Bound} = (1 - f_u) \times 100$$

A potential problem and source of high variability with equilibrium dialysis is protein breakthrough from the protein-rich plasma side to the aqueous side of the dialysis cell, causing false elevation in the buffer compound concentrations. This can be

monitored by taking an aliquot of any remaining buffer, adding acetonitrile to precipitate the protein, and checking the solution for visible particulates. Use of a membrane with appropriate molecular weight cutoff (10 kDa minimum) will help prevent this problem.

During equilibrium dialysis, an equilibration time of 24 h is recommended for the sake of efficiency. However, scientific judgment can be utilized to reduce this time to 4, 6, or 8 h. Equilibrium dialysis devices that allow for agitation may allow for a faster achievement of equilibrium. For definitive data, it is important to assess at least two sequential time points to ensure that equilibrium has been reached. Analytes can be lost during equilibrium dialysis by nonspecific binding to the membrane and apparatus, decomposition of the compound, or solubility issues. Assessment of this loss can be performed in a separate recovery experiment or during determination of equilibration time. As equilibrium dialysis is a comparative technique, the two halves of an individual well should exhibit similar non-specific binding or recovery issues, thereby minimizing negative impact.

23.3.3 Ultracentrifugation

Ultracentrifugation of plasma results in a protein pellet and an essentially protein-free supernatant [22–25]. Plasma is spiked with the test compound. After centrifugation at 200,000 relative centrifugal force (RCF) for 18 h or 550,000 RCF for 4 h at 37°C, the protein bound material is pelleted with the proteins. The unbound drug material remains in the supernatant, which can be analyzed by radiometric or LC-MS techniques depending on whether the test article is radiolabeled. Generally, it is advisable to take a fraction of the supernatant from the center of the tube to avoid lipids, which may lie at the surface, and any drug concentration gradients that may occur as a result of the centrifugation. This technique largely avoids the problem of nonspecific binding to the apparatus; however, it is a low throughput method requiring an ultracentrifuge. For compounds with high plasma protein binding observed with equilibrium dialysis or ultrafiltration, it can afford a suitable alternative because it may reduce the impact of confounding factors such as solubility and nonspecific binding. The main disadvantage of ultracentrifugation relates to the presence of trace amounts of lipoproteins in the “protein-free” supernatant and highly bound drugs may be associated with these macromolecules. Therefore, compounds measured as free drug in the supernatant may actually be bound, and this will lead to an artificially high free fraction determination.

Buffer controls are used to test for nonspecific binding to the tubes. One option is to test at a concentration that is equivalent to 1% (assuming a 99% protein bound compound) of the lowest concentration tested to mimic the free fraction of a highly bound compound.

The equation used for calculations is as follows:

$$\% f_{\text{up}} = \frac{\text{Measured drug concentration post ultracentrifugation}}{\text{Measured drug concentration pre ultracentrifugation}} \times 100$$

23.3.4 Detection—Radiometric or LC/MS/MS

Radiometric detection had been the classic method of quantitation for plasma protein binding until the invention and broad use of LC/MS/MS methodology. The use of

tritium or carbon-14 labeled materials allow a facile determination of absolute recovery from the various plasma-protein-binding devices and a ready measure of drug amount. A potential drawback of radiometric determination occurs for very highly bound drugs and relates to the purity of the label. When the percentage of radiochemical purity of the radiolabel approximates the percentage of plasma protein binding, it is possible that the unbound material measured is actually impure rather than drug material. This puts the results into question. For this reason, the use of LC/MS/MS may afford a more accurate measure for very highly bound drug candidates. The use of standard curves to ensure quantitative measure and comparison to appropriately spiked control samples will allow quantitation for the purpose of recovery calculations.

Historically, highly bound compounds were considered to be those with binding of >95%. With current techniques (either highly pure radiolabel or the use of LC/MS/MS), accuracy within this range has improved considerably and the current limit is considered to be ~99%. If one considers a 10 μ M concentration, 1% unbound material that represents 100 nM, a reasonable limit of detection for LC/MS/MS methods.

23.4 EMERGING TECHNIQUES

Exploratory work continues to investigate new opportunities to improve plasma protein binding beyond the previously described high throughput traditional approaches. These so-called “Emerging Techniques” employ methodologies ranging from chromatography, CE, spectroscopy, biosensors, microextraction, and model artificial membranes. Table 23.1 describes the relative advantages and issues for traditional and screening *in vitro* plasma-protein-binding techniques.

23.4.1 Chromatographic Methods

Significant effort has been invested over the years in assessing plasma protein binding through chromatographic methods based on HPLC columns containing the most abundant plasma proteins, HSA and α -AGP [26–33].

Through the usual chromatographic partitioning processes, binding can be correlated with chromatographic retention time. In a typical experiment, the capacity factor (k') of each compound is calculated based on its retention time, using the following formula:

$$k' = \frac{t_r - t_m}{t_m}$$

where t_r is the retention time of the analyte and t_m is the retention time of an unretained compound, also known as the *column void volume*, and t_m can be assessed using glucose, cesium iodide, or ammonium nitrate. From the k' values, $(k'/k' + 1)$ is calculated and then plotted as a function of the known plasma protein binding, as shown in Fig. 23.3. After the correlation plot is generated, the plasma protein binding of unknown compounds can be calculated based on their retention time on the albumin or α -AGP column. In general, the factor $k'/k' + 1$, rather than k' , shows better linear correlation with plasma-protein-binding values. As shown in the Fig. 23.3, compounds can be binned into low, medium, and high protein-binding categories, to aid in screening. When first establishing the method in one's laboratory, a series of compounds with

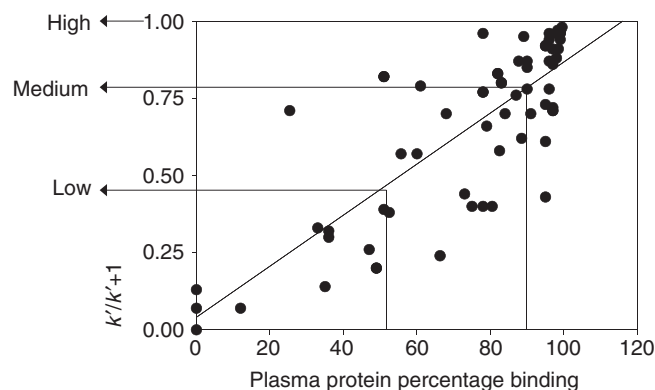


Figure 23.3 Binning of low, medium, and high plasma protein binding by chromatographic retention.

known plasma-protein-binding values should be chromatographically analyzed using an HSA column. In addition, an α -AGP column may be used in series or parallel. The degree of plasma protein binding can be either found in the literature [34,35] or obtained by comparative ultrafiltration or equilibrium dialysis.

Recently, both Hage and coworkers and Wainer and coworkers have published reports on the use of in-house prepared HSA affinity microcolumns for plasma-protein-binding determinations [36–38]. Both frontal affinity and zonal elution chromatography have been utilized, each with its own advantages and disadvantages. The primary stumbling block to widespread application of these techniques within the pharmaceutical industry is the commercial availability of the HSA microcolumns.

In practice, most pharmaceutical laboratories screen only against the HSA column, accepting the risk that binding α -AGP or other less-abundant serum proteins may be completely underestimated. This method generally performs better for compounds exhibiting low to moderate plasma protein binding rather than high (>95%).

23.4.2 Capillary Electrophoresis Methods

The application of CE to the measurement of plasma protein binding represents a logical mechanism to leverage the inherent ability of CE to separate analytes based on electrophoretic mobility. CE techniques utilized for this purpose essentially examine the difference in analyte mobility in bound and unbound states to various proteins. Affinity capillary electrophoresis (ACE) techniques have been utilized for relative rank ordering of compound libraries versus various protein receptors [39], which can be measured during the separation step or against a solid-phase ligand support. This methodology offers the advantages of small sample volume requirements, relatively rapid measurements under equilibrium conditions, and at pH 7.4 “near physiological conditions,” similar to traditional techniques such as equilibrium dialysis. In a study conducted by Ishihama *et al.* [40], this technique was applied to six anionic drugs. Their binding to HSA correlated with values obtained by ultrafiltration over a range of 78.7–99.9% binding, with typical reproducibility <10% CV (coefficient of variation). However, the required drug concentration in solution was 20 μ M.

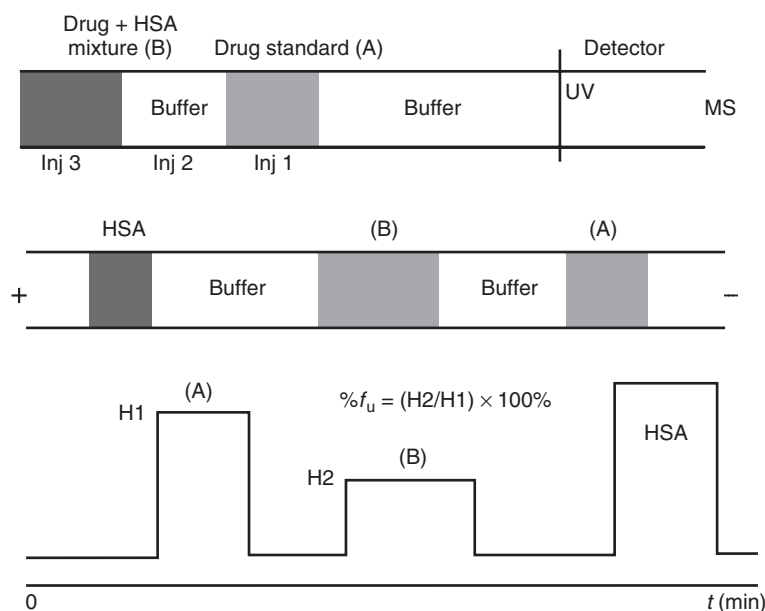


Figure 23.4 Illustration of single-run measurement of protein binding by high performance frontal analysis capillary electrophoresis. H1 and HB are the peak plateau heights of the drug standard and the drug resolved from the drug–protein mixture, respectively. $\%F_u$ is the unbound free fraction of drug to specific protein.

Higher sample volume online approaches can be leveraged by combining frontal analysis with CE. Figure 23.4 shows a schematic of this process, described as frontal analysis capillary electrophoresis (FACE). An injection of neat analyte solution is made, followed by a buffer plug, and subsequently by an injection of analyte + protein mixture. On the basis of binding equilibria as well as relative electrophoretic mobility, the resulting electrophoretogram will contain a free analyte peak, which can be used as a relative standard response against a separate, later eluting peak corresponding to analyte not bound to the protein of interest. Depending on experimental conditions, the unbound analyte peak may or may not be resolved from the protein peak and may instead appear as a shoulder on the larger protein peak. CE is most commonly coupled with UV detection and was leveraged for plasma-protein-binding experiments in a study of protein–drug binding constants for HSA and α -AGP [41,42]. More recently, commercial interfaces to mass spectrometers have been developed and utilized for plasma-protein-binding determinations [43]. The analyte concentrations required can be decreased from the ≥ 1 mM to <20 μ M range. Better correlation of percentage binding values were observed with the plasma was diluted to 10% plasma in buffer. In addition, a dependence of percentage binding on time interval between sample injections was observed. It was hypothesized that because of significant lower concentrations for MS detection compared to UV resulted in differential adsorption of the analyte to the capillary walls, rather than saturation at high concentration. This technique provides rapid screening values but has not yet matured to a stage where precise percentage binding values can reliably be obtained, particularly for highly bound compounds.

23.4.3 Spectroscopic Methods

The 96-well fluorescence approach relies on the detection of intrinsic fluorescence of the tryptophan residues of HSA and α -AGP, the primary plasma protein constituents [44]. As drugs bind to the protein, the fluorescence signal is quenched as a function of analyte concentration. The analyte's dissociation constant, k_D , can then be calculated. Although this method is readily automated, its universality is questionable, since tryptophan residues reflect only a portion of the potential binding sites to both α -AGP and HSA.

23.4.4 Biosensors—Surface Plasmon Resonance

Another high throughput technique for plasma protein binding utilizes the Biacore™ surface plasmon resonance (SPR) technology as shown in Fig. 23.5 [45–48]. Proteins of interest are immobilized onto a gold foil. The drug is exposed to the proteins, binding occurs in real time, causing a change in refractive index of a light beam. The kinetics of both the on-rate and off-rate is captured in a sensorgram. After determining the dissociation k_D values from sensorgrams of multiple solutions containing several concentrations of drug, the corresponding percentage binding can be calculated. The binding to both albumin and α -AGP is combined to give the overall plasma protein binding for these two proteins. If binding to proteins other than albumin or α -AGP are important, the plasma protein binding will be underestimated. The need to purchase Biacore SPR instrumentation as well as instrument and chip synthesis proficiency can be potential limitations.

SPR has the potential to become a very high throughput technique since the use of microfluidics and the generation of protein arrays on the chip allow for highly multiplexed approaches [49]. Although this technique requires a significant amount of time and expertise to establish, once operational, it can provide extremely high throughput results. In addition, the potential to address the critical gap of very high bound plasma-protein-binding determination is an intriguing future prospect.

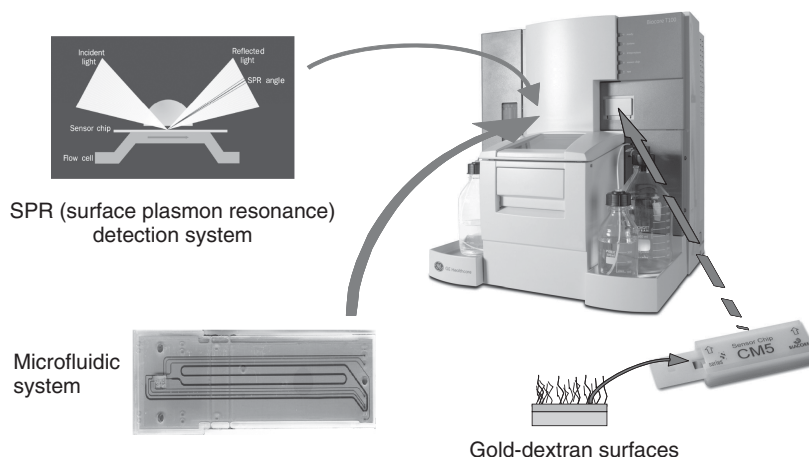


Figure 23.5 Experimental approach and equipment required for Biacore™ analysis of binding by surface plasmon resonance.

23.4.5 Solid-Phase Microextraction

Solid-phase microextraction (SPME), pioneered by Janusz Pawliszyn and coworkers [50], represents one of the more recent developments in general analytical sample preparation. This methodology relies on the extraction of the analyte of interest from liquid or gaseous media onto a solid support, generally a polymeric coating on a metal rod. The microextraction probe is engineered in a manner to enable maximal surface contact and ultimately analyte adsorption. This technique has been utilized to determine plasma protein binding for a set of pharmaceutical compounds including ibuprofen, warfarin, verapamil, propranolol, and caffeine with low, intermediate, and high protein-binding properties. Good correlation was observed between literature and SPME-derived protein-binding values. Figure 23.6 shows a schematic of the SPME experimental design for protein binding. Several variables were optimized including choice of fiber material, pH control mechanisms, and plasma dilution with phosphate buffer saline (PBS). Plasma dilution serves a very useful purpose of allowing highly bound ($>99.9\%$) drugs to be reliably quantitated by decreasing the amount of plasma proteins and correspondingly, the percentage bound. Although commercial devices for this technique are not yet available, exciting possibilities for 96-well parallel extraction designs can be easily envisioned.

23.4.6 TRANSIL™ Membrane and Protein Beads

The TRANSIL™ membrane beads are used to replace erythrocytes [51] in partitioning assays and are uniquely suited to plasma-binding determinations for highly bound or low aqueous solubility drugs [52,53]. In this assay, the analyte is incubated together with plasma and the membrane beads for 30 min. By determining drug concentrations in plasma after incubation, the assay circumvents issues with compounds with poor aqueous solubility. This assay monitors the decrease in analyte plasma concentration rather than the appearance of analyte in another phase (buffer). As essentially an indirect measurement, the need for ultrasensitive quantitation for highly bound drugs is avoided.

To determine the affinity of drugs to plasma, the change in affinity to the membrane beads, induced by changing the plasma concentration, is measured. The fraction

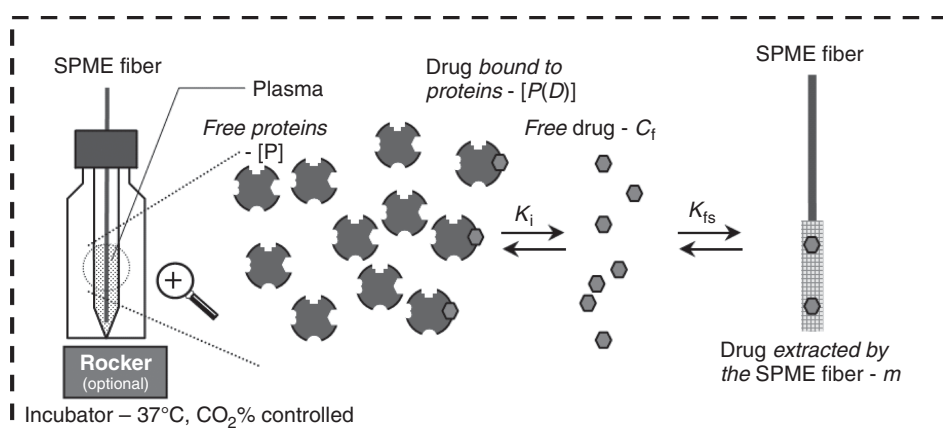


Figure 23.6 Schematic representation of experimental setup for the determination of plasma protein binding by solid-phase microextraction (SPME).

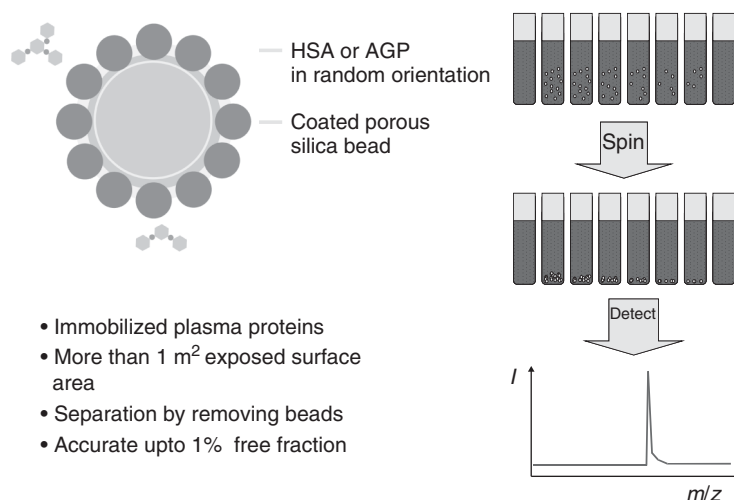


Figure 23.7 Illustration of TRANSIL™ beads and approach for measuring binding using beads embedded with plasma proteins.

bound is calculated from linear regression of membrane affinity (M_A , Y) measurement data obtained from different plasma dilutions (D_{plasma} , X). The calculated slope α and intercept M_{A0} are then inserted into the following equation:

$$f_b = 1 - \frac{1}{1 + \alpha M_{A0}}$$

This approach allows accurate measurement of free fractions of 0.01 or lower.

A rapid technique for predicting plasma binding from affinities (K_d) to HSA and AGP is provided by the TRANSIL protein bead assay system based on HSA and α -AGP mobilized on silica beads. The beads are suspended in a solution of drug and after a few minutes, centrifugation is performed to separate the beads as shown in Fig. 23.7. Versions of the assay exist for HSA, AGP, rat albumin, and HSA and AGP mixed together at a ratio of 24:1 to mimic plasma conditions. Quantitation of analyte remaining in the supernatant may be quickly and reproducibly measured by radiometry or LC/MS/MS, as appropriate

23.5 IMPACT OF HIGH (UNDETERMINABLE) PROTEIN BINDING ON COMPOUND DEVELOPABILITY

It is common that highly lipophilic drugs may exhibit very high plasma protein binding, in excess of 99%. Owing to analytical limitations, the accuracy of determinations above 99% bound are not reliable when radiometry or LC/MS/MS is involved. For radiometric determination, the radiolabel must be >99% pure in order to be confident that the free fraction observed is because of drug and not impurity. Radiolabel with this degree of purity may be very difficult to obtain and is well above the purity typically required for other purposes such as excretion or metabolite profile studies

where 95% radiochemical purity is sufficient. For LC/MS/MS, a 99% plasma protein bound (PPB) compound would yield 1% free, leading to 10 nM LOQ for a study conducted at a typical 1 μ M starting concentration. This LOQ is readily measurable for most NCEs (new chemical entities) but not necessarily with the requisite precision and accuracy to report greater than unit percentage binding values with confidence. Most LC/MS/MS methods rely on relatively simple generic sample extraction methods (protein precipitation) as well as chromatographic and mass spectrometric conditions. The emphasis of method development at the time protein-binding assays are conducted is generally on speed (turnaround time), not exquisite sensitivity. The most pragmatic approach taken, if LC/MS/MS sensitivity cannot be readily improved, is to simply report >99% binding and move on to the next experiment.

The inability to accurately determine the plasma protein binding is an issue because it is the free fraction that is important for the determination of PK/PD and safety margins. Above 99% binding, the corresponding free fraction is 1% or less and differences become pronounced. For example, the difference in free fraction between 99.4% and 99.8% is a factor of 3; 0.6% versus 0.2% free. Analytical methods that provide inadequate sensitivity and corresponding LOQ seriously hamper decision making. Inability to precisely and accurately determine percentage binding values for very highly bound compounds seriously limits accurate PK/PD interpretation from preclinical species or prediction of necessary dosing regimens that will provide efficacious doses in the clinic.

In situations where the PPB is very high, one recommended approach is to conduct the experiment with plasma that is diluted to 10% in phosphate buffer solution. The undiluted plasma result can then be back calculated to plasma binding by the equation [54], where D and $F_{u, \text{measured}}$ represent the fold dilution of plasma and unbound fraction measured with diluted plasma, respectively.

$$\text{Undiluted } f_{\text{up}} = \frac{1/D}{\left[(1/F_{u, \text{measured}}) - 1\right] + 1/D}$$

23.6 CONCLUSIONS

Plasma protein binding is clearly an important ADME parameter required to understand drug efficacy and possibly toxicity. Recent progress in automation as well as increased routine performance of the analytical instrumentation has enabled a greater number of compounds to be assayed earlier in discovery, often at a high enough quality level such that repeated determinations later in development are not needed. Significant challenges still remain unsolved, particularly in cases of high protein binding with low exposure levels that fall below the normal LOQ. As technology improves even further, we can expect more opportunities to understand and leverage this important information to an even greater extent.

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1 Clinical Aspects of Drug Biotransformation: An Overview

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1.1	Introduction	1
1.2	Biotransformation: major components	4
1.3	Control of biotransformation: enzyme induction	11
1.4	Pharmacogenetics: impact on drug efficacy	18
1.5	Inhibition of drug biotransformation: clinical consequences	25
1.6	Patient factors affecting biotransformation	30
1.7	Drug metabolism and drug development	33
1.8	Conclusions	39
	References	39

1.1 INTRODUCTION

1.1.1 Clinical Drug Usage

Over the past 30 years, enormous strides have been made in drug development. The combination of the pharmaceutical industry and government regulatory agencies has to a remarkable extent, produced entirely new drug classes which can not only cure or control many serious and debilitating diseases but also achieve this in many cases with minimal toxicity. Cardiovascular and malignant disease, for example, the largest killers in the developed world, have been treated with a variety of new and demonstrably effective drug regimens, such as the statins, angiotensin converting enzyme (ACE) inhibitors, as well as the taxanes, camptothecins, and tyrosine kinase inhibitors. These drugs have alleviated mortality and morbidity and several previously untreatable and invariably fatal conditions such as AIDS have been clinically redefined since the 1980s through combination drug therapy as serious, but now nonfatal and chronic, yet manageable diseases.

Indeed, the relatively narrow choice of toxic drugs available for other debilitating neurological conditions such as schizophrenia, depression, bipolar disease, and

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epilepsy up to the end of the 1970s has also been vastly widened with the selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) as well as several new neurileptics and antiepileptic drugs (AEDs), which are, in most cases, significantly less dangerous for vulnerable patients, in terms of safety in overdose as well as reduced toxicity. Such advantages also have the added benefit of promoting compliance.

Naturally, none of these advances have been risk free and there are many reasons why drugs may cause adverse effects of various kinds. Some of these problems are very difficult to predict as they are the result of complex pharmacological, physiological, biochemical, and genetic interactions. Such effects may only emerge after many years and sometimes decades of use. The association of long-term β -blocker usage and their promotion of the risk of diabetes [1] was one such problem, and it is likely that the existing risks of several drugs used in mass therapy such as the statins [2] may be compounded by others that may perhaps only emerge in subsequent decades. Sometimes, these risks are balanced by benefit in control of symptoms and increased longevity [3] and as well as other positive off-target effects, such as the possible beneficial effects of statins on dementia [4].

More immediately, there are many factors that can govern day-to-day drug efficacy and adverse reactions that are also difficult to predict. These include receptor numbers and activity (e.g., downregulation), pharmacological interactions, protein and deep tissue binding, as well as abrupt changes in renal clearance. However, the single greatest influencing factor on whether a drug exerts a therapeutic or a toxic effect is its concentration at the receptor sites in the target organ or organs. Of course, receptor concentrations are not necessarily the same as what is broadly termed systemic drug concentrations and any relationship between the two is strongly linked with the physicochemical characteristics of the drug and their influence on its tissue distribution. Nevertheless, systemic concentrations are usually all that can be measured in clinical practice and these concentrations can usually be linked with pharmacological action. This relationship tends to hold for drugs such as AEDs but not for drugs monitored pharmacodynamically, such as warfarin. Assuming dosage is regular and absorption is reasonably complete, systemic drug levels and indeed, drug bioavailability, are themselves dependent on the various processes of biotransformation that occur in the liver, gut, lung, and kidney, which clear the drug either “pharmacologically,” by causing structural changes which reduce or eliminate therapeutic action, or through complete removal from the body in the process of excretion.

1.1.2 Biotransformation and Drug Efficacy and Toxicity

It cannot be overemphasized that biotransformation as a process is a network of coordinated systems that possesses two governing objectives: essentially to “serve and protect.” In terms of “service,” biotransforming enzymes are responsible for the myriad reactions behind the assembly of millions of bioactive endogenous chemicals that, like hormones and other influential small molecules, are responsible for second-by-second homeostatic tasks, through to long-term and permanent comprehensive bodily changes such as those associated with puberty and the maintenance of reproductive capacity. “Protection” involves the control of the cellular environment to ensure that endogenous molecules are confined to half-lives that are commensurate with their purpose, as well as the rapid elimination of interloper molecules such as the classic exogenous

endocrine disruptors, which are now ubiquitous in our environment. As far as biotransformational systems are concerned, drugs fall into the “protect” category and as with any system that has evolved to serve and protect, it must be aware of its environment. Hence, biotransformation is sensitive to challenge by exogenous as well as endogenous chemical entities. Drugs such as many AEDs and some antibiotics such as rifampicin exert powerful enzyme-inducing effects on biotransformation, aimed at expediting their clearance, while inhibitors such as grapefruit juice and azoles shut down crucial aspects of biotransformational capacity to the potential detriment of the individual. Into this dynamic, sensitive and highly efficient system, drug therapy is pitched and considering the high state of evolution of biotransformation, it is remarkable that drug treatment outcomes are as good as they are.

Biotransformation's impact on drug efficacy and toxicity is transmitted through changes in clearance. If an inducer drug is added to a regime, biotransformation of the enzymes that process that agent and indeed many others will increase by several fold to a point of maximal stability, provided the inducer dosage is maintained. The resultant acceleration of the clearance of this drug and many others will cause their respective levels to fall out of the therapeutic window and drug efficacy will diminish and eventually be lost. In contrast, if a potent inhibitor of biotransforming enzymes is added to a regime, then clearance will slow and possibly stop, leading to drug accumulation if the dosage is maintained. This can lead to intensification of the normal profile of pharmacological effects, as well as off-target effects, which are unconnected to the original purpose of the drug, but nonetheless exert considerable impact on the patient. These changes are to some extent predictable and are dose related. Biotransformation can be involved in idiosyncratic drug toxicity through the consequences of the formation of reactive species during metabolic processes. Although biotransformation is not the only mechanism at work in idiosyncratic drug reactions, these effects are notable by their unpredictability and lack of relation to dosage or duration of therapy.

1.1.3 “Real-World” Drug Usage

It could be considered that the patient is vulnerable to problems with drug concentration at two key stages in their therapy. The first is the initial consultation with a healthcare practitioner when drug therapy is prescribed. The second stage might be considered when changes are made in an established drug regime. These two critical stages are themselves influenced by what could be termed underlying factors, such as patient age, gender, pharmacogenetics, diet, and pharmacologically active substance intake, licit, and illicit. What could be termed dynamic factors such as the use of a drug which is either an inhibitor or inducer of biotransformation also applies to these stages.

The potential for these underlying and dynamic factors to influence drug treatment outcomes is very considerable. For example, when a drug regime is initiated, a standard recommended dose may be suitable for the majority of patients, but may lead to accumulation in the elderly and neonates, who possess reduced biotransformation capacity [5]. The prescription of an inhibitor of biotransformation may cause a similar drug accumulation problem. Alternatively, gender can influence rates of biotransformation as females clear many drugs more rapidly than males [6]. These effects, as well as inducing drugs, may seriously jeopardize drug therapeutic success by accelerating clearance from the body.

Of the remaining major influences on biotransformation and prescribed drug outcomes, two are particularly problematic and often interrelated; these include

polypharmacy [7] and the emerging phenomenon of patient self-medication [8]. Of course, these factors may combine, where patients on complex drug regimes may cause serious disruption to their drugs' efficacy and toxicity if they self-medicate with herbal preparations such as St. John's Wort. However, what is of concern is that there are some parallels between the two situations in terms of impact on some selected and vulnerable patient groups. Complex drug regimes are most common in the elderly and those who are under specialized treatment, such as those who have received xenografts, or those under cancer chemotherapy. All these conditions (if being elderly can be described thus) have a powerful impact on patient health, well-being, and particularly mental state. Patients are internally motivated to self-medicate to "assist" therapy, perhaps through taking antioxidant supplements or dietary products during cancer treatment. Conversely, the stress and uncertainty of major therapy may promote the use of herbal antidepressants in cancer chemotherapy and organ graft. Hence, a complex pathology may result, involving factors such as age, gender, and impact of disease that are compounded by polypharmacy. Drug clearance is likely to be strongly affected, leading to problems with toxicity, drug efficacy, treatment outcome, and even life expectancy. Perhaps most worryingly, many patient-motivated changes to diet and medication may occur without the consent or knowledge of the healthcare professionals responsible for the patient's optimum care.

While there are many issues discussed earlier in terms of the management of patient care, what is of paramount importance is the realization and some understanding of the pivotal role of drug biotransformation in patient healthcare outcome. The following chapter will outline some of the major features of the hugely dynamic and almost infinitely adaptable human biotransformation process.

1.2 BIOTRANSFORMATION: MAJOR COMPONENTS

1.2.1 Transporters: Influx

A powerful influence on drug efficacy and toxicity is the process of "first pass," which dictates bioavailability. While for many years the major biotransforming enzymes such as the cytochrome P450s (CYPs) and the glucuronosyl transferases were seen as the major influence on bioavailability, it is now apparent that many more biotransformationally coordinated systems, such as transporters, make a substantial contribution to first pass. The complexity of these systems is rooted in the somewhat contradictory roles that the gastrointestinal (GI) tract is required to play, concerning the simultaneous selective uptake of key nutrients that would not be otherwise absorbed and the role of "gatekeeper" [9], which is necessary to repel potential toxins. Indeed, it cannot be assumed that the most basic step toward a therapeutic effect, that is, drug absorption, is always a simple matter of passive diffusion. While many agents are passively absorbed through membranes due to their lipophilicity, there are others whose physicochemical properties preclude this step, such as amphipathic chemicals. These contain polar as well as nonpolar regions and as such can be excluded from passive membrane diffusion. Hence, amphipathic drugs must avail themselves of transport systems that normally import nutrient amphipathics such as amino acids and bile salts. The organic anion-transporting polypeptides (OATPs) usually exchange such molecules in return for the export of a thiol such as glutathione (GSH). Several amphipathic drugs depend

on the OATPs to be translocated across not only the gut but also the liver. Interestingly, depending on the specific site of the OATPs, diametrically opposed effects on drug bioavailability can result. In the gut, inhibition of OATPs by agents in fruit juices can dramatically reduce the uptake of the antihistamine fexofenadine [10], and these juices can also affect thyroid hormone supplementation [11]. In contrast, further along the process of presystemic absorption, several drugs such as the statins pravastatin and rosuvastatin appear to depend on organic anion-transporting polypeptide 1B1 (OATP1B1) for hepatic uptake from the portal blood supply. One of the best clinical examples of drug–drug interactions (DDIs) concerning the OATPs is the effect of cyclosporine on statin bioavailability. Patients who have received xenografts such as whole organs often have lipid metabolism that is abnormal enough to warrant statin treatment. However, when their main immunosuppressing therapy includes cyclosporine, this drug is a potent inhibitor of hepatic OATP1B1, so the normally extensively metabolized statins evade hepatic uptake and up to 20-fold systemic increases in statin plasma concentrations can result; this can lead to severe muscle toxicity (rhabdomyolysis) [12,13]. Interestingly, not all immunosuppressant drugs cause this effect, as tacrolimus, for example, does not appear to interfere with OATP1B1 [14]. Both influx and efflux transporters (described below) are indirectly sensitive to substrate pressure and are regulated through the same system that controls CYPs and glucuronyl transferases (UGTs) (Section 1.3.1 and 1.3.5).

1.2.2 Transporters: Efflux

The most extensively investigated transporter that is chiefly concerned with expelling drug-like molecules is P-glycoprotein (P-gp, also known as ABCB1). This protein is one of several adenosine-triphosphate (ATP)-binding cassette efflux transmembrane transporters, known as the ABC transporters. They are part of a raft of protective efflux systems that exert a powerful influence on cellular environments. These include the various multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP or ABCG2). P-glycoprotein (P-gp) is intensively studied, as it is ubiquitous in tissues and aside from its gastro-intestinal tract (GIT) protective function, it is one of the first lines of defense against antiproliferative agents in malignant cells and has been implicated in resistance to antiepilepsy therapy. P-gp is often described as promiscuous in its binding of substrates, although there seems to be almost as many apparent inhibitors as substrates of this transporter, so the overall picture as to how different substrates and inhibitors influence each other's penetration into tissues is extremely complex and is not readily predictable. This is partly due to the vast numbers of dietary chemicals that may also interact with P-gp. It is apparent that some deleterious drug interactions are rooted in the role of P-gp. The intensification of cyclosporine-mediated renal toxicity in the presence of coadministered sirolimus is linked with the latter agent's inhibition of P-gp [15]. Some efforts have been made to manipulate P-gp through the use of inhibitors such as verapamil, which in studies with human hepatocytes, can increase drug residence time and promote oxidative metabolism of raloxifene [16]. P-gp is controlled through the pregnane-X-receptor (PXR) induction system (Section 1.3.2.1) and is responsive to drug pressure. It is likely that the major research effort to modulate P-gp clinically will be most effective in the area of cancer chemotherapy resistance attenuation, rather than in drug GIT absorption and metabolism.

1.2.3 Cytochromes P450

1.2.3.1 Introduction. Once a therapeutic agent has either been passively absorbed, perhaps transported by OATPs or P-gp, its next obstacle to surmount before reaching the systemic circulation is probably the ultimate cellular biotransformational force, the CYPs. These enzymes are found in virtually every tissue, but the endoplasmic reticulum of gut and liver cells are particularly rich in cytochromes P450 or CYPs as they are usually termed. These lipophilic enzymes are responsible for more than 70% of drug clearance and possess several unique features that effectively ensure that most present and future pharmaceutical agents can be biotransformed to some degree. CYPs are thought to have originated in bacteria in the early stages of life on Earth and their ability to reduce substrates probably reflects the realities of early life in oxygen-free environments. After life became established and larger species colonized the land, one of the most potent drivers of CYP evolution within animal life is thought to have been the development of the defensive capability necessary to survive various plant toxins as animals established their habitats [17]. Interestingly, present-day bacterial CYPs such as CYPBM3 (102A1) are capable of metabolizing several therapeutic drugs, such as nifedipine and propranolol [18] and only modest bioengineering is required to widen the catalytic capabilities of such bacterial CYPs enormously [19].

CYP evolution has allowed the vast range of eukaryotic life to effectively manage the problems of the manufacture of required endogenous molecules such as hormones, their control, and destruction, alongside the threat from potential xenobiotic endocrine disruptors. To succeed in all these areas over billions of years would almost certainly not be possible using a somewhat restrictive basic “lock-and-key” enzymatic approach. While these enzymes really only act as monooxygenases and place a single oxygen atom into a substrate, the key to CYP success is their ability to apply this single process to a vast array of chemical structures that in turn, leads to a variety of fundamental rearrangements in the structure of the substrate that then causes significant physicochemical changes. These two latter features govern the main pharmacodynamic and pharmacokinetic behaviour associated with a particular drug, toxin, or hormone, usually expediting clearance and subsequent loss of therapeutic impact.

CYPs achieve this through their ability to retain the relative rigidity of their active site, while exhibiting extraordinary flexibility in their ability to bind substrates. Although other biotransforming systems can achieve this, an inkling of the sheer versatility of CYPs has been gained of their flexibility indirectly through the evaluation of many crystal CYP structures where a variety of substrates are bound in unique structural conformations. Hence, CYPs apply the “induced fit” rather than “lock-and-key” model of substrate binding [20], rather like a human hand grasping an object that is aligned toward the palm as the active site [21].

The CYPs are generally identified through their respective gene families (e.g., CYP1 and CYP2) and at least 40% sequence homology aligns a particular CYP to others within that family, while the subfamilies (e.g., CYP1A and CYP2A) are classified according to at least 55% homology. Particular CYP isoforms controlled by a single gene are classified through a number following the subfamily letter, such as CYP1A1 and CYP1A2. Although there are 18 human gene families and 44 subfamilies, many CYPs are engaged with functions other than the metabolism of xenobiotics, such as the complex machinery for the synthesis of the various hormone molecules. Others are

engaged in modulation of brain chemistry and circulatory control. The major families that are of relevance to xenobiotic (i.e., drug and potential toxin) clearance include the CYP1, 2, and 3 families [17].

1.2.3.2 CYP Biotransformational Impact. Alongside most human enzyme systems, the CYP families are mostly highly polymorphic [17], where several alleles exist of the same gene with massively varying degrees of expression and catalytic capability. Indeed, in many cases, particular alleles are absent from considerable proportions of some human populations. Whilst over the millennia, the forces that have shaped differential human expression of CYPs include exposure to various chemical agents in diet and the environment, the net effect is to safeguard the whole population from a single toxin, at the expense of individuals. This enormous strength of our species unfortunately translates into a major problem for drug development, where one size definitely does not fit all.

As CYPs are effectively the major force in drug biotransformation and they affect clearance, drug residence time and efficacy to a critical degree, any changes in their metabolic processes will have profound effects on drug therapeutics. If they are simply not expressed as described above, or inhibited by other coadministered drugs, then their failure to clear their respective substrates will result in drug accumulation. This is initially often translated into “off-target” therapeutic effects that are unintended and often problematic, leading to frank toxicity and even death in the case of torsades des pointes (TdP; Section 1.5.4.2). In contrast, if CYP clearance is accelerated for any reason, drug residence time will fall to the point that therapeutic failure may result. Hence, anything that affects CYP performance will crucially affect therapeutic performance, with potentially serious consequences to the patient.

In terms of the impact of CYPs on drug clearance, it is now an oft-quoted statistic that more than half of all prescription drugs are cleared by one CYP isoform, CYP3A4 [22], which comprises around a third of human CYP content [23]. This CYP is found in large quantities in the gut as well as the liver and is probably the most versatile CYP in terms of substrate range, as it can process very large molecules such as cyclosporine, right down to various steroids. CYP3A4 is complemented by CYP3A5, which is expressed in some ethnic groups, but has a very similar catalytic and expression profile to CYP3A4. Although CYP3A4 is not significantly polymorphic in the real world, in therapeutics, it can be problematic as its basal expression varies significantly. It is also vulnerable to a range of inhibitory drugs [24] that are commonly used and often added to regimes where at least one of the drugs used is a CYP3A4 substrate. Consequently, during drug development, enormous efforts are made to investigate the effects of CYP3A4 inhibition in terms of predicting the possible therapeutic impact on coadministered drugs.

Other major CYPs relevant to drug clearance are the CYP2C and CYP2D sub-families, which include CYP2C9, CYP2C8, and CYP2C19 as well as CYP2D6. With the exception of the latter CYP, the CYP2Cs are highly polymorphic and the whole CYP2 family is believed to be responsible for the metabolism of up to a quarter of all prescription drugs [25]. CYP2C9 is of particular interest for its role in the clearance of several agents, particularly warfarin, phenytoin, and a number of nonsteroidal antiinflammatory drugs (NSAIDs) [26], while CYP2C8 also clears some NSAIDs and taxols. CYP2C19 is well documented for its activation of clopidogrel, as well as clearance of proton pump inhibitors such as omeprazole [26]. While the CYP2C variants

are strongly polymorphic, CYP2D6 is particularly interesting as it metabolizes several popular pharmaceutical groups of drugs, ranging from antipsychotics, antiarrhythmics, opiates, and β -blockers and is highly polymorphic but noninducible.

CYPs are generally assumed to be vital in the clearance of prescription drugs, which usually then leads to loss of pharmacodynamic activity. While most metabolites are diminished in therapeutic effect compared with their parent drugs for structural as well as physicochemical reasons, it is important to appreciate the role of CYPs in prodrug therapeutics. Several agents, such as opiates with CYP2D6 (codeine, oxycodone and hydrocodone) and clopidogrel with CYP2C19, depend on CYP metabolism to liberate their therapeutic effects. This introduces some degree of complexity into the study of the effects of CYP variability in activity and expression on therapeutic effect in terms of clearance of parent or prodrug to active constituent as well as clearance from active parent to inactive metabolite.

1.2.4 Glucuronyl Transferases (UGTs)

The uridine diphospho-glucuronosyltransferases (UGTs) are found in close proximity to the CYPs and their primary role is to catalyze the conjugation of various endogenous waste products, such as steroids and bilirubin, with an activated form of glucose (uridine diphosphate glucuronic acid, UDPGA). This has the effect of making the product heavier and much more water soluble [27]. These enzymes are polymorphic and are also capable of directly conjugating several prescription drugs, such as morphine, azidodeoxythymidine (AZT), and lorazepam. Many publications continue to refer to CYPs as “phase 1” and UGTs as part of “phase 2” of drug metabolism. This can suggest that drug metabolism is a sequential, ordered procedure in most cases, which can be misleading. It is true that some agents are firstly oxidized by CYPs and then the product is more likely to be a substrate for the UGTs. However, many agents are directly conjugated by the UGTs without prior oxidative metabolism, so the phase 1/phase 2 nomenclatures can be somewhat restrictive and it has been suggested that it should be abandoned as misleading [28]. The UGTs are certainly capable of clearing a variety of substrates and they are of very high capacity and relatively low affinity, so they are difficult to saturate. There are more than a hundred variants of these enzymes in mammals [29], but in man, only UGT1A, UGT2A, and UGT2B are likely to be relevant to drug clearance.

Until relatively recently, UGTs have been perceived as mainly associated with the clearance of endogenous metabolites and various harmless drug conjugates. However, it is becoming apparent that, in some cases, UGT-mediated drug clearance has a dominant role in drug treatment outcomes that is much more akin to that usually ascribed to the CYPs. This has been notable in the contribution of UGTs to drug adverse effects, which is becoming better documented. For instance, UGT1A3 is particularly efficient at metabolizing steroids such as estrone as well as bile acids. However, the important role of this isoform in the formation of toxic lactone metabolites of statins such as atorvastatin has only recently come to light and their contribution to the myopathy linked with statins remains to be fully investigated [30]. UGT1A3 is also strongly linked with the metabolism of the antiestrogen tamoxifen and the antiepileptic agent lamotrigine.

Interestingly, one of the most marked impacts on drug effect related to UGTs is paradoxically linked with the absence of a UGT due to a polymorphism, rather than their presence. While UGT1A1 is known for its clearance of bilirubin, it also clears the

active metabolite of the antiproliferative topoisomerase I inhibitor irinotecan, SN-38. Accumulation of this metabolite may improve drug therapeutic effect at the expense of toxicity, such as neutropenia. The polymorphic absence of UGT1A1 predisposes a patient to increased risk of irinotecan-mediated toxicity, although this is to some degree preventable through dosage moderation [31]. Other UGTs may also have a role in patients with UGT1A1 deficiencies, such as UGT1A7, whose level of GIT expression is strongly linked with SN-38-mediated gut mucosal toxicity [32].

1.2.5 Sulfonyletransferases (SULTs)

Of the different sulfotransferases (SULTs), some are membrane bound and metabolize proteins and lipids, while the isoforms responsible for drug and drug-like molecule sulfonation are located in the cytosol of most tissues, particularly the gut, liver, and lungs. The SULTs clear a wide variety of endogenous molecules, such as steroids, thyroid hormones, alcohols, and neurotransmitters as well as many widely used drugs such as paracetamol (acetaminophen) and toxic agents such as aromatic amines and polycyclics [33]. They use a cofactor known as 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and it should be emphasized that they "sulfonate" rather than sulfate their substrates, as they introduce a sulfonate (SO_3^-) to various groups, such as hydroxyl or amine moieties. Although sulfonation is usually a detoxification step, resulting in a more water soluble, easily excretable entity (such as paracetamol sulfate), sulfonation is also responsible for the production of many highly toxic species that are DNA reactive. Interestingly, there is some evidence that they also possess sufficient flexibility in their substrate binding to adopt the CYP-like induced-fit method of substrate processing [33].

The SULTs are, as with the CYPs and UGTs, a superfamily of genes and the main families involved in human metabolism include SULT1, SULT2, and SULT4 [34]. SULT1A1 and 1A2 are very structurally similar, while SULT1A3 has <60% homology with the first two isoforms [34]. SULT1A1 is mainly associated with the clearance of iodothyronines and estrogens as well as paracetamol, minoxidil, and various nitrophenol derivatives. SULT1A2 has some overlap in function, although SULT1A3 is mainly responsible for dopamine metabolism [35]. Sulfonation has long been known to be part of the activation of several carcinogenic aromatic amines as well as 2-acetylaminofluorene [36].

1.2.6 Other Biotransformational Pathways

Aside from oxidative metabolism and conjugation, there are several other minor pathways that make contributions to drug clearance, such as amino acid conjugation (hippurate formation) and various hydrolyzing amidases and esterases, which can occasionally make a marked impact on a particular class of drugs. The various plasma esterases clear some neuromuscular blockers such as succinylcholine as well as heroin and cocaine. The monoamine oxidases (MAOs) are found in the liver and gut (MAO-A) and in the brain (MAO-B) and primarily metabolize monoamine neurotransmitters, such as norepinephrine and dopamine, as well as other amines, such as tyramine and amphetamines [37]. Some of the various antimigraine drugs such as sumatriptan and zolmitriptan are cleared by MAO-A in the liver, while others are metabolized by CYP3A4 [38]. Glutathione-S-transferases (GSTs) are a major protection against reactive species generated from drug metabolites, although they do not make a large contribution to

the clearance of most drugs. They are significant in overdoses with drugs such as paracetamol.

However, several drugs, through their particular structural characteristics, are caught in other biotransformational systems that are part of endogenous molecule homeostasis and the resultant metabolites are not likely to progress excretion, as the product may be more lipophilic than the substrate. This is particularly apparent with acetylation and drugs such as isoniazid, sulfones, and sulfonamides.

1.2.7 Flavin Monooxygenases (FMOs)

These enzymes, sometimes known as the Ziegler enzymes, are found in the endoplasmic reticulum of hepatocytes as well as in the GIT. They differ from CYPs in their mechanism of action, as they transfer two electrons in one step, rather than the CYP-mediated sequential transfer of one electron after another. They tend to oxidize nitrogen- or sulfur-containing drugs, forming *N*-oxides and sulfoxides. The resultant oxidation generally increases the water solubility of xenobiotics and the FMOs overlap with and perhaps complement the CYPs in their ability to promote the clearance of a variety of drugs and toxins. The 2,4-diaminopyrimidine antiparasitic agents pyrimethamine and trimethoprim form *N*-oxides and chlorpromazine and some tricyclic antidepressants also form sulfoxides by this route. Other substrates include methimazole, clozapine, amphetamines, and tamoxifen. There are five FMO forms expressed in man; in the liver, FMO-3 is the most common in adults, followed by FMO-4, with FMO-1 expression being usually recorded as negligible [39]. The kidney, in contrast, is particularly rich in FMO-1 and to a lesser extent FMO-4, with FMO-3 expression being relatively low [40]. There is growing interest in the role of FMOs in drug metabolism, as although they are probably modulated by hormones, they are not directly inducible in man and may provide a more consistent metabolic processing effect across populations. This consistency has aroused interest in drug discovery, as it would be advantageous to develop agents with more predictable clearance routes. Interestingly, the lack of inducibility of the FMOs may not be consistent across species; recent studies have shown some element of induction in mice related to the aryl hydrocarbon receptor (AhR) cytoplasmic receptor system but not rats [41]. A lack of FMO expression is linked with the condition of “fish-odor” syndrome, where dietary trimethylamine cannot be *N*-oxidized to its odor-free metabolite [42].

1.2.8 Biotransformation and “Phase 3”

While the main components of the biotransforming system significantly alter the structure and the physicochemical characteristics of a drug and render it less lipophilic and more water soluble, there remains the problem of physical elimination of the drug-related material. Many biotransformational reactions contain some element of reversibility and metabolites can disrupt homeostasis for a variety of reasons; hence, a comprehensive system of pumps has evolved to accelerate the departure of various polar metabolites from biotransforming cells into the circulation, where they will be eventually renally filtered and cleared into urine. The MRP (also known as ABCC) pumps possess some similarities to P-gp, although they are structurally different and have evolved to exchange polar glucuronide conjugates for GSH. These can be anionic conjugates of endogenous and exogenous steroids as well as xenobiotic conjugates of

various types [43]. Unlike P-gp, the MRPs are found in the basolateral as well as apical areas of cells and their position allows them to be part of the coordinated removal of polar conjugates from the cell [44]. There are nine MRP (ABCC) genes expressed that are linked with drug-related conjugate transport and MRPs 1–5 (ABCCs 1–5) are the most documented [45]. These pump proteins are found in most tissues and these, as well as other ABC transporters are also under scrutiny for their possible role in drug resistance to epilepsy suppression, although there is little direct evidence that the main anticonvulsants are substrates for these pump systems [46]. Expression of MRP is closely coordinated with CYP and conjugation enzyme expression, and this system is extremely responsive to “load,” that is, related to drug or xenobiotic exposure.

1.2.9 Coordination of Biotransformation

Essentially, biotransformation is a flexible and adaptable system for the assembly and disassembly of vital instructional molecules, combined with a key protective function, which defends us against our chemical environment. Enormous strides have been made over the past decade in unraveling the degree of coordination between the mechanisms of detecting molecules that are to be biotransformed, their integration with biotransforming genes, the expression of the appropriate isoforms, followed by the accelerated clearance of the various oxidized and conjugated products of metabolism through to the urine and feces. The next section provides an overview of our current understanding of the ability of biotransformational systems to react to chemical challenges, from within and without.

1.3 CONTROL OF BIOTRANSFORMATION: ENZYME INDUCTION

1.3.1 Introduction

It is, of course, a prerequisite for a successful species that it can engineer major and long-term changes in its body capability as well as short-term modulations in terms of the control of small molecules such as hormones. These endogenous molecules must be part of the orchestration of puberty, as well as the maintenance of the reproductive tract, among other requirements. This needs biotransformational capacity, which can respond to changes that might range from month to month, all the way down to minute by minute.

In addition, a species must respond to threat molecules in its environment, such as changes in the chemical composition of diets, so that it can improve its long-term survival prospects. CYPs and other biotransforming systems have been subject to many evolutionary pressures, such as internal ones related to hormone assembly and breakdown, as well as by the plant toxin evolution process mentioned in Section 1.2.3.1. To endow an organism with sufficient flexibility to cover all these possibilities, some means of regulating CYP expression is essential. This fulfills two main requirements: first, that unnecessary raw materials and efforts are not deployed wastefully to express and sustain CYPs or other biotransformational systems that are inappropriate for the tasks encountered on a day-to-day basis, and second, when specific biotransforming capacity is required, it can be summoned up reasonably rapidly to cope with whatever endogenous or exogenous chemical modulating task is necessary.

Such a system ensures that an individual may respond to a chemical threat by expressing the appropriate specific and increased biotransforming capacity, which leads to a gradual increase in the clearance of the threat. If exposure to that molecule continues to increase, the system will respond commensurately, increasing to a maximal level, rather like expanding a powerful spring. In effect, the capacity to eliminate a threat molecule might increase 10- or even 20-fold in a timeframe of days to weeks. This presents problems in drug development and therapeutic applications, as it means that drug clearance is not stable until the “spring” is fully expanded and this dosage level will vary markedly between individuals. This is linked with the marked variation in background levels of particular biotransforming isoforms. Clinically, the consequences for therapy with an individual agent that acts to “induce” increased drug clearance can be complex. The drug cannot just be prescribed at a single session with a healthcare practitioner; the dose will have to be increased to keep the drug concentrations in the therapeutic window. If the patient is already taking several other agents, it is necessary to review the current regimen and take steps to determine whether the inducer will accelerate the clearance of the other agents and negatively affect efficacy. In the case of some prodrugs, this might mean accelerated production of the active agent, alongside any attendant toxicity and off-target pharmacological effects. Of course, the “spring” will snap back once the inducing drug is discontinued, which causes a marked imbalance between drug dosage and decelerating clearance to emerge over several days.

Although experienced clinical supervision is required, there are many inducers in the drug armamentarium that have been managed successfully for decades. However, vigilance is required, not least because some of the most potent inducers of biotransforming enzymes are available over the counter and patients may or may not inform their healthcare practitioner that they have decided to add these agents to their regime of their own accord.

1.3.2 CYP Induction

If, for convenience, CYPs relevant to clinical drugs are considered as four groups, then the description of the induction process can perhaps to some extent be simplified.

- Our main CYP, CYP3A4 and its close relative CYP3A5, as well as the CYP2Cs (CYP2C8, 9, and 19) and CYP2B6 could be considered in the first group. All these CYPs are responsible for the majority of prescribed drug clearance. They are inducible to a high degree and they share a similar mechanism of induction, which is coordinated through the nuclear receptor system operated by PXR.
- The second group of CYPs, CYP1A (CYP1A1 and CYP1A2) clear only a few agents, are highly inducible, but operate through the cytoplasmic/nuclear receptor aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator protein (AhR/ARNT) system.
- The third group contains a single CYP, CYP2E1, which again is responsible for the clearance of only a small number of drugs, which is inducible through yet another system, which involves the proteasome.
- The final group includes just one CYP again, CYP2D6, which is not conventionally inducible at all.

1.3.2.1 Group 1: Nuclear Receptor-Mediated Induction-PXR/CAR. The respective biotransformational roles of the different CYPs to some extent inform on their modes of induction. The first group (CYPs 3A4 and the 2C series) is linked with hormone metabolism and various xenobiotics. These CYPs are controlled by nuclear receptors, mainly PXR and constitutive androstane receptor (CAR). These biosensors interact with several modulating proteins and receptors as well as through the retinoic acid X receptor (RXR) using the process of heterodimerization. The complexes thus formed migrate to various DNA response sites known as xenobiotic response elements (XREs) on the appropriate CYP gene.

The PXR nuclear receptor is also known as the steroid X receptor and nuclear receptor NR1I2. It controls CYP3A4/5 and transporter expression and appears to have become adapted to act as a xenobiotic as well as an endobiotic sensor. It is essentially expressed only “on demand” and is subject to a complex network of repressor and release proteins that are part of the mechanism that conserves and modulates the ability to respond to chemical challenges [47]. PXR itself is famously promiscuous and its large, spherical binding cavity will capture a remarkably wide structural diversity of endogenous and exogenous substrates, ranging from steroids, dozens of prescription drugs, pollutants, and dietary chemicals [48]. The major endogenous ligands that bind to PXR include bile acids and various steroids. PXR appears also to be extremely important in the prevention of cholestasis related to disorders of bile acid metabolism; indeed, PXR-mediated inducers such as rifampicin and St. John’s Wort have been shown to relieve cholestatic jaundice [49]. In humans, rifampicin is the most potent inducer and the benchmark in the drug industry for categorizing inductive potential, although there are several other potent PXR-modulating ligands (hyperforin, found in St. John’s Wort, dexamethasone, ritonavir, the organochlorine pesticide chlordane, paclitaxel, but not docetaxel), and they exert their inducing action through the subsequent binding of PXR with RXR as well as the complex array of other cobinding factors.

There is considerable evidence that the inductive effects of many chemicals are species specific and it is also related to PXR binding itself; in drug discovery, this has been addressed through the use of humanized mouse models, where human PXR has been inserted into the mouse DNA and the mouse genes deleted. This allows a PXR experimental response to inducers such as rifampicin in the concentration range seen in man [50,51]. PXR is under consideration as a therapeutic target, as there is evidence that some dietary constituents such as the sulforaphane found in broccoli can block its effects [52]. This may allow the active modulation of CYP induction in certain clinical circumstances where the resultant changes in drug levels may prejudice a successful outcome. Indeed, as mentioned previously, human CYP3A4 expression levels show very large variations [53], so the clinical impact of induction varies equally widely, as every individual’s basal expression is different.

CAR (or NR1I3) controls the induction of CYP2C9, which is probably the second most important CYP in terms of drug clearance, as well as CYP2B6. This latter CYP is emerging as being responsible for the clearance of several agents that were thought previously to be metabolized by CYP3A4, such as methadone [54]. This confusion may have arisen as it is not always possible to distinguish which CYP actually bears the main burden of a specific drug’s clearance *in vivo*, while many may metabolize it *in vitro*. The CAR receptor system differs from PXR, in that it generally operates to modulate more endogenous processes which are already strongly expressed. Another

marked difference between CAR and PXR is that there is no direct binding of an inducer to CAR itself, as the interaction appears to be linked with stabilization of the CAR complex [55]. Hence, although CAR-mediated inducers include drugs such as phenobarbitone [55], the CAR system is not as adapted to the sheer variety of xenobiotic structures that is seen with PXR, which probably reflects the narrower scope of the active site of CYP2C9 for the binding of very disparate chemical structures, which is in contrast to that of CYP3A4.

1.3.2.2 Group 2: Cytoplasmic Receptor-Mediated Induction-AhR/ARNT. The AhR is located in the cytoplasm and appears to possess several endogenous functions related to organ development and growth as well as its role in the detection of planar and lipophilic molecules such as polycyclic aromatic hydrocarbons (PAHs [56]). Of the CYPs controlled by the AhR receptor system, some prescription drugs are cleared by CYP1A1 (theophylline) and CYP1A2 (tricyclic antidepressants, R-warfarin, fluvoxamine, and clozapine), although CYP1B1 is more relevant to carcinogenesis. Of these CYPs, CYP1A1 is nonconstituent and is effectively absent unless induced by exposure to PAHs from smoking and the proton pump inhibitor omeprazole. CYP1A2 induction is sufficiently potent as a result of cigarette smoking to accelerate the clearance of agents such as clozapine and fluvoxamine to a clinically significant degree. The AhR receptor system differs from the nuclear receptors most obviously in its cytoplasmic location, where it binds its polycyclic and other substrates. It then associates with several proteins such as heat-shock protein 90, followed by complexing with ARNT [57]. The complex enters the nucleus and induces the expression of the CYP1A series and CYP1B1. This effect is seen in the liver but most notably the lung in smokers.

1.3.2.3 Group 3: CYP2E1 and the Proteasome. CYP2E1 is unusual among CYPs in that it is capable of clearing many small hydrophilic molecules, such as ethanol, pyridine, and acetone, as well as drugs such as chlorzoxazone. It is also found in somewhat unusual sites in cells, such as the mitochondrial membrane, as well as the smooth endoplasmic reticulum. CYP2E1 has long been known to be induced in alcoholism and is strongly linked with the resultant hepatic attrition [58]. CYP2E1 does not use nuclear or cytosolic receptors but is nonetheless efficiently induced by its substrates. Although the mechanism is complex, it is apparent that the substrate itself stabilizes the CYP and prevents its destruction by the proteasome; this can be illustrated in the greatly increased half-life of the CYP in the presence of the substrate [59]. That this CYP has evolved such a mechanism that is distinct from the steroid and polycyclic-oxidizing CYPs again is likely to be strongly linked with homeostasis and adaptation to environment, particularly with respect to highly specific substrates. The induction of CYP2E1 in starvation may conceivably be part of the process whereby potentially intoxicating small molecules are cleared after they result from metabolism that is adapting to severe dietary pressures [21]. That this process of adaptation to diet must necessarily be rapid to promote survival of the organism may be a corollary of the apparently somewhat wasteful continual manufacture and destruction of CYP2E1. It may also be linked with this CYP's tendency to promote oxidative stress [60]. Indeed, from a therapeutic standpoint, it is apparent that CYP2E1's major impact on humans is mainly linked with ethanol abuse rather than a significant contribution to drug clearance.

1.3.2.4 Group 4: CYP2D6 Noninduction. CYP2D6 is the only major human CYP that does not appear to undergo any form of responsive induction. Some have suggested that the evolution of this CYP is strongly linked with defense against plant alkaloids, which often have nitrogen-centered molecules which CYP2D6 appears to readily process. However, if this was the case, it is somewhat perplexing that in perhaps 100 million years, no functioning induction systems have evolved with this CYP, although some studies have reported changes in activity of CYP2D6 in response to some herbal agents [61]. Instead, there is a rather crude fixed response to the presence of multiple CYP2D6 substrates in certain environments. This takes the form of some individuals expressing many copies of the CYP2D6 gene, resulting in large amounts of functional hepatic protein. This can have marked effects on the major groups of CYP2D6 substrates, such as the antipsychotics, antidepressants, antiarrhythmics, and β -blockers, which are cleared by this CYP and the opiates (codeine, oxycodone, hydrocodone and tramadol) which must be metabolized to active opiates.

1.3.3 UGT Induction

Studies over the past decade have underlined the emerging variety of UGT substrates and the value of these isoforms in the clearance of endogenous and exogenous agents. That these isoforms are such a vital factor in human processing of such agents is underlined by the remarkable flexibility in UGT induction response. As well as its role in the control of bile acids, the primary mission of the major UGT, UGT1A1, for example, is in bilirubin conjugation. So the expression of UGT1A1 must adapt to changing bilirubin "load" to ensure that this neurotoxic hemoglobin waste product does not accumulate, so the concentration of such endogenous agents are active regulators of UGT expression. However, CYP inducers such as phenobarbitone are also potent inducers of UGT1A1 [62], and it is clear that the UGTs are regulated by many nuclear receptors, from CAR to PXR, as well as other steroid receptors and even AhR. The UGT1A1 DNA response module, known as gtPBREM, contains multiple binding sites for all these receptors. UGTs thus are sensitive to a variety of inducing drugs, ranging from steroids to rifampicin. In the past, the UGTs have been perhaps overshadowed by the CYPs; however, the advent of potent drugs, such as irinotecan (section 1.2.4), has focused clinical research on the impact of various UGTs on the efficacy and toxicity of this agent. Irinotecan exerts its efficacy through its toxic metabolite SN-38, which is cleared by UGTs, such as UGT1A1 and UGT1A7. The balance between toxicity and efficacy must be attained in each patient, despite both factors being governed by local and systemic concentrations of SN-38, which are, in turn, regulated by UGT expression.

1.3.4 SULT Induction

The mechanisms whereby the various SULTs are controlled both endogenously and exogenously are relatively poorly investigated, even in comparison with the UGTs. Indeed, for many years, they were even not recognized to be inducible. Around the end of the 1990s, it became apparent that, in animal studies, SULTs were inducible through the various nuclear receptors, although there are still few studies in human systems. However, it is emerging that the expression of some SULT isoforms are responsive to several of their main substrates. These include bile acids, peptides, steroids, and neurotransmitters, which may markedly change the expression of the major SULT

isoforms in humans [63]. The major focus of research into SULT metabolism is linked with the bioactivation of potential environmental and dietary carcinogens, rather than drug clearance. However, among these enzymes, SULT2A1 can be induced by steroids and the antifolate agent methotrexate; the latter effect is mediated through CAR, as the CAR agonist 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) is also effective [63]. SULT1A3, which mainly processes catecholamines, is inducible through glucocorticoids [64], while SULT1A1 is inducible through rifampicin [65]; hence, it is likely that the SULTs are controlled by the major nuclear receptors as part of the network of biotransforming enzymes and transporters.

1.3.5 Transporters

The net clinical effects of a major CYP inducer can be measured *in vivo* in terms of the increased rate of clearance of a particular probe drug, although it is much more ambitious to attempt to delineate this impact on clearance in terms of its individual components. Aside from increases in the expression of CYPs and conjugating isoforms, efflux transporters such as P-gp and influx transporters (the OATPs) and the MRPs are also induced to a powerful degree through the same nuclear receptor systems (PXR and CAR), which regulate the rest of the biotransformational response to a chemical or drug challenge. The impact of induction on these transporters is thus multifactorial, distorting the kinetics of the absorption process in the gut through P-gp, while promoting hepatic and gut uptake through the OATPs, followed by accelerated clearance by the induced CYPs to greater quantities of polar metabolites, which, in turn, are cleared more rapidly toward bile and urine. It is likely the most relevant *in vivo* analysis concerning transporter induction and its impact on drug flux is probably best obtained from cellular models [66].

1.3.6 Coordination of Biotransformational Induction: Receptor Cross-Talk

As outlined earlier, the induction of biotransformation requires a coordinated response to the agent, which involves the transporters, CYPs, conjugation isoforms, and MRPs, not to mention the increased expression of cellular protective systems such as the GSTs, which are of greatest relevance in detoxification rather than prescription drug clearance. There is now a great deal of evidence that the various nuclear receptors as well as the cytosolic AhR all act in concert to ensure that every stage of biotransformation is managed to promote not only clearance in the sense that a drug is converted to a less pharmacologically potent metabolite but also physical removal into the urine and the feces. All the major biotransforming enzyme systems as described above have DNA response elements that are responsive to PXR, CAR, and the glucocorticoid receptor (GR). It is known that the nuclear receptors PXR and CAR are regulated by and in turn regulate AhR [67]. These receptors are, in turn, controlled by a “senior” hierarchy of the hepatocyte nuclear factors (HNFs), such as HNF1 α and HNF4 α . HNF1 α regulates a large number of genes linked with glucose, lipid, and protein metabolism and controls the transcription of UGTs after the PXR/RXR system has been activated [68]. HNF4 α acts to transcriptionally regulate CYPs in a similar way and is one of the means by which the various nuclear receptors’ inductive effects are coordinated across a biotransforming tissue and organ [69]. CYPs and other

biotransforming systems are also cross-regulated by numerous transcription factors, such as nuclear factor kappa B (NFκB) [70]. This complex and multifactorial transcriptional regulation system allows living organisms the ability to regulate their own homeostatic processes, while simultaneously protecting themselves from dietary and environmental chemical assaults. Unfortunately, in terms of drug therapy, this well-organized, sensitive, and highly effective system is a formidable obstacle to successful and stable therapeutic intervention and is capable of not only undermining but effectively abolishing drug effect through rapid clearance. It is also less well appreciated that the process of enzyme induction where prodrugs are concerned can lead to excessive adverse reactions and off-target pharmacological effects. To compound this therapeutic hazard, some prescribed agents, such as rifampicin, will accelerate the clearance of a very wide range of drugs cleared by the major CYPs as described previously but also over-the-counter (OTC) medicines can cause this effect also, sometimes as mentioned previously, without the knowledge of prescribers.

1.3.7 Induction: Clinical Consequences

There are many clinical examples of drug interactions that are based on the effect of inducers. Indeed, although this effect is highly undesirable in drug development, a recent study has nonetheless shown that just under half currently used prescription drugs have some inductive capability [71,72]. In terms of therapeutics, the effects of induction could be classified as either expected or unexpected. When a known inducer is deliberately added to a regime, the effects can be anticipated in terms of titrating the increased dosage over a period of time that will be, in effect, customized according to the patient. This requires a considerable measure of clinical input and experience to accomplish while maintaining continuity of clinical care, through continuity of drug concentrations within the therapeutic window. When an inducer is added to a drug regime without the knowledge of the various prescribers who have care of the patient, either by another prescriber or by the patient's initiative, the effects may go unnoticed until failure of drug therapy results or if excessive metabolite production results in toxicity.

As far as anticipated inducers are concerned, rifampicin will induce most of the main CYPs, such as CYP2B6 and CYP2C8/9/19, as well as CYP3A4/5, so accelerating the clearances of hundreds of currently used drugs, including agents such as losartan, propranolol, and verapamil, which can effectively remove their therapeutic effects [71]. Anecdotal evidence suggests that rifampicin is sometimes used in terminal patient care to eliminate multidrug-resistant *Staphylococcus aureus* (MRSA) infections and this can accelerate the clearance of previously stabilized opiate regimes, such as with methadone, requiring major skilled adjustment in dosage. The first generation anticonvulsants such as phenobarbitone, carbamazepine, and phenytoin as well as steroids such as dexamethasone show a broadly similar impact on CYP capability. Other inducers that have a less potent effect on CYP3A4 include the various protease inhibitors (ritonavir and nelfinavir) and the nonnuclear reverse transcriptase inhibitors, such as efavirenz and nevirapine. Most clinicians are aware of the potency of these inducers to cause coadministered drugs to fail to maintain adequate plasma levels and dosage adjustment is essential to maintain therapeutic effects.

Other inducers are less easily detected, as their effects are variable and they are within the realms of patient lifestyle choices. Patients will usually admit to smoking, although they may not divulge the full extent of their tobacco intake, in the same

way that many are somewhat reticent about their ethanol intake also. The inductive effects of smoking really only concern a small number of drugs that are cleared either partly or wholly by CYP1A2, such as clozapine, fluvoxamine, and warfarin [73]. If an individual smoking habit is stable, then they will already be induced to an equally stable extent in terms of their CYP1A2 expression. Hence, whichever dosage is arrived at based on clinical response should not be affected provided they continue to smoke. Of course, patients are exhorted constantly by their families as well as their healthcare practitioners to try to cease or even just reduce their smoking, and although this is desirable for the patient's health, it is important that they are counseled to inform their prescribers when or if their smoking status changes, as a loss of the inductive effect will cause a "smoking" dosage regime resulting in drug accumulation, with a commensurate impact on off-target effects, toxicity, and ultimately patient tolerance.

One of the most powerful inducers of CYP3A4 and P-gp is St. John's Wort, which is available OTC and, as with all herbal preparations, varies enormously in its relative potency. This agent is now known to have clinical effect which is certainly no worse than many prescribed antidepressants and many individuals under treatment for major conditions such as HIV, cancer, or xenograft recipients are likely to become depressed and might resort to St. John's Wort or similar OTC remedies. The potency of this herb is underlined in instances where it has accelerated the clearance of immunosuppressives such as cyclosporine to the point that xenograft rejection has occurred [74]. St. John's Wort also reduces the efficacy of irinotecan, by lowering the plasma concentrations of SN-38, the active metabolite [75], and it also accelerates the disappearance of imatinib, the tyrosine kinase inhibitor used in the treatment of chronic myeloid leukemias [76]. The herb can cause marked adverse reactions in the case of agents such as cyclophosphamide, which rely on CYP-mediated activation to a reactive and toxic metabolite. When the activation process is responsible for both clinical effect and toxicity, a marked shift in toxic metabolite formation may tip the balance toward unacceptable patient toxicity. The key clinical problems with St. John's Wort include its variability in potency and speed of inductive effect as well as the likelihood that patients may not feel that they should inform their healthcare practitioners that they are self-medicating.

Interestingly, recent studies *in vitro* have shown that several anticancer agents, such as ifosfamide, cyclophosphamide, and paclitaxel, are capable of activating PXR and may cause some measure of induction in patients, although this is not always detectable clinically due to the coadministration of inducers during therapy such as steroids [77]. Some studies with higher doses of cyclophosphamide have reported increased elimination that may be linked with autoinduction of metabolism, as this agent is oxidized by several inducible CYPs, including CYP3A4, CYP2B6, and CYP2C19 [78].

Overall, it is apparent that many compounds do bind to PXR, but relatively few agents exert a clinically relevant inductive effect, although those that do present many problems to the day-to-day clinical management of disease states.

1.4 PHARMACOGENETICS: IMPACT ON DRUG EFFICACY

1.4.1 Introduction

It is clear that the success of our species is strongly linked with our genetic variability, which promotes our adaptability, which extends to our biotransformational capabilities.

Although the first studies in this area began with isoniazid in the 1950s and extensive work followed with enzymes such as CYP2D6 and thiopurine-S-methyltransferase (TMPT) from the 1980s onward, the full extent of the pharmacogenetic variation in human biotransformation is still not fully understood. A genetic polymorphism is taken as significant when it affects more than 1% of any given human population and such polymorphisms are usually, but not always, linked with reduced or absent capability in a particular enzyme. The defects can range from specific point mutations leading to the substitution of a critical amino acid for a disadvantageous one which is at or near the active site of the enzyme, to TATA box defects where gene promoters are misread, leading to poorly functional protein. These traits are retained in populations, as they may confer advantage that may ensure the survival of a species if the wild-type, that is, the majority of phenotypes, are adversely affected by a toxin that might require bioactivation, for example. Although polymorphisms benefit species survival, they may be a severe handicap for the individual. The extent of this handicap is related to whether the individual possesses more than one defective allele or copy of the particular gene.

The “wild-type” allele is usually designated *1, followed by those for the various defective alleles, which may be designated *2, *3, and so on. In the case of CYP2C19, the wild-type is quoted as CYP2C19*1, and this allele is fully functional. As one allele is received from each parent, a *1/*1 homozygous individual has full CYP2C19 expression and catalytic activity, so this person would be classed as an extensive metabolizer (EM). Individuals who possess at least one copy of the *2 or *3 alleles together with a copy of the wild-type *1 allele will have reduced expression, such as CYP2C19*1/*2 or CYP2C19*1/*3. These heterozygotes are termed intermediate metabolizers or IMs. Homozygous poor metabolizers (PMs), such as CYP2C19*2/*2, would be termed PMs and would have little or no expression of competent isoform [17]. With many CYPs, there are also small populations where individuals express several copies of the same gene, leading to rapid metabolism with CYP2D6 or there are also ultrarapid alleles, where the CYP is unusually fast metabolizing. With CYP2C19, this is termed *17 and a homozygote for *17 would metabolize a substrate many times more rapidly than the wild-type individuals. Although there are considerable numbers of studies that might quote frequencies of allele appearance in populations, the frequencies of homozygous and heterozygous individuals are the main concerns, as this describes drug clearance capacity in a single individual for a particular isoform.

1.4.2 Pharmacogenetics and Drug Development

As CYPs and other biotransforming systems process more than 80% of drugs in some form or other, population distributions of a particular CYP, for example, have taken on enormous significance in the light of global drug development and marketing. The economic pressures behind modern drug development are such that only such universal marketing may allow a commercial entity sufficient return on the admittedly vast investments necessary to propel a drug to the clinic. However, the lack of homogeneity in human biotransformation is a serious impediment in this global drug marketing drive. If a drug is not cleared by a substantial patient subpopulation, this will promote concentration-dependent toxicity and off-target effects that will reduce patient tolerance. Conversely, if a drug requires metabolic activation before the achievement of clinical effect, then if a population of individuals cannot activate the drug, it will fail to provide therapeutic care and other agents will be substituted.

A key issue is how this information is actually discovered. While many thousands of studies have been published on the huge numbers of individual CYP alleles that can be identified using molecular biological techniques using patient blood samples, there are only comparatively a tiny number of clinical studies on human polymorphisms that actually study the real-life consequences in terms of drug toxicity or failure [79]. Indeed, the numbers of individuals in these studies are minuscule when compared with those routinely included in phase II and III trials for new drugs. Given that many new drugs have been evaluated in anything up to 8000 patients before licensing and still must occasionally be withdrawn due to toxicity, it is clear that a greater emphasis on future clinical work is required to provide the necessary data for predictions on the consequences of biotransformational polymorphisms in human populations.

1.4.3 Biotransformational Polymorphisms

1.4.3.1 NADPH Oxidoreductase. The catalytical activity of CYPs is powered by electrons that are supplied by cytochrome *b*₅ and P450 NADPH oxidoreductase (POR). POR is vital to many processes, but individuals with Antley–Bixler syndrome do have reduced efficiency POR, which can, in turn, compromise CYP3A4 activity and their steroid metabolism [80]. Fortunately, POR polymorphisms appear to be very rare and are not really clinically relevant.

1.4.3.2 CYP3A. Although CYP3A4 clears the majority of prescription drugs, it is generally not subject to significant polymorphic expression, although there has been a report on possible differences between Chinese individuals and Caucasians in terms of CYP3A4 expression, where simvastatin clearance was reduced and its therapeutic effect was commensurately increased [81]. Indeed, along with many other drugs, it is recommended that patients of Chinese extraction and other Eastern races should be at least be started on lower doses of statins due to their general reduced capacity for drug clearance [82]. CYP3A4's close relative CYP3A5 is found in Afro-Caribbean populations and is polymorphic [83].

1.4.3.3 CYP2C9. CYP2C9 is of intense interest, as it accounts for just under a fifth of total hepatic CYPs and is responsible for the clearance of warfarin, several NSAIDs, phenytoin, and many other agents [84]. The major allelic variants of CYP2C9 are the wild-type *1 as well as the defective alleles *2 and *3. The *2 variant is found in 10–13% of Caucasians, while *3 is less common, at around 5–9%; Chinese and Africans have lower frequencies of these alleles [84]. The *2 and *3 variants of CYP2C9 are much less catalytically efficient than the wild-type [85] and this leads to an inability to clear standard doses of anticoagulants, causing excessive bleeding [86]. Interestingly, one particular heterozygous CYP2C9 combination (*1/*3) is strongly linked with males; hence, it is possible that more polymorphisms remain to be discovered which are sex linked [87]. It is often noted that many CYP2C9 substrates have relatively narrow therapeutic indices, so making some investigation into genotype before therapeutic intervention is more desirable to reduce toxicity.

1.4.3.4 CYP2C19. This CYP, formally known as mephenytoin oxidase, has assumed increased importance in recent years, primarily related to its metabolism of several highly successful drugs, including the antiplatelet agent clopidogrel as well as the

proton pump inhibitors. This CYP is also interesting, as aside from its wild-type allele, its two major polymorphic forms (mentioned already in Section 1.4.1) include *2 (more common in Caucasians) and *3, are both nonfunctional. The *3 allele is found in more than 10% of Chinese, but it is rare in other races [84]. Overall, PMs (*2 or *3 homozygotes) of Chinese extraction of this CYP are more common (up to 23%) than in Caucasians (12–23%) and lowest in Africans (4–8%) [88,89]. Consequently, impaired activity of CYP2C19 actually promotes the efficacy of the proton pump inhibitors, while it reduces the efficacy of the anticancer agent cyclophosphamide, as insufficient active metabolite is formed in homozygous CYP2C19*2 individuals [90]. It has also been suggested that CYP2C19 deficiency impairs the efficacy of clopidogrel, which requires metabolism (although several CYPs can activate it) to its active thiol to show efficacy [91]. However, CYP2C19 loss of function has not been shown to significantly impair Caucasian and Latin American patient response in a study with clopidogrel [92], but in another study, CYP2C19 and P-gp status did affect the drug's response [93]. It is probable that the argument will be settled with either prasugrel, which is a relative of clopidogrel and can be activated by many biotransforming systems, or a directly acting agent such as ticagrelor. This latter drug neatly circumvents the polymorphic biotransformational issues and may be more cost effective over its lifetime compared with clopidogrel, as genetic testing is not required for its maximum efficacy [91].

1.4.3.5 CYP2D6. This CYP figures in the clearance of several important groups of drugs, such as the antipsychotics, several antiarrhythmics, and SSRIs as well as the activation of a number of opiates to derivatives that demonstrate morphine-like actions. Thus, CYP2D6 has been the most exhaustively investigated polymorphic CYP and homozygotes for the *4 allele show little or no functional enzyme [94]. In Caucasians, as many as 10% may carry two *4 alleles, which may amount to perhaps 60 million individuals worldwide, although only around 1% of Africans and the Eastern races display this genotype. There is also a deleted gene variant (*5), which is also found in most populations at around 3–5% [95]. It is divided as to whether the PM status actually leads to severe problems in individuals during therapy with certain drugs. It is clear that CYP2D6 is required for optimum activity from tamoxifen, as this prodrug requires activation to potent estrogen antagonists [96]. Other studies have shown that tricyclic antidepressants may show increased toxicity in CYP2D6 PMs [97]. Interestingly, a study that evaluated the effect of PM status on the opiate-like effects of tramadol indicated that despite the lack of CYP2D6 activity, PMs could derive analgesia from the drug [98]. Hence, other CYPs and biotransforming systems, along with additional pharmacological effects with various drugs, may compensate for a lack of metabolism to active agents in *4 and *5 homozygotes.

Individuals with multiple copies of CYP2D6 can clear its substrates extremely rapidly, with opiate toxicity resulting in the case of codeine [95]. It is clear that there are far more studies identifying potentially defective CYP2D6 alleles than are those clinical studies that firmly indicate that PM status predisposes to toxicity and conversely or that PM status abolishes prodrug activation. The significance of CYP2D6 lies mainly in the sheer numbers involved, particularly in the developed world, where therapeutic drug use is more likely than in other populations.

1.4.3.6 Other CYPs—CYP2C8 and CYP2B6. CYP2C8 is mainly known for its metabolism of NSAIDs such as ibuprofen as well as the anticancer agent docetaxel.

Although induction of CYP2C8 is linked with accelerated docetaxel clearance, there appears to be much less of a link between CYP2C8 status and docetaxel clearance or toxicity [99–101]. CYP2B6 is extremely variable and polymorphic [102] and the *6 variant is the commonest defective allele, with only a quarter of the wild-type expression [103]. As touched on earlier, the role of CYP2B6 is gradually emerging in the case of several drugs, such as methadone, which were thought to be cleared by almost entirely by CYP3A4 [54,104]. This is probably linked with studies where known inducers such as rifampicin-caused accelerated methadone clearance and it was assumed that this would be CYP3A4, although most of the PXR/CAR inducers induce all the CYP2Cs, CYP2B6, and the CYP3As. CYP2B6 polymorphisms are associated with altered metabolism in several drugs [105,106] and it is likely that the impact of CYP2B6 polymorphisms on drug toxicity and efficacy is far from full elucidation.

1.4.3.7 FMO Polymorphisms. The major FMO that forms drug-related *N*-oxides in man is hepatic FMO-3, which is subject to a large number of different poorly functional and nonfunctional polymorphic forms, which make up the 1% or so of individuals in Caucasian populations who have trimethylaminuria. These individuals cannot clear trimethylamine to an odorless *N*-oxide and consequently their breath and perspiration smells strongly of fish. Owing to its lack of induction and high capacity, FMO-3 has been considered as a useful clearance route for drugs such as the gastroprokinetic agent itopride, which is free of the problems associated with cisapride. The latter agent was cleared by CYP3A4 and inhibition of this CYP resulted in cardiotoxicity [107]. While FMO-3 polymorphisms do exist, they appear to occur at a lower rate in comparison to other CYPs, such as CYP2D6, so this may be a promising area in drug development. An agent cleared by FMO-3 would not be subject to accumulation except in a small proportion of individuals and the lack of enzyme induction would also be highly desirable, in terms of cost savings in therapeutic monitoring.

1.4.3.8 UGT Polymorphisms. The most significant UGT polymorphism is the TATA box promoter error known as Gilbert's syndrome; this is associated with the allele UGT1A1*28. UGT1A1 is the only known conjugative pathway for bilirubin, which is a neurotoxic metabolite of hemoglobin; so homozygotes for this allele have only about a third of the wild-type capability in clearing this agent. As with other polymorphisms, such as glucose-6-phosphate dehydrogenase deficiency, this can just about be tolerated and does not cause liver damage. However, it does predispose to some cancer risks (related to poor clearance of estrogens) and reduce others [108,109] as well as providing some protection in heart disease due to the antioxidant effects of the mild hyperbilirubinemia that results with this polymorphism [110]. As there is relatively little margin in conjugative capacity in Gilbert's syndrome, it has been established that drugs such as protease inhibitors that are capable of UGT1A1 inhibition can cause marked hyperbilirubemias [111]. Probably the best-known drug toxicity issue with Gilbert's syndrome is in irinotecan therapy, where the active SN-38 metabolite is not cleared due to lack of UGT1A1 expression and toxicity results; there is evidence that other UGT polymorphisms are linked with Gilbert's syndrome, such as UGT1A7 (section 1.2.4), which also markedly affects the toxicity linked with irinotecan therapy [112]. Unfortunately, as SN-38 is responsible for the toxicity as well as the activity against the cancer, so the potential for a strong therapeutic response may rest on the patient's tolerance to the toxicity. There are complex differences in UGT1A1 and other UGT

isoforms across ethnic populations that can lead to significant variation in susceptibility to drug toxicity where UGTs are a primary biotransformational pathway [113].

1.4.3.9 *SULT Polymorphisms.* As mentioned previously, the biotransformational capacity of the SULTs is of interest from the standpoint of predisposition to certain cancers as well as for drug clearance. This is due to the tendency of some of the SULTs, such as SULT1A1, to clear phenolic agents such as not only paracetamol, but also various aromatic amines that can be bioactivated. These agents can attack DNA and lead to malignancy [114]. SULT1A1*2 and *3 are less catalytically competent and stable [115]; hence, expression of SULTs may strongly influence predisposition to certain cancers. There is also evidence that SULT1A1 genotype may influence the effectiveness of tamoxifen, as the *2 variants may negatively affect patient survival, as sulfonation of the drug's hydroxylated metabolites improves its potency [115–117].

1.4.3.10 *Other Biotransformational Polymorphisms.* There are several other biotransformational systems that are polymorphic and have some bearing on the clearance of prescription drugs. Microsomal epoxide hydrolase exists in several catalytic forms and is thought to have bearing on the antiepileptic agent carbamazepine, which is cleared by CYP3A4 to a 10,11-epoxide, which retains pharmacological activity, although it is responsible for some toxicity. More catalytically rapid versions of epoxide hydrolase (such as Arg139) will reduce the therapeutic effect of the drug by clearing the epoxide to a diol, which is not active, while slower versions (such as 113His) run at about half the velocity of the wild-type and will have the reverse effect, possibly increasing toxicity [118,119]. The toxicity of azathioprine is strongly linked with TMPT status, an enzyme that clears the toxic purine metabolites, which confer the activity on the drug. There is evidence that the TMPT phenotype, as detected through a simple functional blood assay, is much more relevant than genotype. This is partly due to concomitant therapy in leukemias where inhibiting salicylates are also used.

1.4.4 Pharmacogenetic Testing: The Future?

1.4.4.1 *Introduction.* There are now several commercially available test systems that can be used to determine genotype in some biotransforming enzyme systems. While this technology is developing rapidly, in terms of reductions in costs as well as sample volumes, the use of these tests has not yet become accepted or widespread in developed countries with the resources to afford them. This is rather surprising in the context of the acknowledged desire of future medicine to become more customized to the patient's individual needs. There are many possible reasons for this and some are discussed below.

1.4.4.2 *Mass or Niche Therapy?* The benefits of pharmacogenetic testing should, in an ideal world, be considered solely in terms of clinical merit. The aim should be to optimize drug therapy to minimize toxicity and maximize drug performance and tolerance. However, cost issues have become increasingly intrusive in drug therapy in general, particularly in the UK, where new drugs are evaluated in terms of efficacy and value for money by the National Institute for Clinical Excellence (NICE). Some authors have analyzed the cost/benefit ratios of certain pharmacogenetic tests and found that several are probably an unattainable luxury [79]. In addition, clinicians

can be reluctant to adopt new tests, partly due to expense and partly due to uncertainty over how to interpret the results. This is borne out by studies which have underlined that CYP2C9 status is not the only contributor to warfarin variability of response and vitamin K epoxide reductase complex subunit 1 (VKORC1) status must also be determined [120]. It is likely that many other tests available currently are also only a single piece in the puzzle as to why a particular drug shows an aberrant effect in an individual or a group of patients. While irinotecan packaging is labeled with warnings linked with the risks of UGT1A1*28 status, the expression of other isoforms, such as UGT1A7, is also a vital component of any patient's response. Similarly, the respective expressions of several genes have a strong impact on tamoxifen therapy, and optimization based on a series of pharmacogenetic tests could markedly improve the efficacy of this agent.

Until our knowledge of all the conflicting haplotypes and their impact on the clearance of a particular drug is understood, many of the recommendations currently given may be somewhat incomplete and it is probably not unreasonable for clinicians to be wary of basing important therapeutic decisions on these data.

There is also the question of whether pharmacogenetic testing would make a cost-effective contribution of niche areas, where small groups of drugs are deployed in a relatively specialized patient population, which is already under close clinical supervision. Such areas include the use of antipsychotics, anticoagulant, and antineoplastic therapy. In the case of sudden death during antipsychotic use, it has been suggested that this is linked to TdP, caused by CYP2D6 polymorphisms. The failure to clear the drugs may have led to sustained high levels in PMs, which, in turn, caused cardiotoxicity. However, recent studies suggest that sudden death in these patients is dose related, but importantly, it is independent of whether older CYP2D6-cleared or newer, non-CYP2D6-cleared agents are used [121]. Hence, although theoretically, some benefit for pharmacogenetic testing of CYP2D6 status should exist, it is likely that other factors are involved in the predisposition of some patients to increased risk of cardiotoxicity. So it is difficult to assess genotypic testing would be of benefit to the experienced clinician. In addition, with drugs used in intractable pain relief in terminal care, such as methadone, the complexity of this drug's clearance may depend on several factors other than CYP2B6 status and to date, caution in escalating the dose may well be just as adequate as knowledge of CYP expression status [122,123].

Anticoagulant therapy as stated previously is linked with VKORC1 as well as CYP2C9 status and in any case, warfarin is monitored pharmacodynamically with great care already and it is possible that genotyping may not be of great benefit when doses are carefully titrated routinely during the initiation of therapy. Anticancer therapy is already closely clinically monitored, with individual patient response assessed for toxicity and efficacy during treatment cycles, although sadly, there is evidence that despite the availability of TMPT status determination for some years, more than 86% of deficient individuals still develop azathioprine-mediated myelosuppression [124].

1.4.4.3 Future Perspectives. Two other issues are relevant in pharmacogenetic testing. With TMPT, the value of a phenotypic test over a genotype test suggests that in many other polymorphisms, it may be misleading or even counterproductive to rely on genotypic testing to make major decisions over therapy. There may be no substitute for an accurate functional determination of the individual's biotransformational activity with a particular drug. In addition, there are also issues relating to investment in pharmacogenetic tests, which may be somewhat hazardous commercially, given that a

rival organization may be on the verge of marketing a radical alternative drug, which does not require any testing to maximize its potency, such as the case of clopidogrel, prasugrel and ticagrelor.

Pharmacogenetic testing hopefully should become more economical and better incorporated into hospital routines, which should be accompanied with a growth in clinical knowledge, experience and the development of relevant standard operating procedures. In time, as more is understood about all the various gene expressions involved in individual drug/patient responses, pharmacogenetic testing of biotransforming enzymes may be of benefit particularly during the initialization of therapy, where it could prevent marked over- and underprescribing, linked to patient variation in biotransformational response.

1.5 INHIBITION OF DRUG BIOTRANSFORMATION: CLINICAL CONSEQUENCES

1.5.1 Introduction

Inhibition of drug metabolism probably poses the greatest physical risk of harm to the patient from several perspectives:

- Unlike the process of induction, the biotransforming system is usually unable to adapt to the inhibition. While the induction process is adaptive, inhibition is similar to blocking the exhaust pipe of the engine of a motor vehicle—it cannot adapt and it just stops. If other pathways are capable of clearing the excess drug, they may well not have the capacity of the main inhibited pathway. While some adaptation might occur eventually, drug accumulation will probably happen much more rapidly, indeed within hours, leading to several potential outcomes. These include major intensification of the pharmacological effect of the agent, as well as off-target effects, followed by frank toxicity if drug levels continue to increase.
- Drugs that already have very high or high first pass will be particularly vulnerable to inhibitors and plasma levels can increase dramatically. Agents such as simvastatin, lovastatin, nimodipine, felodipine, and tacrolimus are good examples.
- The consequences of drug accumulation in narrow therapeutic index drugs can be extremely severe and clinical problems may begin within a very short timeframe, potentially within 2–3 h of drug dosage in some cases.
- If an inhibitor is added to a polypharmaceutical regime, it may retard the clearance of more than one of the drugs in the regime, leading to a complex series of pharmacological and toxicological effects, which may or may not be synergistic.
- Issues related to the practitioner and their knowledge of the patient are also relevant. The prescribing practitioner may not be aware of the inhibitory effects and how rapidly they may overtake the patient. In today's environment of multiple prescribers, one prescriber may not necessarily be fully aware of the patient's regime.
- If the patient ingests a dietary inhibitor, the practitioner will almost certainly not be aware of the developing situation and of course, neither will the patient.

- The usual caution that might govern clinical dosage recommendations, may not necessarily apply when a patient is already subject to a polypharmaceutical regime and just “one more” drug is added to this regime.

All these factors may cause severe injury to the patient in a relatively short time. The main clinical issues of drug inhibition are effectively the intensity, duration, and reversibility of the inhibitor’s effect as well as the specific pharmacological and toxicological impact of the inhibited drug, or drugs, on the patient.

1.5.2 Major Clinically Relevant CYP Inhibitors

1.5.2.1 Reversible Inhibitors: Azoles. Over the past 30 years, successful transplant therapy, from stem cells to complete organs, has been made possible through immunosuppressant drugs, which, in turn has led to major problems in the control of opportunistic fungal disease. The azole drugs have been extremely important in fungal elimination, as alternative drugs such as amphotericin B are much too toxic [125]. All the various azole derivatives, dating from the earliest such as ketoconazole through to more modern agents like such as fluconazole, voriconazole, and posiconazole, are all reversible inhibitors of human CYPs to varying degrees. This is hardly surprising because they were designed as CYP inhibitors of yeasts, blocking 14 α -lanosterol demethylase and leading to toxic accumulation of 14 α -methylated sterols, although they generate reactive species that are also damaging to the yeasts [126]. Their lone pair of electrons in the heterocyclic nitrogen group inhibits through a form of NADPH-mediated, reversible association [127].

The most potent of this class in terms of CYP inhibition is the now obsolete ketoconazole, followed by itraconazole and voriconazole, then fluconazole; these agents tend to inhibit CYP3A4, CYP2C9, and CYP2C19 and their propensity for drug interactions is very high, up to 70% [128]. Interestingly, posaconazole only inhibits CYP3A4 and its inhibitory capacity is not fully elucidated clinically. Voriconazole is cleared by all the CYPs it can inhibit, while the others are less extensively metabolized by CYPs with posaconazole undergoing clearance by UGTs. Itraconazole and posaconazole are also P-gp inhibitors.

The main problem with these drugs is that the major immunosuppressants, cyclosporine, tacrolimus, and sirolimus are all CYP3A4 substrates and when the azoles are used to treat fungal infections, there is potential for accumulation of the immunosuppressants with concomitant organ damage and neurotoxicity [129]. This effect varies according to the azole and the immunosuppressant; with tacrolimus, drug levels must be reduced by 40% to prevent toxicity in the presence of fluconazole [130], while ketoconazole requires a 70% reduction in cyclosporine dosage; indeed, it was proposed that this might be beneficial in reducing the dosage of cyclosporine and making the drug more economical to use [131]. While the ethics of such an approach are questionable, there is a necessity to reduce the dose of cyclosporine and other immunosuppressives during azole therapy to prevent toxicity and the beneficial byproduct is a temporary reduction in the usage and therefore the costs of the immunosuppressants [129,132]. However, extended use of the azoles, as with many other anti-infectives, promotes resistance and it is not ethically or scientifically reasonable to use these drugs to reduce the costs of immunosuppressant therapy [133].

Azoles may interact with many other drugs cleared by CYP3A4, CYP2C19, and CYP2C9. For example, benzodiazepines' sedative effect is greatly enhanced [134] and the CYP3A4-cleared statins may cause rhabdomyolysis in the presence of azoles. There are many drugs that are CYP3A4 substrates that are capable of causing life-threatening TdP and concomitant azole therapy could cause sudden death in such situations.

It is worth noting that despite their ability to inhibit CYPs, azoles are as vulnerable as many other agents are to potent enzyme inducers, particularly the more extensively metabolized drugs such as itraconazole and voriconazole [135,136].

1.5.2.2 Irreversible Inhibitors: Grapefruit Juice. Any part of grapefruit, whether it is tinned, diced, fresh, preserved, or juiced can inhibit CYP3A4 metabolism [11]. While reversible inhibitors such as the azoles may be overcome by substrate, the mechanism-based inhibitors such as grapefruit juice exert a more long-lasting effect, which may continue for up to a week after the last consumption of the juice [137]. It is easy to see that habitual consumption of the juice will virtually shut down the CYP3A4 activity in the gut and as this CYP accounts for the majority of gut CYP activity, this effectively cripples gut drug oxidation; indeed, hepatic CYP3A4 is also inhibited if the juice dosage is sustained and large enough [138]. The inhibition of CYP3A4 appears to inactivate the isoform and necessitate the synthesis of new enzyme and several chemical constituents of the fruit accomplish this. These include various furanocoumarins, such as the bergamottin derivatives, and it is thought that any of these agents will act as inhibitors of CYP3A4 if they contain the 5-geranyloxyfurocoumarin group [139]. The effects of the juice are made more complex by a mild inhibition of P-gp as well as a more potent effect on the OATPs. Overall, grapefruit, Seville orange, pomelos (a grapefruit relative), and other citrus fruits exert complex effects, as they decrease bioavailability through OATP inhibition with drugs such as fexofenadine, as well as inhibiting CYP3A4 clearance and inhibiting P-gp, thus increasing bioavailability [140,141]. The best-documented and severe consequence of grapefruit-mediated CYP3A4 inhibition is fatal TdP from terfenadine accumulation [142]. Statin accumulation and subsequent rhabdomyolysis is also a risk of grapefruit juice ingestion as well as increased bioavailability of felodipine.

1.5.2.3 Other Irreversible Inhibitors. There are many other agents that can act as irreversible inhibitors of CYPs, such as the macrolides erythromycin and clarithromycin [143], the SSRIs fluoxetine, paroxetine and fluvoxamine (particularly CYP2D6) as well as several anti-HIV drugs such as ritonavir and delavirdine and the calcium channel blockers verapamil and diltiazem [144]. Inhibitors often have complex clearance pathways themselves, aside from their effects on other drugs. The antiarrhythmic amiodarone is extensively cleared by CYP3A4, as well as CYP2C8, but it is a mechanism-based inhibitor of CYP3A4 and several other CYPs. Amiodarone can markedly affect clozapine and warfarin clearance [145,146], although its own clearance is also acutely vulnerable to inhibition by grapefruit juice [147]. Illicit agents such as 3,4-methylenedioxymethamphetamine (MDMA; a.k.a, ecstasy) probably make a markedly underestimated impact on patient health through CYP inhibition. The protease inhibitor ritonavir has caused fatal accumulation of MDMA [148], probably through CYP2D6 inhibition, while MDMA itself is a mechanism-based inhibitor of CYP2D6, possibly exerting its effects for up to 10 days [149].

Unfortunately, potent mechanistic inhibitors of CYPs are still appearing in the medical armamentarium, such as the relatively new anticancer agent lapatinib, which is a mechanism-based inhibitor of CYP3A4 and has also caused fatal hepatotoxicity [150]. In general, CYP inhibition, particularly of the major isoforms such as CYP3A4, can jeopardize the future of a new drug, unless it has little or no competition in its therapeutic area. Certainly, one of the contributory factors in the withdrawal of Serzone (nefazodone) was its potent inhibition of CYP3A4, making it difficult to add to existing polypharmaceutical regimes as well as its hepatotoxicity.

1.5.3 Relevant Conjugative Inhibitors

1.5.3.1 UGT Inhibition. There is generally less attention paid to inhibition of conjugative processes rather than CYP inhibition. There are, however, several agents that are capable of inhibiting UGTs, such as ketoconazole, which inhibits UGT1A1 and UGT1A9; ketoconazole can increase SN-38 levels by inhibiting UGT1A1 during irinotecan therapy. The drug can also block lorazepam and AZT glucuronidation, through inhibition of UGT2B7 [151]. The insulin secretagogue repaglinide's glucuronidation is inhibited by gemfibrozil, which is a UGT1A1 inhibitor [152]

1.5.3.2 Sulfonation Inhibitors. The NSAIDs mefenamic and salicylic acids can inhibit sulfonation to a clinically relevant degree [153], while several dietary flavonoids and phytoestrogens are potent inhibitors of sulfonation as well as genistein [154]. Generally, most agents that might be cleared through sulfation would be likely to be cleared through other pathways which would be able to act to remove any accumulation. Hence, sulfation inhibition is more likely to be relevant to carcinogenesis, rather than drug clearance.

1.5.4 Clinical Relevance of Inhibition

1.5.4.1 Introduction. The major feature of CYP inhibition in particular, is how quickly the inhibitor, regardless of its mechanism, can arrest CYP processing. If a drug depends on a particular CYP for clearance, the problem will develop more rapidly than with agents that may be cleared by several independent processes. The inhibition process may occur in less than a few hours, which is in stark contrast to induction, which is measured in days and weeks. The rapid accumulation of the drug or drugs will then expose the patient to a double jeopardy; the drug's specific on- and off-target pharmacological effects, plus they are also likely to be away from medical attention, having just collected their medication and probably returned to their homes or work. The accumulation-driven drug effects will quickly move toward toxicity and factors such as the dosage and the individual target of the drug's effects will govern the degree of toxicity encountered. Some major clinical consequences of toxicity are described below.

1.5.4.2 Torsades des Pointes (TdP). This particular problem is probably the key concern of any drug development programme, in terms of risk of toxicity, as this condition can lead to death in a few hours. Surprisingly, there are dozens of drugs that can cause TdP, although it is usually acutely dose dependent [155], so in everyday drug usage, the risk should theoretically be relatively low. The problem arises due

to myocardial human ether-à-go-go-related gene (hERG) channels, which govern the outflow of potassium ions necessary for adequate cardiac repolarization between contractions. These channels are relatively easy to block, so a variety of disparate agents will occlude them, significantly prolonging the QT interval. This promotes the risk of ventricular arrhythmias, which, in turn, can lead to fatal ventricular fibrillation. QT prolonging drugs include several antihistamines such as astemizole and terfenadine, as well as antipsychotics, gastroprokinetics (cisapride), some tyrosine kinase inhibitors (dasatinib), and some fluoroquinolones. There are often marked differences in hERG receptor affinities between drugs of the same class, with some binding at therapeutic levels (pimozide and astemizole) while others show little or no binding (quetiapine and cetirizine) [155,156]. Many otherwise unexplained deaths are likely to have been linked with hERG blockade and CYP inhibitor combinations and this clinical area is not fully explored or understood. This is highlighted by the previously mentioned (section 1.4.4.2) surprising lack of difference in risk of TdP between old and new antipsychotics in TdP [121].

1.5.4.3 Rhabdomyolysis. This condition is characterized by the destruction of myocytes, releasing myoglobin into the blood. The first sign of this effect is dark colored urine, although the myoglobin then dissociates to form ferrihemate, which is toxic to the kidney. Although it can be caused by mechanical injury, there are many drugs, both licit and illicit that can cause the direct muscle injury that leads to rhabdomyolysis [157]. Statins are probably the best-known causes of this injury, although many other prescription drugs can cause it, such as paracetamol (acetaminophen) and antihistamines [158,159]. Drugs of abuse, such as ethanol, cocaine, and heroin, also cause the condition, but many other agents that induce sedation at higher plasma levels, such as antipsychotics, benzodiazepines, and antidepressants, increase the risk of rhabdomyolysis by reducing patient movement and exacerbating muscle damage [157]. Overall, CYP inhibitors that increase the plasma concentrations of all these agents may promote the appearance of rhabdomyolysis, which can lead to renal failure and death *in extremis*.

1.5.4.4 Sedation. Inhibition of the clearance of many centrally acting classes of drugs will promote their sedative effects and this is likely to be a problem with benzodiazepines, antipsychotics, antidepressants, antiepilepsy drugs and some antihistamines. This is of serious clinical significance if the patient becomes unresponsive and perhaps lives alone. The best-case scenario is that due to the patient's state, no further drugs will be ingested for a considerable period and the drugs are gradually cleared without permanent damage. The worst-case scenario might involve sufficient mental confusion to overdose.

1.5.4.5 Anticancer Agents. Several antineoplastic agents require some form of activation by CYPs to exert their clinical effect, and inhibition of biotransformational processes will hinder their efficacy or promote their accumulation, which can lead to toxicity [160]. Cyclophosphamide's CYP2B6-mediated conversion to an active acrolein derivative is hindered by CYP inhibitors such as ritonavir and efavirenz, which impair efficacy, while the taxols, docetaxel, and paclitaxel are cleared by CYP2C8 and inhibition magnifies their toxicity. In irinotecan therapy, inhibition of SN-38 glucuronidation can lead to severe neutropenia among other toxicities, although efficacy will be

enhanced, provided the patient can withstand the toxicity, as mentioned previously. In general, patients under treatment with anticancer agents can be highly susceptible to toxicity and efficacy problems in the presence of CYP inhibitors although fortunately, their clinical supervision is normally very close.

1.6 PATIENT FACTORS AFFECTING BIOTRANSFORMATION

1.6.1 The Elderly

After their possible polymorphic gene expression, probably the major influencing factor the patient brings to the process of drug therapeutics is their age. It is not completely clear in the literature at what point an individual is classed as “elderly,” although some studies suggest that those aged 70 plus should be thus considered [161]. Indeed, older individuals vary hugely in their general health, robustness, quality of nutrition and other personal habits that might impinge on drug clearance, such as drug or alcohol usage. It could be suggested that for the purposes of drug biotransformation, one does not suddenly become “elderly”; rather, all the processes mentioned earlier contribute to changes in drug clearance that are seen in older individuals and should be considered on an individual basis.

However, in all individuals, certain basic processes apply which are related to age, such as the gradual reduction in hepatic blood flow that can lead to a nearly 50% reduction by the patient’s late 60s compared with that of their early 30s [162,163]. Since many drugs are blood flow dependent in their clearance, such agents will show a proportionally similar reduction in clearance to the decline in blood flow. However, aside from blood flow decline, there is a reduction in the intrinsic clearance of several drugs (paracetamol, naproxen, and valproate) of between 20% and 60% [164]. This is linked to age-related changes in protein binding, as well as a reduction in the activity of some individual CYPs, such as CYP1A2, CYP2C9, and CYP2C19 [165,166], but not others such as CYP2D6 [167].

It might be expected that this mixed effect related to the effects of age on CYPs may be linked with changes in levels in basal hormone secretions; although a study in elderly men with growth hormone supplementation reported that CYP1A2 was induced, but other CYPs were unaffected (CYP3A4 and CYP2D6), while CYP2C19 was inhibited [168]. Processes such as CYP inhibition are likely to cause similar effects in the elderly. Alternatively, regarding induction, a study with St. John’s Wort demonstrated robust increases in CYP3A4 and CYP2E1 expression in a group of individuals with a mean age of 67 years [8]. Hence, it is likely that induction still presents a clinical problem in the elderly when adding inducers to existing regimes or adding to regimes that already contain inducers. Regarding the impact of age on basal expression of the various transporters, this may not be quantifiable due to the extraordinary variation in their expression between individuals [169], although given that induction is probably close to fully functional in an elderly individual’s CYP3A4, P-gp expression at least will probably also respond to induction, so the OATPs may follow suite. Regarding glucuronidation capacity, there is no evidence that this is diminished significantly in old age [170]; drugs that are cleared mainly by this pathway, such as AZT and lorazepam, are likely to show changes in clearance which are commensurate with the age-related blood flow reduction.

Overall, it is recommended that drug dosages should be considerably reduced (often up to half) for the elderly and careful note of concentration-dependent adverse reactions taken to ensure that they are not over medicated. While this applies in the case of the so-called fit elderly, the frail elderly, those with multiple pathologies, present a more considerable challenge. Not only are they likely to be the recipients of polypharmacy, but their general health and capability of their major organs such as renal, hepatic, and GI tract function, is likely to be suboptimal, leading to effects on absorption, distribution (plasma protein level changes), metabolism, and excretion.

1.6.2 Children and Neonates

The state of knowledge of drug clearance in neonates, generally those less than four weeks old, is relatively poor, partly because they are difficult to study for a number of reasons. It is generally accepted that it is highly questionable ethically to carry out clinical research on individuals who by definition cannot give informed consent and are extremely precious to their parents. They are also fragile, have low blood volume (a problem in repeated sampling) and less than stellar renal function, as well as relatively poor biotransforming capacity. It has been suggested that such research is better incorporated into an ongoing treatment programme, with such sedatives as midazolam at low doses [171]. The major CYP in fetal liver is CYP3A7, which has much lower capability than its adult equivalent CYP3A4, although it is more efficient at metabolizing some steroids; interestingly, CYP3A7 is not exclusively fetal, as around 10% of adults express it [172]. Generally, at birth, the neonate CYP hepatic content is less than a third of adults [173], although most CYP activity is on a par with adults within a year or so of birth [174]. Indeed, clearance of CYP3A4 substrates can be faster than adults within a couple of months of birth [175]. Regarding other oxidative pathways, neonates are born with high FMO-1 and low FMO-3 expression, but this reverses rapidly and FMO-3 becomes the dominant form that increases toward adulthood [176].

In contrast to CYPs and other oxidative pathways, UGT capacity is very low at birth and only increases very gradually [177]; indeed, the major UGT1A1, which processes bilirubin, is low in activity around the time of birth, which leads to many babies being born with a degree of mild jaundice, which is much more pronounced in UGT1A1 polymorphisms [178]. Adult levels of the UGTs appear from aged 6 months onward [5], although in general conjugative pathways take longer to mature than oxidative ones. Premature neonates have little drug-metabolizing capacity even in comparison with term neonates, so that they present a particularly difficult therapeutic management problem.

Overall, neonates and children are not miniature adults and have markedly different characteristics, based on dimensions, volumes, intrinsic enzyme capacity, renal performance, and protein binding; indeed, much remains to be discovered about their biotransformational capacity and its impact on drug efficacy and in particular, their vulnerability to drug toxicity [5,175].

1.6.3 Gender

There is considerable evidence in animal models that drug clearances vary between the sexes, partly related to possession or lack of possession of particular CYPs [179].

The effects of gender in humans is not so well characterized, but some CYPs do appear to have higher activities in males, such as CYP1A2, which leads to increased clearance in males with drugs such as clozapine; perhaps surprisingly, smoking is thought to be a larger factor in CYP1A2 activity than gender [180,181]. Overall, females have lower UGT and CYP2E1, higher CYP3A4 and CYP2B6, but the same CYP2C9 and CYP2D6 activities [5,182]. Interestingly, probably the most dangerous drug side-effect in terms of rapidly lethality, TdP, is more common in women due to estrogen effects and so more than 60% of the cases are female [5,182]. Women are also more at risk from other drug adverse reactions, such as rashes [183]. There are other factors that also govern female drug clearance, which include lower total body water, different basal metabolic rates, lower renal glomerular filtration rate, and generally lower body size and volume in comparison with men.

1.6.4 Pregnancy

It is sometimes assumed that only certain highly necessary classes of drug are prescribed during pregnancy, such as antiepileptic agents and antimalarials in appropriate areas. However, a study in Norway revealed that approximately a quarter of fathers and a little less than half of mothers had used prescription drugs in the three months before successful pregnancy and more than half of women continued to use prescription drugs during pregnancy [184]. These agents ranged from antidepressants to hormones, anti-infectives, anxiolytics, and antihistamines. This is highly significant, as aside from the teratogenic risks, drug clearance in pregnancy can also change due to marked distributional changes related to the process. There are steep reductions in the plasma concentrations of several drugs, such as the antiepileptics, as well as transmission of pharmacologically relevant doses to the fetus and neonate through the placenta and maternal milk, respectively [185]. There is little evidence of CYP activity of the placenta, apart from the induction of CYP1A1 in smokers [186]. Transporter activity, such as P-gp, in the placenta is predictably high, with fetal protection being a priority; however, there are marked differences in the transport of even drugs of the same class [187]. There is conflicting evidence that pregnancy affects CYP expression in animals [188,189]. In humans, CYP3A4 is induced by pregnancy, which is related to hormonal changes [190], leading to the suggestion that narrow therapeutic index drugs should be carefully monitored in pregnancy [6]. Overall, although sex differences are less clear in terms of CYPs in humans with respect to animal models, there are clearly enough differences in certain drug clearances to alert clinicians to the possibility that many pharmacokinetic as well as biotransformational factors may conspire to make women's drug therapy potentially more difficult to manage than men's.

1.6.5 Effects of Disease

Multiple pathologies may affect drug clearance, ranging from specific renal or hepatic organ malfunction, which may virtually arrest the clearance of many drugs and metabolites, to more subtle effects such as on the supply of protein for binding globulins or GIT problems that might impair absorption. There are several ongoing global conditions that affect CYP expression, such as diabetes, which induces CYP2E1 activity. This is shown in significantly increased clearance of chlorzoxazone in obese diabetics [191] compared with nondiabetics. However, the major focus in terms of disease on

biotransformational capability is usually in the liver. Any severe disruption of hepatic ultrastructure or metabolism either through disease (hepatitis or cancer) or abuse (cirrhosis) will strongly affect the ability of the organ to fulfill its main biochemical and physiological functions as well as affecting enzyme expression. Hepatitis viral infections, such as the C and B variants, can lead to cirrhosis and hepatocellular carcinoma and they affect liver cellular oxidative defense systems by reducing GSH maintenance, which increases hepatic oxidative stress [192]. Hepatitis C causes down-regulation in the expression of most CYPs, such as CYP2B6, CYP2C9, CYP2C19, CYP2E1, and CYP3A5, but it increases expression of others, such as CYP3A7 [193]. Several autoimmune conditions also lead to antibody formation that is directed at various CYPs, although the mechanisms at work are still not understood [194,195]. In general, CYP capability is reduced in hepatic disease and will affect drug clearance [196].

CYP2E1 expression is strongly linked with the oxidative stress that is associated with alcoholic liver disease [197], and the physical damage inflicted on the vasculature and ultrastructure of the organ is so severe that blood flow is severely restricted. The physical loss of functional hepatocytes, combined with the poor organ perfusion, is the major factor in the drastic loss of biotransformational capacity of the cirrhotic liver. In addition, CYP expression, such as CYP3A and CYP2E1, are both significantly reduced in the surviving hepatocytes [198].

1.6.6 Diet

Aside from the impact of agents such as the various fruit juices (discussed in Section 1.5.2.2), much of the research into the effects of diet on biotransformation is related to the etiology of cancer, such as the generation of toxic species that may damage DNA, through to dietary protective agents, which either inhibit or induce protective or potentially damaging pathways over many years. In terms of impact on drug clearance, unless a diet contains large amounts of cooked food rich in hydrocarbons (CYP1A2 induction) or steroids and phytosteroids (CYP3A family), there are few agents that can be ingested in sufficient quantity to alter drug clearance to a meaningful degree. There is evidence that cruciform vegetables contain potent CYP modulators such as sulforaphane and various indoles, which can clinically induce CYP1A2 capacity when eaten along with other crucifers [199,200]. Broccoli can also induce GSTs, acting to protect against reactive species generation [199]. Regarding the effects of dietary products on CYP induction, there are many agents that interact with PXR and other nuclear receptors; indeed, sulforaphane in broccoli is an antagonist of PXR, while other plant products are inducers [81]. Overall, the net effects on drug clearance of so many agents that are found in typical diets is highly individual and is probably very difficult to predict, given the variability inherent in human biotransformational enzyme expression [201].

1.7 DRUG METABOLISM AND DRUG DEVELOPMENT

1.7.1 Introduction

Drug metabolism is an integral aspect of drug development and has major implications in terms of not only efficacy and toxicity but also of patent protection

for pharmaceutical and biotechnology companies that spend hundreds of millions to develop and market a drug. For example in 1985, Seldane[®] (terfenadine) was the first nonsedating antihistamine to be brought to market by Hoechst Marion Roussel and was perceived in the UK to be safe enough for OTC use. The drug was withdrawn in 1998 in the United States and restricted to named patient only status in the United Kingdom because of the risk of cardiac arrhythmia caused by QT interval prolongation as a consequence of inhibition of CYP3A4-mediated metabolism of terfenadine. Ironically, fexofenadine (Allerga[®]), a carboxylated metabolite of terfenadine, marketed in 1998 as a backup to Seldane[®] by Hoechst Marion Roussel, did not exhibit the QT liability seen with Seldane[®]. In fact, terfenadine could be considered a prodrug of fexofenadine, and if the pharmaceutical company had spent more time/resources to characterize the metabolism of terfenadine and the pharmacology/toxicology of terfenadine metabolites, a safer analog could have been developed and marketed sooner.

1.7.2 The Drug Development Process

Drug development is both arduous and of long duration (10–12 years). It involves (i) the identification of a target, synthesis of possible drug candidates based on structures of known substrates and/or inhibitors of the target; (ii) high throughput *in vitro* screening for binding followed by *in vitro* and *in vivo* evaluation of efficacy in pre-clinical models; (iii) characterization of pharmacokinetics and disposition in rats, dogs, and/or monkeys to select the most promising compound; (iv) assessment of the compound's safety in animal toxicology studies to demonstrate that the drug candidate is reasonably safe to proceed with initial clinical trials in human volunteers (phase 1); (v) demonstration of proof of concept in the intended patient population and conduct DDI studies in phase 2; and (vi) the conduct of two large-scale pivotal clinical trials to demonstrate safety and efficacy to support the marketing application (phase 3). Given that 1 in 10,000 discovery compounds may make it as a drug, one has better odds in Las Vegas.

In silico and *in vitro* methods may be used in the early discovery phase as cost-effective means to “weed out” compounds with metabolic liabilities, such as the formation of reactive metabolites that may be responsible for idiosyncratic drug reactions, most notably hepatotoxicity or drug-induced liver injury (DILI). Yet, there are limitations with *in silico* methods as they rely on actual *in vivo* data. Drug candidates are typically screened *in vitro* for CYP450-mediated metabolic stability and potential CYP450 inhibition; induction studies are typically conducted later in preclinical development. *In vitro* data have been estimated to be predictive of the nature and level of circulating human metabolites from 41% [202] to >70% [203] of the time. Subsequent *in vivo* metabolism studies with unlabeled drug or radiolabeled drug, if available, can provide additional information. Various analytical methods, for example, LC-MS/MS, radiometric, and NMR, are used to analyze samples. During preclinical development, mass balance/metabolite identification studies in the rodent and nonrodent toxicology species are conducted with radiolabeled drug to determine the route(s) of elimination, mass balance, and metabolite identification. Radiolabeled drug is also used for the human mass balance/metabolite identification study, and this study is typically conducted during clinical development. These studies also provide information on clearance mechanisms, such as on renal and/or biliary excretion and metabolism.

1.7.3 Drug Metabolites and Drug Development

The consensus opinion entitled “Contemporary Issues in Toxicology, Drug Metabolites in Safety Testing” was published in 2002 [204]; this article became known as the Metabolites in Safety Testing (MIST) document. Its goal was to define reasonable, scientifically based approaches for the incorporation of metabolite data in the nonclinical safety evaluation of small molecules. In the nonclinical safety evaluation of drug candidates, determination of systemic exposure, that is, maximum observed drug concentration ($C_{\max}$) and area under the concentration–time curve (AUC), is critical for risk assessment and determination of margins of safety to support clinical trials. Unfortunately, metabolites are generally not considered in the risk assessment since their contribution to the overall toxicity profile observed is usually unknown. However, if the incidence and severity of adverse findings/toxicity do not correlate with the systemic exposure to parent drug, then it is likely that a metabolite may be responsible. Thus, the onus would be on the pharmaceutical company to determine the cause.

A major metabolite was defined as “one that accounts for 25% or more of the exposure to circulating drug-related material.” This threshold carries no regulatory authority or implications with respect to the need for safety assessment [205]. However, one must not lose sight that even minor metabolites can and often do cause toxicity.

The MIST document provides recommendations for the three major phases of drug development: (i) nonclinical (a.k.a, preclinical) development [i.e., candidate selection to Investigation of New Drug (IND) filing]; (ii) initial clinical safety, tolerability, and efficacy trials in human volunteers (phase 1 through 2b); and (iii) large-scale clinical trials for product registration (phase 3).

Even though there is no consensus from the members of the Pharmaceutical Research and Manufacturers Association (PhRMA) on the timing of metabolism studies in humans, it is recognized that attempts to identify differences in drug metabolism between animals used for nonclinical safety evaluation and humans be performed as early as possible. The Food and Drug Administration (FDA) and International Congress on Harmonisation (ICH) regulatory guidances do suggest that safety evaluation of metabolites be completed before the start of phase 3 trials. A *de minimis* approach for an IND filing would be to conduct comparative *in vitro* metabolite profiling studies using isolated hepatocytes, liver slices, or hepatic microsomal preparations from the toxicology species (e.g., rat, dog, and monkey) and humans to demonstrate that the toxicology species are being exposed qualitatively to the same metabolites seen in humans and to justify the selection of the nonrodent toxicology species (details on how these studies are conducted are addressed in other chapters of Volume 6). With regard to submission of Investigational New Medicinal Product Dossier (IMPD) in the European Union, it is recommended that *in vitro* studies be conducted before phase 1 to aid in determining whether volunteers of certain genotypes should be excluded and for extrapolation of nonclinical safety data to humans [206]. The use of radiolabeled drug (typically ^{14}C -labeled although ^3H can be used) is recommended to ensure quantitative recovery and facilitate identification of drug-derived constituents. Assurance that the metabolite profile in at least one of the toxicology species is qualitatively similar to that in humans is adequate for nonclinical safety assessment. Metabolite identification is not a prerequisite during preclinical development, but could aid the toxicologist in deciding whether to monitor metabolite(s) in the IND- or IMPD-enabling toxicology studies, especially if toxicity did not correlate with concentrations of unchanged drug in

the exploratory dose range-finding studies. Although it is rare that a metabolite formed in humans is not found in the toxicology species, in those cases where a unique human metabolite is formed or a human metabolite is produced disproportionately (i.e., abundance) in the toxicology species, independent nonclinical safety studies with the human metabolite may be warranted.

The MIST document recommends that *in vivo* metabolite identification be conducted in the toxicology species and in humans sometime in phase 1 through 2b. An *in vivo* metabolism study in which the drug is administered both orally and intravenously provides information on the degree of first-pass metabolism by the GI tract and/or liver. Synthesis of prevalent metabolites is performed for metabolite confirmation and subsequent testing for efficacy in pharmacology models. Metabolism information aids the pharmacokineticist in the design of subsequent studies such as CYP450 reaction phenotyping, *in vitro* drug induction/inhibition, *in vitro* transporter interaction, and clinical DDI studies. Information gained from metabolism, CYP450 reaction phenotyping, *in vitro* drug induction/inhibition, and *in vitro* transporter interaction studies helps assess the DDI potential of a development candidate and whether clinical DDI studies are warranted. Furthermore, additional investigations to assess the contribution of metabolites in the overall toxicity profile of a drug candidate may be warranted in those cases where the therapeutic index is narrow. The MIST document emphasizes that all major human circulating metabolites should be present in at least one of the animal species used in the reproductive toxicology studies. Carcinogenicity studies on administered major metabolite are not recommended or routinely conducted; however, the FDA reserves the right to request such studies when appropriate.

The FDA guidance entitled “Safety Testing of Drug Metabolites” was issued by the Center for Drug Evaluation and Safety (CDER) in February 2008. “The safety of drug metabolites may need to be determined in nonclinical studies because these metabolites are either identified only in humans or are present at disproportionately higher levels in humans than in any of the animal species used during standard nonclinical toxicology testing” [207]. The guidance further states that human metabolites that may pose a safety concern are those found circulating at >10% of parent drug systemic at steady state (in contrast to 25% cited in the MIST document). The FDA recommendation is, however, consistent with ICH Harmonised Tripartite Guideline M3(R2) [208] in terms of the 10% level but ICH M3(R2) does not specify steady state. The confounding factor with radiolabeled mass balance/metabolite identification studies is that a single dose (i.e., nonsteady state) is used. However, an estimate of exposure at steady state could be derived from an accumulation ratio calculated using the elimination rate constant and anticipated dosing interval.

Two approaches for assessing metabolite safety are outlined in the FDA guidance: (i) identification of a toxicology species that forms the metabolite at exposures comparable to or greater than human exposure and evaluation of toxicity of the drug candidate in that species or (ii) if a toxicology species that forms the metabolite cannot be identified, the metabolite should be administered to animals at multiples of human exposure, if possible, for evaluation of toxicity by the intended clinical route. One-to-one coverage of metabolite in one toxicology species would generally be sufficient to qualify the metabolite (this assumes that metabolite exposure in the toxicology species was comparable to human exposure of the metabolite at the maximum recommended therapeutic dose). The duration of general toxicity studies with direct administration

of metabolite should follow ICH M3(R2) [208] and, as a minimum, cover the duration of clinical administration or, as a disproportionate (i.e., abundance) metabolite, its genotoxic potential should be assessed *in vitro* in a mutagenicity and a clastogenicity assay. Embryo-fetal development toxicity studies should also be performed with the metabolite. It is possible that the FDA may accept such a study in only one species that forms the metabolite, while other reproductive studies may be requested by the FDA, depending on the findings in the general toxicity and embryo-fetal development studies. If a drug is administered continuously for at least six months or is used for chronic intermittent treatment and the carcinogenic potential of the metabolite cannot be adequately assessed in carcinogenicity studies with the parent drug, independent carcinogenicity studies should be conducted with the metabolite. With regard to any proposed carcinogenicity studies, the Carcinogenicity Assessment Committee at the FDA should be consulted before study initiation.

Systemic concentrations of metabolites are dictated by the rate and extent of biotransformation of parent, extent of tissue distribution and degree of binding to plasma and tissues, and metabolite clearance rates. All three factors can differ across species, but biotransformation of parent and metabolite clearance are likely to differ the most between animals and humans due to differences in drug-metabolizing enzymes, especially CYP450. [209]. DDIs represent an important source of variability in pharmacokinetics that can affect drug response, in terms of efficacy as well as toxicity. The recommended timing of metabolism and drug interaction studies in FDA guidance is essentially the same in the European Medicines Agency (EMA) Guidelines on the Investigation of Drug Interactions (draft); however, the EMA draft guideline emphasizes that “the potential effects of metabolites on the pharmacokinetics of other drug as well as effects of other medicinal products on the exposure of active metabolites should always be considered” [206].

There have been several articles published on MIST. Some publications have advocated that rather than using a percentage-based approach as recommended in the MIST document, absolute abundance (amount or concentration), the nature of the toxicity mechanism (reversible vs covalent binding), and the biological matrix in which the metabolite was observed (circulatory vs excretory) be used when comparing metabolites across species and making decisions regarding whether to assess the contributions of metabolites to toxicity [209,210]. A fourth tenet, duration of exposure, has recently been added to that decision tree [211,212]. The proposed “new MIST” strategy of considering metabolite structure, amount, biofluid, potential mechanism of toxicity, and duration of exposure is insightful, but the guiding principles proposed may be of limited utility given the number of drugs examined retrospectively in each toxicity class.

The results of independent toxicity testing of an administered metabolite may be misleading and fail to characterize the contribution of metabolite to the overall toxicity of the drug candidate due to differences in the pharmacokinetics and disposition of administered metabolite compared to that formed *in situ* following administration of parent. The discrepancies are not only due to differences between the drug candidate and metabolite in physicochemical properties and interactions with transporters and/or drug-metabolizing enzymes but also by physiological factors such as tissue selective distribution and species differences in transporters and/or metabolizing enzymes [213]. These factors may be mitigated/compensated for, in part, if higher exposure to metabolite can be achieved (e.g., by increasing dose or dose frequency) in nonclinical toxicity studies with the metabolite alone.

1.7.4 Drug Development and Drug Interactions

In developing a drug, the goal is to select a candidate that has a favorable pharmacokinetic profile that entails good absorption/bioavailability, linear pharmacokinetics, an elimination half-life which would support the intended clinical dosing regimen, not extensively metabolised and does not possess pharmacokinetic DDI liabilities. Sometimes, trade-offs are made with regard to the desired pharmacokinetic properties for the sake of efficacy or the lack of available or effective medications for a serious or life-threatening disease state. Refractory epilepsy is a good example. Typically, patients with refractory epilepsy are on multiple drugs. The first generation of AEDs, such as phenobarbital, phenytoin, valproic acid, and carbamazepine, are still commonly used today as baseline therapy and are either CYP450 inducers or inhibitors. As pharmaceutical companies continue to develop newer AEDs for add-on therapy, the goal is to reduce or eliminate DDI potential. Eslicarbazepine acetate, felbamate, lamotrigine, oxcarbazepine, tiagabine, topiramate, and zonisamide are susceptible to inhibition and induction of their own metabolism [214]. Gabapentin [215], pregabalin [216], and vigabatrin [217] are excreted essentially unchanged in the urine and are not CYP450 inducers or inhibitors. As stated by Bialer and White [218], a new AED can be successful if at least one of the following criteria are met: greater efficacy in refractory epilepsy than AEDs in current use, the ability to prevent or delay the onset of epilepsy and/or potential for disease modification; broad use in other nonepileptic CNS disorders, fewer side effects and/or better tolerability than currently used AEDs, and improved ease of use (rapid titration, linear pharmacokinetics, a lack of drug interaction, and once- or twice-daily dosing).

1.7.5 Drug Development and Pharmacogenetics

The CYP450 isozymes (specifically, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5) are the principal oxidative drug-metabolizing enzymes. CYP2C9, CYP2C19, and CYP2D6 are expressed polymorphically and enzymatic activity differs among ethnic groups such that individuals can be categorized as PMs or EMs and dose adjustment would be warranted to avoid toxicity or lack of efficacy.

For example, metabolism of clopidogrel, a prodrug, via CYP2C19 is essential in the management of patients with acute coronary syndrome (ACS). Biotransformation of clopidogrel by the CYP2C19*2 allele, which is present in 30% of some populations, results in reduced production of the active metabolite and is associated with increased risk of adverse events, such as stent thrombosis [219,220]. Development of prasugrel was a major advancement in that biotransformation of prasugrel to its active metabolite was primarily by CYP3A and CYP2B6 [221]. In the case of CYP2D6, Asians, Pacific Islanders, Africans, and African-Americans have a higher percentage (~40% to 50%) of reduced or nonfunctional CYP2D6 relative to Caucasians (26%) [222]. Hence, pharmaceutical companies have avoided developing drugs that are predominantly (>90%) metabolized by CYP2C9, CYP2C19, or CYP2D6.

Since CYP3A4 is responsible for the metabolism of ~60% of marketed drugs, drug candidates that are exclusively metabolized by CYP3A4 or are potent inhibitors of CYP3A4 pose a serious liability for pharmacokinetic drug interactions and would be subject to labeling restrictions with regard to coadministration with other drugs.

The antidepressant nefazodone (Serzone®) is an excellent example (mentioned in Section 1.5.2.3), since the drug is a substrate and inhibitor of CYP3A4 as well as a hepatotoxin due to the formation of a reactive metabolite. The Serzone® product label lists several DDIs, including triazolam, alprazolam, buspirone, atorvastatin, simvastatin, terfenadine, astemizole, cisapride, or pimozide. On the basis of the proposed structure of the glutathione conjugate with nefazodone, a bioactivation sequence involving aromatic *ortho*- or *para*-hydroxylation on the quinolinone followed by oxidation to a quinone-imine was proposed [223]. Serzone® was not surprisingly voluntarily withdrawn from the US market in 2004.

In summary, there is no straightforward or standardized approach to deal with metabolites in safety studies. The number and type of nonclinical studies for drug metabolites could be abbreviated in the case of serious illness (e.g., amyotrophic lateral sclerosis, stroke, and human immunodeficiency virus) or may not be necessary (e.g., cancer) [207]. Using a weight of evidence approach to demonstrate a minimal safety concern, a waiver may be requested from the Agency. In both the MIST document [204,224] and the FDA guidance on Safety Testing of Drug Metabolites, a case-by-case approach to metabolite safety testing is advocated, given the complexity faced in the development of a drug candidate. Although it recognized that there are differences in drug-metabolizing enzymes between animals and humans, the question of how to extrapolate drug metabolism and associated toxicities in animal species to humans challenges the pharmaceutical industry. Finally, metabolism is an important factor that must be addressed during both preclinical and clinical development. Biotransformation can result in the formation of reactive metabolites, reduced or no efficacy, formation of pharmacologically active metabolites, and inducers/inhibitors of major drug-metabolizing enzymes (e.g., CYP450 and UDPGA-transferases).

1.8 CONCLUSIONS

It is to be hoped that this introductory chapter has given the reader some inkling of the sheer scale of the challenges facing the design, development, and successful deployment of clinically effective drugs in human populations. The multiplicity of biotransformational processes that affect drug efficacy and toxicity, ranging from transporters, CYPs, UGTs, SULTs, and their control systems presents huge challenges in the everyday use of potentially life-enhancing medicines. Continued research into human age, gender, genetic, and phenotypic variation, which informs so much of drug impact on patients, will in time improve drug targeting, maximizing efficacy and minimizing toxicity. It is also vital that not only is such research carried out ethically and scientifically, it must also be disseminated to prescribing healthcare professionals the world over to translate clinical research advances into real and sustained gains in patient health during drug therapy.

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2 CYP450 Enzymes in Drug Discovery and Development: An Overview

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2.1	Introduction	1
2.2	Nomenclature and classification of human CYP enzymes	2
2.3	Catalytic activity of CYP enzymes	2
2.4	Common CYP-mediated biotransformation reactions	4
2.5	Species variation in the expression and activity of CYP enzymes	9
2.6	Ethnic variability in expression and activity of cytochrome P450 enzymes	24
2.7	Tools for <i>in vitro</i> – <i>in vivo</i> extrapolation	25
2.8	Summary and future perspectives	27
	Acknowledgment	28
	References	28

2.1 INTRODUCTION

The CYP450 (P450) is a collective name for a very large group of enzymes found in all domains of life and are responsible for the metabolism of a vast array of xenobiotic chemicals, including drugs, carcinogens, pesticides, pollutants, and food toxicants as well as endogenous compounds, such as steroids, prostaglandins, and bile acids [1,2]. The origin of the cytochrome P450 name, first coined in 1962, was from the fact that these enzymes are cellular (cyto) colored (chrome) proteins, which contain heme pigments (P) that absorb light at a wavelength of 450 nm when exposed to carbon monoxide [3,4]. P450 enzymes are predominantly expressed in the liver as well as in extrahepatic tissues such as lungs, kidneys, intestine, brain, and skin. Since their discovery at the end of 1950, P450 research has grown and the multiplicity and complexity of the P450 system has been evident for more than five decades [5]. Over 11,500 members or distinct P450s genes are currently known that are present in the majority of species from all biological kingdoms [6,7].

The P450 enzymes catalyze oxidative as well as some reductive (phase I) reactions. These reactions introduce or unmask a functional group (e.g., –OH, –CO₂H, –NH₂, or –SH) within a molecule to enhance its hydrophilicity. It can occur through direct introduction of the functional group (e.g., aromatic and aliphatic hydroxylation) or by

modifying existing functionalities (e.g., oxidative hydrolysis of the esters and amides, oxidative N-, O-, and S-dealkylation, and reduction of aldehydes and ketones) [8]. As a result, more hydrophilic (water soluble) and polar entities are formed, which are eliminated from the body. In general, metabolism leads to compounds that are generally pharmacologically inactive and relatively nontoxic. However, metabolic biotransformation of drugs at times can lead to the formation of metabolites with pharmacological activity [9] or toxicity [10].

P450 enzymes have long been of interest in the metabolism of pharmaceuticals and other xenobiotics, since these enzymes are responsible for the elimination of majority of the marketed drugs. These reactions account for ~95% of the drug metabolism. In addition, there are a number of endogenous and exogenous factors, such as genetic variation, age differences, hormone levels, diet, and exposure to a variety of drugs, that can influence the expression and catalytic properties of P450 enzymes. Tremendous progress has been made in the last six decades in the characterization, expression, function, and regulation of P450 enzymes in animals and humans [2,11]. In this chapter, we summarize the most recent advances in our knowledge and application of P450 enzymes in drug discovery and development with particular emphasis on their involvement in the metabolism of drugs. In addition, we describe the species and ethnic variation in the expression of P450 enzymes and the tools used to extrapolate metabolism and toxicity in animals to humans.

2.2 NOMENCLATURE AND CLASSIFICATION OF HUMAN CYP ENZYMES

P450 enzymes are categorized into families, subfamilies, and specific enzymes according to their amino acid sequence similarity. P450s that share at least 40% sequence identity are placed within the same family, designated by an Arabic numeral, while those with greater than 55% homology are placed in the same subfamily, designated by a capital letter and those with 97% homology represent individual enzymes, designated again by a number. Individual alleles are designated by appending a star and a number (human cytochrome P450 allele nomenclature committee, <http://drnelson.utmem.edu/Cytochrome450.html>) (Fig. 2.1).

2.3 CATALYTIC ACTIVITY OF CYP ENZYMES

The P450 enzymes are referred to as *hydroxylases*, *monooxygenases*, or *mixed function oxidases* and possess three known types of activities. P450s, acting as hydroxylases, activate molecular oxygen and insert one atom of molecular oxygen into the substrate (S or X) while reducing the other atom of oxygen to water (Eqs. 2.1 and 2.2). As a result, the xenobiotics can undergo hydroxylation, epoxidation, heteroatom (N-, S-) oxygenation, heteroatom (N-, S-, O-) dealkylation, ester cleavage, isomerization, dehydrogenation, and oxidative dehalogenation.



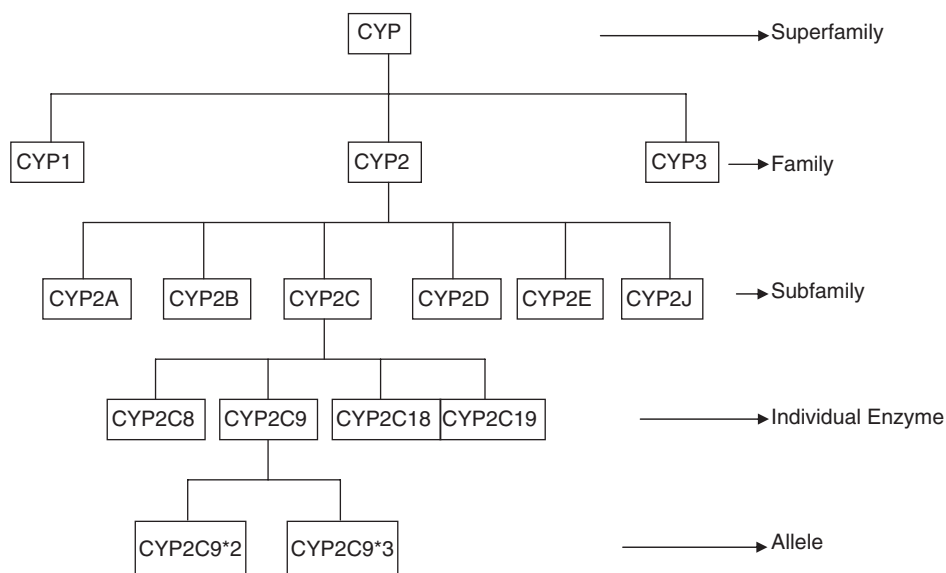
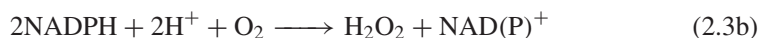


Figure 2.1 Nomenclature of CYP450 enzymes.

The oxidase activity of P450s involves one electron transfer from reduced P450 to molecular oxygen with the formation of superoxide anion radical and H_2O_2 (Eq. 2.3a,b).



The reductase activity of P450s involves direct electron transfer to reducible substrates such as quinones and proceeds readily under anaerobic conditions.

The catalytic cycle of P450 oxidation is a complex multistep processes as follows:

1. P450 enzyme (Fe^{3+}) first binds to a substrate XH to form $\text{Fe}^{3+}\text{-XH}$. This results in lowering the redox potential, which makes the transfer of an electron favorable from its redox partner, NADH or NADPH. This is accompanied by a change in the spin state of the haem iron at the active site.
2. The next step in the cycle is the first reduction of the $\text{Fe}^{3+}\text{-XH}$ to $\text{Fe}^{2+}\text{-XH}$ by an electron transferred from NAD(P)H via an electron-transfer chain.
3. In the third step, an O_2 molecule binds rapidly to the $\text{Fe}^{2+}\text{-XH}$ to form $\text{Fe}^{2+}\text{-O}_2^{\cdot-}\text{-XH}$, which then undergoes a slow conversion to a more stable complex $\text{Fe}^{3+}\text{-O}_2^{\cdot-}\text{-XH}$.
4. The next step in the cycle is a second reduction of $\text{Fe}^{3+}\text{-O}_2^{\cdot-}\text{-XH}$ to $\text{Fe}^{3+}\text{-O}_2^{2-}\text{-XH}$ via the electron donors either NADPH or cytochrome *b5*. This has been determined to be the rate-determining step of the reaction.

5. The $\text{Fe}^{3+}\text{-O}_2^{2-}\text{-XH}$ reacts with two protons from the surrounding solvent, breaking the O–O bond, forming water and leaving an $(\text{Fe-O})^{3+}\text{-XH}$ complex.
6. The Fe-ligated O atom is transferred to the substrate forming a hydroxylated form of the substrate ($\text{Fe}^{3+}\text{-XOH}$).
7. The last step involves the release of product from the active site of the enzyme, which returns to its initial state.

2.4 COMMON CYP-MEDIATED BIOTRANSFORMATION REACTIONS

P450 catalyzed reactions can be classified into four broad categories: (i) hydroxylation reactions where a hydroxyl group replaces a hydrogen atom; (ii) epoxidation reactions where an oxygen atom is introduced into carbon–carbon double or triple bond; (iii) heteroatom oxidation where an oxygen atom is added to a nitrogen or sulfur, (iv) dehydrogenation reactions where two hydrogen atoms are replaced by a double bond [12].

2.4.1 Hydroxylation Reaction

Hydroxylation of an aliphatic carbon or an aromatic ring is one of the most common drug metabolism reactions. The other common biotransformation reaction is the hydroxylation at the α carbon to a hetero atom, which resulted in oxidative cleavage of the molecule.

2.4.1.1 Aliphatic Hydroxylation. For aliphatic hydroxylation, one proposed mechanism is an abstraction of a hydrogen atom by $(\text{Fe-O})^{3+}$ to form a radical intermediate that reacts with the oxygen on the P450 ($\text{Fe-OH})^{3+}$ to yield the alcohol and $(\text{Fe})^{3+}$ (Fig. 2.2).

Drug molecules possess many alkane carbons with abstractable hydrogen atoms and therefore, hydroxylation can possibly occur at any one site that can result in more than one hydroxylated product, as shown for ezlopitant (Fig. 2.3) [13]. However, a product forms preferentially from the most stable radical (resonance stabilized such as benzylic or allylic).

2.4.1.2 Aromatic Hydroxylation. The aromatic hydroxylation occurs by epoxidation of the aromatic ring to form of an arene oxide, which undergoes a 1,2

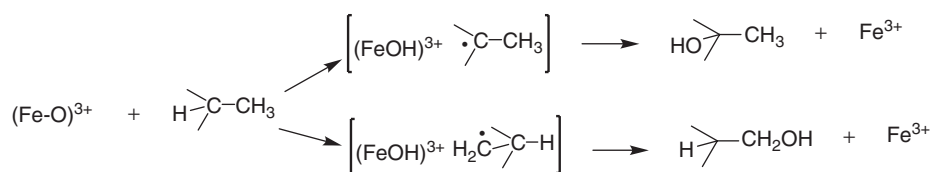


Figure 2.2 Proposed mechanism of aliphatic hydroxylation.

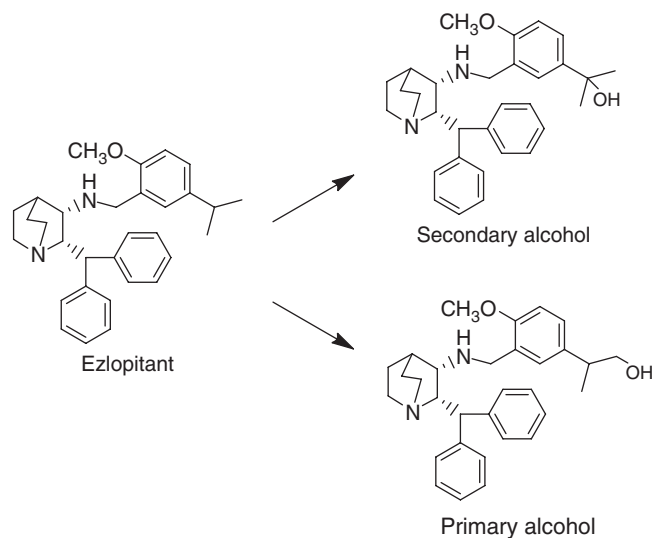
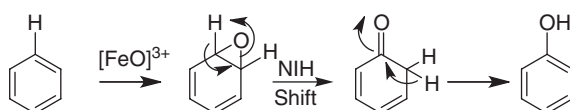
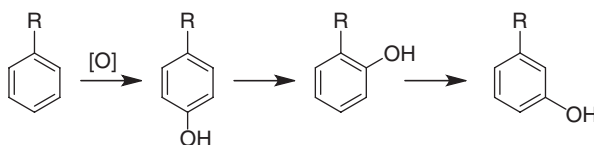


Figure 2.3 Isomeric hydroxylated human metabolites of ezlopitant.

hydrogen shift (NIH shift) and subsequent tautomerization to yield a stable phenol product.

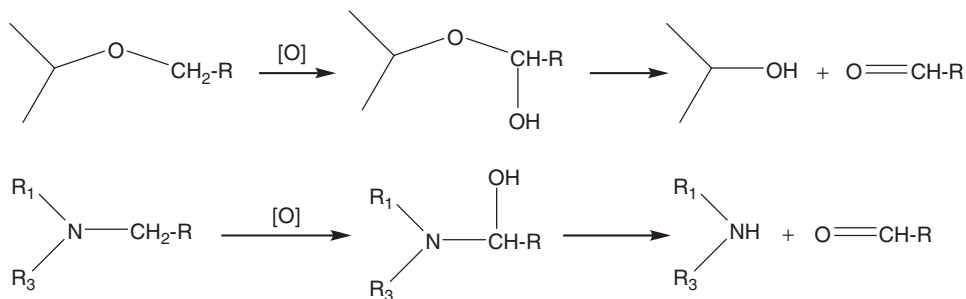


As a result, CYP-mediated aromatic hydroxylation often results in the formation of isomeric hydroxylated products. Owing to resonance stabilization, for monosubstituted phenyl groups, the rate of formation of hydroxylated metabolites is usually: para > ortho > meta.



Oxidation of lasofoxifene is primarily catalyzed by CYP3A4/3A5 and CYP2D6 and leads to formation of isomeric phenols (Fig. 2.4) [14].

2.4.1.3 Hydroxylation at α Carbon to a Hetero Atom (Oxidative O- or N-Dealkylation). Hydroxylation at the α carbon to a hetero atom (O, S, and N) yields an unstable intermediate that decomposes to an aldehyde, and alcohol, thiol, or amine, respectively.



N- and O (S)-dealkylation is a common reaction involving drugs containing a secondary or tertiary amine, alkoxy group, or an alkyl-substituted thiol. For example, ziprasidone, an antipsychotic drug, is metabolized to an aldehyde and benzisothiazole by CYP 3A (Fig. 2.5) [15].

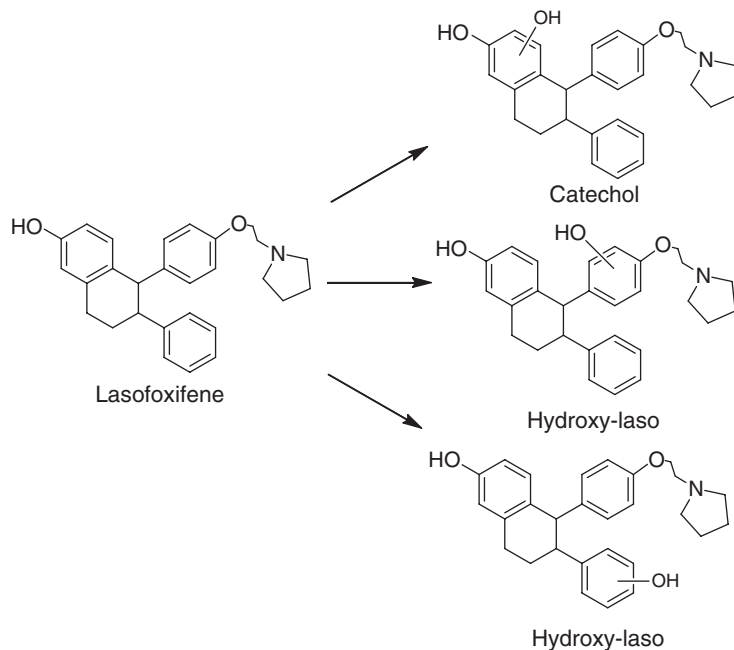


Figure 2.4 Monohydroxylated metabolites of lasofoxifene in humans.

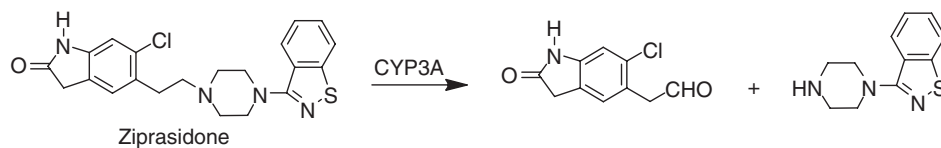


Figure 2.5 N-Dealkylation of ziprasidone.

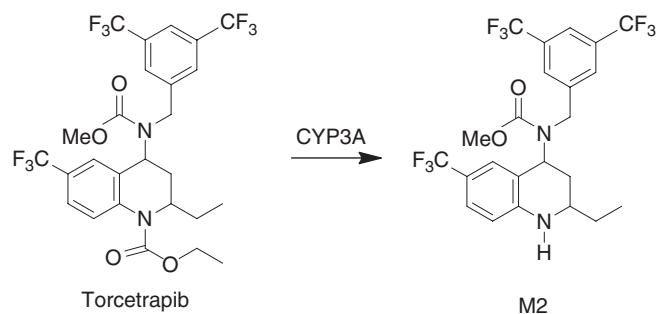
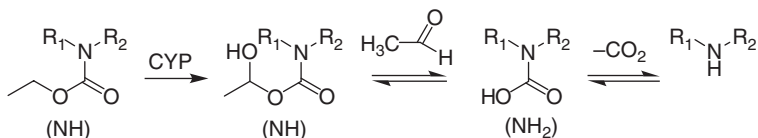


Figure 2.6 Oxidative amide hydrolysis of torcetrapib.

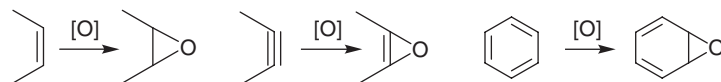
2.4.1.4 Oxidative Ester/Amide Cleavage. Oxidative ester and amide hydrolysis is another common reaction that involves a multistep process: hydroxylation, dissociation of an unstable intermediate, and decarboxylation.



Hydrolysis can also be mediated by non-CYP enzymes, such as hydrolases, through nonoxidative processes. The distinction between the oxidative reaction and nonoxidative hydrolysis is demonstrated by the dependence on NADPH-P450 reductase and NADPH. CYP3A-mediated oxidative hydrolysis of an amide was a major metabolic pathway for the torcetrapib (Fig. 2.6) [16].

2.4.2 Epoxidation

Compounds containing double bonds, triple bonds, and aromatic groups can be subjected to CYP-mediated epoxidation as shown below.



Epoxidation results in the formation of unstable products, which hydrolyze by EHs to form diols or react with nucleophilic groups in macromolecules to initiate toxicological effects. [17]. Epoxides can also be further biotransformed to stable metabolites, as in the case of formation of a carboxylic acid metabolite of erlotinib (Fig. 2.7) [18].

2.4.3 Heteroatom Oxidation

Secondary, tertiary, and aromatic amines are subjected to N-oxidation, which is mediated by a large spectrum of enzymes including CYPs and FMOs.

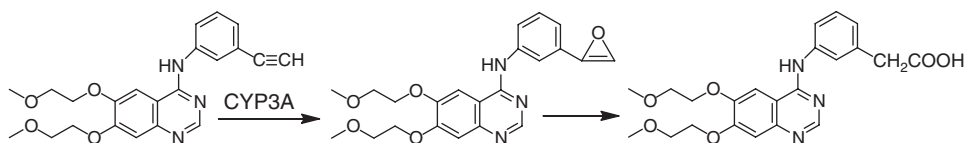


Figure 2.7 Major metabolic pathway of erlotinib in humans.

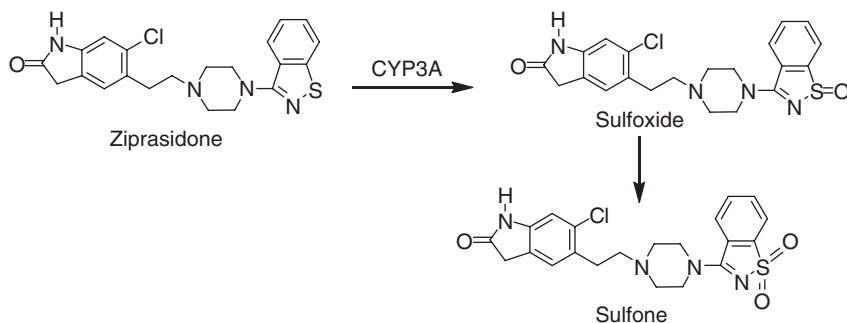
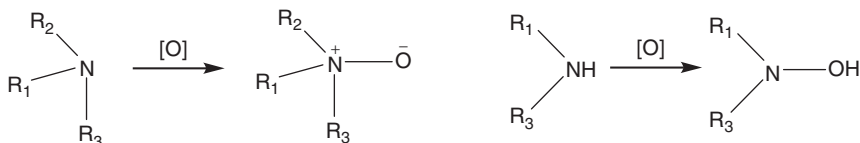


Figure 2.8 CYP3A-catalyzed S-oxidation of ziprasidone.

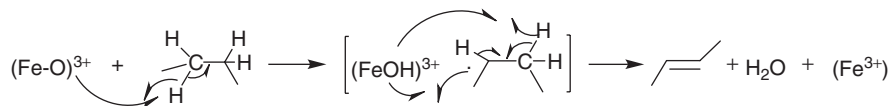
This reaction can result in formation of either the *N*-oxides or the hydroxylamines.



Similarly, thiols are metabolized by S-oxidation to sulfoxide and sulfone as shown for ziprasidone (Fig. 2.8) [15].

2.4.4 Dehydrogenation Reactions

Desaturation often accompanies C-hydroxylation, but there are several examples where the alkene metabolites are known not to be formed from dehydration of the initial alcohol metabolites. Dehydrogenation reactions occur by abstraction of a hydrogen atom by the $(\text{Fe-O})^{3+}$ species to form a carbon-centered radical. Abstraction of another hydrogen atom results in double bond formation as shown below.



We identified an alkene metabolite of ezlopitant, a highly potent and selective NK1 receptor antagonist in humans [13]. It is the result of the desaturation of an isopropyl

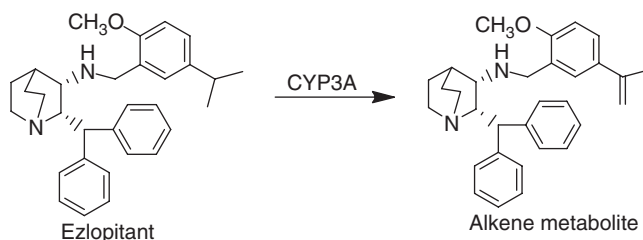


Figure 2.9 CYP3A-catalyzed dehydrogenation of ezlopitant.

group and not from dehydration of the alcohol metabolite. Further, *in vitro* studies using human hepatic microsomes and recombinant human P450 isoforms suggested that the alkene metabolite is formed predominantly by cytochrome P450 CYP3A4/3A5 (Fig. 2.9).

2.5 SPECIES VARIATION IN THE EXPRESSION AND ACTIVITY OF CYP ENZYMES

Animal species at the preclinical stage are often used to predict the pharmacological and toxicological properties, as well as the metabolism of new chemical entities in humans. However, important differences in enzyme expression, selectivity, and catalytic activities of P450s between humans and animals often exist which can limit the direct extrapolation to humans from preclinical species. Therefore, some understanding of the similarities and differences of P450s among species is critical to the identification of relevant drug metabolism and predictive toxicology species. Humans possess 57 CYP genes, mice have 102 genes, while dogs and rats have 54 genes (for details: <http://drnelson.utmem.edu/cytochromeP450.html>). There are relatively small differences in the primary amino acid sequences of P450s across species, although, these small differences can give rise to profound changes in substrate specificity and catalytic activity.

This section focuses on human P450s involved in drug metabolism and their comparison with related P450s in preclinical species (mouse, rat, dog, and monkey).

2.5.1 Human CYP450 Enzymes

The functions of the 57 known human CYP enzymes are summarized into 3 main categories [19]. It has recently been suggested that 15 of the enzymes are mainly involved in the metabolism of clinically used drugs; 27 enzymes are responsible for the metabolism of endogenous compounds such as bile acids, eicosanoids, fatty acids, vitamins, and steroids; and the function of remaining 15 enzymes is unknown. Recent update from Drug Interaction Database of University of Washington (<http://www.druginteractioninfo.org/Query/EnzymeQueries>) indicated that the P450 enzymes from subfamilies CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A are responsible for hepatic metabolism of majority of the marketed drugs. The individual P450 enzymes in these subfamilies involved in the metabolism of drugs are summarized in Table 2.1.

TABLE 2.1 Major P450 Isoforms in Human, Mouse, Rat, Dog, and Monkey

CYP	Human	Mouse	Rat	Dog	Monkey	Typical Substrate and Activity
1A	1A1, 1A2	1a1, 1b2	1A1, 1A2	1A1, 1A2	1A1, 1A2	Phenacetin O-deethylation, caffeine 3-N-demethylation, theophylline-N-demethylation
1B	1B1	1b1	1B1	1B1	1B1	Hydroxylation of 17 β -estradiol benzopyrene
2A	2A6, 2A7, 2A13	2a4, 2a5, 2a12, 2a22	2A1, 2A2, 2A3	2A13, 2A25	2A23, 2A24, 2A26	Coumarin 7-hydroxylation
2B	2B6, 2B7	2b9, 2b10, 2b13, 2b19	2B1, 2B2, 2B3, 2B12, 2B15, 2B31	2B11	2B17	Efavirenz 8-hydroxylation, bupropion hydroxylation
2C	2C8, 2C9, 2C18, 2C19	2c29, 2c37, 2c38, 2c39, 2c40, 2c44, 2c50, 2c54, 2c55	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23	2C21, 2C41	2C20 (2C8), 2C43 (2C9)	Paclitaxel 6 α -hydroxylation (2C8), diclofenac 4'-hydroxylation (2C9), tolbutamide methyl-hydroxylation (2C9), S-warfarin 7-hydroxylation (2C9), S-mephenytoin 4'-hydroxylation (2C19)
2D	2D6, 2D7, 2D8	2d9, 2d10, 2d11, 2d12, 2d13, 2d22, 2d26, 2d34, 2d40	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D15	2D17, 2D19, 2D29, 2D30, 2D42	Bufuralol 1'-hydroxylation, dextromethorphan O-demethylation
2E	2E1	2e1	2E1	2E1	2E1	Chlorzoxazone 6-hydroxylation
3A	3A4, 3A5, 3A7, 3A43	3a11, 3a13, 3a16, 3a25, 3a41, 3a44	3A1 (3A23), 3A2, 3A9, 3A18, 3A62	3A12, 3A26	3A8 (3A4), 3A5, 3A7, 3A43	Midazolam 1-hydroxylation, testosterone 6 β -hydroxylation
4A	4A11, 4A22	4a10, 4a12, 4a14	4A1, 4A2, 4A3, 4A8	4A36, 4A37, 4A38, 4A39	4A11	Lauric acid 12-hydroxylation

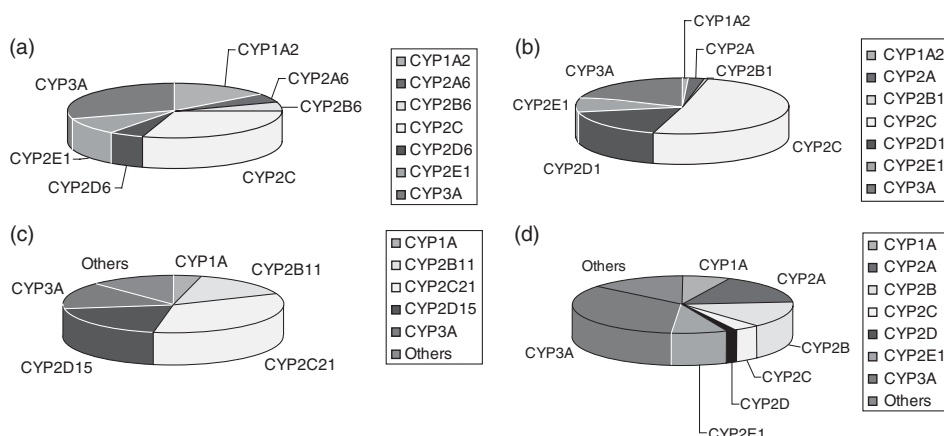


Figure 2.10 Relative abundance of major human (a), rat (b), dog (c), and monkey (d) hepatic P450 isoforms involved in drug metabolism. (See color insert.)

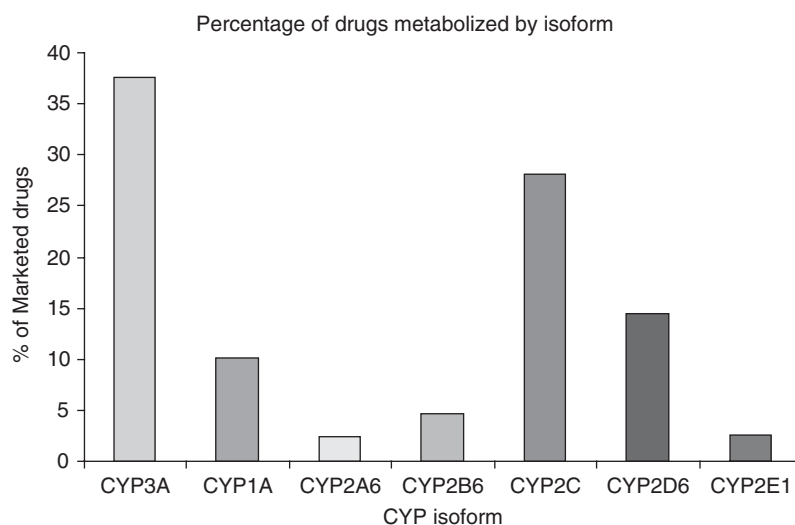


Figure 2.11 Percentage of drugs metabolized by each CYP isoform.

Each P450 enzyme is not evenly expressed in the human liver; indeed, CYP1A1 and CYP1B1 are expressed constitutively at extremely low levels. The relative abundance of major P450 enzymes is listed in Fig. 2.10a. Abundance of an individual P450 does not accurately reflect its contribution in drug metabolism. While CYP2D6 represents only ~5% of CYP enzymes in the liver, but it metabolizes ~12% of the marketed drugs (Fig. 2.11). CYP3A, the most abundant enzyme, constitutes 28% of the human liver and is responsible for metabolism of ~38% of drugs [20]. Recently, the percentage of metabolized drugs for CYP2C has been considerably increased to 28% (Fig. 2.11). The clinically relevant inhibitors and inducers of major human P450 enzymes are listed in Table 2.2.

TABLE 2.2 Examples of Clinically Relevant Inhibitors and Inducers of Major Human CYP450 Enzymes

1A2	2B6	2C19	2C9	2D6	3A
<i>Inhibitors</i>					
Acyclovir	Clopidogrel	Cimetidine	Amiodarone	Amiodarone	Amiodarone
Amiodarone	3-Isopropenyl-	Felbamate	Capecitabine	Bupropion	Azamulin ^a
Cimetidine	3-Methyl	Fluvoxamine	Fluconazole ^a	Chlorpheni-	Bosentan
Ciprofloxacin	diamantine ^a	Isoniazid	Fluoxetine ^a	ramine	Cimetidine
Famotidine	2-Isopropenyl-	Ketoconazole	Fluvastatin	Cimetidine	Diltiazem
Fluvoxamine	2-Methyl	Lansoprazole	Fluvoxamine ^a	Clomipramine	Felbamate
Furaflyline ^a	adamantine ^a	Moclobemide	Metronida-	Diphenhy-	Fluconazole
Levofloxacin	Phencyclidine ^a	Nootkatone ^a	zole	dramine	Grapefruit juice
Mexilitene	Phenylethyl-	Omeprazole	Oxandrolone	Duloxetine	Indinavir
α -Naphtho-	piperidine	Ticlopidine ^a	Paroxetine	Fluoxetine	Itraconazole ^a
flavone ^a	ThioTEPA ^a	Voriconazole	Sulfaphena-	Haloperidol	Ketoconazole ^a
Norfloxacin	Ticlopidine ^a		zole ^a	Indinavir	Macrolide
Propafenone	Voriconazole		Sulfinpyrazone	Methadone	Antibiotics
Verapamil			Voriconazole	Mibefradil	Ritonavir
Zileuton			Tienilic acid	Paroxetine	Roxithromycin
			Zafirlukast	Quinidine ^a	Troleandomycin ^a
				Ritonavir	Verapamil ^a
				Terbinafine	Voriconazole
<i>Inducers</i>					
Carbamazepine	Carbamazepine	Carbamazepine	Phenobarbital	None	Amprenavir
Char-grilled	Efavirenz	Phenobarbital	Nevirapine	identified	Avasimibe
Meat	Nevirapine	Rifampin ^a	Rifampin ^a		Bosentan
3-Methylcho-	Phenobarbital ^a	Efavirenz	St. John's		Carbamazepine
lanthrene ^a	Phenytoin ^a	Ritonavir	Wort		Clotrimazole
β -Naphtho-	Rifampin				Dexamethasone ^a
flavone ^a					Efavirenz
Omeprazole ^a					Etoposide
Lansoprazole ^a					Guggulsterone
					Hyperforin
					Lovastatin
					Mifepristone
					Nevirapine
					Nelfinavir
					Nifedipine
					Omeprazole
					Paclitaxel ^a
					Phenobarbital ^a
					Phenytoin ^a
					Rifabutin
					Rifampin ^a
					Rifapentine ^a

(continued overleaf)

TABLE 2.3 (Continued)

1A2	2B6	2C19	2C9	2D6	3A
					Ritonavir Simvastatin Spironolactone Sulfinpyrazole Topotecan Troglitazone ^a

^aFDA acceptable inhibitor and inducer.

Source: Ref: <http://www.drug-interactions.com> and Chapter 6 of Biotransformation of Xenobiotics [8].

2.5.1.1 CYP1A Family. In this P450 subfamily, all mammalian species possess two conservative and inducible members, namely, CYP1A1 and CYP1A2. CYP1A1 is present predominantly in the extrahepatic tissues such as lung, small intestine, placenta, and kidney, and at a very low level in the liver. CYP1A1 mainly catalyzes activation of polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and heterocyclic amines. For example, the potent carcinogen, benzopyrene is metabolized to an epoxide almost exclusively by CYP1A1. The high variability of CYP1A1 in humans could arise from smoking and diet with high aromatic hydrocarbons such as charcoal-broiled meat and cruciferous vegetables.

In contrast to CYP1A1, CYP1A2 is mainly expressed in the human liver and catalyzes the oxidation of mutagenic and carcinogenic heterocyclic amines. A number of popular drugs, including tacrine, ropinirole, acetaminophen, riluzole, theophylline and caffeine, are metabolized by CYP1A2. CYP1A2 catalyzes the O-dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin [21]. CYP1A2 is also involved in the demethylation of caffeine, which is used as an *in vivo* probe (blood, urine, or saliva) for CYP1A2 activity. CYP1A1 and CYP1A2 both are transcriptionally regulated by aryl hydrocarbon receptor (AhR) [22]. They are inducible not only by food and smoke but also from different drugs such as omeprazole, lansprazole, β -naphthoflavone, and 3-methylcholanthrene [8]. Several studies indicate slightly higher CYP1A2 activity in males compared to females [23]. Its level varies enormously from one individual to another but genetic defects are rare.

There are several clinical relevant inhibitors such as acyclovir, cimetidine, ciprofloxacin, famotidine, fluvoxamine, furafylline, mexilitene, α -naphthoflavone, norfloxacin, propafenone, verapamil, and zileuton for CYP1A enzymes. Coadministration of enoxacin (an inhibitor of CYP1A2) decreases the clearance of warfarin (a CYP1A2 substrate) [24]. Ketoconazole, a well-known 3A4 inhibitor, is also known to inhibit CYP1A1 in humans [25].

2.5.1.2 CYP1B Subfamily. In humans, only one CYP1B1 gene is identified and sequenced by Sutter *et al.* [26]. It is widely distributed and expressed in heart, brain, placenta, lung, liver, kidney and prostate, but in disease conditions, expression levels in tumor cells are much higher compared with normal tissues [27,28]. It is a key enzyme for the estrogen homeostasis, particularly, for the metabolism of 17 β -estradiol, and expressed at high levels in estrogen-related tissues such as mammary, uterus, and ovary [29]. CYP1B1 is also known to participate in the metabolic activation of a

number of procarcinogens, including PAHs, PAH-dihydrodiols, and aromatic amines [30,31]. AhR and AhR nuclear translocator regulate the induction of CYP1B1.

2.5.1.3 CYP2A Subfamily. The CYP2A subfamily includes three members in humans, CYP2A6, 2A7, and 2A13. CYP2A6 constitutes ~4% of the total hepatic P450 in human liver, whereas CYP2A7 and 2A13 are expressed at much lower levels [32]. Both CYP2A6 and 2A13 are active toward many carcinogens and other toxicants. CYP2A6 is mainly involved in O-deethylation of 7-ethoxycoumarin; hydroxylation of coumarin; and oxidation of nicotine, cyclophosphamide, ifosfamide, fadrozole, and aflatoxin [33,34]. Currently, there are nine marketed drugs that are substrates for CYP2A6. Diethyldithiocarbamate [35], methoxsalen, pilocarpine, tranylcypromine, and tryptamine are known to inhibit CYP2A6 in humans. CYP2A6 expression and activity are induced by phenobarbital, rifampicin, dexamethasone, nicotine, and pyrazole. CYP2A13 is predominantly expressed in the human respiratory tract and plays a major role in the activation of aflatoxin B1 [36] as well as in nicotine metabolism [37].

2.5.1.4 CYP2B Subfamily. CYP2B family consists of two members, CYP2B6 and CYP2B7. CYP2B6 is expressed mainly in the liver, whereas CYP2B7 is found in the lung tissue [38]. CYP2B6 is involved in the metabolism of ~5% of the marketed drugs, including bupropion, efavirenz, S-mephenytoin, methoxyflurane, and propofol. CYP2B6 accounts for <1% of total hepatic CYP content in humans. CYP2B6 polymorphism and inductions are known to cause significant interindividual differences in hepatic CYP2B6 expression [39], leading to significant changes in the degree of exposure to a variety of drugs. Several agents, such as ticlopidine, sertraline, clopidogrel, phenylethylpiperidine, 3-isoproprenyl-3-methyl diamantine, and 2-isoproprenyl-2-methyl diamantine, are known to inhibit CYP2B6 in humans. This CYP is inducible by rifampin, phenytoin, phenobarbital, carbamazepine, felbamate, and the herbal preparation St. John's Wort (Table 2.2). Rifampin is known to coinduce CYP2B6 with 3A4 and several CYP2C enzymes by activating constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Recent studies have reported that females express significantly higher amounts of CYP2B6 than males.

2.5.1.5 CYP2C Subfamily. CYP2C subfamily includes four members, CYP2C8, CYP2C9, CYP2C18 and CYP2C19, and all together involved in the metabolism of about 28% of the marketed drugs. They are expressed mainly in the liver and are present at low levels in the small intestine [40]. CYP2C subfamily accounts for 18–25% of the total CYP content in the human liver [41]. CYP2C8 and CYP2C9 are the major enzymes, accounting for ~35% and ~60% of the total human CYP2C, while CYP2C18 and CYP2C19 account only ~4% and 1%, respectively. CYP2C8 is involved in the metabolism of several drugs such as anticancer drugs paclitaxel (Taxol®), amodiaquine, torsemide, and repaglinide and endogenous substrates including retinol and retinoic acid, arachidonic acid (AA) [42]. Specifically, repaglinide was recommended to be a CYP2C8 substrate by the United States Food and Drugs Administration (FDA). Exposure of repaglinide increases from 5.5- to 15-fold in the presence of a CYP2C8 inhibitor, gemfibrozil. CYP2C8 can also metabolize several glucuronide conjugates and this characteristic plays a crucial role in the mechanism by which gemfibrozil

glucuronide inactivates the enzyme. Gemfibrozil glucuronide showed mechanism-based inhibition of CYP2C8 but not gemfibrozil itself [43].

CYP2C9 is responsible for the metabolism of many clinically important drugs including diclofenac, celecoxib (COX 2 inhibitor, celebrex), ibuprofen, flurbiprofen, naproxen, piroxicam, mefenamic acid, glyburide, glipizide, glimepiride and tolbutamide, *S*-warfarin, *S*-acenocoumarol and phenprocoumon, sulfinpyrazone sulfide, torsemide and tienilic acid, candesartan, irbesartan, losartan, and phenytoin. It also metabolizes the endogenous substrates—arachidonic acid, linoleic acid, and serotonin. CYP2C9 is known to form the covalent adducts with the metabolites of tienilic acid, suprofen, and silybin (mechanism-based inhibitor), which in turn inactivates the enzyme [44]. In the presence of CYP2C9, tienilic acid first forms thiophene sulfoxide, which further reacts with water to give 5-hydroxytienilic acid and covalently binds to 2C9 [45]. Detail account of human 2C9 substrates, inducers, inhibitors, and their structure–activity relationship has been recently described by Zhou *et al.* [46].

CYP2C18 is known to play a minor role in drug metabolism. Substrate specificity of CYP2C18 substantially overlaps with other CYP2C enzymes [41]. Tienilic acid derivative with terminal O (CH₂)₃OH functional group is known as *CYP2C18 probe substrate* [47]. CYP2C18 is possibly responsible for the hypersensitivity of phenytoin (commonly used antiepileptic drug) observed in the skin [48]. CYP2C19, the most important member of the CYP2C subfamily, metabolizes 5–10% of prescribed medications with a high degree of stereospecificity. For example, CYP2C19 metabolizes *S*-mephenytoin to hydroxymephenytoin but not the R-enantiomer. CYP2C19 also plays an important role in the metabolism of several proton pump inhibitors including omeprazole and lansprazole. This CYP also preferentially hydroxylates the R-enantiomer of omeprazole and S-enantiomer in case of lansprazole [49]. Many other marketed drugs, including pantoprazole, diazepam, imipramine, and proguanil, are metabolized by CYP2C19. CYP2C19 is highly polymorphic and as many as 20% of Asians are thought to be deficient in CYP2C19 activity.

2.5.1.6 CYP2D Subfamily. CYP2D6 is the only one enzyme of CYP2D family and expressed in various tissues including liver, kidney, placenta, brain, breast, lung and intestine. CYP2D7 and CYP2D8 are inactive pseudogenes [50]. Although CYP2D6 is expressed at a low level in human liver, accounting for about 5% of the total P450 protein, it is, nevertheless, involved in the metabolism of ~12% of the marketed drugs. It is one of the best-studied P450s that exhibits polymorphism at the genomic level. Approximately 7–10% of the Caucasian population shows an inherited deficiency in this enzyme because of the presence of one or several mutant alleles at the CYP2D6 gene locus. The best-known examples of CYP2D6-related polymorphism are sparteine and debrisoquine [51], which are metabolized at different rates among individuals. Depending on the genetic variation, response to the drugs varies. Accordingly, these individuals are categorized into four genotypes, such as poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs). The genetic differences in CYP2D6 are associated with risk for disease like Parkinson's disease, lung cancer, liver cancer, and melanoma [52]. Substrates of CYP2D6 include bufuralolol, dextrometorphane, nortriptyline, propranolol, and several other drugs. Quinidine is a well-known potent selective inhibitor of the CYP2D6.

2.5.1.7 CYP2E Subfamily. CYP2E1 is the only gene of this family and containing ~6% of total human P450 in the liver. It is involved in the metabolism of ~3% of marketed drugs. CYP2E1 is expressed in many tissues including liver, lung, kidney, bone marrow, lymphocytes, and oropharynx (exposed to airborne xenobiotics). It has a dual physiological role both in detoxification and in nutritional support. CYP2E1 was first identified as the microsomal ethanol oxidizing system. It contributes to the metabolism of ethanol and also catalyzes the biotransformation of a large number of halogenated alkane and nitrosamines including aniline, chloroxazone, lauric acid, and 4-nitrophenol [53]. The inducibility of CYP2E1 by ethanol has been reported to occur both in humans and animals. Upregulation of CYP2E1 plays a useful physiological role during starvation/low carbohydrate diet by its capacity to metabolize fatty acid and ability to convert ketone to glucose [54]. Disulfiram and diethyldithiocarbamate are well-known mechanism-based inhibitors of CYP2E1 in humans.

2.5.1.8 CYP3A Subfamily. The CYP3A subfamily is the one of most abundant (~28% of total P450 content) and important drug-metabolizing enzymes. Human CYP3A has been shown to catalyze the metabolism of ~38% of all marketed drugs. In man, CYP3A has four known family members such as CYP3A4, CYP3A5, CYP3A7 and CYP3A43. CYP3A4 is expressed in liver, stomach, lung, intestine, brain, skin, and renal tissues. CYP3A4 expression levels are higher in both liver and small intestine where it metabolizes a large number of therapeutic popular drugs, which includes acetaminophen, codeine, cyclosporine, diazepam, erythromycin, lidocaine, lovastatin, taxol, warfarin, and many more. CYP3A4 has been well studied for its induction or inhibition because of drug–drug, drug–herbal, and drug–food interactions. For example, terfenadine, cisapride, and astemizole (withdrawn 2004) cause ventricular arrhythmias when CYP3A4 inhibitors such as ketoconazole or erythromycin taken along with these drugs. Mibefradil (posicor) is a known mechanism-based inhibitor of CYP3A4. In the small intestine, CYP3A4 plays a major role in the first-pass metabolism (presystemic clearance) of xenobiotics. Catalytic activity of CYP3A4 decreases longitudinally along the small intestine. Generally, the CYP3A4 concentrations in intestine are 10–50% lower than liver [55]. CYP3A4 has large active sites that can interact with two drugs simultaneously [56]. Popular drugs such as testosterone and midazolam both can interact in two distinct sites of CYP3A4 called the *steroid* and *benzodiazepine* sites, respectively. Although both CYP3A4 and CYP3A5 are expressed in liver and intestine, CYP3A5 is the predominant form expressed in extrahepatic tissues. CYP3A5 expression is polymorphic; five allelic variants of CYP3A5 have been reported [57]. There is some ambiguity in reports of the relative rate of drug and steroid metabolism by CYP3A4 and CYP3A5 [58], but an extensive study of recombinant enzymes with 14 compounds showed CYP3A4 was subject to greater inhibition than CYP3A5 [59]. CYP3A7 and CYP3A43 both play a minor role in drug metabolism. CYP3A7 is a fetal enzyme and is involved in the activation of drugs to teratogenic metabolites and CYP3A43 expressed in liver but has very low or restricted activity (0.2–5%) [60].

2.5.1.9 CYP4 Family. CYP4 family consists of 24 subfamilies (CYP4A–CYP4Z), which are expressed in both mammals and insects. This family mainly metabolizes fatty acids and/or eicosanoids. These enzymes oxidize mostly at the terminal methyl group (ω -hydroxylation) and, to a lesser extent, at the methylene group (ω -1 position). For example, CYP4F2 in human and CYP4A2 in rat catalyze hydroxylation of AA

to 20-hydroxyeicosatetraenoic acid (20-HETE), which causes hypertension and subsequently influence renal vascular and tubular functions [61,62]. Humans express 12 members of the CYP4 family, namely, CYP4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, and 4Z1. CYP4A11 is a major hepatic enzyme in humans. It mainly catalyzes the ω - and ω -1-hydroxylation of various fatty acids such as lauric, myristic, and palmitic acid, and it has limited activity toward prostaglandins [63]. CYP4F2 and CYP4F3 were also identified in the human liver. Both have high specificity of ω -hydroxylation of leukotriene B₄. The immunosuppressant drug fingolimod and antiparasitic drug DB289 are predominantly hydroxylated by CYP4F [64,65]. The CYP4F2 in kidney also plays an important physiological role to produce 20-HETE, which effectively increases blood pressure. The elevated urinary 20-HETE and hypertension have been reported in Chinese populations with CYP4F2 variant [61].

2.5.2 Mouse CYP450 Enzymes

With good knowledge of mouse genetics and well-developed techniques for manipulating the genome of this species, the mouse is the most widely used animal model, in either native or genetically modified form, in pharmacology and toxicology and in the areas of pharmacokinetics and pharmacodynamics. This model is more practical and popular for research because of its low body weight, the rapid breeding time, and low maintenance costs compared with larger species. In mouse, ~90 functional CYP genes and ~21 pseudogenes with more than 80 enzymes are expressed and detected (www.drnelson.utm.edu/mouse). The major P450s from each subfamily involved in drug metabolism are listed in Table 2.1. The total P450 level in the liver is quite similar across commonly used mouse strains such as CBA, CD-1, and C57bl/6 [66].

CYP1A1/2 and CYP2E1 are also conserved and expressed in mouse. CYP1A2 is mainly expressed in the liver. The expression level of CYP1A1 in the liver is much lower than that of CYP1A2. Mouse CYP1A2 also specifically catalyzes phenacetin O-deethylation. The higher activity in male mice than female is attributed to relatively high expression level of CYP1A2 in the male mice [66]. Furafullyline selectively inhibits mouse CYP1A2, similar to human CYP1A2. Although mouse CYP2E1 is considered very conservative across species, however, some expression variability among different strains has been observed. The CD1 mouse has the lower level of CYP2E1 than other strains. Within two human CYP2E1 substrates, chlorzoxazone and *p*-nitrophenol, the latter seems to be a more specific substrate for mouse CYP2E1 because mouse CYP3A is also involved in 6-hydroxylation of chlorzoxazone.

In the mouse CYP2A subfamily, four members have been identified, CYP2A2, CYP2A5, CYP2A12, and CYP2A22. CYP2A5 shows the highest similarities to human CYP2A6 and CYP2A13 in amino acid sequence and substrate specificity. Like CYP2A6, it is mainly expressed in liver, kidney, brain, and olfactory mucosa [67]. It exhibits a high catalytic activity on 7-hydroxylation of coumarin and shares substrate pool of human CYP2A6, including testosterone, nicotine, cotinine, and carcinogenic nitrosamines [68]. CYP2A24 has high sequence similarities to CYP2A5 but its activity seems lower than CYP2A5. It is expressed mainly in the liver and dominant in the female mouse. The function of other two members in CYP2A subfamily is still unknown.

In mouse CYP2B subfamily, CYP2B9 and CYP2B10 are two major enzymes. CYP2B9 is dominant in female mice and its levels are negligible in male mice at

the age of about 13 weeks. CYP2B10 is detected in the liver and intestine in both male and females. The specific probe substrate of human CYP2B6 seems not to be as useful for mouse CYP2B enzymes. For example, 7-ethoxy-4-trifluoromethylcoumarin was specifically dealkylated by human CYP2B6, but this dealkylation activity in mouse liver microsomes appears not correlated with CYP2B enzyme level [66]. CYP2B19 is highly expressed in the outer, differentiated cell layers of mouse epidermis and plays an important role controlling epoxyeicosatrienoic acid formation [69]. Mouse CYP2B subfamily is inducible by andrographolide and nonylphenol [70].

Like human and rat, the mouse has the largest number of members in CYP2C subfamily including CYP2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50, 2C54, 2C55, and unpublished enzymes. CYP2C29, 2C37, 2C38, 2C39, 2C44, 2C50, 2C54, and 2C55 are expressed in the liver and CYP2C37 is the most abundant. The common CYP2C substrate tolbutamide is catalyzed by CYP2C29, CYP2C37, CYP2C38, and CYP3C39, but not by CYP2C44. Mouse CYP2C38 and CYP2C39 are two closely related enzymes with 92% amino acid sequence identity but they do not share similar catalytic activity. Mouse CYP2C39 specifically catalyzes 4-hydroxylation of retinoic acid and control this acid level in the liver [71]. Several CYP2C enzymes, including CYP2C50, 2C54, and 2C55, play an important physiological role by oxidizing AA and linoleic acid to regio- and stereo-specific epoxy- or hydroxymetabolites [72]. CYP2C40 is abundant in kidney and intestine. Most mouse CYP2C enzymes, such as CYP2C37 and CYP2C29, are inducible by phenobarbital and phenytoin via CAR. CYP2C44 has the lowest similarity (50–60%) of amino acid sequence to other enzymes and is mainly responsible for the metabolism of AA to 11,12-epoxyeicosatrienoic acids, which increases sodium renal uptake and blood pressure. It is not inducible by phenobarbital.

At least nine mouse CYP2D genes (Table 2.1) have been discovered. The expression level and enzyme function on most of them remains unknown [73]. CYP2D22 is one of the most abundant CYP2D enzymes in the mouse liver [74] and shows 77% identity to amino acid sequence of human CYP2D6, although it appears to have different substrate specificity and inhibition property [75].

In the mouse CYP3A subfamily, six members have been identified (Table 2.1). CYP3A11, CYP3A25, and CYP3A41 are predominantly expressed in the liver, while CYP3A13 is expressed more in the intestine than liver [76]. Among them, CYP3A11 has the closest similarity (75%) to amino acid sequence of human CYP3A4 and catalyzes testosterone 6 β -hydroxylation as well [77]. The female mouse has higher testosterone 6 β -hydroxylation activity than male mouse across most common strains [66] due to higher expression levels of CYP3A41 and 3A44 in female than male mouse [78]. The mouse CYP3A11, not CYP3A13, is inducible by phenytoin and dexamethasone.

CYP4A10, CYP4A12, and CYP4A14 were identified in mouse liver and kidney. They are homologs of rat CYP4A1, 4A8, and 4A2/3, respectively. Like other CYP4A members, they catalyze the oxidation of fatty acid and eicosanoids. CYP4A12, with 75% amino acid sequence identity to human CYP4A11, has the highest 20-HETE synthase activity, which regulates renal blood pressure. The disruption of CYP4A14 gene in mouse results in increase of androgens in plasma and CYP4A12 expression level in the kidney, ultimately causing hypertension [79]. On the other hand, the disruption of CYP4A10 gene in mouse causes increase of CYP2C44 expression or its mediated epoxyeicosatrienoic acids formation, which activates kidney epithelial sodium channel, and ultimately increase sodium reabsorption and blood pressure [80]. CYP4A10

and CYP4A14 were highly inducible in the liver and kidney of both genders by peroxisome proliferator, methylclofenapate. However, CYP4A12 is sexually dimorphic. Methylclofenapate induces the transcription of CYP4A12 in the liver of female mice but reduces the enzyme level in the liver of male mice [81].

2.5.3 Rat CYP450 Enzymes

More than 90 cytochrome P450 enzymes have been identified in rats from P450 genes and pseudogenes (<http://drnelson.utmem.edu/CytochromeP450.html>). Major P450 enzymes involved in the metabolism belongs to same P450 subfamilies as humans. Each rat enzyme is summarized in Table 2.1. The relative abundance of major CYP enzymes in the liver is shown in Fig. 2.10b. The expression level of CYP2C is dominant in adult rat and the contents of CYP2A, CYP2C, and CYP3A are sex dependent. Adult male rats expressed CYP2C11 and CYP3A2 as the major isoforms. However, adult female rats mainly expressed CYP2C12 and did not have CYP2C11 or CYP3A2.

Two isoforms of CYP1A subfamily, CYP1A1 and CYP1A2, are well conserved in mammalian species. Similar to humans, CYP1A2 in rats is mainly located in the liver and the relative expression levels are lower than those of humans. CYP1A1 is almost undetectable in the liver but is dominant in other tissues such as small intestine [82]. Rat CYP1A1 and CYP1A2 shared similar substrates as humans, catalyzing PAHs and carcinogenic heterocyclic amines. Subtle difference in catalytic activity and enzyme inhibition of CYP1A2 exists between human and rat. For example, human CYP1A2 does not show any selectivity for the metabolism of 7-methoxy or 7-ethoxyresorufin, whereas rat CYP1A2 preferentially catalyzes the O-dealkylation of 7-ethoxyresorufin. Both human and rat CYP1A2 enzymes have high catalytic affinity for phenacetin O-deethylation. Furafylline is a potent mechanism-based inhibitor of human CYP1A2, while it shows 40-fold less inhibition for rat CYP1A2 [83]. Rat CYP1A subfamily is also inducible by PAHs, cruciferous vegetables, cigarette smoke, and drugs at variable extent through the Ah receptor. The antiulcer drug, omeprazole, has been reported to specifically induce human hepatic CYP1A enzymes but its inducible effects were not observed in rodents [84,85].

Like CYP1A1, rat CYP1B1 is constitutively expressed mainly in extrahepatic tissues such as lung and adrenal glands. Interestingly, both rat and human CYP1B1 have 80% homology in their amino acid sequences. Both human and animal forms are involved in metabolic activation of PAHs to reactive metabolites, which are associated with mutagenesis and carcinogenesis [30,86]. CYP1B1 is also inducible through Ah receptor.

The rat CYP2A subfamily contains CYP2A1, CYP2A2, and CYP2A3. CYP2A1 is expressed in the liver of male and female rats, and the expression level decreased in mature rats [87]. The expression of rat CYP2A1 is regulated by the growth hormone. CYP2A2 is exclusively expressed in adult male rats [88]. The total CYP2A1/2 contribution is ~2% of the total CYP contents in the liver. Although CYP2A1 and CYP2A2 show 60% amino acid sequence homology to human CYP2A6, they have different substrate specificity. The endogenous steroids are major substrates of rat CYP2A, while human CYP2A6 mainly catalyzes the metabolism of many xenobiotics but not steroids. For example, CYP2A1 and CYP2A2/3 catalyze the 7 α - and 15 α -hydroxylation of testosterone, respectively. CYP2A1 is inducible by phenobarbital and

3-methylcholanthrene, while CYP2A2 is not. Rat CYP2A3 is an extrahepatic enzyme and predominantly expressed in the olfactory mucosa and at relatively low levels in the lung [89]. CYP2A1 and CYP2A2 appear not to metabolize foreign molecules; in contrast, CYP2A3 activates 4-nitrophenol, hexamethylphosphoramide (a nasal procarcinogen), and 2, 6-dichlorobenzonitrile to cause tissue-specific toxicity in the olfactory mucosa of rodents.

The rat CYP2B subfamily contains three isoforms, CYP2B1, CYP2B2, and CYP2B3, and they share ~75% homology with the human CYP2B6. The expression level of CYP2B in the rat was relatively low and contributes to <5% total CYP content in the liver. It is expressed in hepatic and other tissues [90] and mainly involved in the metabolism of foreign molecules. CYP2B1 and CYP2B2 shared 97% amino acid sequence homology with very similar substrate specificities. In most cases, substrates overlap with those of human CYP2B6. For example, testosterone and lidocaine are oxidized by CYP2B1/2 and CYP2B6. CYP2B subfamily activates many compounds such as anticancer drugs, cyclophosphamide, and ifosfamide [91,92] and natural carcinogens such as aflatoxin B₁. The expression of CYP2B is regulated by hormones and glucocorticoids, which may lead to different CYP2B levels between male and female rats [93]. Like CYP2B6, CYP2B1 and CYP2B2 were induced via CAR and PXR.

Like human CYP2C subfamily, multiple CYP2C enzymes, such as CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13, CYP2C22, and CYP2C23, are expressed in rats. This subfamily comprises of about ~50% of the total CYPs in liver [87]. In contrast to humans, the expression of CYP2C enzymes in rats is sex dependent. CYP2C11 and CYP2C13 are expressed in the liver of only male rats while CYP2C12 is expressed only in the liver of mature female rats. CYP2C enzymes are also expressed in extrahepatic tissues such as kidney, brain, and intestine but at lower levels [94–96]. CYP2C6 is not sex specific and expressed in relatively low levels in liver and intestine. CYP2C23 is expressed mainly in rat kidney and plays an important role in the metabolism of eicosatrienoic acid to form 11,12-epoxyeicosatrienoic acid and hydroxy-eicosatrienoic acid, which are involved in the regulation of renal vascular tone and salt excretion [97]. CYP2C enzyme catalyze a variety of compounds including endogenous compounds such as testosterone and foreign molecules such as diclofenac, (-)-verbenone [98], and perfluoro-octanesulfonic acid derivatives [99]. CYP2C6 is inducible by phenobarbital, while other isoforms are not inducible.

Six enzymes (CYP2D1, CYP2D2, CYP2D3, CYP2D4, CYP2D5, and CYP2D18) have been identified from *CYP2D* gene subfamily in the rat. Like human CYP2D6, this subfamily is expressed in various tissues such as liver, kidney, and brain [100]. Among these enzymes, CYP2D1 exhibits close homology of human CYP2D6, catalyzing similar substrates such as bufuralol and debrisoquine. Bufuralol 1'-hydroxylation and 8-hydroxylation of mianserin are catalyzed by most CYP2D enzymes, and CYP2D2 showed the highest affinity to bufuralol 1'-hydroxylation [101,102]. The substrate specificity of other rat CYP2D enzymes remains unknown.

CYP2E1 is a conservative P450 enzyme across species. Rat CYP2E1 shows more than 80% amino acid sequence homology to human CYP2E1 and represents ~10% P450 contents in the liver. Similar to human, rat CYP2E1 is capable of activating nitrosamines and many organic volatile procarcinogens such as benzene, carbon tetrachloride, and chloroform. The rat seems to be the best model for evaluating CYP2E1 activity in the human. It is also inducible by acetone and ethanol.

CYP3A1 (3A23), CYP3A2, CYP3A9, CYP3A18, and CYP3A62 have been identified from *CYP3A* gene subfamily in rats. The expression of these enzymes appears to be sex dependent and regulated by growth hormones in a similar manner as other sexually dependent rat P450s [103,104]. CYP3A1 and CYP3A18 are male dominant while CYP3A9 is female dominant in the liver [105]. Rat CYP3A2 is equally expressed in both male and female rat liver at the young age, but it is expressed exclusively in male rat liver at their adult stage [87]. In contrast to humans, rat *CYP3A* subfamily represents only ~20% P450s in rat liver. CYP3A62 is dominant P450s in the rat intestine. Rat CYP3A1, CYP3A2, CYP3A9, and CYP3A62 show 71–75% similarity in amino acid sequence of human CYP3A4 and CYP3A5 [104]. 6 β -Hydroxylation of testosterone and 1- and 4-hydroxylation of midazolam are common substrates for all CYP3A forms. However, some human CYP3A substrates such as nifedipine are not catalyzed by rat CYP3A [33]. Unlike human CYP3A, rat CYP3A is not inhibited specifically by ketoconazole [83]. The species difference in mechanism-based inhibition of CYP3A has also been reported [106]. Human CYP3A can be induced by both rifampin and dexamethasone, while rat CYP3A was induced by dexamethasone only [84].

The rat CYP4A subfamily has similar catalytic properties as human CYP4A. It oxidizes both endogenous and foreign compounds, including steroids, fatty acids, leukotriene B₄, and AA. Rat CYP4A1–3 enzymes are present mainly in the liver and share 72–96% amino acid sequence similarity with human CYP4A11 [107]. They mainly catalyze ω -1 and ω hydroxylation of fatty acids and the substrate specificity and ω -regioselectivity appear different from human CYP4A [63]. Rat CYP4F1 is expressed predominantly in liver and kidney and, catalyzes hydroxylation of leukotriene B₄ and AA, similar to human CYP4F [108].

2.5.4 Dog CYP450 Enzymes

The dog is another animal species used in pharmacokinetic and toxicology studies during the development of both human and veterinary medicines. It possesses the same subfamily of CYP450 enzymes including CYP1A1/2, 2A13, 2A25, 2B11, 2C21, 2C41, 2D15, 3A12, and 3A26. The expression levels of major CYP enzymes in male dog liver are shown in Fig. 2.10c [109]. Like other species, although dog CYP450 isoforms show a high degree of similarity in amino acid sequence to homologs of human CYPs, their enzyme activity and specificity are not always the same.

CYP1A1/2 is present relatively in low levels (~4%) in the dog liver and has 84% similarity in amino acid sequence. Ethoxyresorufin and phenacetin are good substrates for dog CYP1A1/2, but it does not catalyze other human CYP1A2 substrates such as caffeine, theobromine, and estradiol [110]. Dog CYP1A1/2 is also inducible by PAHs such as β -naphthoflavone and 3-methylcholanthrene [111].

Dog CYP2A13 and CYP2A25 are expressed in the liver while human CYP2A13 is mainly expressed in respiratory tissues, including nasal mucosa, trachea, and lung. Human CYP2A13 catalyzes tobacco-related *N*-nitrosamines efficiently [112] but little is known about the substrate of dog CYP2A subfamily.

Unlike human CYP2B6 or rat CYP2B1, CYP2B11 is predominant in the dog liver and comprises 10–20% of total CYP450 contents. Similar to rat CYP2B1, dog CYP2B11 also catalyzes the hydroxylation of 16 α - and 16 β -testosterone [113]. Dog CYP2B11 is induced by phenobarbital and it also activates anticancer prodrugs,

cyclophosphamide and ifosfamide. Although it shares ~78% similarity in amino acid sequence to human CYP2B6 [114], CYP2B11 has the different substrate specificity. For example, it catalyzes 4'-hydroxylation of mephenytoin and N-demethylation of dextromethorphan, which are mainly catalyzed by human CYP2C19 and CYP3A, respectively. *N*-(α -Methylbenzyl)-1-aminobenzotriazole is identified as a potent mechanism-based inhibitor of CYP2B11 [115].

CYP2C21 and CYP2C41 have been identified in dog liver and both comprise ~20% total P450 contents in liver. They exhibit 67–76% similarity in amino acid sequence to human CYP2C subfamily. 16 α -Hydroxylation of testosterone is catalyzed by CYP2C21 [116]. However, specific human CYP2C9 and CYP2C19 substrates, *S*-warfarin and mephenytoin, respectively, are not catalyzed by dog CYP2Cs; instead, they are catalyzed by dog CYP3A12 and CYP2B11, respectively [117]. CYP2C41 is more homologous to human CYP2Cs than CYP2C21. The unique polymorphism of CYP2C41 in dog liver was observed and this enzyme was found only in 10–16% of the tested dogs, which may provide variability of metabolic clearance in dogs when compounds are metabolized by CYP2C subfamily [118].

CYP2D15 is expressed in dog liver and comprises ~10% of total P450 contents. Its enzymatic activity is similar to human CYP2D6. Two typical human CYP2D6 catalytic reactions are bufuralol 1'-hydroxylation and dextromethorphan O-demethylation, mainly used for monitoring enzymatic activity of dog CYP2D15 [116]. It is also selectively inhibited by quinidine [119]. The cDNA of dog CYP2E1 was first cloned in 2000 [120], and its activity seems to be similar to human CYP2E1.

CYP3A12 and CYP3A26 are two identified dog CYP3A isoforms and comprise ~15% of total P450 contents in the liver. CYP3A12 shares about 80% similarity in amino acid sequence of human CYP3A4, and it selectively catalyzes 6 β -hydroxylation of testosterone. The catalytic activity of CYP3A12 is also inhibited by ketoconazole and troleandomycin. Dog CYP3A12 is reported to catalyze other human CYP subfamily substrates such as diazepam, dextromethorphan, and *S*-warfarin [116,117]. CYP3A26 shares similar substrates as CYP3A12, but its catalytic activity seems to be less than CYP3A12 [121]. The mRNA expression level of CYP3A26 is higher than CYP3A12 in the liver but lower in the duodenum [122]. Both enzymes are inducible probably via PXR but ligands seem have different binding specificity on human and dog PXR [123]. CYP3A12 is inducible by rifampin but not by dexamethasone while both ligands can induce human CYP3A4. Ketoconazole is identified as potent and selective reversible inhibitor of CYP3A12 with K_i at 0.13–0.33 μ M.

2.5.5 Monkey CYP450 Enzymes

The monkey is commonly chosen as a nonhuman primate to conduct pharmacology, toxicology, and pharmacokinetic studies. Cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*) are commonly used. CYP1A, CYP2B, CYP2C, CYP2D, CYP2E1, and CYP3A subfamilies have been characterized in monkeys, similar to humans and rats. The expression levels of major P450 enzymes in untreated monkey are shown in Fig. 2.10d [124,125]. The characteristics of these P450 enzymes described here are mainly obtained from cynomolgus monkeys. Most of these CYP enzymes show a high sequence identity (>93%) to the homologous of human CYPs and metabolize all the typical substrates of the corresponding human CYP subfamily [126].

CYP1A1/2 and CYP2E1 are well conserved and expressed in the monkey. Homologies of these enzymes between human and monkey reached above 94% in amino acid sequences [127]. In contrast to human and rat, CYP1A1 content is higher than CYP1A2 in the monkey liver [128]. Catalytic activities of these enzymes are very similar to those of the human. Monkey CYP1A1, CYP1A2, and CYP2E1 catalyze the 7-ethoxycoumarin O-deethylation, phenacetin O-deethylation, and chlorzoxazone 6-hydroxylation, respectively, similar to other species. Surprisingly, monkey CYP1A2 is not inhibited by furafylline [119].

Monkey CYP2A family contains CYP2A23 and CYP2A24. Both exhibit above 95% similarity in amino acid sequence of human CYP2A6. Both catalyze 7-hydroxylation of coumarin, a typical CYP2A substrate.

CYP2B17 is the only one identified CYP2B enzyme in the monkey. It exhibits >94% similarity in amino acid sequence to human CYP2B6 and similar catalytic activity to pentoxyresorufin O-dealkylation [126].

The monkey CYP2C subfamily comprised of four members, CYP2C20, CYP2C43, CYP2C75, and CYP2C76. CYP2C20 has a high sequence identity and enzymatic activity to human CYP2C8. It shows 92% identity of amino acid sequence of CYP2C8 and catalyzes the same substrate, paclitaxel. CYP2C43 has both 93% and 91% sequence identity to human CYP2C9 and CYP2C19, respectively. CYP2C43 catalyzes 21-hydroxylation of progesterone and 4'-hydroxylation of *S*-mephenytoin, similar to human CYP2C19, but it does not catalyze the human CYP2C9 substrate, tolbutamide. Therefore, CYP2C43 appears functionally related to human CYP2C19. CYP2C75 has 92–93% sequence identity to human CYP2C9 and CYP2C19. In contrast to other CYP2C isoforms, monkey CYP2C76 exhibits only ~70% similarity of amino acid sequence of other human and monkey isoforms [129]. It also catalyzes the tolbutamide 4-hydroxylation and testosterone 2 α -hydroxylation but at a relatively lower rate compared to CYP2C75.

The monkey CYP2D subfamily is comprised of five enzymes but each enzyme is expressed in different strain. CYP2D17 is expressed in the liver of cynomolgus monkey and has 93% identity of amino acid sequence to human CYP2D6. It catalyzes 1'-hydroxylation of bufuralol at higher activity than human CYP2D6 [126]. CYP2D19 and CYP2D30 were identified in the liver of different female marmosets, and they showed homologies of 93% in their amino acid sequence. CYP2D30 exhibits selective 4-hydroxylation of debrisoquine and stereoselective 1'(S)-hydroxylation of bufuralol, similar to human CYP2D6. In contrast, CYP2D19 prefers catalyzing 5,6,7,8-hydroxylation of debrisoquine and 1'(R)-hydroxylation of bufuralol [130]. Whether both isoforms exist in the same marmoset liver remains unknown. CYP2D29 was found in Japanese monkeys (*Macaca fuscata*) and has 96% identity of amino acid sequence to human CYP2D6. The variant CYP2D42 was detected in the rhesus monkey.

CYP3A8 is a major enzyme of monkey CYP3A subfamily. It has 93% identity of amino acid sequence to human CYP3A4 and represents ~20% of total P450 contents in the liver. Like CYP3A4, it catalyzes 6 β -hydroxylation of testosterone, 1'-hydroxylation of midazolam, and N-demethylation of erythromycin. Since total P450 content per milligram liver microsomes protein ratio is higher in monkeys than in humans, CYP3A8 has four to five times higher catalytic activity than CYP3A4. Other enzymes of the human 3A subfamily, namely, CYP3A5, 3A7, and CYP3A43 were also reported in the monkey [131].

CYP4A11, CYP4F2, CYP4F3v2, and CYP4F11/12 are also detected in liver, jejunum, and kidney of monkeys. Their cDNAs are highly identical to human homology and assumed to have similar functions.

2.6 ETHNIC VARIABILITY IN EXPRESSION AND ACTIVITY OF CYTOCHROME P450 ENZYMES

As discussed earlier, one of the major factors influencing a patient's response to any medication is drug metabolism. The metabolism of a drug is governed primarily by the P450s and many of these enzymes show genetic polymorphism, which can affect the levels of their expression and catalytic properties. Therefore, ethnic variations and polymorphisms in P450 enzymes have been used to predict drug metabolism/toxicity, efficacy, and selection of doses. Optimization of genetic basis for the difference in drug responses is crucial to address the ethnic variation in P450 activity.

There are wide varieties of drugs that have shown a good correlation between decreased drug clearance/decreased activity of metabolizing enzymes and their adverse reactions after normal doses. It is now well known that genetic variation provides the molecular basis of variability in drug-metabolizing enzymes mostly 2C9, 2C19, 2D6, and 3A. There are at least three categories in genetic variation (i) UMs, which metabolize substrate so quickly because of gene duplication. This is so effective in maintaining high drug clearance, that therapeutic levels often cannot be reached in clinical practice in these individuals; (ii) PMs, these inherit genetic material that is devoid of effective enzyme expression, which results in a lack of metabolism of CYP substrates; and (iii) IMs, these are heterozygotes with partial expression of appropriate CYPs, which result in intermediate drug clearance. The most severe side effects are often noticed with IMs and PMs, and substrates that require metabolic activation may demonstrate suboptimal clinical efficacy.

CYP2D6 polymorphism is of the major concern as many of the antipsychotic drugs possess narrow therapeutic indices and are primarily metabolized by CYP2D6. Approximately 7–10% of Caucasians, 0–5% Africans, and 0–1% Asians lack CYP2D6 activity because of the presence of one or several mutant alleles at the *CYP2D6* gene locus, and these individuals are known as *PMs*. This results in a poor response to codeine therapy, for example, because of their low formation of the active metabolite, morphine, catalyzed by CYP2D6. Compared with IMs or UMs, PMs demonstrate markedly greater AUC values for parent drugs that are metabolized by CYP2D6, and therefore require lower doses to achieve therapeutic effects. Codeine metabolism is considerably lower in Chinese than in Caucasian individuals [132]. Another study showed that codeine's clearance via O-demethylation is significantly higher in white Americans than in Chinese. Indeed, with coadministration of a CYP2D6 inhibitor, quinidine, the production of morphine is markedly greater in Caucasians than in the Chinese population [133].

Other polymorphic P450 enzymes are CYP2C9 and CYP2C19. Approximately 20% of Asians and 3% Caucasians are PMs of CYP2C19 [134,135]. The patients carrying allelic variants CYP2C9*6 or CYP2C19*2 are shown to be susceptible to neutropenia in the treatment of anticancer drug indinavir, which is metabolized primarily by CYP2C9 and 2C19. Caucasian patients require higher warfarin (metabolized by CYP2C9) doses than Asians to attain a comparable anticoagulant effect. Conversely, Chinese patients

need 50% lower average doses than the Caucasians patients. The average maintenance dose for Japanese patients is also much lower than for US patients [133]. Pharmacokinetic studies of diazepam, a substrate of CYP2C19, showed higher clearance for Caucasians relative to Asians, while levels of desmethyldiazepam, the major active metabolite higher in Asians [136]. Clopidogrel, an antiplatelet prodrug, is activated by CYP2C19. CYP2C19 PMs are at high risk of treatment failure with clopidogrel [137]. There are tremendous variations in responsiveness to metabolic activation of clopidogrel attributed from genetic differences. The FDA has shown concern (a black box warning) about clopidogrel's efficacy in patients who are PMs of CYP2C19, which may influence estimated 2–14% American and almost 50% of Asian population.

Several drugs have shown the ethnic variability in drug response, and there is no clear FDA guidance requiring pharmaceutical companies to conduct clinical studies in different population. Personalized drug therapy based on genetic information will improve drug efficacy and reduce adverse drug reactions. Currently, therapeutic drug monitoring approach is focused on the pharmacogenetic information of patient to optimize dose regimens in a noninvasive manner before drug administration. Kim *et al.* [138] have recently reported a detailed study using a decision matrix, which included ethnic frequency of clinically relevant polymorphic P450 enzymes, metabolic profiles, and adverse drug reactions. This is termed as the *pharmacogenetic drug monitoring (PDM) system*. The system facilitated the identification of 17 drugs for which PDM provides the greatest potential benefit at one Korean Hospital. The disadvantage of the system particularly for pharmaceutical company is that it will be more time consuming and expensive to develop drugs and doses depending on the ethnic variability. It has been proposed to use genetic test of CYP2C19 to choose right patients of clopidogrel and provide alternative treatment for PM patients of CYP2C19. However, the long genetic test time (normally three days) prevents its practical use from the acute coronary syndrome treatment drugs such as clopidogrel, which is required immediately for patients. The alternative druglike ticagrelor does not have genetic polymorphism issue and could be favored by patient if genetic tests are required for clopidogrel.

2.7 TOOLS FOR *IN VITRO*–*IN VIVO* EXTRAPOLATION

Since the significant difference exists in the enzyme expression and catalytic activities of P450 between humans and animals, it brings challenges to understand if drug-metabolism-associated toxicities in preclinical species are relevant to humans and the safety assessment of human metabolites is properly covered in preclinical species used for safety assessment of drug candidates. Many valuable tools have been developed to extrapolate metabolism and toxicity in animals to humans. *In vitro* system, human liver microsomes, recombinant human P450 enzymes, human hepatoma-derived cell lines (HepG2), and cultured human hepatocytes are routinely used in preclinical studies to determine metabolic profiles, potential drug–drug interactions, induction/inhibition, polymorphic metabolism, and P450-mediated genotoxicity of new drug candidates. *in vivo*, genetically modified mice have also been used for assessing the drug-metabolizing enzymes and drug-induced toxicity. This section briefly describes the application of each system and their limitations.

Human liver microsomes and recombinant human CYP isoforms expressed in baculovirus-insect cells are widely used to conduct inhibition studies and phenotyping

of P450s. These assays are relatively easy to handle, offer high throughput capability, and are widely used for the mechanistic studies on P450 catalytic activities. However, these assays have limitations, because the integral cell environment is disrupted and the result may not reflect the real function of P450s *in vivo*.

Primary culture of human hepatocytes usually cultivates cells between two layers of collagen (sandwich culture); therefore, both biological functions and morphology of liver are preserved. Thus, primary culture of human hepatocytes is currently considered the closest *in vitro* model to predict functions of P450s and other drug-metabolizing enzymes *in vivo*. It has been used for metabolic stability, drug induction studies, and gene-expression-based hepatotoxicity of new drug candidates [139]. However, the donors of primary human hepatocytes are unavoidably variable because of intrinsic individual differences; therefore, the expression levels and enzymatic activities of P450s could be different from study to study. Meanwhile, hepatic functions decline within few hours under conventional culture conditions [140]. The liver slices have the closest resemblance to *in vivo* cytoarchitecture and their functionality is also close to the situation *in vivo*. However, its short life time (~ 1 day), individual variability, and erratic supply of human liver tissue limit its applications.

Recently, a microscale culture system has been developed for human liver cells. This micropatterned system maintained hepatocytes morphology and functions for several weeks [141]. Thus, this system may provide more reliable information on metabolic profiles, P450 induction, and hepatic toxicity of new drug candidates.

Human hepatoma-derived cell lines such as HepG2 cells are also frequently used as *in vitro* models for studying the metabolism and toxicity of drugs. The cells retain many liver-specific functions and have unlimited lifespan. However, they do contain very low levels of all major drug-metabolizing enzymes [142,143]. Thus, it is usually necessary to insert recombinant human P450 isoforms into the HepG2 cells in order for the catalytic activity and metabolic bioactivation of each human P450 to be studied. The international working group on genotoxicity testing recommended that the use of genetically engineered hepatoma cell lines stably expressing P450 enzymes is an optimized system because the P450s in traditional induced rat liver S9 did not resemble human P450s. This transfected HepG2 cell system could also identify the P450 isoform contributing the genotoxicity. The expression of single or multiple P450s at well-controlled level via adenovirus system suggests that transfected HepG2 cells could be used for P450 phenotyping, induction, and genotoxicity studies [144].

The recent genetically engineered mouse models, including transgenic and humanized models, provide potential to evaluate the role of human P450 enzymes and their relevance to toxicity *in vivo* [145]. In most cases, the transgenic models express human P450 enzymes on a wild-type mouse background; the knockout mice models often deactivate individual P450 in host mouse; and humanized models express human P450 enzymes in the absence of the host (mouse) P450 orthologs. For example, species difference in the acetaminophen-induced hepatotoxicity has been successfully demonstrated by using wild-type, *Cyp2e1*^(-/-), and humanized CYP2E1 mice [146]. At the dose of 400 mg/kg acetaminophen, all wild-type mice exhibited severe hepatic necrosis, and 9 out of 10 humanized CYP2E1 mice showed moderate centrilobular hepatotoxicity; however, the *Cyp2e1*^(-/-) mice had no detectable histological hepatic necrosis. These results suggest that CYP2E1 bioactivation was responsible for hepatotoxicity of acetaminophen, and human CYP2E1 appears less active than *Cyp2e1* in the induction of hepatotoxicity. So far six humanized mice models, which expresses individual

human CYP1A1, CYP1A2, CYP2E1, CYP2D6, CYP3A4, and CYP3A7 enzymes, have been developed [147]. Although these models mimic P450-mediated drug metabolism in humans, it is still difficult to quantitatively extrapolate metabolic information to humans because the expressed human P450 enzyme level, enzymes distribution in mice tissues, and physiology in humanized mice (such as hepatic blood flow) are still significantly different from those of humans.

2.8 SUMMARY AND FUTURE PERSPECTIVES

Cytochrome P450 enzymes play an important role in the metabolism of foreign molecules (drugs and environmental chemicals) and endogenous compounds. This large enzyme family with broad substrate diversity facilitates to eliminate xenobiotics from body and catalyze catabolism of endogenous compounds for their physiological functions. Over the past 20 years, our understanding of genes, structures, subfamily diversities, functions, and regulations of human P450s is considerably advanced and 30 crystal structures of 8 cytochrome P450s have been published [148]. This has resulted in much useful published literature and FDA guidance on P450 inhibition and its impact on drug–drug interactions. The accumulated knowledge of P450s has significantly improved the contribution of drug metabolism studies to the drug discovery and development.

Meanwhile, many questions and challenges on P450s and other drug metabolism enzymes still exist. The overwhelming substrate diversity of cytochrome P450s seems to arise from their intrinsic physiological roles to eliminate foreign molecules from body. Thus, their substrate specificity and inhibition selectivity are still not predictable, despite the availability of the crystal structures of these enzymes. The large scale screening of P450 substrates and inhibitors experimentally is, however, routine practice during the drug discovery. While the existence of P450 differences between human and animals is well recognized, how to extrapolate drug metabolism and associated toxicities in preclinical species to humans remains an important question in the pharmaceutical industry. The better understanding of involvement of P450s in rodent carcinogenicity studies on new chemical entities may improve prediction of human relevance.

The metabolic activities of human P450s *in vitro* have been well established, and their catalytic activity on new chemical entities and metabolite formation has been qualitatively predicted well *in vivo*. But quantitative predictions are still inaccurate, probably because of limitations in our knowledge of the interplay of P450s with other conjugating enzymes and transporters. The humanized animal models and living culture systems will have the potential to integrate drug metabolism enzymes and transporter interactions and differentiate species differences.

Knowledge of polymorphism of major human P450 has grown considerably in recent years. The genotype, age, sex, race, and environmental and personal lifestyle all influence P450's expression and function. Indeed, remarkable progress has been made in the understanding of on genetic polymorphism of P450s, such as CYP2D6 and CYP2C19. Personalized drug therapy based on P450 expression variation in patients is attempted to optimize dose regimen and subsequently improve drug efficacy [138]. However, there is still much to learn for the pharmaceutical industry in the design and

safe marketing of drugs of certain drugs in individual human populations, as well as the wider issue of personalized drug therapy.

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3 DDI Risk Assessment and Evaluation in Pharmaceutical Development: Interfacing Drug Metabolism and Clinical Pharmacology

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3.1	Summary	1
3.2	Introduction and medical importance	2
3.3	Framework for DDI risk assessment and evaluation in drug development	4
3.4	NME as precipitant: DDI risk assessment for CYP inhibition	5
3.5	NME as Precipitant: Mechanism-based CYP inactivation DDI	13
3.6	NME as Precipitant: CYP induction DDI	20
3.7	NME as precipitant: clinical pharmacologic evaluation	27
3.8	NME as Object of DDI	32
3.9	Physiologically based pharmacokinetic modeling and simulation of DDIs	39
3.10	Design, Analysis and Interpretation of Clinical DDI Study Results	41
3.11	Examples illustrating translation of DDI knowledge to prescribing guidance	48
3.12	Concluding remarks	51
	Acknowledgment	53
	References	53

3.1 SUMMARY

Scientifically guided risk assessment and clinical pharmacologic evaluation of pharmacokinetic (PK) drug–drug interactions (DDI) are key components of contemporary drug development and play an integral role in the overall benefit/risk evaluation of new molecular entities (NMEs). DDI risk assessment and clinical evaluation should consider the potential for the NME to alter the PK of coadministered drugs (NME as the precipitant) and also the potential for coadministered drugs to alter NME PK (NME as the object). Risk assessment begins with *in vitro* and mechanistic studies of the NME's potential to alter drug disposition via inhibition or induction of drug-metabolizing enzymes and transport proteins, and additionally, studies that elucidate

the clearance mechanisms of the NME itself. This is followed by a mechanistic and quantitative translation of *in vitro* parameters to estimate clinical PK consequences of a potential DDI (i.e., expected increase in object drug exposure). When the risk for DDI cannot be definitively dismissed as low based on translational assessment from the results of *in vitro* studies, controlled clinical DDI studies are designed to evaluate the clinical pharmacologic correlates of the *in vitro* data. Finally, the results of clinical DDI studies will need to be interpreted in the context of knowledge of the therapeutic index of the object drug, gained from exposure–response understanding for both safety and efficacy endpoints. This chapter provides a technical overview and strategic perspectives on each of these aspects of DDI risk assessment and clinical evaluation, with the use of examples that illustrate applications in drug development and the translation of scientific knowledge to prescribing guidance, in the context of currently available regulatory guidance and published scientific opinion.

3.2 INTRODUCTION AND MEDICAL IMPORTANCE

DDI represent an important mechanism of adverse drug reactions making their evaluation a key element of contemporary drug discovery, development, and regulation, given their potential impact as an integral component of clinical therapeutics. DDI profiling and risk forecasting when incorporated into the overall framework of multidimensional optimization can help identify candidates with likely low clinical DDI risk in the discovery stage and subsequently enable development of rational clinical pharmacologic DDI evaluation strategies to manage risk in clinical drug development. Finally, the ultimate goal of nonclinical and clinical DDI studies is to permit integration of DDI knowledge acquired in the development phase into prescribing guidance in a manner that enables optimal postmarketing risk management following marketing authorization.

A clinically significant DDI occurs when the therapeutic or toxic effects of a medication are altered by administration with another drug. DDIs are broadly classified into two categories: (i) PK interactions wherein one drug alters the absorption, distribution, metabolism, or elimination of another coadministered drug resulting in altered blood/target organ levels and potential effects on efficacy and/or safety; and (ii) pharmacodynamic (PD) interactions wherein one drug alters the pharmacologic effect (efficacy and/or safety) of another coadministered drug without affecting its PK. This chapter discusses DDIs resulting from PK mechanisms, with a focus on metabolic interactions and approaches for risk assessment and clinical pharmacologic evaluation of such interactions in contemporary drug development.

The medical importance of DDIs, and, in particular, the need for scientifically guided premarketing risk evaluation can be readily appreciated from the results of a review of drugs withdrawn from the US market from 1998 through 2003 because of their involvement in major clinical DDIs with commonly coadministered agents [1]. A notable example is the calcium channel blocker mibefradil that was used in the medical management of hypertension and angina. Mibefradil, a strong mechanism-based inactivator of cytochrome P450 3A (CYP3A) and an inhibitor of the efflux transporter P-glycoprotein (P-gp) produced dangerous and occasionally fatal interactions with sensitive substrates of CYP3A, such as the calcium channel blocker felodipine. This led to

the United States Prescribing Information (USPI) for mibefradil to be revised to recommend the contraindication of concomitant use of 26 drugs spanning multiple therapeutic areas. Mibefradil was voluntarily withdrawn in June 1998 by the manufacturer within a year following approval because of recognition of these serious interactions produced by this drug and the complexity of prescribing information needed to administer the drug safely. Additionally, considering that other pharmacotherapeutic options without similar serious DDI risks were available for the medical management of the conditions for which the use of mibefradil was indicated, the overall benefit/risk ratio for mibefradil was unfavorable. This example illustrates the importance of early assessment of risk for NMEs to produce DDIs with coadministered agents via effects on their PK (e.g., via metabolic inhibition by the NME). Other notable examples of drug withdrawals due to unacceptable DDI liabilities include the nonsedating antihistamine drugs, terfenadine and astemizole, and the prokinetic agent cisapride that was used for the management of gastroesophageal reflux disease. All three drugs were associated with two common properties. These drugs were inhibitors of the ion channel hERG, which plays a critical role in cardiac repolarization, and were additionally cleared almost exclusively via metabolism by CYP3A such that large and clinically significant increases in their exposure could occur on coadministration of inhibitors of this enzyme. This combination of an unfavorable off-target pharmacologic effect associated with proarrhythmic risk and exquisite sensitivity to metabolic inhibition resulted in major clinically deleterious interactions. The clinical consequence was an increased risk for the fatal cardiac arrhythmia *torsades de pointes* when coadministered with many commonly administered therapeutics, including antibiotics such as erythromycin and consumer products such as grapefruit juice. This set of examples illustrates the importance of an adequate level of premarketing characterization of DDI risks in the context of the NMEs safety profile and therapeutic index.

Although CYP enzyme-based DDIs have received the greatest amount of attention, it should be noted that DDIs at the level of non-CYP enzymes can also be clinically significant. An example of a serious DDI involving inhibition of a non-CYP enzyme that has resulted in fatal outcomes is the case of interactions between the antiviral drug sorivudine and the cytotoxic anticancer agent 5-fluorouracil (5-FU) or 5-FU prodrugs that are metabolized by the enzyme dihydropyrimidine dehydrogenase (DPD). This interaction is explained by mechanism-based inactivation (MBI) of DPD by a metabolite of sorivudine resulting in an essentially irreversible and long-lasting impairment of DPD activity and 5-FU clearance [2–4]. Another example of clinically significant DDIs resulting from inhibition on non-CYP enzymes is the interaction between the xanthine oxidase (XO) inhibitor allopurinol and 6-mercaptopurine (6-MP) or its imidazolyl derivative, azathioprine. Inhibition of XO-mediated metabolism of 6-MP by allopurinol has been shown to result in a fivefold increase in systemic exposure of orally administered 6-MP [5], necessitating a reduction in the dose of 6-MP or azathioprine in patients requiring coadministration with allopurinol [6].

As a consequence of serious medically significant DDI reports, as exemplified by the above discussed examples, risk assessment and clinical management of DDIs has gained importance across the sectors of medical practice, pharmaceutical research and development, and drug regulation. This is especially important considering the increase in pharmacotherapy options for patients and the emergence of polypharmacy, especially in certain therapeutic areas such as oncology, HIV medicine, and in the geriatric population [7]. Finally, the identification of clinically important interactions with food

products such as grapefruit juice, and importantly herbal products, is also a serious issue. This is partly because they are unfortunately often perceived as innocuous substances without risks. These factors have highlighted DDI awareness and magnified its importance as a public health issue [1,8]. In the face of these drivers of awareness and recognition of the importance of DDIs, there have been significant scientific advances over the last two decades, resulting in critical enablers that now permit a rational and mechanistic approach to drug interaction risk assessment and clinical pharmacologic evaluation. These enablers include: (i) biological and chemical reagents and *in vitro* technology platforms to study drug metabolism and DDIs, made possible by molecular cloning of the human drug-metabolizing enzymes and transporters; and (ii) advances in the science of quantitative *in vitro*–*in vivo* extrapolation (IVIVE) through modeling and simulation to forecast clinical pharmacologic correlates and optimize designs of clinical DDI studies from the results of nonclinical studies. Finally and importantly, advances in quantitative clinical pharmacology have enabled exposure–response understanding of the efficacy and safety profiles of drug candidates during the course of their clinical development. Such exposure–response characterization is necessary for the quantitative and objective estimation of therapeutic index, which is critically important to assess the clinical significance of drug interactions and contribute to prescribing guidance [1].

3.3 FRAMEWORK FOR DDI RISK ASSESSMENT AND EVALUATION IN DRUG DEVELOPMENT

A simple conceptual framework for DDI risk assessment and clinical evaluation in drug development is illustrated in Fig. 3.1. For representative purposes, interactions affecting clearance processes are illustrated in this figure, although this framework is equally applicable for interactions resulting from modulation of drug absorption. As shown in this framework, there are two broad categories of DDIs that require consideration as part of drug development. First, it is important to assess the risk for clinically meaningful alterations in exposure of an NME when it is coadministered with other (marketed) agents that may alter its PK (i.e., NME as the *substrate* drug and the *object* or *victim* of the interaction). A second assessment is of the risk for the NME to produce clinically significant alterations in the PK of concomitant agents, especially those with a narrow therapeutic range (i.e., NME as the *precipitant* or *perpetrator* agent because of its ability to act as a clinically meaningful *inhibitor* or *inducer*). The following sections of this chapter discuss the scientific considerations, experimental methodologies, and strategies employed in answering each of these questions and ultimately integrating the knowledge gained from these assessments into the overall clinical pharmacologic characterization of an NME to inform prescribing guidance. While the focus of this chapter is on metabolic DDIs, it is important to note that transporter-mediated DDIs also represent an important emerging area, and there have been many recent examples of clinically important interactions resulting from modulation of absorption as well as clearance processes via effects of coadministered agents on drug transporter expression and/or activity. A detailed discussion of experimental approaches and strategies for transporter-based DDI risk assessment, and their clinical evaluation is not within the scope of this chapter and the reader is referred to a recent review for current opinion on this topic [9]. Finally, although the focus of this chapter is on DDI risk assessment

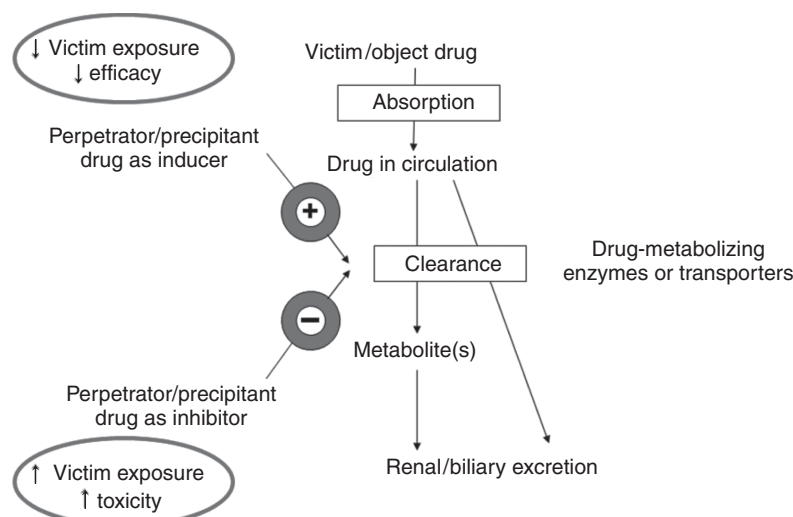


Figure 3.1 Conceptual framework for DDI Risk assessment and evaluation in drug development, considering the NME as both the victim or object drug and as the perpetrator or precipitant drug.

and evaluation for small molecule drugs, it should be noted that the topic of PK DDIs with biotherapeutics (e.g., therapeutic antibodies) is an emerging area of science. There have been many recent reports of DDIs between therapeutic antibodies and small molecules resulting, for instance, from indirect immunomodulatory mechanisms that can alter the expression and activity of drug-metabolizing enzymes. For an overview of this emerging area and current expert opinion, the reader is referred to recent review articles [10–13].

3.4 NME AS PRECIPITANT: DDI RISK ASSESSMENT FOR CYP INHIBITION

The assessment of CYP-inhibitory DDI potential of an NME in drug development is a two-step process: (i) *In vitro* estimation of inhibitory potency; and (ii) Translation of *in vitro* inhibitory potency to project potential clinical pharmacologic correlates (i.e., drug interaction magnitude).

3.4.1 *In vitro* CYP inhibition Studies

The *in vitro* experimental models, methodology, and associated data analytic methods for the assessment of the potential for an NME to produce inhibition of the activity of the major drug-metabolizing human CYP isoforms have been developed and refined over the last two decades and are now well established to permit their routine integration into drug discovery and development [14–19]. The most commonly applied approach involves measuring the effect of increasing concentrations of the NME on the metabolic rate in pooled human liver microsomes, of selective index reactions that are

representative of the activities of individual drug-metabolizing CYP isoforms, to estimate IC_{50} values as measures of inhibitory potency. Clinically important CYP isoforms from the standpoint of human drug metabolism and DDIs include CYPs 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4/5 (referred to as 3A). Examples of commonly utilized index reactions for each of these enzymes have been published [15,16,19]. These studies are typically conducted at a substrate concentration equal to the K_m of the index reaction, such that the apparent K_i can be approximated as $0.5 \times IC_{50}$ under the conservative assumption of competitive inhibition. For enzymes that are inhibited sufficiently to enable IC_{50} estimation, a more complete enzyme-kinetic analysis of inhibitory mechanism for definitive estimation of the apparent K_i can be performed following the IC_{50} analysis. Such an experiment will provide definitive information on the kinetic mechanism of inhibition and the associated inhibition constant [20], thereby minimizing the impact of assuming competitive inhibition in subsequent *IVIVE* of clinical interaction magnitude. Such additional refinement, while useful, especially when the underlying mechanism of inhibition is atypical/complex [21], may not be a critical requirement from a pragmatic standpoint for meeting the overall strategic goal of DDI risk assessment in drug development, as estimates of K_i from IC_{50} appear to be sufficiently informative in assessing the risk for PK DDIs and in guiding development of the optimal clinical DDI evaluation strategy [22,23].

An important consideration in the design of *in vitro* CYP inhibition DDI studies is the use of experimental conditions to minimize the extent of nonspecific microsomal binding of the NME. Originally identified as an important factor that can influence estimation of *in vitro* K_m values and thereby confound the accuracy of *in vitro*–*in vivo* scaling of drug clearance [24–27], nonspecific microsomal binding has been established as a source of overestimation of IC_{50}/K_i estimates (i.e., underestimation of inhibitory potency) in CYP inhibition studies [20,28–30]. With the use of sensitive LC/MS/MS bioanalytical methods, it is possible to keep the human liver microsomal protein concentrations to <0.2 mg/mL [19,22] such that the extent of microsomal binding would likely be minimized for most NMEs [e.g., microsomal unbound fraction ($f_{u,mic}$) ≥ 0.8]. If this is not feasible or when dealing with NMEs that are expected to be meaningfully bound even at low microsomal concentrations based on their physicochemical properties (e.g., highly lipophilic bases) or other available data, it would be important to account for this phenomenon and apply necessary corrections for microsomal unbound fraction in the calculation of the unbound inhibitory potency (e.g., $K_{i,u} = f_{u,mic} \times \text{observed } K_i$). Of note, the importance of considering microsomal binding in the calculation of K_i values for basic drugs is recognized in the European Medicines Agency (EMA) draft guideline [31]. This is best done via *in vitro* measurement of the unbound fraction of the NME at the microsomal concentration employed in the CYP inhibition studies [20,28,29]. Alternatively, initial estimates of $f_{u,mic}$ can be calculated from physicochemical properties of the NME using *in silico* models, which have undergone considerable refinement with improvement in their predictive accuracy in recent years [32–34] in relation to the first described model [35]. For highly lipophilic drugs (e.g., $\log P/D > 3$), uncertainty in estimating $f_{u,mic}$ is still high enough that it is advisable to directly measure this parameter for such NMEs [33].

Pooled human liver microsomes represent the most commonly used and generally preferred *in vitro* system for the conduct of CYP inhibition studies. However, it should be noted that CYP inhibition studies can also be performed using human hepatocytes, and there are certain situations where such evaluations may be helpful,

as is discussed later in the section on “mechanism-based inactivation” (Section 3.5). Specifically, when extensive non-CYP-mediated and/or nonmicrosomal metabolism of the NME is expected or known, use of hepatocytes for estimating inhibitory potency for CYP inhibition by the NME may need to be considered as it is possible that there could be *in vitro* system-dependent differences in outcomes, with the outcomes in hepatocytes rather than human liver microsomes being more representative of the clinical outcomes [36].

3.4.2 DDI Risk Assessment from *in vitro* CYP Inhibition Data

Following estimation of inhibitory potency (K_i or IC_{50}), the next step is to put these *in vitro* data in context of the exposures/PK properties of the NME at the clinical dose(s) to forecast the level of risk for DDI with substrates of the enzyme being inhibited, to enable development of risk management plans in later phases of clinical drug development and to guide the strategy/design of clinical DDI studies. Ideally, the objective of IVIVE is to permit prediction of the magnitude of increase in exposure of a coadministered object drug and where possible, a more specific prediction of the nature of the altered plasma concentration–time profile and associated population variability. Substantial progress has been made in the IVIVE of CYP-inhibitory DDI over the last decade, with some recently published examples of successful retrospective predictions from large databases [20,22,37,38]. However, uncertainty still remains in certain key parameters that are critical to the prediction of DDI magnitude (e.g., PK metric that is best suited to serve as a surrogate of enzyme-available inhibitor concentration), as will be reviewed later. Therefore, the quantitative prediction of CYP-inhibitory DDI in drug development remains an emerging area of science without a clear consensus on the best approach that balances scientific rigor and an adequately conservative predictive performance. The approach currently recommended by the FDA Draft DDI Guidance [39], consistent with leading scientific opinion on the topic [14], involves a simple and empirical, yet useful approach intended to provide a conservative categorization of the level of risk based on the $[I]:K_i$ ratio, where $[I]$ is the total systemic maximum plasma concentration of the NME achieved following administration at the highest clinical dose/frequency (e.g., steady-state C_{max}). The underlying scientific rationale for this risk level classification approach is based on the hyperbolic relationship (Fig. 3.2) between $[I]:K_i$ and fold increase in AUC of an orally administered substrate drug whose clearance is entirely mediated 100% via metabolism by the enzyme that is inhibited by the NME—assumptions that intentionally err on the conservative side to project the maximum possible magnitude of a DDI (Eq. 3.1).

$$\frac{AUC_{inhibited}}{AUC_{control}} = \frac{CL_{int,control}}{CL_{int,inhibited}} = 1 + \frac{[I]}{K_i} \quad (3.1)$$

The $[I]:K_i$ ratio cut-offs of <0.1 (corresponding projected maximum increase in AUC of <1.1 -fold) and >1 (corresponding projected maximum increase in AUC of greater than twofold) are to be used for qualitative risk level classification as “low” and “high,” respectively, and are not intended to serve as quantitative predictions of DDI magnitude. The application of this classification is simply to eliminate the need for unnecessary follow-up clinical DDI studies for enzymes that are inhibited with an inferred low DDI risk, and to sequentially prioritize the conduct of clinical DDI studies

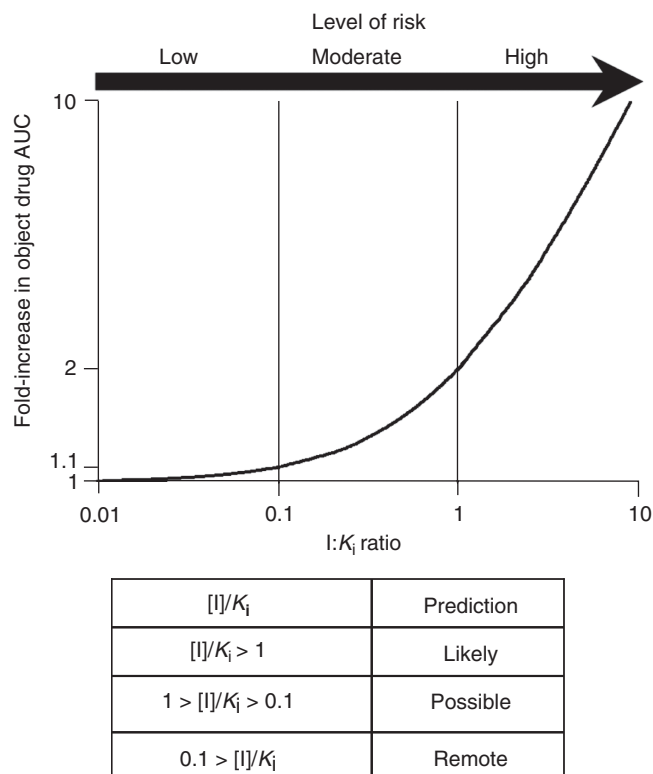


Figure 3.2 Relationship between the $I:K_i$ ratio for CYP inhibition by an NME and the associated level of DDI risk assessed based on the maximum expected fold increase in systemic exposure of an orally administered object drug whose oral clearance is mediated solely by hepatic metabolism by the inhibited enzyme.

for enzymes that are inhibited with decreasing levels of DDI risk, as will be discussed later. Consider, for example, the anticancer drug everolimus, an mTOR (mammalian target of rapamycin) inhibitor, indicated for the treatment of advanced renal cell carcinoma. Everolimus inhibits CYP2D6 activity *in vitro*; however, the clinically observed mean steady-state $C_{\max}$ at the recommended 10 mg daily dose is more than 12-fold below the CYP2D6 inhibitory K_i , based on which the everolimus USPI concludes that an effect of everolimus on the metabolism of CYP2D6 substrates is unlikely [40]. This example illustrates how *in vitro* data, when clearly indicative of low DDI risk, can inform prescribing guidance without the need for unnecessary clinical DDI studies.

The approach to DDI risk assessment using the $[I]:K_i$ ratio alone (with $[I]$ defined as the clinically observed systemic $C_{\max}$ of the NME), while simple and pragmatically straightforward, is not without limitations. The assumptions underlying this simple classification system have been addressed in multiple quantitative enrichments to the framework that have built in considerations of the appropriate metric of $[I]$, route of administration of the victim drug, and potential for extrahepatic metabolism. Finally, if the goal is to make quantitative predictions of DDI magnitude that extend beyond risk

level categorization, it is important to consider the fraction of victim drug clearance that is mediated by metabolism via the inhibited enzyme ($f_{m(CYPi)}$). Each of these aspects is reviewed briefly in the following subsections.

3.4.3 Considerations in Defining [I]

A key consideration in DDI risk assessment from *in vitro* enzyme inhibitory potency is the selection of the best PK surrogate of the enzyme-available inhibitor concentration in the liver. The selection of an appropriate surrogate for [I] has been an extensively debated topic for nearly two decades, with multiple reports of variable predictive success resulting from use of various surrogates—some more scientifically valid and appealing than others. Considering that this continues to be an emerging area of science without global regulatory consensus guidelines available at the time of writing of this chapter, this chapter does not provide any definitive recommendations on the preferred approach to estimate [I] for DDI predictions in drug development but will only discuss the relative merits and limitations of the available approaches. For metabolism by liver microsomal enzymes, enzyme-available inhibitor concentration in the hepatocyte should in principle equal the unbound concentration in hepatic cytosol around the enzyme, which is unfortunately impossible to directly measure in humans. Pharmacologic principles and specifically the unbound drug hypothesis would suggest that the unbound plasma concentration of the inhibitor would be expected to be in equilibrium with the unbound concentration within the hepatocyte (in the absence of active hepatic uptake), suggesting that it may be the most appropriate surrogate for [I]. However, multiple reports have consistently demonstrated that the use of unbound systemic exposure (either steady-state average concentration over the dosing interval or steady-state peak concentration) results in substantial underpredictions of DDI magnitude, with unacceptably high false negative prediction rates, such that major clinically important DDIs would be missed. Multiple reasons can potentially explain the poor performance of unbound systemic exposure as a surrogate for [I]. For orally dosed inhibitors that undergo first-pass hepatic extraction, significant portal/systemic concentration gradients can be expected. Therefore, the unbound hepatic inlet concentration has been proposed as a suitable surrogate for [I] based on the recognition that use of systemic unbound concentrations can underestimate the transiently high concentrations that will be “seen” by the drug-metabolizing enzymes in the liver following an oral dose of the NME, and therefore result in underprediction of DDI magnitude. This can be readily calculated using Equation 3.2 where $I_{\max}$ is the systemic maximum concentration of the inhibitor, f_u is its plasma free fraction, f_a is its fraction absorbed, k_a is its absorption rate constant, and Q_h is hepatic blood flow [41,42].

$$I_{\text{inlet,max,u}} = f_u \times \left(I_{\max} + \frac{f_a \cdot \text{dose} \cdot k_a}{Q_h} \right) \quad (3.2)$$

Another potential reason for underprediction of DDI magnitude with the use of unbound systemic concentrations is the active hepatic uptake of certain inhibitors that can result in unbound hepatocellular concentrations in excess of the corresponding unbound systemic concentrations. Considering these complexities and uncertainties, and in the interest of having a conservative, yet simple approach to DDI risk assessment from *in vitro* CYP-inhibitory potency data, two distinct approaches are currently

advocated by the FDA and the EMA draft DDI guidance documents, respectively. The regulatory draft guidance document from FDA conservatively recommends the use of total systemic $C_{\max}$ of the CYP-inhibitory NME for calculating the $[I]:K_i$ ratio, with a 10-fold safety factor as discussed earlier (i.e., risk for DDI cannot be excluded unless $[I]:K_i$ ratio is <0.1) [39]. The EMA draft guideline that was recently released for comment considers plasma protein binding and recommends unbound systemic $C_{\max}$ as the surrogate of $[I]$ but accordingly recommends the use of higher safety factors to account for factors such as portal/systemic concentration gradients and uncertainties in K_i estimation, and thereby minimize false negative risk assessments. A 50-fold safety factor is recommended (i.e., risk for DDI cannot be excluded unless $[I]:K_i$ ratio is $\leq 1/50$), with a higher safety factor of 250-fold (i.e., risk for DDI cannot be excluded unless $[I]:K_i$ ratio is $\leq 1/250$) for drugs with $>99\%$ plasma protein binding [31]. In addition to consideration of the systemic unbound exposure of the inhibitor, the EMA draft guideline also recommends consideration of intestinal exposure when the enzyme being inhibited is known to have pronounced intestinal expression (e.g., CYP3A). Two measures of intestinal exposure are recommended to be calculated: a maximum luminal concentration calculated as the maximum dose taken at one occasion divided by 250 mL and a predicted maximum concentration in the enterocyte using Equation 3.3 [43], where Q_{ent} is the enterocytic blood flow (~ 248 mL/min):

$$I_{\text{gut}} = \frac{f_a \times \text{Dose} \times k_a}{Q_{\text{ent}}} \quad (3.3)$$

Safety factors of 10-fold (for the calculated maximum luminal concentration) and 50-fold (for the calculated concentration in the enterocyte) are recommended to be jointly applied (in addition to the previously discussed approach to risk assessment based on the systemic unbound $C_{\max}$) for translational assessment of clinical DDI risk from *in vitro* CYP-inhibitory potency data [31]. The performance characteristics of these suggested safety factors remain to be investigated.

3.4.4 Quantitative Predictions of DDI Magnitude: Victim Drug-Specific Considerations

An important factor that determines the magnitude of a DDI is not only the potency of inhibition of the drug-metabolizing enzyme by the NME and the NME's exposure at clinical dose(s) but also the fraction of total clearance of the object drug that is mediated by metabolism via the specific inhibited enzyme (Eq. 3.4; Fig. 3.3), denoted variably by f_m , $f_{m(\text{CYP})}$, $f_{m(\text{CYPi})}$, or the contribution ratio (CR) in previous investigations of CYP-based DDIs [22,23,44–48].

$$\frac{\text{AUC}_{\text{inhibited}}}{\text{AUC}_{\text{control}}} = \frac{1}{(1 - f_{m(\text{CYPi})}) + \frac{f_{m(\text{CYPi})}}{(1 + \frac{I}{K_i})}} \quad (3.4)$$

The approach to DDI risk assessment based solely on $I:K_i$ ratio (Fig. 3.2) conservatively assumes that $f_{m(\text{CYPi})}$ of the object drug is unity, although this is seldom the case even for highly selective probe substrates of the major human CYP enzymes, with estimated values of $f_{m(\text{CYP1A2})}$ of ~ 0.95 for caffeine, $f_{m(\text{CYP2C9})}$ of ~ 0.91 for *S*-warfarin, $f_{m(\text{CYP2C19})}$ of ~ 0.87 for omeprazole, $f_{m(\text{CYP2D6})}$ of ~ 0.9 for desipramine,

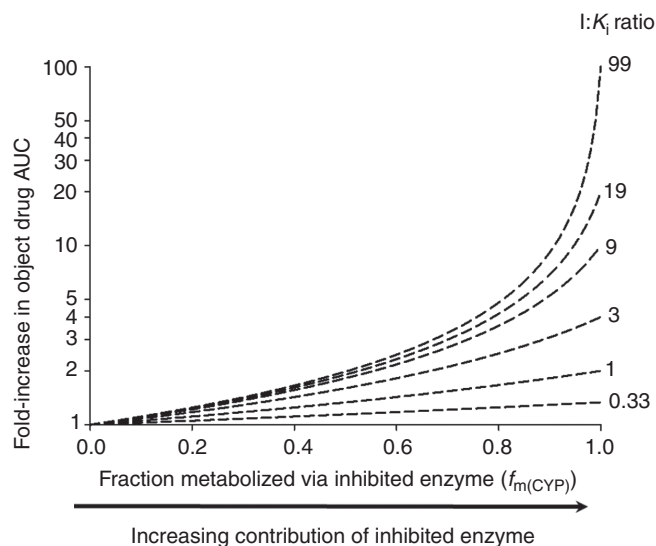


Figure 3.3 Fraction of total clearance of the object drug that is mediated by metabolism via the specific inhibited enzyme ($f_{m(CYP_i)}$) and the precipitant's I:K_i ratio as determinants of clinical DDI magnitude.

and $f_{m(CYP3A)}$ of ~ 0.93 for midazolam, as reviewed previously [49]. Considering the nonlinear relationship between $f_{m(CYP_i)}$ of the object drug and predicted interaction magnitude for a given I:K_i ratio (Fig. 3.3), sensitivity of the IVIVE of DDI to the assumption of $f_{m(CYP_i)}$ of unity can be quite large and result in a substantial overprediction of DDI magnitude, even when dealing with a highly selective substrate with $f_{m(CYP_i)} \geq 0.9$. Accurately defining $f_{m(CYP_i)}$ of the object drug with a high level of precision (e.g., 0.85 vs 0.95) is not straightforward. One approach involves the derivation of $f_{m(CYP_i)}$ from the results of clinical DDI studies with a strong and selective inhibitor (e.g., ketoconazole for CYP3A), and has been utilized for estimating $f_{m(CYP3A)}$ of midazolam and other CYP3A substrates [22,48]. It is important to note that the assumption of complete and specific inhibition of the target enzyme by a strong inhibitor is inherent to such an approach. The adequacy of this assumption of complete inhibition of the target enzyme depends on the potency of the inhibitor in relation to time course of inhibitor concentrations achieved in the DDI study and the mechanism of inhibition (reversible vs mechanism based). Another approach that is probably scientifically most appealing (provided all necessary data are available to enable its application) involves a calculation of $f_{m(CYP_i)}$ from various sources of data, including a quantitative knowledge of the clearance mechanisms of the object drug (e.g., as gained from the results of a radiolabeled mass balance study with adequate quantitative tracking of the chemical fate of the object drug in excreta) and quantitative *in vitro* reaction phenotyping of its biotransformation pathways. This approach has been used successfully in estimating $f_{m(CYP1A2)}$ of theophylline and clozapine [22,49]. With this approach, it is important to note that although the identification of major versus minor contributing enzymes can be expected to be consistent between the complementary approaches of *in vitro* reaction phenotyping, quantitatively equivalent estimates of relative contributions of each enzyme are not always obtained, introducing uncertainty in the derived $f_{m(CYP_i)}$.

Finally, the most straightforward approach is in the setting where the target enzyme is polymorphically expressed and clinical PK data are available in extensive metabolizer (EM) and poor metabolizer (PM) populations. In such cases, $f_{m(\text{CYPi})}$ can be readily estimated as one minus the inverse of the ratio of exposure of the object drug following oral dosing in PM versus EM subjects. Such an approach has been used successfully in estimating the $f_{m(\text{CYPi})}$ of commonly used probe substrates of CYP2C9, CYP2C19, and CYP2D6 [22,45,49]. While the certainty in estimation of $f_{m(\text{CYPi})}$ can be considered to be high in these cases when EM/PM PK data are available, the same is not true when dealing with the other methods of estimating this input parameter, where the associated uncertainty is relatively greater. The topic of defining $f_{m(\text{CYPi})}$ is discussed further in a later section in the context of risk assessment for DDI with the NME as the object drug, but the principles are equally applicable to defining this critical parameter of the object drug to enable quantitative predictions of the magnitude of CYP-inhibitory DDI produced by an NME as the precipitant.

3.4.5 Quantitative Predictions of DDI Magnitude: NME-Specific Considerations

When using Equation 3.4 to make quantitative predictions of DDI magnitude, a key question as discussed earlier for qualitative (categorical) risk assessment is the selection of PK surrogate for [I] of the NME inhibitor. Various measures of [I] (e.g., total systemic $C_{\max}$, unbound systemic $C_{\max}$, total maximum hepatic inlet concentration, and unbound maximum hepatic inlet concentration) have been investigated in prospective and retrospective exercises of IVIVE of CYP-inhibitory DDI without general consensus on the surrogate for [I] associated with best predictive performance. In a large retrospective analysis based on a primary database of *in vitro* CYP-inhibitory potencies by Obach and colleagues [22], use of unbound maximum hepatic inlet concentration (Eq. 3.2) provided the best predictive accuracy relative to other metrics of [I], including total systemic $C_{\max}$, unbound systemic $C_{\max}$, and total maximum hepatic inlet concentration. In contrast to these observations, use of the unbound maximum hepatic inlet concentration of the inhibitor resulted in an overall underprediction of DDI magnitude in another retrospective analysis by Brown and colleagues [20], also based on a primary database of *in vitro* CYP-inhibitory potencies. This latter study found total systemic average concentration to be associated with the best predictive accuracy (91% of predictions within twofold of the observed DDI magnitude), which is unfortunately at odds with expectations from the unbound drug hypothesis and is therefore harder to explain from a mechanistic standpoint. The case of inhibition of CYP2C8 by the leukotriene receptor antagonist montelukast represents an example that supports the importance of considering plasma protein binding of the inhibitor during IVIVE of DDI magnitude. Montelukast has been identified to be a potent CYP2C8 inhibitor with a K_i of ~ 9 nM [30]. Total systemic plasma concentrations of montelukast at the 10 mg/day therapeutic dosing regimen (steady-state average concentration of ~ 0.4 μM ; steady-state $C_{\max}$ of ~ 0.9 μM) are in far excess of the *in vitro* K_i for CYP2C8 inhibition, such that nearly complete inhibition of CYP2C8 should be anticipated. However, clinical DDI studies with CYP2C8 substrates such as repaglinide [50] and pioglitazone [51] have consistently demonstrated that montelukast does not alter the PK of these agents, confirming the lack of CYP2C8 inhibition by montelukast *in vivo*. Montelukast is highly plasma protein bound ($>99\%$) and the estimated unbound hepatic inlet $C_{\max}$ calculated using Equation 3.2 is <11 nM, translating to a <2.2 -fold DDI magnitude even if

100% of clearance of the substrate drug is via CYP2C8-mediated metabolism [30]. As plasma protein binding of montelukast is not precisely known and exact estimates of $f_{m(\text{CYP2C8})}$ of rosiglitazone, pioglitazone, and repaglinide are difficult to obtain considering involvement of nonmetabolic OATP1B1 transporter-mediated clearance of these agents, it is difficult to more precisely predict the quantitative extent of DDIs with montelukast. Nevertheless, it is clear that predictions that do not consider the plasma protein binding of montelukast would substantially overpredict the risk for clinical DDIs with CYP2C8 substrates.

For CYP3A substrates administered orally, the quantitative prediction of DDI magnitude following administration of a CYP3A inhibitor should consider inhibition of intestinal metabolism in addition to hepatic metabolism of the object drug, due to the established expression of CYP3A in the small intestine and clinically important contribution to the clearance of many (but not all) substrates. The previously discussed IVIVE analysis by Obach and colleagues [22], accounted for inhibition of intestinal first-pass metabolism for CYP3A inhibitory DDIs, based on inclusion of the fold increase in the intestinal bioavailability as a multiplicative term together with the hepatic component. This is implemented using Equation 3.5 [52], where F_G is the baseline value of intestinal bioavailability of the object drug, I_g is the estimated intestinal concentration of the inhibitor (calculated using Eq. 3.3).

$$\frac{\text{AUC}_{\text{inhibited}}}{\text{AUC}_{\text{control}}} = \frac{1}{F_G + \frac{(1-F_G)}{\left(1 + \frac{I_g}{K_i}\right)}} \times \frac{1}{(1 - f_{m(\text{CYPi})}) + \frac{f_{m(\text{CYPi})}}{\left(1 + \frac{I}{K_i}\right)}} \quad (3.5)$$

The contribution of CYP3A inhibition in the intestine to the overall magnitude of a DDI clearly depends on the baseline F_G of the object drug. For highly potent inhibitors, the $I_g : K_i$ ratio may be large enough such that the first term in Equation 3.5 can be approximated as the inverse of baseline F_G , as has been done in the above discussed analysis by Brown and colleagues [20] for the CYP3A-inhibitory azole antifungal agents. For a detailed review of the assumptions and considerations associated with IVIVE of intestinal inhibition in the overall prediction of DDI magnitude, the reader is referred to a recent comprehensive review on this topic [53].

3.5 NME AS PRECIPITANT: MECHANISM-BASED CYP INACTIVATION DDI

Many clinically important PK DDIs result from impairment of metabolic clearance via MBI of CYP enzymes. This can be readily appreciated from an examination of the strong and moderate inhibitors of the major human drug-metabolizing enzyme CYP3A identified in the 2006 FDA draft guidance on drug interaction studies [39]. Fourteen out of 19 identified clinically significant CYP3A inhibitors are either established or putative mechanism-based inactivators of the enzyme, ranging from the natural product grapefruit juice to prescription drugs spanning several therapeutic classes, including antiretroviral agents (e.g., ritonavir, saquinavir), antibiotics widely used in general practice (e.g., clarithromycin, erythromycin), the calcium channel blockers diltiazem and verapamil, and the antidepressant agent nefazodone.

3.5.1 *In vitro* Diagnosis of MBI

The *in vitro* CYP inhibition studies and conceptual framework to IVIVE of DDI magnitude discussed in the previous section are only applicable to the setting of reversible CYP inhibition and are not appropriate when the investigational agent is a mechanism-based inactivator of a drug-metabolizing enzyme. Reversible CYP inhibition is only concentration dependent (at a given substrate concentration), whereas MBI is characterized by both concentration *and* time-dependent inactivation of the enzyme, secondary to metabolic activation of the inactivator (often described as a suicide inhibitory mechanism). An efficient approach to diagnosis of MBI by an NME is a test of enhancement of its apparent inhibitory activity following preincubation with human liver microsomes (or specific recombinant CYP isoforms) and NADPH before incubation with the isoform-selective index substrate. If the test compound is a mechanism-based inactivator, the above described preincubation process will result in a leftward shift in the IC_{50} curve (relative to a control experiment conducted alongside with the cofactor NADPH omitted in the preincubation period) and a correspondingly smaller estimate of the IC_{50} [54,55]. For simple reversible inhibitors, the IC_{50} curves will be expected to be essentially indistinguishable when interpreted in context of experimental variability, or may actually demonstrate an apparently greater IC_{50} following preincubation, as a consequence of metabolic consumption of the reversible inhibitor during the preincubation. If the above described diagnostic test is positive indicating time-dependent inhibition, further DDI risk assessment requires a full *in vitro* kinetic analysis to estimate the parameters characterizing MBI by the NME. An abbreviated empirical IVIVE approach using the “shifted IC_{50} ” estimated following preincubation of the inactivator with human liver microsomes and NADPH, in the context of the reversible inhibition model (i.e., using one-half of the “shifted IC_{50} ” in place of K_i in Eq. 3.5) has been explored [55,56]. The “shifted IC_{50} ” is inversely related to the $k_{inact} : K_I$ ratio, which is a measure of the inactivator’s intrinsic efficiency [57,58]. However, the performance of quantitative DDI predictions using the “shifted IC_{50} ” is suboptimal, and it is therefore recommended that the appropriate mechanistic model for IVIVE of MBI based on *in vitro* parameters derived from a full enzyme-kinetic characterization of inactivation be utilized for definitive quantitative predictions and clinical DDI risk assessment [56].

3.5.2 *In vitro* Kinetic Characterization of MBI

The two primary kinetic parameters of relevance to IVIVE of MBI DDI are a capacity term reflecting the maximum rate of loss of enzymatic activity that the inactivator is capable of producing (k_{inact}), and a potency term (K_I) reflecting the concentration of the inactivator at which half-maximal inactivation rate ($0.5 \times k_{inact}$) is achieved. The inactivator at varying concentrations ($[I]$) is preincubated with the source of enzyme (human liver microsomes or recombinant CYP preparation) and NADPH for varying durations of time (t) before dilution into a secondary metabolic incubation (activity assay) containing NADPH and an index substrate of the target CYP isoform at concentrations in excess of its K_m . Dilution following the preincubation and use of saturating substrate concentrations in the activity assay help minimize any surmountable reversible inhibition, which may otherwise potentially confound estimation of the true kinetic parameters of CYP inactivation. The time course of loss of CYP activity over the duration of the preincubation is analyzed to estimate the apparent first-order

inactivation rate constant λ at each concentration of inactivator.

$$\text{Activity} = \text{Activity}_{t=0} \cdot e^{-\lambda t} \quad (3.6)$$

The parameters k_{inact} and K_I characterizing the hyperbolic relationship between λ and $[I]$ are subsequently estimated using either a nonlinear regression approach or the less preferred approach involving linear transformations of the data (e.g. Kitz–Wilson analysis).

$$\lambda = \frac{k_{\text{inact}} \cdot [I]}{[I] + K_I} \quad (3.7)$$

The design of *in vitro* inactivation kinetic studies and subsequent data analysis should be performed in a manner that minimizes bias and increases precision of the estimated parameters [59]. For a detailed review of best practices for the experimental designs and data analytical approaches to *in vitro* studies of CYP inactivation, the reader is referred to a recent review that provides an expert opinion on the topic from a PhRMA perspective [60].

A complete elimination of bias in parameter estimates may be difficult to achieve using conventional experimental and data analytical approaches for inactivation kinetic characterization, as illustrated via theoretical simulations of the fidelity of such traditional approaches [61]. For example, metabolic depletion of the inactivator during the preincubation (as a consequence of which, nominal concentrations may overestimate the actual concentrations during the preincubation) as well as possible inactivation of the enzyme during the metabolic incubation with probe substrate (i.e., inadequate “quenching” of inactivation by dilution when the metabolic incubations with the probe substrate are conducted for relatively long durations) can introduce bias in k_{inact} and K_I estimates. These considerations emphasize the importance of using the lowest required microsomal concentration during the preincubation and the shortest possible duration of incubation for the metabolic activity measurement. Other features of the experimental protocol (e.g., dilution factor employed between the preincubation and incubation steps) can additionally influence the estimated inactivation kinetic parameters as illustrated in a series of studies on CYP2D6 inactivation by 3,4-methylenedioxymethamphetamine [62,63]. To address these issues, Yang and colleagues [64] have proposed a mechanistically based experimental protocol to eliminate the bias introduced in the estimation of inactivation kinetic parameters with the above described conventional approach. This approach involves three separate experiments to assess the metabolism of the inactivator, its ability to cause competitive inhibition, and its ability to cause time-dependent inhibition, followed by a simultaneous analysis of all resulting data to permit a model-based extraction of MBI kinetic parameters [64]. A variation of this approach referred to as *progress curve analysis* has been described and applied to estimate and distinguish the potencies of reversible inhibition and MBI of the CYP1A2 inactivators resveratrol, furafylline, and dihydralazine within the same experiment [65]. The complete time courses of substrate turnover were characterized in the absence of the inactivator and in the presence of varying concentrations of the inactivator. The reversible K_i was estimated based on the concentration–effect relationship for inhibition of initial velocity of substrate metabolism and the inactivation kinetic parameters (K_I and k_{inact}) were estimated based on kinetic analysis of the entire progress curve of substrate turnover

followed by characterization of the concentration–effect relationship for inactivation using the time-averaged inactivator concentration over the duration of the incubation [65]. Additional research is required to fully evaluate the performance characteristics of these novel promising approaches across CYP isoforms and their application for MBI DDI risk assessment.

3.5.3 Considerations in *In vitro* System Selection for MBI Kinetic Studies

In vitro studies of MBI can be performed using recombinantly expressed CYP enzyme isoforms, human liver microsomes, or hepatocytes. It should be noted that MBI kinetic parameters estimated using certain recombinantly expressed CYP preparations may not be reflective of those estimated using native human liver microsomes [66]. Therefore, caution should be exercised in their use for DDI risk assessment, as has been demonstrated for CYP3A inhibitory DDIs produced by macrolide antibiotics [67]. A recent study suggested a better predictive performance of IVIVE based on *in vitro* inactivation kinetic parameters determined in primary human hepatocytes compared to human liver microsomes [68]. Nevertheless, when viewed in the context of multiple reports that suggest reasonably successful classification of the level of DDI risk using time-dependent inhibition studies in human liver microsomes, and the relative convenience and opportunity for “standardization” of this experimental system (e.g., use of pooled human liver microsomes to represent the “population average” distribution of enzymes), the most commonly utilized system for studies of MBI for DDI risk assessment is human liver microsomes. Consistently, human liver microsomes represent the recommended *in vitro* system for estimation of kinetic parameters of inactivation in the expert opinion from the PhRMA [60].

It should, however, be noted that if substantial non-CYP- and/or nonmicrosomal-mediated metabolism of the NME is expected and if such metabolism modifies the inactivation effects of the NME, data from human hepatocytes rather than human liver microsomes may be more translatable to the clinical setting. This has been demonstrated recently for gemfibrozil, bupropion, and ezetimibe [36]. Gemfibrozil produces clinically relevant inhibition of CYP2C8 through MBI of the enzyme by its acyl glucuronide. Bupropion produces clinically relevant inhibition of CYP2D6 through metabolism-dependent inhibition of the enzyme by its reduced metabolites *erythro*- and *threo*-hydrobupropion. As hepatocytes represent a more complete biotransformation system compared to human liver microsomes, potent inhibition of CYP2C8 and CYP2D6 by gemfibrozil and bupropion, respectively, are readily observed following preincubation of hepatocytes with these drugs but are not observed using human liver microsomes as the *in vitro* system, where direct evaluation of the metabolites is necessary to observe potent enzyme inhibition [36]. The reverse is true in the case of ezetimibe, where potent MBI of CYP3A4 is observed in human liver microsomes, whereas this is not the case when using hepatocytes as the *in vitro* system, because of turnover of ezetimibe by direct glucuronidation, which occurs *in vivo*, resulting in the lack of clinically relevant CYP3A inhibition [36].

Considering the relatively large microsomal concentrations generally used in the preincubation step in *in vitro* kinetic studies of MBI, nonspecific microsomal binding can be significant, introducing bias in K_I estimates, jeopardizing their applicability in subsequent IVIVE of DDI magnitude. Therefore, correction of apparent K_I estimates for microsomal binding can be important in getting unbiased estimates of inactivator

potency for highly lipophilic basic inactivators that can be substantially bound to microsomal phospholipids. For example, failure to consider nonspecific microsomal binding of paroxetine results in a greater than threefold underprediction of the magnitude of interactions with CYP2D6 substrates [69].

3.5.4 DDI Risk Assessment and IVIVE for MBI

The theoretical framework that guides IVIVE of DDI magnitude resulting from CYP inactivation was first described by Hall and colleagues in 2000 [70]. As with IVIVE of reversible inhibition, the first step in IVIVE of MBI is to estimate the effect of the inactivator on intrinsic clearance of metabolism via the inactivated enzyme. Considering that steady-state levels of CYP enzymes *in vivo* are maintained by a balance of zero-order production (k_{syn}) and first-order degradation (rate constant, k_{deg}), the perturbation of this system by an inactivator (i.e., introduction of an additional mechanism of loss of active enzyme characterized by the apparent first-order rate constant λ) without an effect on enzyme synthesis rate would be expected to result in the following derivation of the expression for fold reduction in intrinsic clearance:

$$\frac{CL_{int, control}}{CL_{int, I}} = \frac{E_{ss}}{E_{ss, I}} = \frac{k_{syn}/k_{deg}}{k_{syn}/k_{deg} + \lambda} = \frac{k_{deg} + \lambda}{k_{deg}} = \frac{k_{deg} + \frac{[I] \cdot k_{inact}}{[I] + K_I}}{k_{deg}} \quad (3.8)$$

The model described above provides the fundamental basis of IVIVE of MBI DDI and is shown in Fig. 3.4. The 2010 EMA draft guideline on DDIs recommends use of the derived expression in Equation 3.8 to predict the effect of NMEs that display time-dependent inhibition *in vitro* on intrinsic clearance of the enzyme being inactivated. If $\geq 30\%$ inhibition is predicted using this equation (with the estimates of $[I]$ and safety factors recommended in the guideline as discussed previously for reversible inhibition), *in vivo* inhibition may not be excluded and a clinical DDI study is recommended [31].

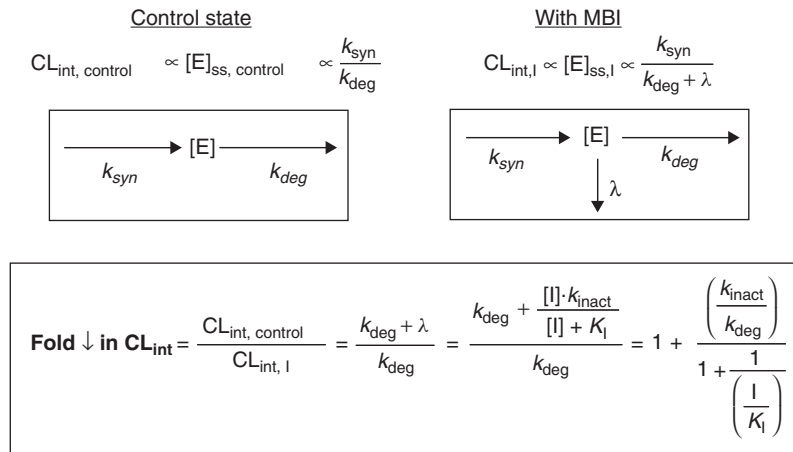


Figure 3.4 Schematic illustration of the enzyme-kinetic mechanistic model underlying mechanism-based inactivation.

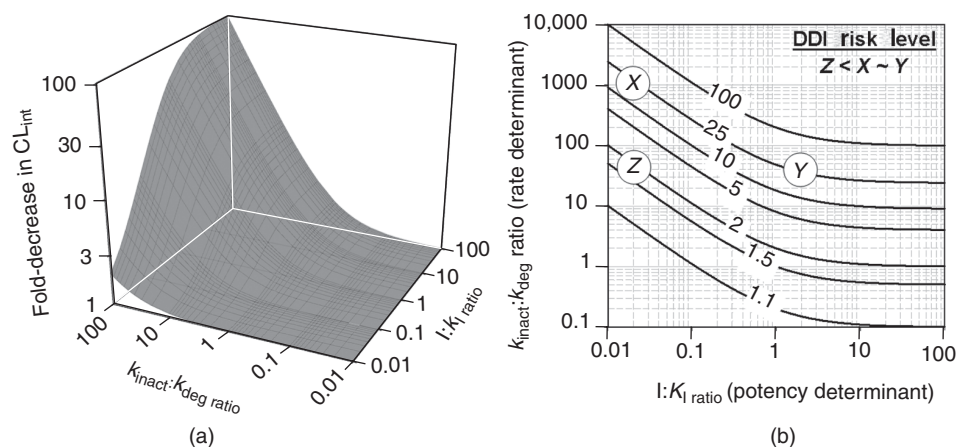


Figure 3.5 (a) MBI surface plot showing the fold reduction in intrinsic clearance as a function of $I:K_I$ and $k_{\text{inact}} : k_{\text{deg}}$. (b) Contour map positioning as a tool for DDI risk assessment via MBI. See text for details.

Rearranging the terms in the derived expression in Equation 3.8, a modified form of this expression can be obtained and is shown in Equation 3.9 [71].

$$\frac{CL_{\text{int,control}}}{CL_{\text{int,I}}} = 1 + \frac{\left(\frac{k_{\text{inact}}}{k_{\text{deg}}}\right)}{1 + \frac{1}{\left(\frac{I}{K_I}\right)}} \quad (3.9)$$

It follows from Equation 3.9 that the fold decrease in intrinsic clearance of metabolism via the enzyme being inactivated is a complex nonlinear function of $I:K_I$ and $k_{\text{inact}} : k_{\text{deg}}$ (Fig. 3.5a) and that various combinations of $I:K_I$ and $k_{\text{inact}} : k_{\text{deg}}$ can result in identical fold reductions in intrinsic clearance. MBI interaction contour maps can thus be generated to serve as a visual aid to guide risk assessment for DDI from *in vitro* inactivation kinetics, initial estimates of k_{deg} , and of enzyme-available inactivator concentration *in vivo*. Each contour is a slice of the surface at a given magnitude of fold decrease in intrinsic clearance of the enzyme, and tracks combinations of $I:K_I$ and $k_{\text{inact}} : k_{\text{deg}}$ producing identical fold reductions in intrinsic clearance (Fig. 3.5b). Therefore, rank ordering of the level of DDI risk associated with individual compounds within or across chemical series can be readily done based on the interaction contour on which they fall (or are closest to), which may not be readily apparent based on inspection of the four independent input parameters (k_{inact} , K_I , k_{deg} , $[I]$). This is illustrated in Fig. 3.5b where three hypothetical compounds characterized as inactivators (X with $I:K_I$ of 0.02 and $k_{\text{inact}} : k_{\text{deg}}$ of 1000; Y with $I:K_I$ of 2 and $k_{\text{inact}} : k_{\text{deg}}$ of 40; and Z with $I:K_I$ of 0.02 and $k_{\text{inact}} : k_{\text{deg}}$ of 40) are compared on a MBI contour map. Despite vastly different values of $I:K_I$ and $k_{\text{inact}} : k_{\text{deg}}$, the level of DDI risk associated with X and Y can be inferred to be comparable because they both fall close to the same contour (tracking an ~25-fold reduction in intrinsic clearance), albeit at different positions as dictated by their relative potencies and rates of inactivation. In contrast, compound Z represents a significant improvement over X or Y with respect to risk for MBI DDI, as is evident from its position on a contour

tracking a less than twofold reduction in intrinsic clearance. An important lesson learned from examination of compound X is that despite a very small $I:K_I$ ratio of 0.02 (which would typically be considered to be associated with negligible risk and a remote possibility of a clinically meaningful interaction if the inhibition mechanism were reversible), the resulting fold reduction in intrinsic clearance is predicted to be very large (~ 20 -fold), as a consequence of very rapid inactivation that is three orders of magnitude faster than the rate of degradation of the inactivated CYP isoform. This emphasizes the importance of not only potency considerations but also rate considerations when making inferences regarding the level of DDI risk associated with MBI. Making comparisons of K_I against clinical exposures of the inactivator is inappropriate for risk assessment unless considered in context of the $k_{\text{inact}} : k_{\text{deg}}$ ratio. Examples include CYP2D6 inactivation by paroxetine, CYP2C19 inactivation by ticlopidine, and CYP3A4/5 inactivation by erythromycin, verapamil, and diltiazem, which are all associated with clinically significant DDIs despite the corresponding $I:K_I$ ratios being below 0.1 [55,71].

For the quantitative prediction of DDI magnitude with CYP inactivators, the fold decrease in intrinsic clearance of the inactivated enzyme (Eq. 3.9) takes the place of the “ $1 + (I/K_I)$ ” term in Equation 3.5, resulting in the following equation (where $k_{\text{deg(intestine)}}$ and $k_{\text{deg(liver)}}$ are the first-order turnover rate constants for the intestinal and hepatic enzyme):

$$\frac{\text{AUC}_{\text{inhibited}}}{\text{AUC}_{\text{control}}} = \frac{1}{F_G + \frac{(1-F_G)}{\left(1 + \frac{\left(\frac{k_{\text{inact}}}{k_{\text{deg(intestine)}}}\right)}{1 + \frac{1}{\left(\frac{I}{K_I}\right)}}\right)}}} \times \frac{1}{(1 - f_m(\text{CYPi})) + \frac{f_m(\text{CYPi})}{\left(1 + \frac{\left(\frac{k_{\text{inact}}}{k_{\text{deg(liver)}}}\right)}{1 + \frac{1}{\left(\frac{I}{K_I}\right)}}\right)}}} \quad (3.10)$$

Use of this equation has resulted in reasonable predictions of the magnitude of DDIs produced by CYP inactivators based on retrospective analyses from primary *in vitro* databases that suggest that unbound systemic exposure appears to be the best surrogate for hepatic [I], with the use of Equation 3.3 for estimation of intestinal [I] [55,56].

3.5.5 CYP Turnover Half-Life: A key parameter for IVIVE of MBI DDI

It is readily apparent on inspection of Equations 3.8–3.10 that a key drug-independent physiological parameter that is necessary to translate *in vitro* data on CYP inactivation to predict clinical pharmacologic consequences is the degradation/turnover rate constant (k_{deg}) of the enzyme *in vivo*. While relatively good estimates of *in vivo* k_{deg} are available for certain human CYP enzymes (e.g., hepatic CYPs 1A2, 2C8, 2D6, 2E1, and intestinal CYP3A), the same is not true for other enzymes where *in vivo* estimates are either lacking (e.g., CYP2C9, CYP2C19) or are available with a high degree of uncertainty reflected by a wide range of estimates across literature reports that have estimated this parameter using complementary methods and data analytic approaches with varying associated assumptions (e.g., hepatic CYP3A4). A detailed review of the methods used to estimate k_{deg} , their merits and limitations, and a critical analysis of available data in this regard is outside the scope of this chapter and the reader is referred to a recent detailed overview [72]. For CYP1A2, a half-life of 39 h is estimated

based on time course of deinduction on smoking cessation [73]. For CYP2D6, a half-life of 51 h has been derived by noncompartmental deconvolution of the steady-state serum half-life of paroxetine from the time course of recovery of CYP2D6 activity following cessation of treatment with the inactivator [69,74]. In the case of CYP2E1, the time course of both deinduction (during the withdrawal phase in alcoholics) and recovery following disulfiram inactivation have been characterized and both estimates yield comparable CYP2E1 turnover half-lives of 60 h [75] and 51 h [76], respectively. A recent report estimated the half-life of hepatic CYP2C8 to be 22 h based on analysis of the time course of recovery of repaglinide oral clearance following inactivation by gemfibrozil [77]. The turnover half-life of intestinal CYP3A (likely reflective of the kinetics of enterocyte turnover in the intestinal epithelium) has been estimated as 23 h based on the time course of recovery following inactivation by grapefruit juice ingestion [78]. For hepatic CYP3A, a wide range of estimates (24–140 h) is available based on analysis of clinical data of the time course of induction or deinduction, or of recovery from inactivation using CYP3A substrates of varying specificity and different methods of data analysis ranging from nonlinear regression of time course of induction/deinduction [72,79] to more sophisticated analyses using mechanistic population PK models describing autoinduction [80]. CYP3A turnover half-lives of 28 h [81,82], 36 h [37,55,71,83], 48 h [68], and 72 h [56,84] have been used in previous analyses of IVIVE of MBI DDI. Considering the uncertainty in half-life of certain human CYP enzymes and hepatic CYP3A, in particular, a useful approach to IVIVE of CYP3A MBI is to select a certain reasonable initial estimate (within the 24–72 h range, for instance) and then examine the sensitivity of the predicted drug interaction magnitudes around this value. The key question is whether the prediction for a given inactivator using the initial estimate of k_{deg} falls on the dynamic range of the sensitivity curve or on a relatively flat portion of the curve, as this would translate to a relatively low versus high confidence in prediction, respectively. The previously discussed MBI contour mapping approach can be useful in visually interpreting the results of analyses of sensitivity of the outcome of IVIVE of MBI DDI to uncertainty in k_{deg} when reliable estimates of this parameter are not available [49].

3.6 NME AS PRECIPITANT: CYP INDUCTION DDI

Induction of drug-metabolizing enzymes is an important mechanism of clinically significant DDIs. Increases in enzyme content secondary to either increased expression or protein stabilization by an NME can result in an increase in intrinsic clearance of metabolism by the induced enzyme, leading to decreased systemic exposure of coadministered drugs that are substrates for the enzyme that is induced. Although this typically manifests itself as a reduction in therapeutic efficacy (e.g., loss of oral contraceptive efficacy due to decreased exposure by enzyme-inducing antiepileptic drugs) and increased dosage requirements (e.g., higher cyclosporine A dose requirement in patients taking St. John's wort) in the setting of induction, increases in metabolism can also result in an alteration of the safety profile when dealing with drugs with active and/or toxic metabolites. Unlike reversible inhibition, induction DDIs are time dependent in their onset and offset, complicating their clinical management both after initiation of treatment with an inducer and in the deinduction period following cessation of treatment with the inducer.

3.6.1 Molecular Mechanisms of Induction and *In vitro* Assay Platforms

The major mechanism of induction of drug clearance is via increased expression of drug-metabolizing enzymes and transport proteins by the inducer via increased transcription. Therefore, risk assessment for induction DDIs requires the use of whole cell systems with intact transcriptional and translational machinery as opposed to inhibition DDI studies that can be readily performed in human liver microsomes. The major molecular mechanisms of induction DDI involve binding to and activation of receptors that are involved in the regulation of transcription of genes encoding drug-metabolizing enzymes and/or transporters. Although many such receptors and transcriptional mechanisms have been identified as regulators of gene expression, the three receptors that mediate gene transcription that is induced in the majority of clinically relevant induction DDIs include (i) the aromatic hydrocarbon (Ah) receptor, (ii) the constitutive androstane receptor (CAR), and (iii) the pregnane X receptor (PXR). For a detailed overview of the molecular mechanisms of enzyme induction, the reader is referred to recent review articles [85–87].

The *in vitro* system that is currently considered as reliable for evaluating the potential for an NME to produce induction of drug-metabolizing enzymes is cultured human hepatocytes, either as primary cultures of fresh human hepatocytes or as attachable cryopreserved hepatocytes, as reflected in the recently published perspective of the PhRMA [88]. It is recommended for *in vitro* induction studies in human hepatocytes that the potential for an NME to produce induction of CYPs 1A2, 2B6, and 3A4 be evaluated, as the genes encoding these enzymes are considered to be representative sensitive targets that respond to induction via the AhR, CAR, and PXR, respectively. Significant overlap and cross talk between the CAR and PXR systems is well established, with coinduction of CYPs 2B6 and 3A4 by prototypic inducers such as rifampin. Accordingly, it was originally considered to be sufficient to test NMEs for potential for induction of CYPs 1A2 and 3A4, to enable risk assessment for induction DDIs that may result from increased expression of AhR and CAR/PXR target genes, respectively, as reflected in the recommendations in the 2006 FDA Draft Guidance [39]. However, more recent studies have indicated that although human PXR regulates both CYP3A4 and CYP2B6 with less selectivity, human CAR exhibits marked selectivity for CYP2B6 over CYP3A4 such that selective CAR activators can induce CYP2B6 substantially without appreciable levels of CYP3A4 induction [89]. Additionally, some inducers of CYP2B6 and CYP3A4 (e.g., carbamazepine, nevirapine, efavirenz) produce their effects primarily via CAR rather than PXR activation [90]. On the basis of these advances in our understanding of the molecular mechanisms of induction, it is now generally accepted that a comprehensive and definitive *in vitro* evaluation of risk for an NME to produce DDIs via CYP/transporter induction should include analysis of the potential for induction of CYPs 1A2, 2B6, and 3A4 in human hepatocytes [88], or in an appropriately qualified cell line that maintains inducible regulation via the AhR, CAR, and PXR mechanisms as also noted in the 2010 EMA Draft DDI guideline [31].

Although recognized as the gold standard *in vitro* system for induction DDI risk assessment, limitations do exist in the availability of high quality human hepatocytes as reproducibly performing biological reagents for *in vitro* induction assays. While interindividual variability in responsiveness still requires consideration, the challenges related to supply limitations and the intermittent availability of fresh human hepatocytes have been substantially offset by the availability of cryopreserved human hepatocytes

and establishment of experimental approaches that enable their reliable use as models for induction studies [91]. In an effort to qualify biological reagents for *in vitro* induction studies with reproducible performance characteristics that model the performance of high quality human hepatocytes, hepatocyte-derived cell lines have received considerable attention as surrogate models of human hepatocytes for *in vitro* induction studies.

Successful use of the immortalized hepatocyte cell line Fa2N-4 has been described in the *in vitro* assessment of enzyme induction for PXR target genes (e.g., CYP3A4, MDR1, CYP2C9) and AhR target genes (e.g., CYP1A2) [92], with promising *in vitro*–*in vivo* correlations to enable induction DDI predictions [93]. However, since this immortalized hepatocyte cell line does not express CAR, induction of CYP2B6 is not observed in response to CAR-selective inducers, and induction of CYP3A4 in Fa2N-4 cells following treatment with CAR-selective inducers is either blunted or absent when compared to cryopreserved human hepatocytes [94,95]. Additionally, the level of expression of several hepatic transporters in Fa2N-4 cells is substantially lower than in human hepatocytes. Specifically, expression of the uptake transporters OATP1B1 and OATP1B3 is substantially reduced, as a result of which the apparent potency for rifampin induction of CYP3A4 in Fa2N-4 cells is 10-fold lesser than that observed in cryopreserved human hepatocytes [94], consistent with rifampin being a substrate for OATP-mediated hepatic uptake, which regulates its intracellular concentrations and apparent induction potency [96]. Therefore, Fa2N-4 cells may represent a good surrogate for primary human hepatocytes for evaluating the potential for an NME to produce AhR- or PXR-mediated induction, but not CAR-mediated induction, limiting their utility as a definitive *in vitro* model for CYP induction studies in a drug development setting [88].

The differentiated hepatoma cell line HepaRG appears to be a promising surrogate for human hepatocytes, based on recent studies that have demonstrated expression, activity, and inducibility of CYPs 1A2, 2B6, and 3A4 [97,98], consistent with expression of AhR, CAR, and PXR under optimally cultured conditions [99]. Unlike Fa2N-4 cells, where CYP2B6 induction is not observed following treatment with CAR-selective inducers such as phenytoin or phenobarbital due to lack of CAR expression [94], induction of CYP2B6 by these inducers has been observed in HepaRG cells [98]. Additionally, an excellent correlation between parameters characterizing the maximum magnitude, potency, and efficiency of inducers ($E_{\max}$, EC_{50} , and $E_{\max}:EC_{50}$ ratio, respectively) in primary human hepatocytes versus HepaRG cells has also been described [100]. The experience gained from the above discussed examples of studies on the Fa2N-4 and HepaRG cell lines suggest that a comprehensive qualification of induction of CYPs 1A2, 2B6, and 3A4 using appropriate positive controls that measure regulation of all three pathways (AhR-, CAR-, and PXR-mediated induction) is critical to the use of any such cell line as a surrogate in place of primary human hepatocytes for *in vitro* induction studies.

Assays that measure binding to nuclear receptors and those that measure transactivation of reporter gene expression can be useful in the drug discovery setting due to their relatively higher throughput compared to definitive hepatocyte-based induction assays. Additionally, nuclear receptor transactivation assays can be useful in elucidating the molecular mechanism of the observed induction in hepatocytes (e.g., does an NME produce induction primarily via PXR or CAR activation?). However, since the mechanisms of induction are complex and involve multiple nuclear receptors with cross talk between the mechanisms, these assays alone are not considered sufficient in

excluding the potential for enzyme induction by an NME in drug development settings to support clinical DDI risk assessment [88].

3.6.2 Endpoints in *In vitro* Induction Studies

The general approach to *in vitro* induction studies in human hepatocytes (or adequately qualified cell lines with appropriate scientific justification of suitability, as discussed earlier) involves treatment with the NME for two to three days, with daily replacement of the culture medium containing the NME. Usually, a range of NME concentrations including the therapeutic systemic concentrations and including up to an order of magnitude higher concentration are evaluated to assess concentration response and the studies are conducted in hepatocytes from at least three different donor livers. At the end of the incubation period, the endpoints typically measured include (i) activity of CYPs 1A2, 2B6, and 3A4 *in situ* or in microsomes prepared from the hepatocytes using isoform-selective index reactions; and/or (ii) mRNA expression of CYPs 1A2, 2B6, and 3A4 using techniques such as reverse transcription-polymerase chain reaction [88]. Many CYP3A4 inducers are additionally time-dependent inhibitors of the enzyme's activity, making it important to include mRNA measurements in addition to enzyme activity assessments to aid appropriate mechanistic interpretation of the results, translation of relevance to the clinical setting, and appropriate clinical DDI study design, as reflected in the PhRMA perspective [88]. In fact, the EMA draft guideline mandates the inclusion of mRNA measurement in *in vitro* induction studies for the interpretation of study results if inhibition of the studied enzyme may not be excluded at the concentrations used or if a downregulation is suspected based on activity measurements [31]. A classical example of a drug producing complex simultaneous inhibition/induction effects is the HIV protease inhibitor ritonavir, with *in vitro* induction studies showing increases in mRNA expression of CYP3A4 due to PXR-mediated induction and decreased CYP3A4 activity due to time-dependent inactivation of the induced enzyme [101]. The clinical pharmacologic picture is consequently characterized by complex dose- and time-dependent interactions that are additionally dependent on the PK properties of the substrate drug. For example, in the case of the CYP3A substrate alprazolam, the net effect was a clinically significant level of inhibition of oral clearance (~2.5-fold increase in AUC) following short-term low dose treatment with four doses of 200 mg ritonavir administered BID, considered to be representative of a dosage schedule that may be used to initiate treatment with ritonavir [102]. In contrast, mild and clinically insignificant level of induction of alprazolam oral clearance (12% decrease in AUC) was observed as the net effect following a 10-day treatment with usual therapeutic dose of 500-mg BID of ritonavir as reflected in the ritonavir USPI [103]. Depending on the substrate drug's PK properties, the outcome of multiple-dose treatment with therapeutic doses of ritonavir can be variable, with substantial impairment of oral clearance (i.e., net inhibition rather than induction) observed even in the setting of a week-long treatment with high dose (500-mg BID) ritonavir for substrates such as sildenafil [104]. These consequences are obviously not straightforward to predict from the *in vitro* results alone, making it important to measure both mRNA and activity-based endpoints in the *in vitro* induction studies especially when there is a potential for concurrent inhibition. It is important to recognize that the use of CYP3A4 activity and/or mRNA measurement as an endpoint in *in vitro* induction studies is to serve as a sensitive marker of PXR-mediated induction and its associated pleiotropic

phenotype. For instance, if CYP3A4 induction is not observed in an *in vitro* induction study in human hepatocytes following treatment with an NME at concentrations up to 10 times the mean systemic $C_{\max}$ at clinically relevant doses, one can not only conclude that the risk for the NME to produce DDIs via CYP3A induction is low but also that the risk for it to produce DDIs via induction of other coinduced PXR targets such as CYPs 2C8, 2C9, 2C19, and MDR1 P-gp is also low as per the 2006 FDA Draft guidance [39]. With an NME that has properties such as ritonavir (i.e., PXR-mediated inducer and mechanism-based inactivator of CYP3A), if activity data alone were measured and used in risk assessment, it could result in a false negative risk assessment for induction DDIs. The conclusion that the risk for induction DDI is low may still be generally valid for potential interactions with CYP3A substrates since the net effect of inhibition may likely predominate and the time-dependent inhibition studies would lead to a clinical DDI evaluation of the effect of the NME on the PK of a sensitive CYP3A substrate to assess clinical relevance. However, a bigger impact is on risk assessment for induction of non-CYP3A targets of PXR, where a low risk for induction DDI with substrates of such enzymes or transporters (e.g., oral contraceptives, digoxin, warfarin) may be erroneously concluded in the absence of mRNA measurements that would be required for observing induction of gene expression, which would not be reflected by CYP3A4 activity measurements alone. Therefore, it is critical that the conclusion of lack of CYP induction be unbiased by factors such as concurrent inhibition or inactivation that could jeopardize the ability to pick up induction potential of an NME.

A recent analysis of CYP3A4 inducers evaluated the performance characteristics of mRNA versus enzyme activity as endpoints in cryopreserved human hepatocyte induction studies with respect to their sensitivity and specificity in the identification of the risk for induction DDIs and inferred that measurement of CYP3A4 mRNA levels represents the most sensitive endpoint for detecting induction in human hepatocytes [105]. Across inducers, the maximum fold increase in CYP3A4 mRNA expression observed in cryopreserved human hepatocytes was generally greater than the corresponding maximum observed fold increase in CYP3A4 catalytic activity [105], consistent with similar findings in a previous study using fresh human hepatocytes [100]. This is explained, at least in part, by concurrent inhibition of CYP3A4 by the inducer. Interestingly, when using mRNA expression of CYP3A4 as the endpoint, even with CAR-selective inducers such as phenytoin and phenobarbital, the fold increase in mRNA of CYP3A4 was consistently higher than the fold increase in mRNA of the other PXR/CAR-regulated drug-metabolizing enzymes examined (CYPs 2B6, 2C9, 2C19, 3A5), leading to the conclusion that CYP3A4 mRNA measurement may suffice as a single endpoint for evaluating risk for induction via the PXR and/or CAR pathways [105].

3.6.3 DDI Risk Assessment from *In vitro* CYP Induction Data

The translation of the results of *in vitro* induction studies performed in human hepatocytes (or adequately qualified cell lines that are responsive to PXR-, CAR-, and AhR-mediated induction, as discussed earlier) to clinical PK DDI risk assessment is an emerging area of science. Empirical cut-offs for fold increase in mRNA or activity over vehicle control and for the percentage of the observed induction by a strong inducer positive control (e.g., rifampin for CYP3A) tested alongside in the same experimental system have been used for classifying *in vitro* positives versus negatives for induction DDI risk assessment. For instance, observation of <40% of the positive

control's effect with use of a strong prototypic inducer as the positive control (e.g., rifampin for CYP3A) has been suggested as sufficient for classifying an NME as an *in vitro* negative for induction, eliminating the need for further clinical evaluation of DDI risk [14]. These approaches unfortunately lack a mechanistic basis and in the absence of a formal statistical optimization of the cut-off values for the specific experimental system, they should be used with caution. A recent study evaluated the performance characteristics of various preselected cut-offs for mRNA and activity endpoints in the categorization of risk for clinically relevant CYP3A induction DDIs using *in vitro* CYP3A4 induction data on 20 clinically significant CYP3A inducers and 15 noninducers in cryopreserved human hepatocytes [105]. This retrospective analysis concluded that a cut-off of fourfold increase in mRNA expression provided 98% sensitivity while maintaining a specificity of $\sim 70\%$ and importantly also demonstrated that the previously suggested cut-off of “40% of the positive control's effect” was associated with an unacceptably low sensitivity (i.e., high false negative rate) [105]. A similar observation questioning the conservativeness of the “40% of positive control” cut-off has been made in a previous study in primary human hepatocytes [106]. Opportunities exist for statistical refinement/optimization of empirical cut-offs using approaches such as receiver operator characteristics analyses that have been used with success in optimizing cut-offs for I:IC₅₀ ratios for assessment of risk for digoxin DDIs via P-gp inhibition [107].

3.6.4 Quantitative Predictions of Induction DDI Magnitude

For a detailed overview of approaches to quantitative IVIVE of induction DDIs, the reader is referred to a recent review article [108]. The first step in this process is to characterize the concentration–effect relationship for the induction observed *in vitro* (either for the mRNA increase and/or increase in enzyme activity), which is typically accomplished using a typical $E_{\max}$ model. IVIVE is then performed assuming that the fold increase in enzyme expression or activity estimated from the *in vitro* concentration–effect relationship at the *in vivo*–relevant concentration of the inducer directly translates to fold increase in intrinsic clearance of metabolism via the induced enzyme *in vivo* [109]. For an orally administered object drug without appreciable intestinal extraction (or under the assumption of lack of meaningful effect of the inducer on its intestinal extraction ratio), the following equation mechanistically allows the translation of the estimated *in vitro* concentration–effect relationship to a predicted interaction magnitude and is essentially similar in form to Equation 3.4 that was presented previously for inhibition DDI:

$$\frac{AUC_{\text{induced}}}{AUC_{\text{control}}} = \frac{1}{(1 - f_{\text{m(CYPi)}}) + \left(f_{\text{m(CYPi)}} \cdot \left(1 + \frac{E_{\max}[\text{Ind}]}{EC_{50} + [\text{Ind}]} \right) \right)} \quad (3.11)$$

With enzyme activity as the endpoint in the *in vitro* induction assay in fresh human hepatocytes, use of unbound systemic steady-state maximum concentration of the inducer to estimate [Ind] and use of unbound EC₅₀ of the inducer *in vitro* (calculated from the *in vitro* induction EC₅₀ and the measured unbound fraction of the inducer in hepatocytes), Equation 3.11 has been successfully used to predict the magnitude of CYP3A induction DDIs [106]. Interestingly, when mRNA expression rather than enzyme activity was used as the *in vitro* endpoint, the performance of

IVIVE was poorer, with a trend for overprediction of the magnitude of clinical DDIs, explained by a higher $E_{\max}$ for mRNA expression compared to enzyme activity as the *in vitro* endpoint. This is not surprising considering that the fold increase in mRNA (a measure of the extent of induction) may not necessarily quantitatively equal the fold increase in enzyme content (which is governed by the efficiency of mRNA translation to protein), which ultimately determines enzyme activity. Nevertheless, this does not necessarily mean that enzyme activity rather than mRNA is a better endpoint for concentration–effect analysis of *in vitro* induction data to enable IVIVE. As discussed earlier, since certain CYP3A inducers also produce some level of inhibition of the enzyme’s activity either via reversible or time-dependent mechanisms, an advantage of the mRNA endpoint is that it is a “pure” reflection of the induction effect unbiased by effects of the inducer on enzyme activity, the IVIVE of which should ideally be performed using approaches previously discussed for inhibition DDIs. If, however, mRNA is used as the endpoint, since a one-to-one correspondence between fold increase in mRNA and fold increase in enzyme content/activity cannot be assumed, there is a need for introducing an empirical scalar (multiplicative adjustment factor on $E_{\max}$ in Equation 3.11) that can be derived using a calibration approach using positive controls within the same experimental system, as has been described by Fahmi and colleagues [83]. In their model, Fahmi and colleagues additionally consider induction of intestinal metabolism, as well as concurrent reversible inhibition and time-dependent inhibition, taken together in the extrapolation of net *in vivo* DDI magnitude using a more general form of Equation 3.10 where the composite multiplicative effect of the inhibition, inactivation, and induction of intrinsic clearance of the affected enzyme is considered [83].

In addition to the above discussed mechanistic/semimechanistic IVIVE approaches to the prediction of induction DDIs, empirical *in vitro*–*in vivo* correlation approaches have also been described. These approaches involve establishment of a descriptive correlation relating the *in vitro* induction data and clinical exposures of the inducer to the observed clinical DDI magnitude for a database of prototypic inducers using a fit-for-purpose empirical mathematical model. Such a model is developed from a database of *in vitro* induction parameters, human exposure information, and clinical DDI outcomes. It is intended to provide a quantitative knowledge management framework that can aid forecasting of the clinical DDI magnitude associated with an NME from *in vitro* induction results and human exposure of the NME at clinically relevant doses (or predicted human exposures, if applied before entry into humans). With the immortalized cell line Fa2N-4 as the *in vitro* system, Ripp and colleagues described application of a relative induction score (RIS) approach for predicting the magnitude of clinical DDIs of inducers with midazolam (sensitive CYP3A substrate) and ethinyl estradiol (substrate for CYP3A as well as other PXR-inducible enzymes) [93]. The parameters $E_{\max}$ and EC_{50} describing the concentration–effect relationship for the fold increase in CYP3A4 mRNA *in vitro* were estimated for each inducer. The RIS for each inducer in the database was then calculated by substituting the unbound clinical $C_{\max}$ of the inducer for the concentration term into the estimated *in vitro* concentration–effect relationship for CYP3A4 induction. The RIS therefore is simply a calculated fold increase in CYP3A4 mRNA at the unbound $C_{\max}$ of the inducer based on the concentration–effect relationship characterized for the inducer *in vitro*. The relationships between the RIS and percent decrease in object drug (midazolam or ethinyl estradiol) AUC were fitted to empirical sigmoid $E_{\max}$ models. These models provide quantitative frameworks to guide prediction of clinical DDI magnitude with

midazolam and ethinyl estradiol from *in vitro* induction parameters (EC_{50} and E_{max}) and the NME's clinical PK data [93]. Although first described using Fa2N-4 cells as the *in vitro* model, the RIS approach has been subsequently qualified as applicable using cryopreserved human hepatocytes as well [110]. Using HepaRG cells as the *in vitro* model, Kanebratt and Andersson described an *in vitro*–*in vivo* correlation relating the ratio of total systemic exposure (AUC) of the inducer to the concentration of the inducer that produced a twofold increase in mRNA expression of CYP3A4 *in vitro* (F_2) as the predictor variable and the clinical DDI magnitude expressed as percent decrease in AUC of a selective and sensitive CYP3A substrate as the dependent variable. Similar to the RIS-based approach, the relationship between the AUC/F_2 ratio and percent decrease in object drug AUC was fitted to a hyperbolic E_{max} model to provide a quantitative framework to guide prediction of clinical DDI magnitude from *in vitro* induction data (F_2) and clinical exposures (AUC) of an NME [98].

3.7 NME AS PRECIPITANT: CLINICAL PHARMACOLOGIC EVALUATION

The key goal of the *in vitro* DDI risk assessment program evaluating the effects of an NME on the activity and expression of CYP enzymes is to guide development of a scientifically appropriate clinical DDI evaluation strategy. Importantly, the *in vitro* data are utilized to eliminate the need for unnecessary DDI studies in cases where it can be concluded that there is a remote possibility of a DDI via CYP inhibition or induction based on the CYP-inhibitory potency or concentration response for CYP induction viewed in the context of clinical exposures of the NME. For instance, there are many recent examples of approved drugs across therapeutic areas where an $I:K_i$ ratio of <0.1 (Fig. 3.2) has been used to conclude that clinical DDIs via inhibition of the particular CYP isoform are unlikely without the need for specific clinical DDI studies. When the *in vitro* data cannot be used to exclude the risk for a potential DDI, prioritization of clinical DDI studies is necessary such that the conduct of clinical DDI studies that are more likely to result in clinically meaningful interactions is planned ahead of the conduct of less critical DDI studies that may result in smaller magnitude interactions.

For CYP-inhibitory DDIs, developing the strategy for clinical pharmacologic evaluation of DDIs is performed using the rank-order approach [22,23], a central assumption of which is that the rank order of potencies of inhibition of multiple CYP isoforms by an NME will be preserved *in vivo* such that the rank order of clinical DDI magnitude with isoform-selective probe substrates would be similar to the rank order of *in vitro* potencies versus each of those enzymes. The use of this approach in guiding clinical DDI strategy is illustrated schematically in Fig. 3.6. Consider, for example, a situation where an NME produces concentration-dependent *in vitro* inhibition of multiple CYP isoforms (*A* through *E*), with *A* being the most potently inhibited enzyme (lowest IC_{50}) followed by *B*, *C*, *D*, and *E* in that order. In such a situation, since *A* is the most potently inhibited enzyme, the clinical pharmacology plan should typically consider a DDI study with a probe substrate for enzyme *A* as the first clinical DDI study. If the results of this DDI study indicate the absence of an interaction, it would not be necessary to conduct DDI studies with probe substrates for enzymes *B*–*E* since they are less-potently inhibited *in vitro* and the resulting clinical DDI magnitude would

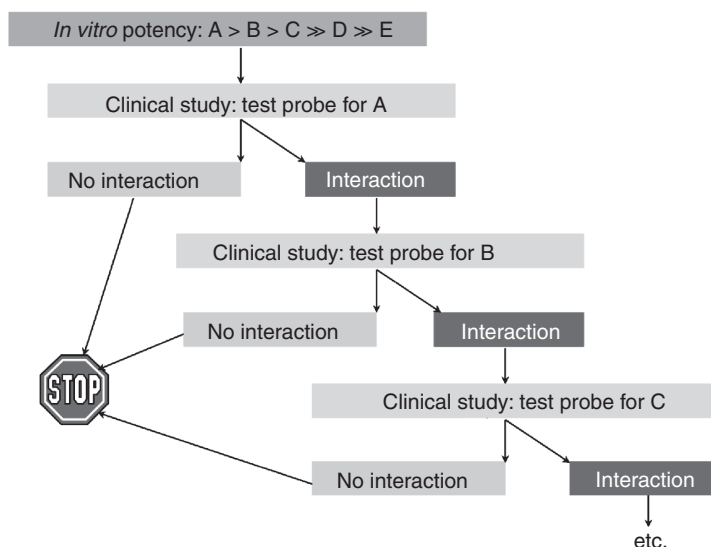


Figure 3.6 Rank-order approach for developing clinical DDI evaluation strategy based on *in vitro* CYP inhibition potency data.

therefore be expected to be lesser than that observed on inhibition of enzyme A. If, however, the clinical DDI study evaluating the effect of the NME on a selective probe substrate for enzyme A reveals a clinically meaningful interaction, the next DDI study to be considered is with a probe substrate for enzyme B (the enzyme with the second lowest IC_{50}), with the need for a DDI study with a probe substrate of enzyme C evaluated based on the results of the DDI study with the substrate of enzyme B. This iterative process is repeated until the lack of a clinical DDI is demonstrated, at which point it can be concluded that no additional DDI studies are necessary. This is analogous to a bridging approach where the first DDI study demonstrating the lack of an interaction serves as the link between the *in vitro* and clinical settings, enabling extrapolation of the *in vitro* data for less-potently inhibited enzymes to the conclusion that clinical DDIs via inhibition of these enzymes is unlikely. The fidelity of the rank-order approach has been retrospectively examined by Obach and colleagues [22,23] from a primary *in vitro* database of CYP isoform-specific inhibitory potency data for 21 drugs for which the results of clinical DDI studies with selective substrates of at least 3 CYP isoforms were available in the published literature. A twofold clinical DDI magnitude (i.e., ratio of exposure of the probe substrate in the presence of the CYP inhibitor in reference to that observed in the absence of the CYP inhibitor) was used to categorize an interaction as positive for purposes of this exercise. This analysis indicated that in 18 out of the 21 cases examined, retrospective application of the above described iterative rank-order approach would have been successful, supporting the performance of this strategy to define the clinical DDI strategy in a drug development setting.

There is precedence for the use of the rank-order approach to inform regulatory decision making. For example, *in vitro* CYP inhibition studies indicated that temsirolimus is an inhibitor of both CYP2D6 ($I:K_i$ ratio of 0.38) and CYP3A4 ($I:K_i$ ratio

of 0.19) [111]. A clinical DDI study was performed to evaluate the effect of temsirolimus at the clinically recommended dose of 25 mg on the PK of the CYP2D6 substrate desipramine. This study concluded the lack of an effect of temsirolimus at the recommended clinical dose on CYP2D6 activity in humans based on 90% confidence intervals for the ratio of geometric least square means of desipramine AUC and $C_{\max}$ (when administered with temsirolimus in reference to when administered alone) being contained within the 80–125% range [112]. The Clinical Pharmacology Review of the temsirolimus NDA concluded that based on the $I:K_i$ ratios from *in vitro* studies, if temsirolimus did not produce clinically meaningful inhibition of CYP2D6, it can be extrapolated that temsirolimus would not inhibit CYP3A *in vivo* [111]. Accordingly, the USPI for temsirolimus indicates that no clinically significant effect is anticipated when temsirolimus is coadministered with agents that are metabolized by CYP2D6 or CYP3A4 [113]. Another example illustrating the application of the rank-order approach is for the Bcr-Abl kinase inhibitor nilotinib. Nilotinib was an *in vitro* inhibitor of multiple human liver microsomal CYP isoforms including CYPs 2C9, 2C8, 3A, 2D6, and 2C19 in decreasing order of potency, with corresponding $I:K_i$ ratios of 32.6, 18.2, 9.6, 2.9, and 1.1, respectively, calculated using the mean steady-state $C_{\max}$ of nilotinib at the recommended dose of 400 mg BID as the measure of $[I]$ [114]. As the clinical DDI implications of these *in vitro* observations were not fully elucidated at the time of approval of this anticancer agent, follow-up clinical DDI evaluation was required as a phase 4 postapproval commitment. On the basis of the observed rank order of CYP-inhibitory potency indicating inhibition of CYP2C9 with the highest potency, it was recommended in the Clinical Pharmacology Review of the nilotinib NDA that the sponsor conduct a DDI study evaluating the effect of multiple-dose administration of nilotinib on the PK of a sensitive CYP2C9 substrate (e.g., *S*-warfarin). Further, it was indicated that if a significant interaction was demonstrated, additional DDI studies evaluating the effects of multiple-dose administration of nilotinib on the PK of a sensitive CYP2C8 substrate and/or a sensitive CYP3A substrate may be needed [114], suggesting application of the rank-order approach in defining the appropriate clinical DDI evaluation strategy based on the results of *in vitro* CYP inhibition studies.

It is important to note that the rank-order approach is not without assumptions: one, is that the factors that determine quantitative translation of the *in vitro* K_i to the magnitude of the clinical DDI are similar across all CYP isoforms. However, this may not be the case when comparing across enzymes that may be expressed in the small intestine and liver (e.g., CYP3A) and those that are mainly expressed in the liver (e.g., other CYP isoforms). This is because inhibition of intestinal presystemic extraction of the probe drug can contribute to the overall magnitude of the observed interaction via CYP3A inhibition in addition to the impact of inhibition of hepatic metabolism, depending on the baseline F_G of the probe (Eq. 3.5). Consider, for example, an NME that inhibits CYP1A2 and CYP3A4, with a marginally greater potency of inhibition of CYP1A2 versus CYP3A4. If a DDI study with a sensitive CYP1A2 substrate (e.g., caffeine) demonstrates lack of an interaction, the possibility of *in vivo* inhibition of CYP3A may still be difficult to completely exclude for orally administered CYP3A substrates subject to substantial intestinal extraction (e.g., midazolam, simvastatin, buspirone). This is because the rank order of $I:K_i$ ratio using systemic $C_{\max}$ of the NME as the estimate of $[I]$ may not directly translate to the rank order of *in vivo* DDI magnitude due to the impact of higher intestinal concentrations of the inhibitor that may contribute to a greater effect on the PK of an orally administered CYP3A substrate

than would be expected based on considerations of systemic exposure alone. It is of interest that the 2010 EMA DDI Draft Guideline specifically addresses this caveat of the rank-order approach and does not permit extrapolation of the lack of inhibition *in vivo* for orally administered CYP3A substrates based on a lower potency of CYP3A inhibition and lack of an *in vivo* interaction with a probe substrate of a more potently inhibited enzyme [31]. Despite this caveat and the recommendation to avoid extrapolation to CYP3A for orally administered substrates, the rank-order approach, nevertheless, provides a useful framework to streamline clinical development planning by guiding clinical DDI strategy, prioritizing the conduct of clinical DDI studies and eliminating the need for unnecessary studies.

3.7.1 Probe Substrate Drug Selection in Clinical DDI Studies

A critical decision in clinical DDI study design for the evaluation of the CYP-inhibitory or inducing effect of an NME is selection of the probe substrate of the affected enzyme. Ideally, the probe drug should be a sensitive and selective substrate of the enzyme whose inhibition or induction by the NME is being investigated *in vivo*. A probe drug can be considered a sensitive substrate of a CYP enzyme if its exposure has been shown to increase greater than or equal to fivefold by a known inhibitor of that enzyme [39]. The reason it is important to select a sensitive probe substrate is because if lack of interaction is demonstrated with a sensitive substrate, it can be presumed that less sensitive substrates will also be unaffected, eliminating the need for conducting additional DDI studies to evaluate the potential clinical relevance of DDIs with less sensitive (but perhaps more clinically relevant) coadministered agents that are metabolized by the same enzyme as the probe drug. Consider, for instance, the drugs theophylline and caffeine. Both methylxanthine derivatives are substrates of CYP1A2, with a major portion of their clearance mediated via oxidative metabolism by this enzyme. It is estimated that ~95% of *in vivo* clearance of caffeine is via CYP1A2-mediated metabolism, whereas the relative contribution of CYP1A2 to theophylline clearance is lower, with an estimated contribution of ~80% as inferred from the results of human radiolabeled mass balance/metabolism and *in vitro* reaction phenotyping studies [49]. As indicated by Equation 3.4, the maximum possible magnitude of a DDI resulting from inhibition of CYP1A2 with caffeine as the substrate ($f_{m(CYP1A2)}$ of ~0.95) would be ~20-fold, whereas this ceiling for maximum possible magnitude of a CYP1A2-inhibitory DDI with theophylline ($f_{m(CYP1A2)}$ of ~0.8) would be calculated to be ~5-fold. Accordingly, DDI studies with the strong CYP1A2 inhibitor fluvoxamine have revealed a 5- to 12-fold decrease in caffeine oral clearance on coadministration with fluvoxamine [115,116], while the effects of fluvoxamine on theophylline AUC are more modest, characterized by an ~3-fold increase in AUC [117,118]. Although theophylline has a narrow therapeutic range and the consequences of inhibition of its clearance by an NME would be greater from a medical standpoint compared to the implications of impairment of caffeine clearance, the above discussed scientific considerations justify the use of caffeine rather than theophylline as a sensitive CYP1A2 probe substrate in the design of clinical DDI studies aimed at evaluating an NME as a potential inhibitor of this enzyme. If a caffeine DDI study establishes the lack of an interaction, it can be presumed that the exposure of theophylline will not be affected by the NME due to the greater sensitivity of caffeine compared to theophylline as a CYP1A2 substrate *in vivo* and conduct of a

theophylline DDI study would be unnecessary. In contrast, if the exposure of caffeine is increased meaningfully (e.g., greater than or equal to twofold), it cannot be presumed that the PK of theophylline will be unaffected. If the NME is being developed in a therapeutic area or patient population where coadministration with theophylline is likely, it may be necessary from a medical standpoint to conduct a DDI study with theophylline to quantify the magnitude of the specific interaction and provide prescribing guidance.

When dealing with CYP3A, due to the expression of the enzyme both in the liver and in the small intestine, the sensitivity of a probe substrate of this enzyme depends not only on the contribution of CYP3A to the overall hepatic metabolism of the probe but also on the extent of contribution of intestinal CYP3A to its presystemic extraction following oral administration (Eq. 3.5). Therefore, two critical parameters: $f_{m(\text{CYP3A})}$ and F_G determine the sensitivity of substrates of CYP3A. Consider, for example, three substrates of CYP3A: midazolam, alprazolam, and buspirone. The rank order of sensitivity of these three drugs to CYP3A-inhibitory DDIs is as follows: buspirone > midazolam > alprazolam. This is readily apparent on examination of Fig. 3.7, where the effects of various CYP3A inhibitors (ketoconazole, itraconazole, grapefruit juice, nefazodone, erythromycin, diltiazem, verapamil, and ritonavir) on midazolam AUC on the X-axis is compared against their corresponding effects on buspirone (circle symbols) or alprazolam (square symbols) AUC on the Y-axis. This analysis indicates that alprazolam is generally less sensitive to CYP3A inhibition than midazolam (square symbols are below the line of identity) but that buspirone is generally more sensitive than midazolam (circle symbols are above the line of identity). These observations are explained by the PK properties of these object drugs, with differences in sensitivity of buspirone and midazolam being explained by buspirone having a smaller F_G than midazolam (0.21 versus 0.5) and differences in the sensitivity of alprazolam and midazolam being explained by differences in $f_{m(\text{CYP3A})}$ (alprazolam: 0.74, midazolam: 0.93) as well as F_G (alprazolam: 0.99, midazolam: 0.5). Clearly, CYP3A is a major contributor to alprazolam clearance *in vivo* such that coadministration with strong inhibitors such as ketoconazole can result in clinically significant increases in alprazolam exposure of approximately fourfold increase in AUC [119] with such coadministration not recommended in clinical practice [120]. However, alprazolam is not a sufficiently sensitive CYP3A substrate and is therefore not a suitable probe for evaluating the potential CYP3A inhibitory effect of an NME in a clinical DDI study. In contrast, both midazolam and buspirone represent sensitive probe substrates of CYP3A for use in clinical DDI studies as coadministration with CYP3A inhibitors (e.g., ketoconazole, ritonavir, itraconazole) has resulted in fivefold or greater magnitudes of DDI with these substrates (Fig. 3.7).

Examples of selective probe substrates for the major human drug-metabolizing CYP isoforms include caffeine and tizanidine for CYP1A2; omeprazole for CYP2C19; desipramine for CYP2D6; and midazolam, triazolam, buspirone, simvastatin, and felodipine for CYP3A [39]. For some CYP isoforms, established sensitive probe substrates are lacking and in these cases, representative reasonably selective substrates will need to be used. In some cases, although the clinically dosed substrate drug is a racemic mixture, the PK of specific enantiomers will need to be characterized using chiral bioanalytical methods due to stereoselective metabolism of individual enantiomers by specific enzymes. For example, although racemic warfarin is administered as the probe substrate for evaluation of CYP2C9 inhibitory effect of an NME, the PK of *S*-warfarin will need to be characterized as only this enantiomer is

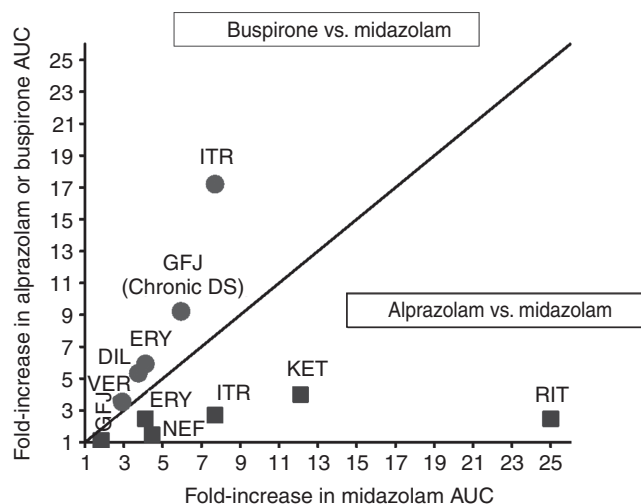


Figure 3.7 Illustration of differential substrate sensitivity to metabolic inhibitory DDIs, based on examination of the effects of different CYP3A inhibitors on the AUCs of three representative CYP3A substrate object drugs midazolam, alprazolam, and buspirone. The squares indicate the relationship between the effects of these inhibitors on alprazolam AUC (Y-axis) and their corresponding effects on midazolam AUC (X-axis). The circles indicate the relationship between the effects of these inhibitors on buspirone AUC (Y-axis) and their corresponding effects on midazolam AUC (X-axis). The line represents the line of identity ($X = Y$). The CYP3A inhibitors included are diltiazem (DIL), erythromycin (ERY), single-strength grapefruit juice (GFJ), chronic double-strength grapefruit juice (GFJ, chronic DS), itraconazole (ITR), ketoconazole (KET), nefazodone (NEF), and ritonavir (RIT).

selectively metabolized by CYP2C9 [31]. Finally, in some cases, specific pathways of metabolism of the probe substrate (as opposed to total clearance) are known to be selectively catalyzed by the enzyme of interest because of the existence of additional metabolic pathways catalyzed by other enzymes. In these cases, a metabolite/parent exposure ratio rather than parent drug AUC is used as the endpoint for DDI studies. For example, hydroxylation of the antidepressant bupropion is a specific index of CYP2B6 activity, as supported by *in vitro* metabolism studies of this pathway in human liver microsomes and using recombinantly expressed human CYP isoforms [121]. Consistently, use of the hydroxybupropion/bupropion exposure ratio as an endpoint in clinical DDI studies is supported by its ability to identify the CYP2B6 inhibitory potential of the antiplatelet agents, ticlopidine and clopidogrel, that produced 90% and 70% decrease, respectively, in the hydroxybupropion/bupropion AUC ratio [122].

3.8 NME AS OBJECT OF DDI

The preceding sections of this chapter have provided an overview of the approaches used for assessment of the risk for DDIs produced by the NME as the precipitant drug and their clinical pharmacologic evaluation, either as a potential inhibitor or inducer of the mechanisms of disposition of coadministered agents. The scientific considerations

underlying risk assessment for DDIs resulting from modulation of disposition of the NME and strategies for their clinical pharmacologic evaluation in drug development will now be discussed using representative examples.

3.8.1 Determinants of DDI involving the NME as the Object Drug

The cornerstone for assessment of risk for PK DDIs for an NME as the object drug is a quantitative understanding of the relative contributions of each mechanism to total human clearance at the level of its specific molecular determinants, that is, specific enzyme or transporter isoforms, denoted variably by f_m , $f_{m(\text{CYP})}$, $f_{m(\text{CYPi})}$, or the CR in previous investigations of CYP-based DDIs [22,44–48,123,124], as is evident from the previously discussed Equations 3.4 and 3.11. Derivation of the $f_{m(\text{CYPi})}$ for an NME to define the contribution of a specific enzyme to its overall clearance is a two-step process. The first step involves an estimation of the *in vivo* contribution of specific biotransformation pathways to total drug clearance in humans, and the second step involves an estimation of the relative contribution of specific enzyme isoforms to each of these *in vivo* biotransformation pathways using *in vitro* reaction phenotyping approaches. The preferred approach for the first step of this two-step process is a radiolabeled mass balance study that includes identification and quantification of metabolites recovered in excreta (urine and feces) to enable construction of the overall human biotransformation scheme for the NME and estimate relative contributions of each primary biotransformation pathway to the overall clearance of the drug [125]. The second step involves an *in vitro* analysis of the principal biotransformation pathways identified in the first step, using an appropriate *in vitro* system that models human biotransformation of the NME, to define the relative contribution of each molecular determinant (e.g., CYP isoform) to the clearance of the NME via that biotransformation pathway. The results from these two distinct steps will need to be integrated to derive the $f_{m(\text{CYPi})}$ for each contributing enzyme isoform to enable DDI risk assessment for the NME as the object drug. For example, if two primary biotransformation pathways A and B are identified for an NME based on analysis of excretory metabolites in a radiolabeled mass balance study, with relative contributions of 30% and 60% to total clearance, and if reaction phenotyping studies on pathways A and B indicate 30% and 50% contributions of CYP3A, respectively, it can be inferred that ~40% of the total clearance of the NME is expected to occur via CYP3A-mediated metabolism (calculated as the sum of 30% of 30% for pathway A and of 50% of 60% for pathway B). Similarly, if the reaction phenotyping studies indicate that the contributions of CYP2D6 to pathways A and B are 50% and 10%, respectively, it can be inferred that ~20% of the total clearance of the NME is expected to occur via CYP2D6-mediated metabolism.

3.8.2 Strategy for Clinical DDI Evaluation

On the basis of current regulatory guidance [31,39], clinical DDI studies evaluating the effects of strong inhibitors/inducers of a participating enzyme are not necessary if the relative contribution of the enzyme to overall clearance is <25%. Accordingly, in the above discussed scenario, the clinical pharmacology plan for such an NME should typically include DDI studies with a strong CYP3A inhibitor (e.g., ketoconazole) and strong CYP3A inducer (e.g., rifampin), whereas studies evaluating the effect of a strong

CYP2D6 inhibitor (e.g., paroxetine) or of the impact of the CYP2D6 polymorphism (e.g., EM vs. PM study) would not be required, as a complete inhibition of CYP2D6 or lack of activity of this enzyme would not be expected to result in a clinically meaningful exposure alteration (contribution <25% would translate to a <1.33-fold increase in exposure on complete inhibition of the enzyme, based on Equation 3.4).

If more than one enzyme is predicted to contribute meaningfully (>25%) to the overall clearance of an NME, DDI studies with strong inhibitors and inducers of each of these pathways should be typically planned. However, a step-wise approach can be used starting with the DDI study evaluating the effect of a strong inhibitor of the highest contributor enzyme followed by conduct of DDI studies with strong inhibitors of the less important contributors (analogous to the rank-order approach described earlier for CYP inhibition studies for the NME as the inhibitor). For each of the contributing enzymes, if a strong inhibitor produces a large and clinically meaningful increase in NME exposure, the need for additional DDI studies with less potent (e.g., moderate) inhibitors of that enzyme will need to be considered to guide DDI risk management and inform prescribing guidance. An example that illustrates this is for the CYP3A substrate everolimus, where the results of DDI studies with strong and moderate CYP3A inhibitors have informed prescribing guidance [126]. The value of such characterization in informing prescribing guidance is discussed later. If a contributing enzyme is polymorphically expressed, with existence of PM genotypes that are deficient in enzyme activity, a PK study evaluating the effect of the polymorphism on the NME's PK (e.g., in EMs vs PMs) can serve the purpose of a DDI study with a strong inhibitor and an additional strong inhibitor DDI study would not be necessary. However, it is important to note that patients with PM genotypes for the major molecular determinant of clearance of the NME may still be at risk of DDIs resulting from inhibition of the alternative "minor" clearance mechanism(s). Although these alternative clearance pathways may be considered minor contributor(s) to total clearance in EMs, they will be important contributor(s) in PMs. For example, consider a drug that is cleared primarily via metabolism by the polymorphic enzyme CYP2D6 ($f_i(\%)$ 80%), with the remainder of its clearance occurring via metabolism by CYP1A2. Although CYP2D6 EMs will not be at risk of DDIs via inhibition of CYP1A2, it is important to note that clinically relevant increases in exposure to the NME can still be produced by CYP1A2 inhibitors (e.g., fluvoxamine) in the CYP2D6 PM subpopulation. As will be discussed later, population-based simulations integrating *in vitro* quantitative metabolism phenotyping data together with the available clinical PK characteristics of the NME in EM and PM subpopulations represent a powerful approach to DDI risk forecasting in such special populations and can additionally inform the need for and design of DDI studies in such special subpopulations.

3.8.3 Risk Assessment Strategies in Early Clinical Development

Although the above described two-step process that requires data from a human radiolabeled mass balance study is the gold standard approach for definitive DDI risk assessment and development of the clinical DDI strategy for the NME as the object drug, it is not always practically feasible in early clinical development planning to complete a radiolabeled mass balance study ahead of initiation of clinical development in patient populations (i.e., ahead of phase 2 proof-of-concept clinical studies). Nevertheless, early assessment of DDI risk is necessary to enable appropriate risk management

strategies in clinical studies in patient populations. Some key questions that will need to be answered include: (i) Are certain DDIs expected to be either large in magnitude or clinically meaningful (e.g., with strong inhibitors of an enzyme that is expected to be a major contributor to clearance of the NME), to require exclusion of specific categories of concomitant medications? (ii) Is the expected magnitude and clinical significance of certain DDIs small enough to permit inclusion of patients on certain concomitant medications (e.g., patients on weak or moderate inhibitors of a contributing enzyme) provided adequate safety monitoring is included? (iii) Is there advice that can be provided on alternate concomitant medications within a particular therapeutic category that may be best suited from the standpoint of DDI risk minimization (e.g., sertraline or citalopram in place of paroxetine or bupropion as a selective serotonin reuptake inhibitor antidepressant for an NME that is expected to be metabolized by CYP2D6; azithromycin or amoxicillin as potential antibiotics in place of clarithromycin for an NME that is expected to be metabolized by CYP3A). In oncology drug development, DDI risk assessment and the development of risk management strategies will need to begin sooner than in many other therapeutic areas considering that phase 1 first-in-human studies are commonly done in patients with advanced cancer that are often on multiple concomitant medications, especially when dealing with cytotoxic NMEs that cannot be studied in healthy volunteers [127]. Therefore, from a practical standpoint, alternate methods for an earlier provisional estimation of $f_{m(CYPi)}$ are often necessary before initiation of phase 2 clinical studies, and in some cases, possibly even ahead of first-in-human studies if drug development is to initiate in patient populations. *In vitro* metabolite profiling and identification studies using radiolabeled substrate and human biomaterials (e.g., human liver microsomes, hepatocytes, S9 fractions) are generally successful in identifying the primary biotransformation pathways of drugs in humans [128]. To enable identification of the primary biotransformation pathways in humans with reasonable confidence in the absence of data from a human mass balance study, a bridging approach may be used that leverages *in vitro* and *in vivo* metabolism data from preclinical species using radiolabeled NME together with *in vitro* metabolite profiling using human biomaterials. The observation of *in vitro* to *in vivo* correspondence in the biotransformation pathways of the NME in preclinical species (e.g., rat, dog) can increase confidence in use of *in vitro* metabolite profiling studies using human biomaterials (e.g., human liver microsomes, human hepatocytes) to estimate the contributions of individual metabolic pathways (e.g., oxidative vs conjugative) to overall human clearance and identify pathways of importance. These data can then be used to design *in vitro* quantitative reaction phenotyping studies of the principal biotransformation pathways to estimate the relative contributions of individual enzyme isoforms to the overall clearance of the NME.

3.8.4 Quantitative Reaction Phenotyping of NME Metabolism

Irrespective of the approach used to defining the contribution of specific biotransformation pathways to NME clearance (*in vivo* radiolabeled mass balance study in humans as the gold standard vs a provisional estimate using radiolabeled *in vitro* metabolite profiling studies with human biomaterials), the second step of the two-step process of estimating $f_{m(CYPi)}$ involves quantitative reaction phenotyping of the principal biotransformation pathways. *In vitro* drug metabolism studies have reached an impressive level of sophistication to permit derivation of a quantitative phenotype

(i.e., description of the percent contributions of each enzyme to total metabolism *in vitro*) of CYP-mediated biotransformation of drug candidates. This is possible using the complementary approaches of selective chemical and/or antibody-mediated inhibition, scaling from kinetic studies on recombinantly expressed individual CYP isoforms, and correlation analyses of metabolic rates of the NME against the content/activity of individual CYP isoforms in a panel of human liver microsomes. A detailed overview of reaction phenotyping strategies and experimental approaches is not within scope of this chapter, and the reader is referred to review articles on the subject [14,129–134].

Although the *in vitro* prediction of *in vivo* glucuronidation-mediated clearance and reaction phenotyping of conjugative metabolism is an emerging area of science, significant progress has been made in recent years with availability of recombinantly expressed uridine diphosphate glucuronosyltransferase (UGT) isoforms, characterization of isoform-selective substrates/index reactions, and limited examples of isoform-selective inhibitors [135]. For example, optimization of experimental conditions for successful quantitative phenotyping of the relative contributions of parallel pathways of direct glucuronidation versus oxidative CYP-mediated metabolism to the overall metabolism of drugs has been recently described [136]. Once the relative contribution of glucuronidation to total hepatic metabolism is defined using such an approach, the relative contribution of specific molecular determinants can be further assessed with the use of recombinantly expressed UGT isoforms, with the use of selective UGT inhibitors where available, and with studies using genotyped liver microsomal samples for UGTs displaying polymorphic expression. A recent example of such an application is the use of kinetic studies on human liver microsomes with functional UGT2B17 (*1/*1 genotype) versus UGT2B17-deficient liver microsomes (*2/*2 genotype) coupled with kinetic studies of recombinantly expressed UGT isoforms to reveal an important contribution of UGT2B17 to the metabolism of the histone deacetylase inhibitor anticancer drug vorinostat [137]. Application of a two-stage approach involving (i) estimation of the relative contribution of glucuronidation versus CYP-mediated metabolism to total metabolic clearance and (ii) reaction phenotyping of the glucuronidation and oxidation components, should therefore permit derivation of initial estimates of the contribution of each molecular determinant (i.e., individual UGT and CYP isoforms) to the overall hepatic clearance of an NME.

3.8.5 Considerations of Pharmacokinetic Properties of the NME in Risk Assessment

Factors that need to be considered in translational assessment of the level of DDI risk include the route of administration and the expected hepatic extraction ratio. For intravenously administered high clearance drugs, clearance is largely dependent on hepatic blood flow, and the PK of such drugs will therefore be relatively less sensitive to alterations in hepatic metabolic intrinsic clearance by coadministered inhibitors or inducers of metabolic enzymes. In contrast, intravenously administered low clearance drugs whose clearance is largely dependent on hepatic intrinsic clearance are relatively less sensitive to changes in hepatic blood flow and will be more sensitive to DDIs via inhibition of hepatic metabolism [138]. Unlike intravenously administered drugs, the systemic exposures of orally administered drugs that are primarily cleared via the hepatic route will be equally sensitive to DDI via inhibition of hepatic metabolism irrespective of their baseline extraction ratio. However, the specific

nature of the PK interaction (i.e., change in shape of the oral dose PK profile due to a DDI) will be different, with the half-life being altered (reflecting alteration of systemic clearance) without an appreciable change in peak plasma concentrations for low clearance drugs and the peak concentrations being principally affected (reflecting an alteration of bioavailability) without an appreciable alteration of half-life for high clearance drugs. Additionally, when CYP3A is identified as a contributor to the human liver microsomal metabolism of an NME, intestinal first-pass extraction of the NME can occur following oral administration, inhibition of which can increase DDI magnitude beyond what would be predicted solely from the *in vitro* liver microsomal metabolic phenotype. Considering the above discussed multiple factors (route of administration, extraction ratio, intestinal extraction, etc.) that influence translation of $f_{m(CYPi)}$ to the projected maximum DDI magnitude, physiologically based IVIVE permits integration of all available data on the NME and inhibitor of interest, to predict clinical DDI magnitude [124,139], as will be discussed later.

3.8.6 Considerations of Transporter Contribution to NME Disposition

Uncertainty in the quantitative translation of the drug-metabolizing enzyme phenotype derived from *in vitro* studies can be expected to be higher when the NME is also a substrate for nonmetabolic transporter-based clearance mechanisms. The Biopharmaceutics Drug Disposition Classification System (BDDCS) provides a useful framework to guide assessment of the importance of intestinal and hepatic transport as well as enzyme-transporter interplay to the NME's disposition and sensitivity to DDIs, resulting from metabolic and/or transporter inhibition [9,140]. Additionally, knowledge of the BDDCS class of the NME can help guide expectations regarding the level of confidence in IVIVE of metabolic DDI risk based solely on the NME's metabolic phenotype derived from *in vitro* metabolism studies. For instance, the confidence in predictions from *in vitro* metabolism data alone may be relatively high for BDDCS class 1 (i.e., highly soluble and highly permeable/extensively metabolized) NMEs. This is because transporter effects are expected to be minimal in the gut and liver, and it should therefore suffice to consider the impact of intestinal and hepatic drug-metabolizing enzyme inhibition for class 1 NMEs. In contrast, considerations of the contributions of intestinal efflux and hepatic uptake and efflux, as well as enzyme-transporter interplay are particularly important for BDDCS class 2 (i.e., poorly soluble and highly permeable/extensively metabolized) NMEs [141]. Importantly, for BDDCS class 2 NMEs that are substrates of both intestinal drug-metabolizing enzymes (e.g., CYP3A, intestinal UGT isoforms) and efflux transporters (e.g., P-gp), large clinically relevant DDIs can result from coadministration with dual inhibitors of the metabolizing enzyme and efflux transporter (e.g., ritonavir, a potent inhibitor of CYP3A and P-gp). Predicting the clinical correlates of the dynamic interplay of metabolism and transport for BDDCS class 2 drugs requires careful consideration of expectations at each anatomic site of the interaction (e.g., intestinal vs hepatic extraction), given the inverse orientation of P-gp and CYP3A in these organs [142]. Notable progress has been made in the IVIVE and phenotyping of hepatic uptake and efflux clearance through the use of cryopreserved hepatocytes, heterologously expressing cellular models of coupled vectorial transport, and vesicular models for *in vitro* kinetic studies of drug uptake by transporters such as OATP1B1 and OATP1B3 and efflux by transporters such as P-gp, BCRP, and MRP2.

Application of a relative activity factor-based scaling approach has enabled quantification of the contributions of individual organic anion transporting polypeptide (OATP) isoforms to overall hepatic uptake clearance [143–146], with simulation of clinical PK correlates made possible through use of physiologically based PK (PB-PK) models [147–149]. Despite these emerging scientific developments, approaches for estimating the clinical PK correlates of inhibiting individual molecular mechanisms of clearance for drugs cleared via mixed mechanisms (metabolism and transport) remain to be characterized from a standpoint of translational validity. Of note, the examples illustrating the application of the aforementioned methods for transporter identification and phenotyping have been primarily focused on nonmetabolized drugs (primarily BDDCS class 3 drugs) and additional evaluations are necessary to qualify more general applications of the framework for metabolized compounds that may additionally be substrates of active efflux and/or uptake transporters.

3.8.7 Risk Assessment for DDIs with Inducers

An important difference between susceptibility of an NME as an object drug to induction versus inhibition DDI is that when an enzyme is a minor contributor (e.g., $f_{m(CYPi)} < 25\%$) to total clearance of an NME, while the magnitude of an inhibitory DDI will be relatively small even on essentially complete inhibition of the enzyme (< 1.33 -fold), the magnitude of an interaction resulting from strong induction of that enzyme can reach clinically significant magnitudes [123]. Therefore, the relative contribution of inducible enzymes will need to be very small to conclude that the risk for induction DDI is low (e.g., resulting in a $< 25\%$ decrease in NME exposure). For example, it has been shown that even if the contribution of CYP3A to the overall clearance of an object drug is only 25%, the enzyme-inducing anticonvulsants phenytoin and carbamazepine can be expected to produce an ~ 40 – 60% decrease in exposures of the object drug and the strong CYP3A inducer rifampin can be expected to produce an $\sim 65\%$ decrease in the object drug exposure, due to the high intrinsic efficiency of these agents as CYP3A inducers [123].

Additionally, it is important to recognize that induction of multiple drug-metabolizing enzymes and transporters occurs in a pleiotropic manner via common upstream nuclear receptors that regulate the expression of a battery of drug-metabolizing enzyme encoding genes. Therefore, even though the relative contribution of CYP3A per se may be well below 25%, if other PXR-inducible enzymes such as CYPs 2C9 and 2C19 or drug transporters such as P-gp contribute additionally to the overall clearance of the NME, the possibility of clinically significant decreases in exposure following administration of strong PXR agonist inducers such as rifampin cannot be excluded. A recent example illustrating this concept is the interaction between the strong CYP3A inducer rifampin and the antibiotic linezolid, which is not directly metabolized by CYP enzymes, not a substrate of P-gp, and is primarily metabolized by a nonenzymatic chemical oxidation mechanism. In a clinical DDI study, rifampin produced an $\sim 30\%$ reduction in linezolid exposure, with *in silico* simulations demonstrating that an interaction of such magnitude can be explained by a CYP3A contribution to linezolid clearance of $< 10\%$ [150]. Therefore, when determining the need for a clinical DDI study for an NME with a strong inducer (e.g., rifampin), it may be important to consider such studies even if the contribution of inducible clearance pathways is $< 25\%$, because of the possibility that the contribution

of the inducible clearance mechanism can be large enough in the induced state to meaningfully increase the overall clearance of the NME and result in clinically relevant decreases in systemic exposure.

3.9 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING AND SIMULATION OF DDIs

The approaches discussed thus far, while useful for initial risk assessment and quantitative predictions of expected interaction magnitude, nevertheless, represent a simplification of the problem of IVIVE of DDIs, as they assume a single constant value of $[I]$, substrate concentrations below the K_m , and do not consider the time-varying concentration of the inhibitor or inducer, or the object drug in the context of typical clinical dosing regimens. These aspects can only be incorporated when the theoretical frameworks for IVIVE of CYP inhibition or induction DDI discussed in the previous sections of this chapter are implemented in the context of a PB-PK model. Use of such a model-based approach allows prediction of not only the fold increase in exposure (AUC) of the object drug but also simulation of the expected PK profile following administration of the metabolic inhibitor or inducer, enabling forecasting of effects on parameters such as peak plasma concentration and half-life. With the availability of automated software to enable such simulations from *in vitro* data the application of full PB-PK model-based methods in drug discovery and development is increasing. The increased use of such model-based approaches is exemplified by many recent publications highlighting the utility of these approaches in the prediction of DDI magnitude [37,38,68,124,139,151,152]. This framework allows simultaneous modeling of object and precipitant drug PK including complexities such as nonlinear PK characteristics, metabolite kinetics and interactions resulting from effects of metabolites in addition to the parent perpetrator agent, and can additionally permit incorporation of multiple mechanisms of interactions (e.g., reversible inhibition, MBI, induction) when dealing with mixed mechanism precipitants with complex properties [38,81,82,153]. In addition to enabling simulation of the typical effect of the metabolic inhibitor on the full PK profile of the object drug, tools such as the Simcyp[®] population simulator allow forecasting of the associated intersubject variability of the DDI in the population because variability in demographic (e.g., body weight), physiological (e.g. liver blood flow), biochemical (e.g., abundance of individual CYP isoforms in the liver, serum albumin concentration), and genetic (e.g., population frequency of CYP2D6 EM vs PM genotypes) parameters are all considered together with drug-specific *in vitro* input parameters (e.g., CYP inhibition K_i , K_m for metabolism of the object drug by individual CYP isoforms) in forecasting outcomes of the DDI in the population [43,154,155]. The use of PB-PK model-based predictions of the outcome of DDIs can be particularly important in guiding the design of clinical DDI studies ahead of conducting them. Simulation studies can be particularly valuable in estimating the required duration of washout between periods of crossover DDI studies as well as guiding the minimum necessary duration of dosing of the precipitant drug to elicit the maximum possible interaction magnitude, both in context of the investigational agent as the object and as the precipitant. A recent example of such an application is in the evaluation of the impact of dose, dosing duration, and dosing frequency of ketoconazole as a strong CYP3A inhibitor on the magnitude of DDI with CYP3A substrates of varying PK characteristics to develop

general recommendations on the optimal dosing regimen of ketoconazole for use as a strong CYP3A inhibitor in clinical DDI studies based on the object drug's characteristics [156]. These approaches can be particularly useful in evaluating whether the magnitude of the interaction can be offset by altering the timing of administration of the object drug and the metabolic inhibitor [157], enabling scientifically guided risk management strategies in later-phase clinical trials, where appropriate, based on the PK properties and clinical dosing regimens of the object and precipitant drugs.

When dealing with the investigational agent as the object drug, physiologically based modeling and simulation can be particularly useful in projecting the impact of DDIs in special patient populations (e.g., patients with renal impairment or genetically distinct subpopulations such as CYP2D6 PMs), where the relative contributions of individual clearance mechanisms to the overall clearance of the investigational agent can be different from those in the general patient population. Consider, for example, a drug that is metabolized by CYP2C19 (major contributor) and CYP3A4 (minor pathway with <20% contributor to total clearance). The effects of CYP3A4 inhibitors can be expected to be minimal and not of clinical significance in patients with functional CYP2C19, whereas this cannot be expected for CYP2C19 PMs where loss of functional activity of CYP2C19 can result in this drug essentially behaving as a "pure" CYP3A substrate, with potentially major risks for clinically important DDIs with strong CYP3A inhibitors. Conducting a DDI study in PMs of CYP2C19 can be operationally challenging, considering the low frequency of the PM genotypes in Western populations. The challenges can be even worse if the investigational agent is a cytotoxic antineoplastic agent that cannot be dosed in healthy volunteers, which would then necessitate the conduct of such a DDI study in a genetically defined special population of cancer patients. Simulations of the expected effect of strong inhibitors (e.g., ketoconazole) on the PK of such a drug in CYP2C19 PMs, using *in vitro* reaction phenotyping data and available clinical PK data in EM versus PM subjects as prior knowledge can represent a powerful approach to forecasting the level of DDI risk in such special populations. Whether such simulations can be solely used in lieu of a dedicated DDI study will, of course, depend on the specific case under consideration and factors such as the population frequency of the special subpopulation, predicted magnitude of the interaction, sensitivity of the predictions to uncertainties in input parameters or key assumptions, as well as the clinical impact of the predicted interaction based on exposure–response understanding of safety of the drug in question. Nevertheless, having the results of such simulations in a drug development setting can guide determination of the timing and design of such special population DDI studies and facilitate scientific discussions with regulatory agencies on the proposed clinical DDI risk assessment and evaluation strategy. Another setting where PB-PK model-based simulations can be helpful is in the forecasting of level of DDI risk via a new route of administration to guide product labeling. An example of such an application is the use of a model-based approach to predicting the magnitude of effect of the strong CYP3A inhibitor ritonavir on the PK of intravenously administered sildenafil, with qualification of the model based on its ability to adequately predict the interaction between oral sildenafil and ritonavir [158]. This analysis provided support for the conclusion that the magnitude of the effect of CYP3A inhibitors on intravenous sildenafil can be expected to be lesser than for oral sildenafil, resulting in the following text in the Clinical Pharmacology section of the USPI: "*Predictions based on a pharmacokinetic model suggest that drug–drug interactions with CYP3A inhibitors will be less than those observed after oral sildenafil administration*" [159].

3.10 DESIGN, ANALYSIS AND INTERPRETATION OF CLINICAL DDI STUDY RESULTS

When designing a clinical DDI study between two drugs, factors requiring consideration include the PK properties of the object and precipitant drugs, the mechanism of the expected interaction (e.g., reversible inhibition, time-dependent inhibition, induction) and wherever possible, the expected magnitude and time course of the interaction (e.g., as predicted from the *in vitro* data and available understanding of the PK properties of the object and precipitant drugs). When evaluating the effect of an NME as an inhibitor or inducer on the PK of a selective probe substrate of an enzyme, the most commonly employed approach is to estimate the effect of multiple-dose administration of the NME on the PK of a single dose of the probe substrate. The NME is typically administered at the highest dose and administration frequency of clinical relevance to estimate the maximum possible magnitude of the interaction. The NME should be dosed until PK steady-state conditions are achieved before administration of the probe substrate. Dosing of the NME should be continued through the period of PK characterization of the probe substrate to maintain the condition of metabolic inhibition or induction. When evaluating the effect of a probe inhibitor or inducer on the PK of an NME, the most commonly applied design involves evaluating the effect of steady-state administration of the selected inhibitor or inducer on the single-dose PK of the NME, provided the multiple-dose PK of the NME are predictable from its single-dose PK profile (i.e., there are no time-dependent nonlinearities in the PK of the object NME).

3.10.1 Common Clinical DDI Study Designs

Many different designs are used for clinical DDI studies [160]. Examples of commonly employed DDI study designs are shown in Fig. 3.8 and will be discussed here. A widely utilized and robust DDI study design is the randomized crossover design (Fig. 3.8a). In this design, subjects are randomized to one of two sequences: a or b. Subjects randomized to sequence a are administered a single test dose of the object drug for PK characterization in period 1 followed by administration of the object drug for PK characterization during multiple-dose administration of the precipitant (at PK steady state of the precipitant) in period 2 to enable assessment of the effect of the precipitant on the object's PK. Dosing of the precipitant drug is continued through the period of PK assessment of the object drug. In sequence b, the order of treatments is reversed such that the single-dose PK of the object drug are evaluated in the setting of steady-state precipitant coadministration in period 1, followed by evaluation of the single-dose PK of the object drug in the absence of the precipitant in period 2. Periods 1 and 2 should be separated by an adequately long washout period that permits complete washout of both the object drug and the precipitant and ensures recalibration to baseline conditions of metabolic activity at the start of the second period. When the precipitant is a reversible CYP inhibitor, the duration of washout can be generally determined easily as five half-lives of the precipitant (and of any known CYP-inhibitory circulating metabolites). In contrast, when the precipitant is a mechanism-based inhibitor or an inducer, the washout period will typically need to be longer than anticipated from PK considerations alone, as the time course of recovery from CYP inactivation or of deinduction, as discussed previously, are dependent not only on the half-life of the precipitant drug but also importantly governed by the biological turnover half-life of

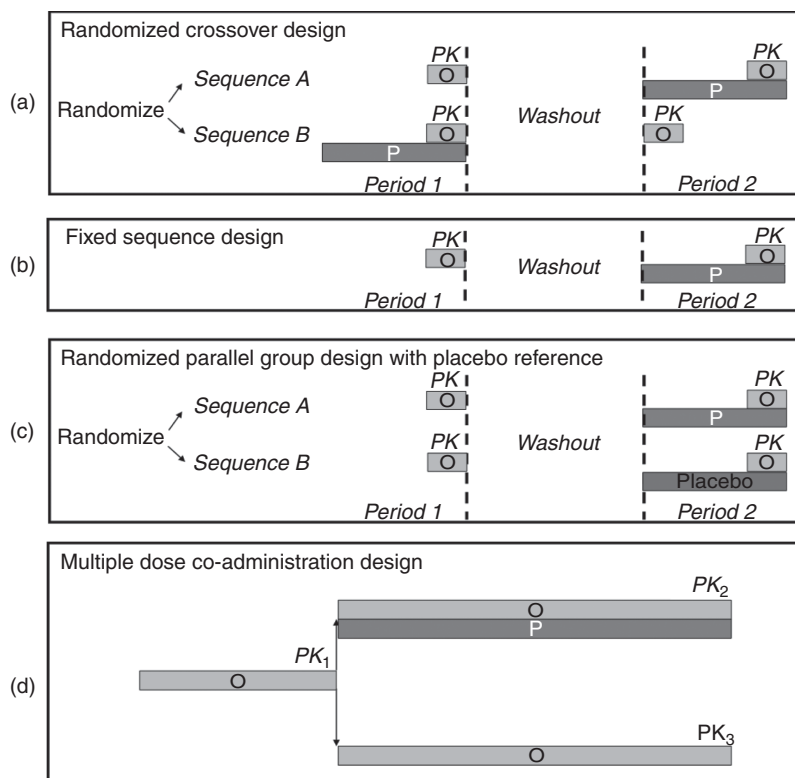


Figure 3.8 Schematic illustration of clinical DDI study designs (O, object drug; P, precipitant drug; PK, pharmacokinetic sampling).

the enzyme that is subject to inactivation or induction. The duration of the washout phase should be adequately long for scientific robustness of the study design. However, in the interest of operational feasibility, the washout period should not be longer than necessary, and optimization is essential. This can be done using physiologically based PK modeling and simulation of DDIs. In some cases, a randomized crossover study design may not be feasible because of a very long half-life of one or both drugs that would make it impractical to incorporate a sufficiently long washout phase between the study periods. In these cases, a fixed-sequence study design (also sometimes referred to as a *one-way crossover design*) is often used where the PK characterization of the object drug alone precedes the PK characterization of the object drug administered with the precipitant (Fig. 3.8b). The duration of the washout period with such a design needs to consider only the half-life of the object drug.

The robustness of the randomized crossover study design over the fixed-sequence design stems from the randomization to one of two sequences such that any potential confounding impact of period and/or sequence effects can be minimized. The risk of a sequence/period effect on PK endpoints is generally low (provided washout is adequate and there is no carryover between periods), given the objective nature of these measurements. Therefore, in most cases, a fixed-sequence study design will be appropriate. In fact, the fixed-sequence design was the most commonly utilized study

design (70% of all studies evaluated) for DDI studies based on an analysis of New Drug Application submissions to the US FDA during the period from December 1995 to November 1996 [160]. However, if the DDI study includes measurement of subjective PD endpoints (e.g., CNS depressant effects using a visual analog scale) in addition to PK endpoints, the randomized crossover design is recommended to minimize potential subjective influences on the study conclusions. This may be applicable, for instance, in DDI studies aimed at evaluating the effect of MDR1 P-gp inhibition on the PK of an NME that is known to be a P-gp substrate. In addition to increasing systemic drug exposure by an increase in the extent of oral absorption (via inhibition of intestinal efflux transport) and/or a decrease in renal and/or biliary clearance, it is possible that P-gp inhibition at the blood–brain barrier may additionally increase CNS drug availability beyond the extent that would be expected based on increases in systemic drug exposure alone. There is currently little clinical evidence to support the clinical significance of such DDIs that may increase human CNS drug distribution via inhibition of P-gp at the blood–brain barrier, although some examples have been reported in experimental clinical pharmacology studies [161,162]. There is little rationale to advocate direct assessment of CNS drug distribution via invasive approaches such as sampling of cerebrospinal fluid in DDI studies evaluating the effects of a P-gp inhibitor (e.g., quinidine) on the PK of a P-gp substrate NME. Nevertheless, if the NME is a P-gp substrate with potential for target or off-target CNS PD effects, it may be useful to include CNS PD and/or safety evaluations in addition to systemic PK measurements in DDI studies evaluating the effect of a P-gp inhibitor. Considering the subjective nature of such endpoints, it would be important to utilize a randomized crossover design instead of a fixed-sequence design in such DDI studies, to permit unbiased evaluation of the PD and/or CNS safety data of special interest. If a randomized crossover design is not feasible (e.g., due to a long half-life of the precipitant drug) and if the potential bias introduced by a fixed-sequence design is to be avoided (e.g., if subjective PD endpoints are being measured), an alternate design that may be used is the randomized parallel group design with a placebo reference (Fig. 3.8c). In this design, subjects are randomized into one of two groups. One group of subjects undergo PK characterization of the object drug alone in period 1 followed by PK characterization of the object drug in the setting of coadministration with the precipitant drug in period 2, and a second group of subjects undergo PK characterization of the object drug alone in period 1 and of the object drug following blinded administration of a placebo in period 2. Such a design provides the ability to separate out the effect of a potential DDI from any confounding period effects.

When evaluating the effect of a probe inhibitor or inducer on the PK of an NME as the object drug, if the PK of the object NME are characterized by time-dependent nonlinear kinetics (e.g., autoinduction or autoinhibition of clearance) and if understanding the effect of enzyme inhibition or induction on the PK of the NME following multiple-dose administration are of primary clinical relevance, the DDI study may need to be designed as a steady-state coadministration study because conclusions from a single-dose DDI study may not be translatable to the clinically relevant setting of multiple-dose administration. As an example, the exposure of single-dose imatinib is increased by the strong CYP3A inhibitor ketoconazole by 40% in a healthy volunteer DDI study [163]. However, a subsequent DDI study in cancer patients in the clinically relevant setting of multiple-dose administration of imatinib failed to reveal an effect of short-term ritonavir (a CYP3A inhibitor that is comparably potent as ketoconazole, with

short-term administration selected to avoid its CYP3A induction effects) on the steady-state exposure of imatinib [164]. Although the mechanism underlying this difference is not entirely clear, it is possible that it may be explained by CYP3A inhibition by imatinib [165] that may translate to an autoinhibition of the CYP3A-mediated component of its oral clearance, thereby resulting in a potentially decreased contribution of CYP3A to steady-state imatinib oral clearance compared to the corresponding contribution following single-dose administration. When designing a multiple-dose coadministration DDI study, the steady-state PK of the NME would need to be characterized following multiple-dose coadministration with the probe inhibitor or inducer and compared to the steady-state PK of the NME administered alone. The multiple-dose coadministration of the probe inhibitor or inducer will need to be performed for a sufficiently long period until attainment and characterization of the new PK steady state of the NME under maximally inhibited or induced conditions of its metabolism. As discussed earlier, optimization of study duration can be achieved using physiologic model-based IVIVEs and comparison of the performance of competing study designs via simulations. One approach to a multiple-dose coadministration DDI study is to utilize a randomized crossover design (similar to that shown in Fig. 3.8b, except that multiple dosing of the NME object drug would be employed instead of single-dose administration). Such a design can be potentially onerous to execute as the study duration will be quite long (especially for drugs with long half-lives), as it would be comprised by the time taken for achievement of PK steady state of the NME alone, the time taken for achievement of PK steady state of the NME in the setting of coadministration of the precipitant drug, and the interperiod washout, which would need to be long enough to ensure return to baseline metabolic conditions and ensure complete washout of both the drugs. Therefore, when multiple-dose coadministration is required for a steady-state DDI evaluation, the use of a fixed-sequence design may be preferred as a simpler alternative (similar to that shown in Fig. 3.8a, except that multiple dosing of the NME object drug would be employed instead of single-dose administration). Even in the setting of a fixed-sequence design, if the half-life of the object drug is long, the total study duration for a steady-state DDI study can be excessive and potentially impractical. In such cases, alternate study designs can be considered, which eliminate the interperiod washout phase. An example of such an alternate design for a multiple-dose coadministration DDI study is shown in Fig. 3.8d. In this design, multiple-dose administration of the object NME is initiated in all subjects and continued until PK steady state is approximately achieved, at which point a PK characterization over the steady-state dosing interval is performed (PK₁). Following completion of this PK assessment of the NME alone, one-half of the subjects continue multiple-dose administration of the object NME alone and another half of the subjects continue multiple-dose administration of the object NME, with coadministration of the probe inhibitor or inducer. The assignment of subjects to the treatment of NME alone versus NME plus probe inhibitor or inducer can be based on randomization that is performed at entry into the study. Both groups of subjects continue their respective multiple-dose study treatments for a prespecified duration that is designed to be adequate to allow achievement of the new PK steady state of the NME when coadministered with the probe inhibitor or inducer. At this point, all subjects undergo PK characterization of the object NME again (PK₂ representing the assessments in subjects coadministered the NME with the probe precipitant; PK₃ representing the assessments in subjects that continued administration of the NME alone). Statistical analysis of the PK₂/PK₁ ratios of systemic exposures

in reference to the PK_3/PK_1 ratios of systemic exposures of the object NME should permit estimation of the magnitude of the interaction.

3.10.2 Statistical Considerations in Design and Analysis of Clinical DDI Studies

A key consideration in the design of clinical PK DDI studies is the determination of an appropriate sample size to enable an informative analysis of the resulting data to guide interpretation of the clinical significance of any observed interaction. DDI studies are generally designed with a sufficient number of subjects that can permit estimation of the magnitude of the interaction with a reasonable level of precision, based on prior knowledge of the variability in PK of the object drug. The operational metric of variability is the within-subject variance in the object drug's PK endpoints (e.g., AUC and C_{max}) for designs where each subject serves as his/her own control and undergoes PK characterization of the object drug alone as well as in the setting of coadministration of the precipitant drug. For less commonly utilized parallel group DDI study designs, the operational metric of variability is the total variance in PK endpoints thereby translating to a larger sample size. The level of precision desired in the estimate of interaction magnitude depends on the broader strategic objective of the DDI study. If the objective is to claim lack of an interaction in proposed labeling and if the exposure–Response relationships for efficacy and safety of the object drug have not been adequately understood, the study would need to be sized to include a sufficient number of subjects for the 90% confidence intervals for the ratio of geometric mean AUC and C_{max} (object plus precipitant in reference to object alone) to be contained within the 80–125% range (default bioequivalence range). However, if the intent is to estimate the magnitude of the interaction with a level of precision that is sufficiently high to allow decisions to be made regarding the potential clinical significance of the interaction to inform risk management strategies and prescribing guidance, sizing the study to show bioequivalence may not be necessary. When dealing with the NME as the object drug, the desired level of precision in estimating the magnitude of the effect of a probe inhibitor or inducer should be ideally based on no-effect boundaries derived from quantitative clinical pharmacologic understanding of the therapeutic index of the object drug [1]. When robust exposure–response relationships for the therapeutic and adverse effects of the NME are available, such understanding provides a powerful framework to prospectively define clinically relevant no-effect boundaries (as opposed to defaulting to the standard bioequivalence criteria) and to interpret the results of the DDI study to guide next steps. PK data from a DDI study are typically analyzed using noncompartmental methods to calculate individual values of PK parameters (e.g., AUC and C_{max}) of the object drug when administered alone and when administered with the precipitant drug. Statistical analysis of PK parameters is generally done using a mixed effects analysis of variance on log-transformed AUC and C_{max} , with estimation of the ratio of geometric mean AUC and C_{max} (object plus precipitant in reference to object alone) and associated 90% confidence intervals. This approach of estimation of interaction magnitude is preferred over hypothesis testing and use of p -values for the assessment of the statistical significance of a DDI, as has been illustrated using representative examples from a review of analyses of DDI studies in New Drug Application submissions to the US FDA during the period from December 1995 to November 1996 [160]. As a hypothetical example, consider the following two scenarios: Study A yielding an estimated ratio of geometric mean AUCs of 89%,

with a 90% confidence interval ranging from 85% to 95% and a p -value of <0.05 ; and Study B yielding an estimated ratio of geometric mean AUCs of 120%, with a 90% confidence interval ranging from 60% to 220% and a p -value $\gg 0.1$. Although Study A revealed a “statistically significant” interaction based on a p -value of <0.05 , this is simply a function of the low within-patient variability in the object drug’s exposure resulting in the ability of the study to demonstrate statistical significance in the small observed decrease in object drug exposure (by 11% on average). However, the 90% confidence interval for the ratio of geometric mean AUC is contained within the 80–125% range, and the observed mean decrease in exposure of 11% would be of no clinical relevance in most cases. Therefore, the conclusion of statistical significance based on a p -value alone is not particularly meaningful or informative to guide next steps in terms of whether the observed interaction requires risk management via dosage increases and/or other precautionary guidance to prescribers. In fact, no further clinical action would typically be necessary for DDIs of this magnitude, and this inference can be reached based on the point estimate of interaction magnitude and the associated 90% confidence interval as a measure of precision in its estimation. In contrast, although the p -value for Study B was $\gg 0.1$, a conclusion of the lack of interaction based solely on the “lack of statistical significance” is not appropriate. Unfortunately, the results of Study B cannot be considered as adequately informative to guide next steps unless exposure–response considerations can be used to justify a wide therapeutic range of the object drug such that decreases in exposure by up to 40% or increases in exposure as large as 2.2-fold would not be of clinical relevance. The poor precision in the estimated interaction magnitude in Study B is likely due to high within-patient variability in the object drug’s exposure. Therefore, although the point estimate of interaction magnitude (1.2-fold increase in AUC) is relatively low, the large uncertainty in its estimation precludes definitive conclusions regarding whether or not there is a clinically meaningful DDI. Again, the p -value is of no value in reaching this conclusion, and it would be inappropriate to use it to suggest the lack of a meaningful interaction. The ability to show statistical significance or the failure to demonstrate statistical significance should not drive interpretation of clinical DDI study results and the use of a confidence interval approach (90% confidence interval about the geometric mean ratio of the AUC and $C_{\max}$ with and without the interacting drug) as opposed to testing for statistical significance is considered as appropriate in the analysis and interpretation of DDI study results. This is reflected in expert opinion articles [166] as well as the Draft FDA and EMA DDI guidance documents [31,39].

3.10.3 Assessment of Clinical Relevance of DDIs

A key strategic objective of clinical DDI studies performed using probe substrates or inhibitors is to utilize the results to evaluate the clinical relevance of potential interactions of the NME with other object drugs with similar clearance mechanisms or precipitant drugs with similar CYP or transporter inhibitory or inductive properties. As discussed previously, use of a sensitive substrate of the target CYP isoform (when investigating the NME as a potential precipitant) or a strong inhibitor or inducer of the contributing CYP isoform (when investigating the NME as the object drug) in the probe DDI study are important to enable assessment of relevance of the interaction with similarly or less sensitive substrates, or with similarly or less potent inhibitors or inducers. This is important because the conduct of each and every possible DDI study

is neither practical nor warranted unless a specific drug combination is of substantial clinical relevance in the target patient population such that specific investigation of a particular interaction will yield medically important information to guide prescribing. For example, consider an NME that is an inhibitor of a drug-metabolizing enzyme, as confirmed in a clinical DDI study evaluating its effects on the PK of a selective and sensitive substrate of the inhibited enzyme. If this NME is expected to be frequently coadministered in clinical practice with a specific object drug with a narrow therapeutic range that is metabolized by the inhibited enzyme, an exact recommended dose reduction or a precise dose titration plan for the object drug of interest may be desirable. This may not be possible to derive based solely on the results of the DDI study conducted with a probe substrate. In such a situation, it can be useful to perform an additional DDI study with the specific object drug of relevance to the intended setting of clinical use of the NME, with the primary objective of contributing to specific prescribing guidance in patients requiring such coadministration.

Some key questions that often need to be considered as part of evaluating the implications of the findings from a clinical DDI study and informing prescribing guidance include:

1. Can the drugs be safely coadministered through appropriate risk management (e.g., safety monitoring, dose reduction) or should use be contraindicated?
2. If the interaction is clinically meaningful, is a dosage adjustment necessary or is it sufficient to advise cautious coadministration with appropriate clinical safety monitoring?
3. If the NME is the object drug, are additional DDI studies needed with other clinically relevant and/or less potent inhibitors or inducers to inform prescribing guidance?
4. If the NME is the precipitant drug, are additional DDI studies needed with other clinically relevant and/or less sensitive substrates of the affected enzyme to inform prescribing guidance?

Modeling and simulation can be particularly helpful in answering these questions with quantitative rigor. The answers to questions 1 and 2 are best developed based on the observed DDI magnitude viewed in context of the clinical safety profile of the drug, exposure–response relationships for efficacy endpoints, and exposure–response relationships for adverse effects. If recommendation of a dose modification of the NME object drug is considered appropriate, integration of DDI study results into population PK/PD models for safety and efficacy of the NME can permit simulation of the performance characteristics of the proposed dose-modification guidelines, an approach analogous to that described for developing dosing recommendations for special populations of patients with impaired renal or hepatic function [167,168]. Consider, for instance, a scenario where the results of a clinical DDI study of an orally administered NME with a strong inhibitor of its metabolism indicate a substantial exposure increase such that a dose reduction of the NME would be necessary to allow coadministration with strong inhibitors. The results of the DDI study can be used to calculate the reduced dose of the NME based on the fold increase in NME exposure estimated in the clinical DDI study. However, in many such instances, only a limited number of dose strengths of the NME may be available and delivery of the calculated reduced dose

may require a unit dose strength that is not available. In such situations, modeling and simulation can represent a valuable approach to test the performance characteristics of alternate dose reduction guidelines that may potentially be supported by the available dose strengths of the NME without having to manufacture and qualify biopharmaceutical performance of lower dose strengths. The key question is whether the proposed dose reduction guideline can be expected to result in a population distribution of NME exposures that would not compromise the benefit/risk balance in patients receiving the strong enzyme inhibitor, which can be addressed using simulations that integrate the DDI study results into the results of population PK/PD models for safety and efficacy endpoints.

Questions 3 and 4 can be addressed using PB-PK modeling and simulation leveraging the available *in vitro* data on both clinically studied and unstudied interactions together with the PK data from the completed clinical DDI studies to simulate the expected outcomes from unstudied interactions. Sensitivity analyses evaluating the impact of unknown or inaccessible model parameters can be particularly helpful in assessing the level of uncertainty in predictions to guide the decision of whether additional clinical DDI studies are of added value.

3.11 EXAMPLES ILLUSTRATING TRANSLATION OF DDI KNOWLEDGE TO PRESCRIBING GUIDANCE

The interpretation of clinical significance of DDIs and the use of clinical DDI study results to guide product labeling, both for the NME as the object drug and for the NME as the precipitant drug, can be better appreciated using some representative examples. If a DDI is characterized by a large clinically significant exposure increase or decrease, and/or a large intersubject variability in interaction magnitude, recommendation of coadministration of the interacting drugs through use of dose modification and/or clinical safety monitoring guidelines may sometimes be difficult. This is because it may be questionable whether a positive benefit/risk balance is preserved when indicating use of the drug in patients receiving such major interacting concomitant medications. This is particularly the case when the object drug has a narrow therapeutic range such that serious unacceptable toxicities can be expected at higher exposures that may be observed as a consequence of DDIs. In such cases, the combination may be contraindicated or alternatively it may be recommended that such concomitant use be avoided. However, if the interaction magnitude is not excessive, it may be possible to utilize the results of controlled clinical DDI studies to provide pharmacokinetically derived dose-modification recommendations in prescribing guidance. These concepts are illustrated using three representative examples (tizanidine, everolimus, and darunavir/ritonavir) that demonstrate the translation of knowledge gained from DDI investigations to the development of risk management guidelines in prescribing guidance.

Example 1: Tizanidine. The centrally acting α_2 -adrenergic agonist tizanidine indicated for the management of spasticity is an ultrasensitive substrate of CYP1A2. When administered with the strong CYP1A2 inhibitor fluvoxamine, a 33-fold mean increase in tizanidine AUC is observed, with a 12-fold mean increase in $C_{\max}$, and a nearly 3-fold prolongation of half-life [169]. Importantly, these PK changes were accompanied by serious clinical consequences of hypotension

(characterized by a mean systolic blood pressure of ~80 mm Hg when coadministered with fluvoxamine) and marked drowsiness and psychomotor impairment reflecting exaggerated PD secondary to the large increase in tizanidine exposure. Additionally, the variability in interaction magnitude was also significant with the 33-fold mean increase in AUC comprised of a range of 14- to 103-fold across individual subjects. In another DDI study that evaluated the effect of another strong CYP1A2 inhibitor ciprofloxacin on tizanidine's clinical pharmacology, a 10-fold mean increase in AUC was observed with clinically significant potentiation of its hypotensive and sedative effects [170]. Considering the large magnitude of these exposure increases and their associated serious clinical consequences that can jeopardize patient safety, it is clear that coadministration of tizanidine with fluvoxamine or ciprofloxacin would not preserve a positive benefit/risk balance for patients. With 2 mg being the lowest available dose strength and 8–12 mg three times a day representing the highest recommended dose of tizanidine, the challenges associated with recommending a dose reduction that would permit safe use of tizanidine in patients requiring fluvoxamine or ciprofloxacin treatment become immediately apparent. Accordingly, the USPI for tizanidine contraindicates its use in patients receiving the strong CYP1A2 inhibitors, fluvoxamine or ciprofloxacin [171]. Additionally, based on *in vitro* studies of the enzymology of tizanidine metabolism that have identified CYP1A2 as the principal determinant of its biotransformation [172] and knowledge of CYP1A2 inhibitory drugs, an extension of DDI risk assessment to other CYP1A2 inhibitors is also possible. Accordingly, the USPI also includes the following statement warning prescribers regarding potential interactions that may be expected with other CYP1A2 inhibitors: “*Because of potential drug interactions, concomitant use of tizanidine with other CYP1A2 inhibitors, such as zileuton, other fluoroquinolones, antiarrhythmics (amiodarone, mexiletine, propafenone, and verapamil), cimetidine, famotidine, oral contraceptives, acyclovir, and ticlopidine should ordinarily be avoided. If their use is clinically necessary, they should be used with caution.*” [171]. This example illustrates the extension of DDI risk assessment to other precipitant drugs based on understanding of the underlying mechanisms and molecular determinants of established interactions.

Example 2: Everolimus. As another example, consider the anticancer drug everolimus, a substrate of CYP3A4 and P-gp. Ketoconazole (strong CYP3A inhibitor and P-gp inhibitor) produced a 15-fold increase in everolimus AUC, and erythromycin and verapamil (moderate CYP3A inhibitors and inhibitors of P-gp) produced 4.4- and 3.5-fold increases in everolimus AUC, respectively [126]. Considering the relatively large magnitude of the observed interaction with ketoconazole, the everolimus USPI recommends that concomitant use of strong CYP3A4 or P-gp inhibitors be avoided [40]. If patients require coadministration with a moderate CYP3A4 inhibitor, the everolimus USPI recommends use with caution at a reduced dose of 2.5 mg/day (one-fourth of the usual recommended dose of 10 mg/day), with a dose increase to 5 mg/day, which may be considered based on patient tolerance [40]. Finally, in a DDI study with the strong CYP3A inducer rifampin, a 64% reduction of everolimus AUC was observed [173]. Accordingly, the USPI recommends that concomitant use of strong CYP3A inducers be avoided and that if such combined use cannot be avoided, a dose increase from 10 to 20 mg/day may be considered

in 5 mg increments [40]. This example illustrates how adequately designed clinical DDI studies can inform the development of pharmacokinetically derived dose-modification guidelines for use in clinical practice when coupled with adequate safety monitoring.

Example 3: Ritonavir-Boosted Darunavir. The examples discussed above represent relatively simple illustrations of the concepts of translating the results of clinical DDI studies to guide prescribing information. There are many such examples across multiple therapeutic areas. Of note, particularly complex dose-modification guidelines are recommended for some antiretroviral agents where clinical use is indicated in the setting of multiagent combination therapy with other antiretroviral agents that have complex inhibitory or inducing effects on CYP enzymes as well as drug transporters. As an example, consider the prescribing information for ritonavir-boosted darunavir that exemplifies the translation to prescribing guidance, of knowledge collectively gained from *in vitro* drug metabolism and DDI studies, understanding of human disposition mechanisms of darunavir as well as coadministered agents, and the results of clinical DDI studies with probe substrates and inhibitors/inducers as well as commonly coadministered drugs. Darunavir is a CYP3A substrate and ritonavir coadministration boosts darunavir exposure by 11-fold due to the strong CYP3A inhibitory effect of ritonavir [174], representing a beneficial and necessary DDI in the context of its clinical use. In fact, to achieve sufficient exposures for antiretroviral therapy, darunavir should only be used with 100-mg BID ritonavir [175]. However, the state of nearly complete deficiency in CYP3A activity produced by regular use of ritonavir in patients receiving treatment with ritonavir-boosted darunavir results in clinically important DDI implications that complicate coadministration of other therapeutic agents that are substrates of CYP3A. In particular, serious safety concerns can be expected when the coadministered agent (i.e., object drug) is highly dependent on CYP3A-mediated metabolism for its clearance and additionally has a narrow therapeutic range. Accordingly, the USPI for darunavir provides a list of such drugs classified by therapeutic class, whose coadministration with darunavir/ritonavir is contraindicated (e.g., alfuzosin, dihydroergotamine, ergonovine, ergotamine, methylegonovine, cisapride, pimozide, oral midazolam, oral triazolam, lovastatin, simvastatin, and sildenafil for treatment of pulmonary arterial hypertension) together with rationale for the contraindication and the potential clinical consequences of coadministration based on knowledge of the safety profile of these object drugs [175]. For other object drugs that are substrates of CYP3A or CYP2D6 (since ritonavir is also an inhibitor of CYP2D6), the USPI offers cautionary guidance and dosage recommendations, where appropriate, based on knowledge of the therapeutic range and safety profile of the object drug and sensitivity to CYP3A or CYP2D6 inhibitory DDIs. Examples of such drugs include but are not limited to CYP3A substrates such as atorvastatin, cyclosporine, and tacrolimus, and CYP2D6 substrates such as risperidone, thioridazine, and metoprolol [175]. Additionally, because of pleiotropic induction of drug-metabolizing enzymes by ritonavir via PXR-mediated mechanisms, exposures of object drugs such as ethinyl estradiol (substrate of CYP3A and phase 2 metabolizing enzymes including UGTs and sulfotransferases) and *S*-warfarin (CYP2C9 substrate) were decreased by 44% and 21%, respectively, in DDI studies performed with oral contraceptives and warfarin, respectively. These findings translate, respectively, to

recommendations for alternate methods of nonhormonal contraception in patients treated with darunavir/ritonavir and monitoring of the international normalized ratio in patients receiving warfarin in combination with darunavir/ritonavir therapy [175]. Finally, with respect to DDI risk assessment for darunavir as the object drug, the USPI contraindicates coadministration with rifampin and St. John's wort based on knowledge of the clinically significant CYP3A inducing effects of these agents that may translate to a decrease in darunavir exposure, which may lead to loss of therapeutic effect and development of resistance [175].

3.12 CONCLUDING REMARKS

DDIs represent an important source of variability in PK that can impact drug response, including both the desired therapeutic effects and the unintended adverse effects. This chapter has provided an overview of the scientific principles, experimental approaches and clinical pharmacologic strategies applied in the investigation of PK DDI characteristics of investigational small molecule drugs during their development, and the application of knowledge gained from such evaluations to contribute to prescribing guidance. This chapter focussed on metabolic DDIs, although it should be noted that the general principles discussed here are also applicable to PK DDIs via nonmetabolic mechanisms (e.g., transporter-mediated DDIs; absorption DDIs via physicochemical or physiologic mechanisms), the investigation of which are equally important. DDI risk assessment and evaluation in drug development needs to consider the complementary scenarios of the NME as a victim or object and as a perpetrator or precipitant. A conceptual framework that illustrates the various considerations around DDI risk assessment and evaluation is provided in Fig. 3.9. The central goal of clinical pharmacologic understanding in drug development is to enable definition of the right dose of the right drug to the right patient populations. To this effect, it is important in drug development to characterize the impact of the various intrinsic and extrinsic sources of PK and PD variability that can influence the therapeutic as well as adverse effects of the drug, thereby altering the overall benefit–risk balance. This is illustrated on the scheme shown on the left panel of Fig. 3.9. DDIs represent an important source of variability, but their effects and clinical relevance will need to be interpreted in the context of other factors that may influence PK (genetic variation in drug-metabolizing enzymes or transporters, effects of food, age, body size, eliminating organ function, etc.) and PDs (PD DDIs, genetic variation in drug target proteins, altered sensitivity due to age or comorbid disease, etc.). Forest plots represent a powerful graphical framework to provide a concise, yet comprehensive, visual illustration of the comparative effects of various intrinsic and extrinsic factors (including the effects of co-administered agents) on the PK exposure parameters of a drug, as applied in recent USPI examples [176]. When utilized in a drug development setting and to guide development of proposed product labeling, such visual frameworks can permit an integrated assessment of the relative magnitude of various DDIs for the NME as the object drug, also in relation to other sources of PK variability including other extrinsic factors as well as intrinsic factors. The right panel of Fig. 3.9 provides a pyramid view of the integrated approach to DDI risk assessment and evaluation in drug development that was focussed on in this chapter. The base of the pyramid reflects the scientific considerations and approaches

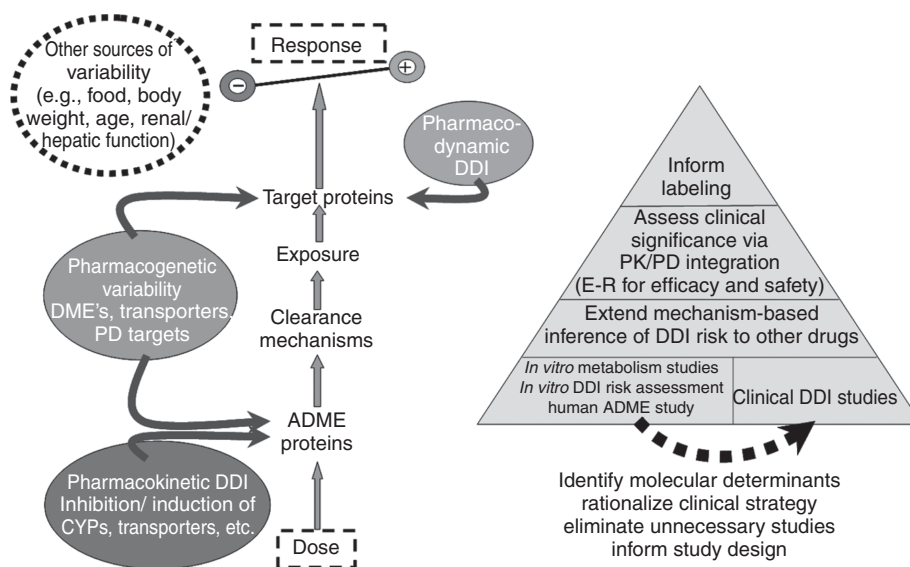


Figure 3.9 Integrated approach to strategic integration of DDI risk assessment and evaluation in contemporary drug development.

(experimental and clinical pharmacologic) employed in DDI risk assessment and evaluation that provide the data that serve as the foundation for translation to clinical significance. Of note, knowledge gained from mechanistic *in vitro* drug metabolism and DDI studies and understanding of clearance mechanisms of the object drug, represents critical prior information to enable DDI risk assessment and evaluation of the need for further clinical DDI evaluation. Importantly, understanding of the enzymology of metabolism and disposition of the NME and its effects on the activity and expression of drug-metabolizing enzymes and transporters *in vitro* together with quantitative IVIVE can eliminate the need for unnecessary clinical DDI studies and streamline the clinical DDI evaluation program. Such understanding can permit forecasting of DDI risk with unstudied objects (for NME as precipitant) and unstudied precipitants (for NME as object) based on knowledge of the clearance mechanisms of coadministered agents and knowledge of the effects of the coadministered agents on the clearance mechanisms of the NME, respectively. The quantitative translation of results from *in vitro* DDI studies to predictions of clinical pharmacologic correlates represents an emerging area of research, and this chapter has provided an overview of some of these approaches based on published expert opinion and emerging regulatory guidance (US and EU) in this area. Ultimately, the clinical relevance of PK changes resulting from DDIs will need to be assessed in the context of exposure–response understanding for the efficacy and safety characteristics of the object drug to contribute to product labeling. As is readily apparent from the above discussed integrated approach, DDI risk assessment and evaluation is a highly interdisciplinary science. Strong partnerships and collaborations across drug metabolism scientists, clinical pharmacologists, physicians, pharmacometricians, statisticians, pharmacogeneticists, and regulatory scientists engaged in the cross-functional mission of contemporary drug development are therefore critical to success.

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4 Current and Future Tools for Predicting Clinical Drug–Drug Interactions

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4.1 Summary	1
4.2 Introduction to the clinically significant drug–drug interactions (DDIs)	2
4.3 Biological and analytical tools used to investigate pharmacokinetics-based DDIs	6
4.4 High throughput screening (HTS) and predictive modeling of DDIs	15
4.5 Role of genetic polymorphism and pharmacogenetic considerations in prediction of DDIs	19
4.6 Conclusions and future perspectives	19
References	20

4.1 SUMMARY

The failure of lead candidate drugs in clinical trials is often because of the drug's toxicity, related to poor pharmacokinetic (PK) behavior, or the potential to be involved in drug–drug interactions (DDIs) in human patients. The prediction and accurate assessment of clinically relevant DDIs for novel drug candidates represents one of the major tasks of contemporary drug development. Understanding underlying mechanisms of DDIs and obtaining relevant ADME (adsorption, distribution, metabolism, and excretion) characteristics early in drug development helps to improve successful selection of candidate compounds. For more than two decades, information regarding the potential ability of a new drug to participate in clinically relevant dispositional DDIs leading to adverse drug reactions has been required during clinical trials. At present, such requirements include not only listing potential DDIs but also providing detailed information on the molecular mechanisms, enzymes, and molecular and cellular targets involved, as well interactions with influx and efflux drug transporters. The process includes identifying the enzymes responsible for the metabolism of a new pharmaceutical compound, determining mechanisms of transport across cellular membranes and drug transporters

involved, and determining PK parameters that show utility in predicting DDIs that may possibly lead to adverse drug reactions in humans. In this chapter, we focus on details of this process based on analysis of (i) mechanisms leading to clinically significant DDIs, including enzyme- and transporter-mediated interactions, and (ii) currently available analytical tools, including *in vitro* assays and high throughput screening (HTS) technologies and virtual screening, to assess and predict PK-based, clinically relevant interactions in humans. We proceed with the evolution of current approaches to predict DDIs for novel chemical entities and drug candidates in development and conclude the chapter with analysis of modern trends in the field.

4.2 INTRODUCTION TO THE CLINICALLY SIGNIFICANT DRUG-DRUG INTERACTIONS (DDIs)

4.2.1 The Incidence of Clinically Significant DDI

DDIs occur when the pharmacological effects of one drug are altered by the presence of another drug in the plasma. Clinically significant DDIs can lead to adverse reactions, life-threatening toxicity, or diminished drug efficacy in human patients [1]. In some cases, clinically significant DDI related to severe toxicity have led to the withdrawal of drugs from the market [2]. Clinically significant DDI present serious problems in both ambulatory and hospitalized patients that may lead to increased morbidity and mortality and contribute to the increasing cost of treatment. Indeed, it has been estimated that 2–3% of all hospitalized patients experience adverse reactions caused by DDI [3], and global medical reports indicate DDI in all types of hospitalized patients [4,5], with high incidence of DDI-related adverse effects in intensive care [6,7], psychiatric [8], cancer [9], cardiovascular [10], and HIV patients [11], as well as in patients in pediatric wards [12] and nursing homes [13]. Moreover, according to the National Ambulatory Medical Care Survey and the National Hospital and Ambulatory Medical Care Survey, ~4.5 million adverse drug effects related to ambulatory visits occur in the United States each year, the majority of these in outpatient office practices [14]. An overall high incidence and growing cost of treatment of the toxicity related to DDI in all groups of patients presents serious health and economic concerns [7,15,16]. In some cases, DDI may not result in immediate toxic reactions but cause increased patient exposure to suspected human carcinogens, leading to an increased risk of cancer and mortality in these groups of patients, hence contributing to an indirect increase in the cost of medical treatments [17]. In addition, DDI may blunt therapeutic effects of known anticancer treatments [18] or diminish therapeutic effects by preventing prodrug activation [19], thereby negatively affecting therapeutic effectiveness of the medications. A growing proportion of the aging population and the increased number of patients requiring multiple drugs or polypharmacy regimens (e.g., a practice of coadministering of several drugs to treat multiple health ailments simultaneously) have contributed to the increased incidence of DDI [20]. For instance, more than one-third of all patients over the age of 57 take five or more prescribed drugs [21], while the average number of chronic medications prescribed for cardiac patients varies between six and eight drugs [22]. The issue becomes even more pronounced in the elderly, where over 25% of all hospitalizations in geriatric patients over 80 years involve adverse drug reactions related to DDIs as a result of polypharmacy treatment [23], which presents significant a problem affecting over

40% of the geriatric population in the United States [24]. In all groups of patients, the problem of adverse drug reactions and toxicity associated with DDIs is further complicated by growing numbers of available new drugs and medications, available both by prescription and over the counter. The latter group may include, in addition to drugs, nutraceuticals, dietary supplements, and natural health products with a potential to cause drug–food and drug–nutrient interactions [25,26]. All these facts, including growing elderly populations, the increased incidence of polypharmacy, and the growing number of new medications, have contributed a unique challenge for modern drug development programs, which now face the necessity to perform drug efficacy and safety assessments for novel drug candidates in development in consideration not just with their single use, but with their potential coadministration with a whole spectrum of other established and novel therapeutic agents, including herbal products, nutraceutical compounds, and vitamins [27]. Recent clinical evidence has thus prompted the development of the novel screening strategies based on the advanced tools and assays for the evaluation and prediction of possible clinically significant DDIs (potential drug–drug interactions, pDDIs) [28].

4.2.2 The Importance of Preclinical Assessment of DDI Potential

Prediction and possible prevention of adverse reactions and toxicity caused by DDIs present critical issues in modern pharmacotherapy and pharmaceutical drug development and now are included as a part of preclinical assessment of drug safety and toxicity for drug candidates in development [2]. The importance of preclinical assessment of DDIs is highlighted by the regulatory requirement of DDI information to be included as an integral part of risk assessment of therapeutics during drug development (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf>). A significant effort has been made by academic institutions and the pharmaceutical industry to characterize and explore underlying mechanisms of adverse drug reactions and *in vivo* toxicity stemming from clinically significant DDIs that alter toxicity or efficacy of the administered drug. Such efforts, combined with a growing knowledge and understanding of molecular mechanisms of DDI-related adverse drug effects and toxicity, have led to a progressive development and advancement of *in vitro* and *in vivo* analytical tools, assays, and methodologies for prediction and preclinical assessment, as well as predictive modeling of such interactions [24,29]. Many of these tools and methods have now become a part of routinely adopted practices for *in vitro* screening aiming for prediction of DDI-related adverse drug reactions in preclinical drug development. Moreover, screening for the ability of a novel drug candidate to cause significant DDI has shifted to earlier stages in a drug development process and become a central part of the pharmaceutical industry's strategy to reduce attrition rate and the overall cost of bringing new drugs to the market [30]. Such a strategy has also prompted development of novel screening platforms as well as spurring advancements of modern technologies including HTS [31], ultrahigh throughput screening (u-HTS), microarrays, and virtual screening [32,33]. The evolution of the current approach to evaluate DDIs at the preclinical stage, together with analysis of available methods, screening platforms, and tools for prediction of clinically significant DDIs, are described in more detail in this chapter.

4.2.3 Common Types and Mechanisms of Clinically Significant DDIs

Drug action includes desirable (therapeutic) and undesirable (toxic) drug effects and is regarded in terms of a therapeutic window for a particular drug and patient. Coadministration of two different drugs can result in increased undesirable drug effects compared to individual drug use and lead to adverse drug reactions and toxicity stemming from clinically significant DDIs. DDIs based on changes in PK or pharmacodynamic (PD) parameters are classified as PK- or PD-based DDIs, respectively. PD-based DDIs are caused by the coadministered drug interference at the target site and are not directly related to changes of drug concentration in the body or plasma. PK-based DDIs arise from changes in PK parameters (e.g., $C_{\max}$ or AUC) and are related to changes in drug or drug metabolite concentration in a body as a result of coadministered drug interference with one of the ADME parameters (Fig. 4.1). Changes in drug concentration or exposure arising from PK-based DDIs that lead to increased plasma drug concentration may vary significantly, from two- to threefold to more than an order of magnitude [34]. Significant changes in the concentration of unchanged drug or drug metabolite may cause severe toxicity and adverse drug reactions and are classified as clinically significant DDIs. Drugs with a narrow therapeutic range (NTR) or low therapeutic index are more likely to be involved in serious drug interactions. In some cases, the serious adverse effects of the drug therapy can be attributed to a change in concentration and rate of production of reactive metabolites, as in case of thiabendazole bioactivation, leading to clinically significant DDIs [35]. Drug interactions that affect PK behavior occur at several sites within the body, including in the gastrointestinal mucosa and the liver and at drug-binding sites on plasma proteins, such as albumin and α 1-acid glycoprotein. These interactions include changes in the rate/extent of absorption as a result of changes in the function of drug transporters, alterations in the metabolism of compounds by way of inhibition or induction of metabolic enzymes, and changes in the plasma concentration of one drug by competition with another for plasma protein binding [36]. The focus of this chapter is on the *in vitro* tools for prediction of the

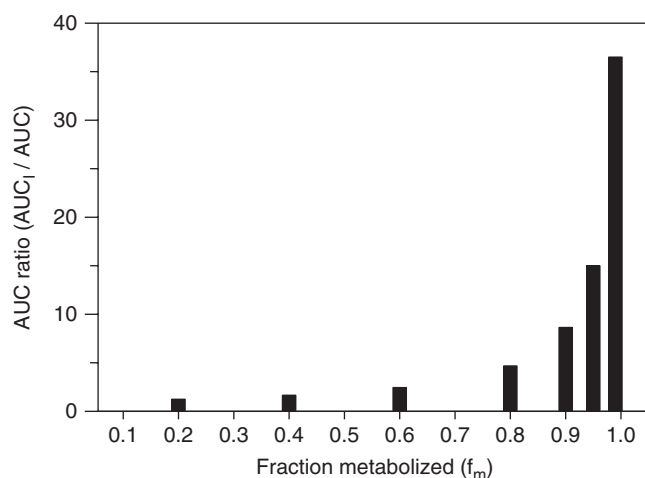


Figure 4.1 The dependence of AUC ratio on the fraction of dose eliminated by hepatic metabolism (f_m) when $I = 6 \mu\text{g/mL}$ and $K_i = 0.1 \mu\text{g/mL}$.

PK-based DDIs, and specifically on pDDIs arising from inhibition and induction of major drug-metabolizing enzymes (DMEs) and major drug transporters [37]. Detailed information on adverse drug reactions related to changes in drug absorption profile and interactions with multiple classes of drug transporters is presented in the chapter titled *Conjugation, Transport, and Elimination of Drugs in Humans*.

4.2.4 Involvement of Drug-Metabolizing Enzymes in Drug Interactions

Two of the most common mechanisms of PK-related DDIs involve inhibition or induction of the major DMEs, leading to an increased concentration of drug or metabolite in the patient or to a loss of drug efficacy [36]. Several classes of DMEs are involved in the process of drug biotransformation in humans, catalyzing oxidative and conjugative phases of the drug metabolism process. The cytochrome P450s (CYP450s) are the major group of enzymes involved in the oxidative phase (formerly phase I) of drug metabolism in humans. UDP-glucuronosyltransferases (UGTs) are the main superfamily of conjugative drug metabolism (phase II according to a previous classification). In humans, CYP450 and UGTs are two main classes of DMEs that, together with drug transporters, are involved in the majority of PK-based DDIs [34].

The CYP450 superfamily consists of multiple, intracellular membrane-bound DMEs that express broad, often overlapping, substrate specificities among its different members [38]. According to modern nomenclature, 18 families of human CYP450 genes are divided into 43 subfamilies, with 15 human CYP450 primarily involved in xenobiotic metabolism, all of them being from CYP1, CYP2, and CYP3 families [39]. The CYP450 subfamilies 1–3 are responsible for 70–80% of all oxidative metabolism reactions. In fact, up to 90% of their metabolic activity is attributed to only six enzymes, namely, CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 [40]. All members of the CYP450 subfamilies can be induced (with the exception of CYP2D6) or inhibited by certain drugs. Alterations in CYP450-mediated metabolic pathways can lead to clinically significant DDIs stemming from inhibition or induction of the individual CYP450 enzymes [41]. At present, significant effort by many ADME programs is devoted to identifying compounds that may alter the major CYP450 oxidative pathways causing clinically significant DDIs [42].

It has been well documented that DDIs involving inhibition of major human CYP450 enzymes represent one of the most common mechanisms leading to increased toxicity and adverse drug reactions in humans [34]. Since 1997, the FDA and other regulators have emphasized the importance of documenting the potential for new orally administered drug candidates to be involved in metabolism-based, PK DDIs with coadministered medicines before a candidate reaches first-in-human studies. Many of these interactions have received considerable attention because some successful drugs (e.g., terfenadine, cisapride) caused severe adverse effects when coadministered with certain anti-infectives [43]. Such interactions involved inhibition of the important members the CYP450 superfamily of DMEs. At present, the FDA provides recommendations for classification of the severity of inhibition for major human CYP450 enzymes. According to the FDA guidance, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 inhibitors are qualified as strong [fivefold increase in area under the plasma concentration–time curve (AUC) of substrates for specific CYP enzymes], moderate (two- to fivefold increase in AUC), and weak (1.25-fold to twofold increase in AUC). In addition, sensitive substrates for a particular CYP450 enzyme

that cause fivefold increase in AUC in the presence of specific CYP450 inhibitors, or substrates with a NTR, are also defined. For NTR substrates, even small increases in exposure levels may lead to serious safety problems. This classification system can be used for selection of substrate or inhibitor drugs to be used in drug interaction studies or for labeling purposes. For instance, if a novel chemical entity is labeled as a “sensitive CYP3A substrate” or an “NTR CYP3A substrate,” there may be a warning in the label regarding a potential drug interaction with strong or moderate CYP3A inhibitors (CDER Drug Development and Drug Interactions Website, <http://www.fda.gov/Drugs/DevelopmentApprov>) [36].

The conjugative phase of drug metabolism includes reactions of glucuronidation, sulfation, methylation, and acetylation, which may either follow the oxidative phase or be independent of oxidative metabolism by CYP450. Reactions of conjugative drug metabolism quantitatively represent the most important pathways involved in drug biotransformation [44]. In conjugative biotransformation, glucuronidation accounts for up to 35% of all conjugative drug metabolism reactions [45] and is catalyzed by members of the UGT superfamily of DMEs. According to modern nomenclature, the UGT superfamily consists of at least 20 human isozymes encoded by two gene families and three subfamilies (UGT1A, UGT2A, and UGT2B) [46]. UGTs catalyze the conjugation of endobiotics such as bilirubin, steroids, and xenobiotics including drugs, pesticides, carcinogens, and phytochemicals with uridine diphosphoglucuronic acid (UDPGA), resulting in the formation of a glucuronide product (metabolite). The resulting glucuronide is more water soluble compared with the parent compound, and this property facilitates faster metabolite elimination. Changes in the glucuronidation rate of a specific compound due to inhibition or induction of the specific UGT enzyme(s) can affect PK parameters for other drugs that are eliminated primarily via a glucuronidation pathway, resulting in important pharmacological consequences leading to potential toxicity and clinically significant DDIs. Clinically significant pDDIs related to inhibition of human UGTs have recently received considerable attention [45,47]. In addition to xenobiotics, UGT-related adverse drug effects have been attributed to drug interactions with endogenous compounds [48], phytochemicals, and nutritional and herbal supplements [49]. In all cases, for new lead compounds eliminated via the glucuronidation pathway, an accurate assessment of the metabolism rates and PK parameters to predict and prevent UGT-related DDIs is recommended [50].

4.3 BIOLOGICAL AND ANALYTICAL TOOLS USED TO INVESTIGATE PHARMACOKINETICS-BASED DDIs

4.3.1 DDIs Related to Competitive and Noncompetitive Inhibition of CYP450s

It is important to remember that if a candidate compound is metabolized by a particular CYP450 enzyme it will inhibit that enzyme to a degree that depends on the “affinity” of the drug for the catalytic site of the enzyme (K_m) and the concentration of the compound under investigation. However, a compound is not necessarily metabolized by a CYP450 enzyme merely because it inhibits that enzyme. An excellent example of this apparent contradiction is quinidine, which is a potent inhibitor of CYP2D6 but is metabolized by other CYP450 enzymes, including CYP3A4 [51].

When they occur, metabolism-based PK interactions involving inhibition can cause a large increase in exposure to one of the drugs administered by acting as an alternate substrate or an inhibitor, which frequently leads to the onset of adverse, instead of therapeutic, effects [52]. This type of interaction most frequently arises from the competition between two compounds for hepatic CYP450. These interactions can also occur via noncompetitive (allosteric) interactions or by time-dependent metabolic inactivation of an enzyme by one of the drugs. Metabolic interactions can also occur in the small intestine. So the purpose of investigating drug candidates as CYP450 inhibitors *in vitro* is to qualitatively predict their potential to change *in vivo* PK behavior. For example, if polymorphically expressed CYP2D6 is inhibited strongly by a candidate compound, the following are factors that must be considered when predicting the possible significance of the inhibition:

- The extent to which the candidate is cleared by CYP2D6
- The potential for saturating the metabolic capacity of CYP2D6 (easier in poor metabolizers than in extensive metabolizers)
- The possibility of administering the candidate drug with other medications that are CYP2D6 substrates
- The PK behavior of the candidate drug (the potential inhibitor)
- The possible magnitude and potential consequences of alterations (typically increases) in the PK behavior of other CYP2D6 substrates, specifically exposure.

The first factor links reaction phenotyping (enzyme identification) and enzyme inhibition studies. For example, *in vivo*, for a given concentration of a potential inhibitor, as the fraction of the coadministered medicine metabolized (f_m) increases, the AUC increases nonlinearly, until at $f_m = 0.99$, the AUC can typically increase by 35-fold [52]. This was the case for terfenadine when it was coadministered with normal doses of ketoconazole [43]. After conducting an extensive analysis of the utility of these enzyme inhibition studies, Obach *et al.* [53,54] concluded that CYP450 *in vitro* inhibition data are valuable in designing clinical drug interaction study strategies and can be used to predict the magnitude of these interactions.

In order to complete a semiquantitative prediction of the effect of a candidate drug on the PK behavior of an index drug, it is important to determine K_i , the inhibition constant, of the candidate compound for each of the major human liver CYP450 enzymes. K_i is an intrinsic inhibition constant that defines the affinity of the test compound (as opposed to the probe substrate) for the enzyme in question. The value of K_i is much more reproducible from one laboratory to another than IC_{50} , which is specific to a given set of experimental conditions. It is also important to determine the free concentration of candidate drug in the plasma (I). At present, the FDA recommends that the ratio I/K_i should be used to qualitatively predict the probability of significant PK interactions. For example, given an index drug with $f_m = 0.70$, if $I/K_i = 0.1$ for the candidate drug, then the AUC ratio will be 3.2. If $f_m = 0.99$ and $I/K_i = 0.1$, the AUC ratio will be 36.5. However, if $I/K_i = 1.0$, the AUC ratio will be ~ 1.6 , and when $I/K_i = 1000$, the AUC ratio would be ~ 3.99 . So, the FDA position on the value of I/K_i in predicting the clinical significance of interactions is that if its value is 10 or more, it is likely that PK interactions will occur and that necessary clinical drug interaction studies need to be planned. As f_m increases, however, the rise in I/K_i will cause greater increases in AUC (Fig. 4.2).

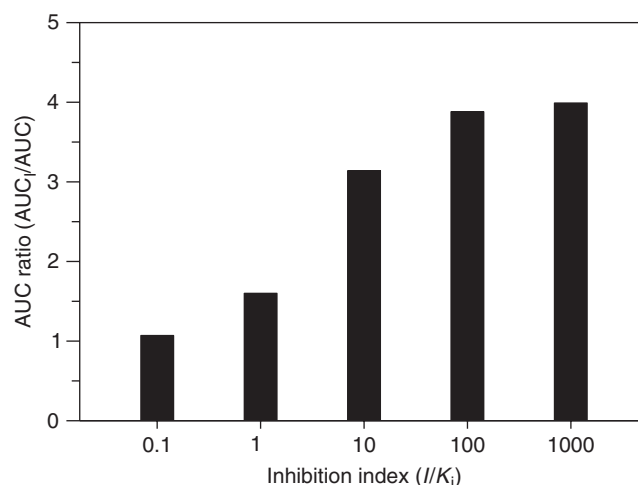


Figure 4.2 The dependence of AUC ratio on the inhibition index (I/K_i) when $f_m = 0.75$.

4.3.2 DDIs Caused by CYP450 Induction

Drug interactions can also result from an increase in the expression of individual DMEs, particularly the hepatic CYP450 enzymes. DDIs caused by induction of CYP450 can impact the efficacy and safety of coadministered drugs. Therefore, it is important to understand the potential of a new compound to cause enzyme induction and how to use *in vitro* induction data to predict potential for DDIs *in vivo*.

The expression of most of the clinically relevant microsomal CYP450 enzymes in human liver are under the control of xenobiotic-activated nuclear receptors and may be induced by exposure to medications, components of food and herbal supplements, and occupational/environmental contaminants. The notable exception to this generalization is CYP2D6. Many drugs currently on the market in the United States are capable of inducing one or more CYP450 enzymes at therapeutic doses. The effect of enzyme induction is to reduce exposure to drugs that are substrates for the enzyme(s) undergoing induction by increasing the metabolic clearance. In this way, an inducer may change the PK behavior of a second drug, or it may increase its own rate of clearance (autoinduction). The resulting diminution of exposure can reduce efficacy or eliminate desired therapeutic effects.

Hepatic microsomal CYP450 induction is subject to important species-related differences [55,56], such that induction studies conducted with nonhuman species can yield misleading results, making accurate human predictions risky. Furthermore, induction studies conducted in nonhuman species tend to be expensive and require a substantial amount of test compound, unless this objective is incorporated into a repeat-dose toxicity study. However, these difficulties have been overcome by the development of robust human hepatocyte culture methods and the general availability of fresh and cryopreserved cells for this purpose [57]. The constitutive expression of CYP450 genes in cultured hepatocytes invariably falls for the first 48–72 h after seeding, regardless of culture conditions. However, the responsiveness of these genes to appropriate

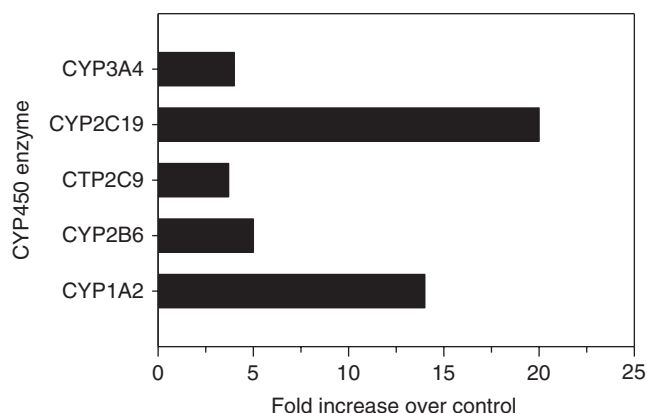


Figure 4.3 A decision tree for CYP450-based drug–drug interaction studies.

inducers is preserved if culture conditions are selected carefully [58–60]. Optimal culture conditions permit the hepatocytes to exhibit near-normal cellular physiology, gene expression, intercellular contacts, and bile canaliculi formation [61]. Thus, primary cultures of human hepatocytes serve as a selective and sensitive model for predicting the induction of CYP450 *in vivo*. General industry guidance and recommendations for the conduction of CYP450-based inhibition and induction studies for new drug candidates in development are summarized in Fig. 4.3.

Enzyme induction may be measured in several ways. After exposure of human hepatocyte cultures to pharmacologically relevant concentrations of test compound, or vehicle, for two to three days, CYP450-specific mRNA and protein are typically measured and compared. Monitoring enzyme activity against CYP450-specific substrates, directly in cultures or in microsomes harvested from cultures, may also be used to detect enzyme induction. In the best case, there is a clear relationship between nominal test compound concentration and the fold increase in CYP450 activity (Fig. 4.4). In this model, α -naphthaflavone, phenobarbital and rifampin are considered potent prototypical human CYP450 inducers, against which the effect of a test compound is compared. For example, typically, naphthaflavone induces the expression of CYP1A2, phenobarbital induces CYP2B6, and rifampin induces CYP3A4 and CYP2C19. Phenobarbital and rifampin are also capable of inducing members of the CYP2C and CYP2E subfamilies. However, there is cross talk between the nuclear receptors that regulate these enzymes, which may result in a pleiotropic response to some inducers [59]. The best example of this is the effect of phenobarbital on liver function and CYP450 induction [62]. In many cases, the demonstration of CYP450 induction *in vitro* provides an explanation for qualitative changes in PK behavior observed in patients receiving the drug in question. Although the human hepatocyte model is sensitive and specific, it does not consistently and accurately predict the magnitude of PK changes *in vivo* because of the multitude of other factors that can alter drug disposition. However, one method has been suggested for quantitatively predicting the effect of CYP450 induction on PK behavior based on results from *in vitro* experiments using cultured human hepatocytes [63]. The resulting *in vitro*–*in vivo* relationship for eight drugs characterized as

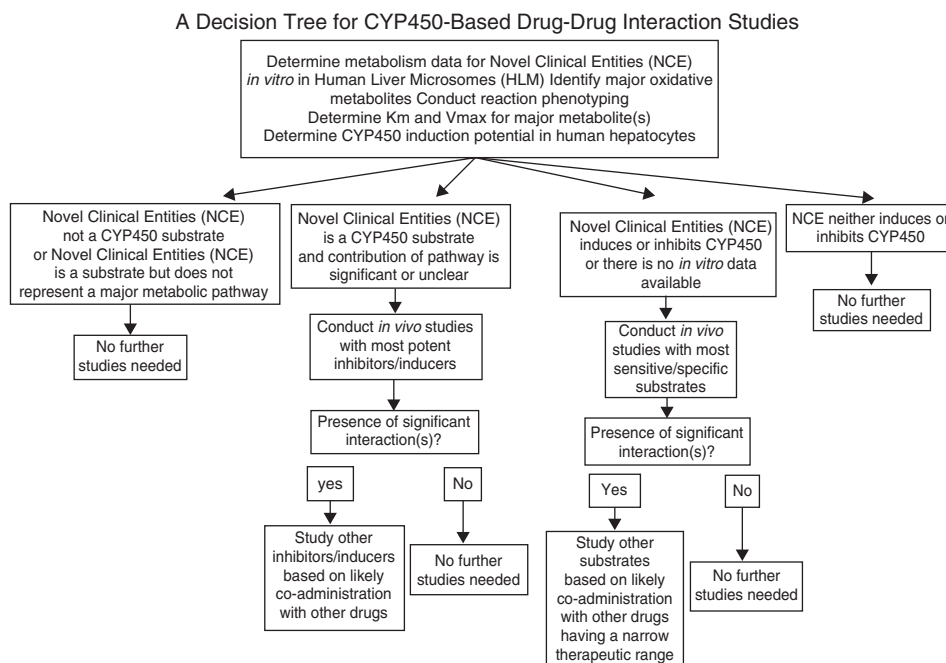


Figure 4.4 Typical fold induction in cultured human hepatocytes of CYP1A2 by 25 μM omeprazole, of CYP2B6 by 500 μM phenobarbital, and of CYP2C9, CYP2C19, and CYP3A4 by 10 μM rifampin.

inducers of CYP1A and CYP3A enzymes, and several enzyme-selective substrates suggested that the ratio E_{max}/EC_{50} for induction determined in cultured human hepatocytes correlated moderately well ($r = 0.768$, $p < 0.05$) with the change in intrinsic hepatic clearance calculated from PK data before and after administration of the inducing drug. This represents a respectable prediction, however, imperfect.

More recently, the activation and translocation of the nuclear receptors that control the transcription of these enzymes have been used as indicators of induction [64,65]. These receptors include aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR). PXR is the most promiscuous of these [66], and it frequently functions as part of a heterodimer with CAR. Once in the nucleus, these xenosensors bind to xenobiotic responsive elements (proximal and distal promoter sequences) on the target genes and cause an increase in transcription. The heterodimerization of these transcription factors is responsible for the cross talk observed in CYP450 induction. Our understanding of the molecular events involved in induction is incomplete, as is the development of a practical method using this phenomenon to predict the magnitude of human liver CYP450 induction and its effect on PK behavior. One reason for this delay is that hepatocyte culture conditions must support the near-normal expression of these nuclear receptors and no consensus yet exists on ideal or optimal culture conditions. For example, the expression of PXR is also under the influence of the glucocorticoid receptor [62].

4.3.3 DDIs Due to Inhibition of Conjugative Drug-Metabolizing Enzymes

Glucuronidation is an important metabolic pathway of the conjugative phase of drug biotransformation. The variety of substances catalyzed by UGTs includes bioflavonoids, carcinogens, environmental pollutants, and many prescribed drugs from all therapeutic classes [67]. In most of the cases, drug glucuronidation results in detoxification of the parent compound and production of the less harmful metabolites, but in some cases it can lead to the production of reactive metabolites, such as in the bioactivation to genotoxic acyl glucuronide metabolites of carboxylic acid drugs capable of damaging cellular DNA via glycation and/or glycooxidation [68]. UGTs also catalyze glucuronidation of many important endobiotic compounds, including bilirubin, steroids, and bile acids [46]. Therefore, xenobiotics and endobiotics that are eliminated primarily via glucuronidation could be affected by changes in the rate of glucuronidation and production of toxic metabolites, resulting in toxicity and adverse drug reactions in patients. The human hepatic UGTs participate in the clearance of ~15% of the top 200 drugs [69]; there are multiple examples of involvement of this class of DMEs in clinically significant DDIs [70,71]. However, DDIs related to inhibition of human UGT enzymes are less characterized compared to DDIs caused by inhibition of major human CYP450 enzymes. In part, it is related to difficulties in obtaining active UGT enzymes in highly purified form for protein crystallography and modeling studies, obstacles in the development of UGT assays for multiple UGT isoforms, insufficient information about potent and specific isoform-specific inhibitors, and clinical importance of UGT polymorphisms [72]. With further advancements of the UGT field, gaps of knowledge related to the role of UGTs in clinically significant DDIs are expected to be filled, as in the case of CYP450s. At present, there are no consensus requirements regarding the assessment or regulation of UGT-related DDIs in clinical studies. However, an accurate assessment of the specific UGT isozyme involvement, rates of metabolism, and potential to cause UGT-related DDIs is now recommended for all new lead compounds eliminated predominantly via the glucuronidation pathway.

4.3.4 DDIs Due to Competition during Absorption or Elimination of Orally Administered Drugs Involving Transporters

Many human tissues, including the small intestine, liver, kidney, and central nervous system, express a wide variety of drug transporters. Drug transporters mediate the transmembrane passage of drugs and can act as potential sites for the interaction of two or more drugs. This interaction can alter PK behavior significantly, similar to the interactions involving DMEs discussed previously. Members of the solute carrier (SLC) class of membrane transporters are typically located on a basolateral membrane of cells (e.g., hepatocyte sinusoidal membrane). The ATP-binding cassette (ABC) transporters are usually found on an apical membrane (e.g., biliary canalicular membrane, duodenal enterocyte membrane, and renal distal tubule membrane) and facilitate transport of a wide variety of exogenous and endogenous substances across cellular membranes against a concentration gradient via ATP hydrolysis. Pharmaceutical compounds that happen to be good substrates for the export transporters may exhibit poor or variable PK behavior, including limited or unpredictable bioavailability and altered elimination. The export transporters may also alter trough plasma drug concentrations and the

disposition of substrates to peripheral tissues and compartments, thereby changing the dose–response relationship and altering therapeutic effects.

Among the SLC superfamily of drug transporters, several members of the organic anion transporting polypeptide (OATP) family play an important role in the determination of the drug PK parameters. Some drugs, such as cyclosporine, may inhibit OATP transporters, therefore causing PK-based DDIs [73].

The multidrug export transporter P-glycoprotein (PGP) (ABC-type drug efflux pumps, e.g., ABCB1 = P-glycoprotein = MDR1), is the most extensively studied member of the ABC transporter family and is an important site for potential transporter-mediated drug interactions [74]. It is present in the small intestine, blood–brain barrier, distal renal tubule, and placenta. Generally, understanding of PGP and the biological and chemical tools with which to study its contribution to drug interaction potential are more advanced compared to other transporters of the ABC and SLC families.

4.3.5 DDIs Mediated by Inhibition and Induction of PGP

Like many of the human hepatic microsomal CYP450 enzymes, the substrate specificity of PGP is very broad, which makes the prediction of PGP-mediated drug interactions from *in vitro* studies quite difficult. Compounds that interact with PGP may be organized into three classes, based on their ability to stimulate the ATPase activity of PGP and whether they are substrates or inhibitors of the transporter [2,52,68,75–78].

New medicinal compounds may be tested to determine if they are PGP substrates or inhibitors by conducting a bidirectional permeability study in monolayers of polarized cells expressing PGP. These include Caco-2 cells, MDR1-transfected Madin–Darby canine kidney cells, and LLC-PK1 and MDR1-transfected LLC-PK1 cells. These experiments include reference substrates that exhibit low to moderate apparent basal-to-apical membrane permeability ($P_{appA-B} = 2\text{--}30 \times 10^{-6}$ cm/s) and a net flux ratio (P_{appB-A}/P_{appA-B}) of at least 2.0 and inhibitors that exhibit low values of K_i or IC_{50} ($>10\text{ }\mu\text{M}$). Examples of acceptable reference substrates with which to compare new compounds include digoxin, loperamide, and vinblastine, and examples of suitable reference inhibitors include cyclosporin A and elacridar [79]. Unfortunately, because multiple efflux transporters may be expressed in model cell systems and some compounds may inhibit multiple transporters, further experiments should be conducted. However, if the efflux ratio of the new compound is greater than 2.0, and two or three known PGP inhibitors significantly reduce the net flux ratio, the compound is likely to be a PGP substrate. If a significant proportion of the efflux activity is not inhibited by reference PGP inhibitors then other efflux transporters may contribute to the efflux activity. Presently, the results described in the latter case are somewhat difficult to interpret, as our understanding of the many other human efflux transporters is quite limited relative to PGP. The results of studies designed to identify new compounds as PGP substrates may be used to design *in vivo* studies for the investigation of PGP interactions. Clinical examples of PGP-mediated drug interactions include the altered PK of digoxin caused by quinidine and the alteration of fexofenadine PK caused by ketoconazole and erythromycin [79]. Clearly, the involvement of drug transporters can have a significant effect on PK behavior [80]. The expression of PGP in the intestinal epithelium is moderated by the same nuclear receptor, PXR that regulates the expression of CYP3A4. Therefore, repeated oral administration of rifampin or St. John's Wort

can result in an increase in the efflux of PGP substrates from the intestinal epithelium, thus changing the PK behavior by reducing the bioavailability of substrate drugs and reducing therapeutic effects. However, structurally, the ligand-binding domains among animal species are quite divergent from each other and from the human PXR. Furthermore, Caco-2 cell monolayers, so useful in estimating intestinal permeability and absorption, are not suitable for this purpose, possibly due to a lack of human PXR activity in this cell line. Alternately, the human cell adenocarcinoma cell LS180/WT and its adriamycin-resistant (LS180/AD) or vinblastine-resistant (LS180/V) sublines may be used to study PGP induction. However, in general, methods for *in vitro* evaluation of PGP induction, and predicting the effect of this phenomenon on PK behavior, are not well established. In addition, it is very difficult to predict the magnitude of the effect that PGP induction will have on PK variables such as bioavailability.

4.3.6 Impact of OATP Transporters on PK Parameters and DDIs

The OATP family of human drug transporters consists of 11 members, of which OATP1B1, 1B3, 2B1, and 1A2 are the most studied for their potential involvement in transporter-mediated DDIs and changes in PK behavior. Among these, the OATP1B1, 1B3, and 2B1 transporters are most frequently expressed in the sinusoidal membrane of hepatocytes, while OATP1A2 is found on the luminal membrane of enterocytes and at the blood–brain barrier, making these all of these organs potential sites for transporter-mediated DDIs.

OATP transporters are involved in the influx transport of both endogenous (e.g., bile acids, conjugated steroids, steroid hormones and their metabolites, and thyroid hormones) and exogenous compounds, including many that are widely prescribed. Several clinically important drugs identified as substrates for these transporters include antibiotics, HMG-CoA inhibitors (statins), antineoplastic agents, and nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., diclofenac, ibuprofen, and lumiracoxib) [73,81,82]. Simultaneous administration of two or more drugs that are substrates of these transporters may alter drug PK parameters, leading to clinically significant DDIs. Common mechanisms of the OATP transporter-mediated DDI may involve inhibiting the specific transporter (e.g., cyclosporine) [83] or altering its expression by the induction [73,84]. Alternative allosteric mechanisms of drug interactions with transporter proteins leading to pDDIs have also been suggested. In addition, OATP1B1 and OATP1B3 may be indirectly involved in the regulation of the expression of several DMEs and transporters mediating transport of ligands for xenobiotic sensing nuclear receptors including PXR and CAR [85]. However, determining the role of a single transporter protein in transporter-mediated DDIs may be challenging because OATP transporters exhibit broad substrate specificity across various members of the family and many of their substrates are also substrates for the CYP450 family of DMEs and PGP [73].

4.3.7 Importance of a Combined Approach

In vitro metabolic studies are now widely accepted as an integral part of drug development, as the results of these studies can be used to establish the need for, and design of, subsequent *in vivo* assessments of potential drug interactions. The evaluation of the potential of a new pharmaceutical compound to be involved in DDIs, and the possible impact of such interactions on its PK behavior, is a necessary part of the

drug development process and regulatory review before market approval [36]. Three fundamental questions require answers. Will other drugs alter the exposure to the new drug? Will this new drug change exposure to other drugs? Will these interactions alter the exposure significantly enough to require dose adjustment of the new drug or other coadministered medicines?

Until two decades ago, the liver was thought to be the only important site of first-pass metabolism for orally administered drugs, but many reports have been published in the interim concerning the important role of the small intestine in influencing the bioavailability of many medications [86]. Although the liver remains an important site of drug metabolism, duodenal and jejunal enterocytes express several enzymes belonging to the CYP450, glutathione-S-transferase [87], and UGT superfamilies [41]. During the past decade, the commercial availability of human biological products (e.g., intestinal and liver microsomal preparations, cryopreserved human hepatocytes, recombinant human DMEs, and cell lines expressing human DMEs, nuclear receptors, and drug transporters) has helped improve predictions of PK behavior and reduced the previous uncertainty associated with using nonhuman analogs of these tools. The use of these has been combined with the development of novel fluorescent and luminescent technologies to assess drug metabolism and inhibition.

As mentioned previously, CYP450 enzymes are important in this context because of their involvement in the elimination of the majority of marketed drugs. Owing to this broad specificity, it is possible that two or more CYP450 enzymes can contribute to the metabolism of a single candidate compound and that many therapeutically unrelated drugs can be metabolized by the same enzyme. When multiple CYP450 enzymes metabolize the same compound, their relative contribution is determined by the ratio $V_{\max}/K_m$, the intrinsic clearance (e.g., oxycodone, CYP2D6, and CYP3A4). When a single CYP450 enzyme determines the rate of elimination of many drugs, they typically exhibit very different affinities and rates of reaction (e.g., serotonin reuptake inhibitors and CYP2D6).

Human *in vitro* systems are commercially available to investigate drug interactions for the purpose of predicting if new compounds could be involved in clinically significant CYP450-related PK interactions, by assisting in the design of meaningful and cost-effective clinical drug interaction studies [52,53]. These tools include characterized human liver microsomes, recombinant human DME and enzyme-selective probe substrates, and inhibitors combined with sensitive and specific bioanalytical assays. Quantitative assays used at this stage may include multiple approaches, such as radiometric assays based on isolation of radiolabeled metabolites [88], high performance liquid chromatographic (HPLC) methods including rapid LC/MS approaches for metabolite analysis [89], and use of fluorogenic CYP450 substrates [34]. Predictions of potential PK-based DDIs are derived from two types of *in vitro* studies. The first is an enzyme inhibition study using pooled human liver microsomes, which are employed to determine if a candidate drug is capable of inhibiting any of the clinically relevant CYP450 enzymes and to what degree. The second is an enzyme induction study, which is conducted in cultured human hepatocytes and is typically used to predict the effect of a test compound on the level of expression, and therefore activity, of clinically relevant CYP450s. Consensus recommendations for appropriate experimental protocols used for CYP450 inhibition studies have been refined and published [36,90,91]. These include recommendations for CYP450-selective probe substrates with which to measure the activity of each enzyme and for potent and selective reference inhibitors with

TABLE 4.1 Human Liver CYP450 Enzymes, Probe Substrates, and Preferred Inhibitors

CYP450	Proportion of Drugs Metabolized (%)	Preferred/Acceptable Reactions	Preferred Inhibitor
CYP1A2	5	Phenacetin O-deethylation or 7-ethoxyresorufin O-deethylation	Furafylline
CYP2B6	25	Bupropion hydroxylation, orfavirenz hydroxylation	Ticlopidine
CYP2C8	1	Taxol 6-hydroxylation or amodiaquine N-deethylation	Quercetin
CYP2C9	11	Tolbutamide methyl-hydroxylation or diclofenac 4'-hydroxylation	Sulfaphenazole
CYP2C19	4	S-mephenytoin 4'-hydroxylation or omeprazole hydroxylation	Ticlopidine
CYP2D6	30	Bufuralol 1'-hydroxylation or dextromethorphan O-demethylation	Quinidine
CYP3A4	52	Testosterone 6 β -hydroxylation and midazolam 1-hydroxylation	Ketoconazole

which to compare the results of the candidate compound and methods for carrying out required experiments (Table 4.1). No such recommendations have yet been proposed for conducting human CYP450 induction studies *in vitro*.

4.4 HIGH THROUGHPUT SCREENING (HTS) AND PREDICTIVE MODELING OF DDIs

4.4.1 HTS Assays for Drug Metabolism: General Approach

Traditionally, ADME studies have been performed with a limited set of compounds late in the development process. The new paradigm in drug discovery includes developing predictive ADME assays amenable to HTS of large numbers of molecules to save both time and money by eliminating undesirable compounds before they reach development [30,92]. For example, incorporation of ADME parameters into the design of combinatorial libraries is now recommended [92]. An example of the successful application of HTS to evaluate and predict possible DDIs is the development of screening platforms for oxidative and conjugative DMEs and drug transporters [72,93]. The advisability of obtaining absorption and metabolism profiles at the earliest stages of drug discovery for the large number of compounds in combinatorial libraries has led to a development of the new generation of assays with higher throughput capabilities along with increased sensitivity and reproducibility. The analytical tools allowing such capability are represented by assays for prediction of DDIs related to inhibition or induction of major human CYP450 enzymes and currently exist in 96-, 384-, 1586-well plates and microarray formats [94]. Performing these assays early in drug development process facilitates elimination of molecules with unwanted metabolic properties and

helps medicinal chemists to produce better clinical candidates with optimized ADME properties [95].

4.4.2 HTS Assays for CYP450

The approach that currently demonstrates the most promise for high throughput inhibitor screens in early discovery is based on the use of fluorogenic substrates, which can be conducted in a low volume homogenous format, requires no postreaction separation steps, and is amenable to automation and further miniaturization, including 384, 1536, and 3456 high density formats and microarray development [94].

Fluorescent HTS methods employ fluorescent P450 substrates that are efficiently metabolized by specific CYP450 isozymes to yield a product with altered fluorescent properties, usually increased fluorescent intensity [96]. Metabolism assessments with fluorogenic substrates are based on the ability of a new compound to inhibit the metabolism of the fluorogenic reporter substrate in the P450 reaction and is dependent on the kinetic parameters of such an interaction [97,98]. The hits from these competitive inhibition screens must be further evaluated to determine whether they are inhibitors or substrates for the indicated isozyme. Several of the fluorescent CYP450 substrates have been applied for the development of 96-well compatible fluorometric assays employing different human CYP450 enzymes [97,98], including polymorphic variants for selected CYP2C9 isoforms [99]. In addition, fluorometric assays in 384-well format for the CYP3A4 and CYP2D6 isozymes have also been developed [100]. Progress in the field led to the resolution of many undesirable features present in early generations of the fluorogenic CYP450 substrates, including their poor aqueous solubility requiring addition of high concentration of the organic solvents, high background fluorescence (e.g., the fluorescence of unmetabolized substrate), and low signal to noise ratios, which originally limited their applications for low volume ultrahigh throughput formats. Recently, highly miniaturized formats for primary screening assays involving several important pharmaceutical targets have been developed [101]. The ability to perform primary screening assays in microwell plates at 1- to 3- μ L volumes has made significant impact on the early stages of drug discovery by accelerating and reducing the cost of the drug discovery process [102]. The u-HTS assays were successfully developed and applied to characterize potency of many drug discovery targets *in vitro*, including pharmaceutically important class of G-protein-coupled receptors (GPCRs) [103]. Later it became apparent that with all the HTS leads being produced and characterized in the u-HTS mode, u-HTS assays of similar speed, precision, and high throughput capabilities for the CYP450 metabolism and inhibition assays involving the principal human CYP450 enzymes CYP3A4, 2D6, 2C9, 2C19, and 1A2 would be beneficial. Following this trend, highly miniaturized CYP450 screening assays were designed to enable facile analysis of CYP450–drug interactions in higher density microplate formats [97,104,105]. In particular, the development of the CYP450 assays in 1536-well plate format became possible following progress in the design and synthesis of novel class of fluorogenic CYP450 substrates characterized by improved solubility, higher metabolic rates, and lower fluorescent background [50,96]. With application of these substrates, IC_{50} values obtained in the 1536-well assays for CYP3A4, 2D6, 2C9, 2C19, and 1A2 demonstrated a high degree of correlation with data obtained in 96-well assays and conventional HPLC-based assays and were used for the study of enzyme-specific DDIs based on CYP450 inhibition. In addition to fluorogenic assays, luminogenic

assays that couple CYP450 enzyme activity to firefly luciferase luminescence were developed in order to screen for CYP450 metabolism and inhibition and related DDIs [106]. These assays, more appropriate for HTS CYP450 screening in early drug discovery, demonstrated improved sensitivity and decreased interference with optical properties of the compounds libraries. Both fluorogenic and luminogenic substrates have also been applied to measure CYP450 induction in cells [106,107], although high throughput (HT) capabilities of such assays in current formats are somewhat limited. Coincidentally, toxicological and pharmacological implications of CYP450 induction remain much less investigated. Recent reports indicate that altered expression of these enzymes may be of clinical significance and could result in increased toxicity because of the accumulation of toxic metabolites, drug side effects, or by altering the therapeutic efficacy of a coadministered drug. Therefore, identifying compounds involved in CYP450 induction, in addition to CYP450 inhibitors, would allow broadening the overall prediction of the potential for CYP450-related toxicities and DDIs. One of the promising opportunities in the field lies in the possibility of a simultaneous assessment of CYP450 inhibition and induction, allowing identification of CYP450 substrates and inducers and enabling in-depth characterization of their interactions with the CYP450 enzymes, including possible toxicity stemming from such interactions [107]. In future, similar approaches can also be used to create easy read-out assays for the assessment of multiple effects of P450 induction and inhibition for all inducible members of the human CYP450 family. Several promising technologies that show good predictability in a field of CYP450 induction and are amenable to miniaturization, automation, and further development in an HTS format include quantitative nuclease protection assay (qNPA) and PXR transactivation screening assay [108].

4.4.3 HTS Assays for UGTs

Until recently, most of the screening for UGT activity, including screening in the HTS format, was directed toward searching for novel isozyme-selective substrates of high affinity and specificity and not for potent UGT inhibitors [50]. The recent discovery of potent UGT inhibitors, such as the isoform-specific inhibitors for UGT2B7 [109] will have a significant impact toward the design of a future strategy for UGT-HTS screening process. As with CYP450s, one of the possible strategies for UGT screening now includes first-tier binding assays to determine all of the potent modulators of UGT binding activity. This may be combined with second-tier functional assays to determine whether a modulator is a substrate or inhibitor of a specific isoform, and additionally to predict potential UGT-mediated DDIs.

Finally, availability of an active UGT isoform in purified format may facilitate the progress of other methods for detection of UGT activity. For example, a screening method based on the quantification of adenine dinucleotides has been introduced for detection of activity of soluble enzymes, such as kinases and nonmembrane bound soluble bacterial glycosyl transferases [110]. Application of this screening technology in combination with the fluorescent probes designed to reduce light scattering effect [111] was successful in assays using purified, soluble protein preparations. However, these technologies are much more difficult to apply to lipid-containing preparations of membrane proteins, such as UGTs, because of the possibility of a compound partitioning into the hydrophobic membrane fraction, thereby causing unforeseen changes in fluorescent properties.

4.4.4 *In Silico* Assessment: Application of Predictive Modeling and Simulations

Over the years, the development of HTS assays for CYP450 was focused on the early identification of potent inhibitors of major CYP450 isoforms, with the likelihood of causing clinically significant DDIs and adverse drug reactions later in the drug development process as a part of the pharmaceutical industry's strategy to reduce the attrition rate in bringing new drugs to the market [100]. Further progress with the CYP450 HTS platform has led to the development of automated u-HTS assays [105] to screen multithousand-member libraries of diverse compounds and to an appearance of cell-based assays to screen for CYP450 induction [107]. Data obtained in all these assays were used for building predictive CYP450 QSAR models and development and validation of *in silico* screening [112]. A multitude of factors contributed to the availability and success of virtual screening platforms, including advancements in expression, cloning, purification, and reconstitution procedures, allowing the production of large quantities of recombinantly produced active CYP450 enzymes used in protein crystallography and modeling studies [113]. Compared to CYP450, enzyme modeling studies and virtual screening for UGT superfamily of DMEs are less advanced. However, ongoing achievements in the analysis of the recently obtained data in UGT-HTS applications for the screening of diverse chemical libraries are expected to facilitate further progress of the field, including advancements in the UGT modeling studies [114,115]. The identification of novel isozyme-selective UGT substrates and inhibitors, as well as the delineation of the role and significance of particular UGT isozymes, may play an important role in the prediction of clinically relevant DDIs. These advances also include *in silico* modeling of the novel UGT substrates and inhibitors [116].

4.4.5 Limitations and Challenges of Current Approaches

One of the shortcomings of the competitive displacement assays is that compound inhibition potency may be dependent on the nature and concentration of the reporter substance used in the reactions [23]. A valuable approach to HTS is based on the use of a mixture of substrates (substrate cocktail) in the microsomal incubations to simultaneously determine the activities of a number of CYP450 isoforms, including CYP1A2, 2A6, 3A4, 2C9, and 2D6 isoforms, has been recently reported [117]. A sensitive, high throughput ultraperformance liquid chromatography/tandem mass spectrometric (UPLC/MS/MS) method for evaluation of CYP450 inhibition has allowed determination of specific CYP450 inhibition related to DDIs with a panel of test compounds and calculation of the IC_{50} values. In both screening platforms, the possibility of ranking a compound's potency based on their apparent IC_{50} or *in vitro* inhibition constants (K_i) values as an initial step is viewed as a valuable approach to select a set of most potent modulators from a large number of compounds in a compound library for further characterization in secondary assays and additional *in vitro*–*in vivo* correlation experiments [118]. Data obtained in HTS assays have powered the development of computational tools and quantitative structure–activity relationship models aiming to develop new classes of drugs with the least potential of clinically significant DDIs. Despite significant progress in modeling, in the current state of the art, there is a need to enhance the accuracy, interpretability, and confidence in the computational models applicable to drug metabolism in the drug discovery process [119].

4.5 ROLE OF GENETIC POLYMORPHISM AND PHARMACOGENETIC CONSIDERATIONS IN PREDICTION OF DDIs

Both UGTs and CYP450 are highly polymorphic enzymes, and certain functional polymorphisms in these families of enzymes are associated with multiple cases of drug toxicity [120]. For carriers of such polymorphic variants of DMEs, alterations in PK parameters leading to DDIs may have an elevated significance. Several members of the CYP450 superfamily have a high incidence of genetic polymorphisms in human populations; DDI related to CYP2D6, CYP2C19, and CYP3A4 polymorphisms have been reported [121]. Among the UGTs, UGT1A1 is a highly polymorphic enzyme. The alteration of PK parameters and DDIs for the carriers of genetic polymorphisms associated with UGT1A1 [96,122] are of particular importance. One of the common polymorphic variants is UGT1A1*28, which is correlated with decreased transcriptional activity and is associated with mild hyperbilirubinemia or Gilbert's syndrome [123]. Recent evidence suggests that individuals with Gilbert's syndrome, who take medications metabolized by glucuronidation, such as some anticancer agents (irinotecan) and acetaminophen, suffer from increased drug toxicity [10]. Several other UGT1A1 polymorphisms (UGT1A1*33, UGT1A1*34, and the -3156G>A variant) have also been linked to impaired metabolism or increased toxicity of anticancer agents [124,125]. DDIs related to UGT genetic polymorphisms may have more pronounced significance in specific segments of clinical populations, including pediatric and geriatric patients. In pediatric patients, a number of cases of UGT-related DDIs leading to adverse drug reactions and toxicity in children, such as chloramphenicol and other antibiotic agents, have been attributed to changes in specific UGT expression during development (UGT ontogeny) [77]. Therefore, assessment of UGT activity is especially significant in pediatric drug therapy to prevent DDIs in pediatric patients. Glucuronidation is also a major drug metabolism pathway for elimination of many prescribed drugs in geriatric patients, and data related to UGT-based DDIs are important for choosing the proper dose and dose schedule and prevention of DDIs common in the elderly [76].

4.6 CONCLUSIONS AND FUTURE PERSPECTIVES

The current state of technology for the prediction of clinically significant DDIs has led to some important innovations as the field continuously evolves. The existing trend to miniaturize drug metabolism assays and extend them to different classes of targets including enzyme polymorphic variants is expected to progress into the future. For example, one of the emerging issues in the development of miniaturization technologies in screening for adverse drug reactions *in vitro* is the appearance of CYP450 microarrays [20,126]. Several groups have developed gel immobilization techniques of CYP450-containing microsomes, with apparently full preservation of enzyme activity. Another example presents the development of a nanoparticle biosensor for CYP3A4 based on label-free localized surface plasmon resonance spectroscopy [127] and ultimate miniaturization achieved by alginate gel microarray representing a 2000-fold scale down from the traditional 96-well plate format [126].

In general, technologies that have led to the progress in miniaturization of HTS-ADME assays are likely to cross over into other areas where testing of biological responses of vast amounts of individual molecules is required. In general, advances

in these areas will result in further reduction in reaction volumes and concentration of reactants and increased assay rates. The key factor here will be the emergence of standardized robotic hardware capable of handling and reading the nanoliter volumes of solutes and advancement of bioinformatics tools for the data manipulation and analysis. Specific examples of such HTS applications have now been described in the area of genotoxicity environmental testing [94].

Further expansion of virtual screening technologies and computational modeling of major ADME parameters and prediction of relevant DDIs is now a growing field [128], and it is expected to continue to grow in the future [129]. A combination of virtual screening and computational modeling with both high throughput and high content screening technologies allows more biologically relevant data to be obtained from such screens. Similar approaches may be developed as an attempt to systemically investigate complex drug effects on multiple aspects of ADME involving inhibition and induction of both oxidative and conjugative DMEs and drug transporters. In the future, such systematic approaches employing all DMEs and transporters could be applied to investigate multiple complex mechanisms, allowing more complete and careful assessment and accurate prediction of clinically relevant DDIs.

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5 Predicting Human Biotransformation Pathways

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5.1	Summary	1
5.2	Introduction	2
5.3	<i>In silico</i> tools	4
5.4	<i>In vitro</i> tools to make predictions	6
5.5	<i>In vivo</i> tools to make predictions—animal studies	12
5.6	<i>In vivo</i> human studies	18
5.7	Conclusions	22
	References	23

5.1 SUMMARY

Knowledge of the human biotransformation pathways is critical for safe and effective dosing of a new molecular entity (NME). Predicting human metabolism begins in the early discovery phase where *in silico* and *in vitro* tools are utilized to predict the extent of metabolism, the site of metabolism, and the metabolites formed. These studies may compare the *in vitro* metabolism across species. As development progresses, additional studies are conducted both *in vitro* and *in vivo*, first in animals and then in humans. *In vitro* studies are conducted most often with human liver microsomes, liver slices, or hepatocytes. These studies focus on determining which metabolites are formed and which enzyme systems are responsible for the metabolism. The *in vitro* studies are also utilized to determine if a compound inhibits or induces CYP450 enzymes. Expressed enzyme systems provide an *in vitro* system enriched in specific CYP450 enzymes. Other tissues such as intestinal microsomes may also be included in some studies.

In vivo studies start with determination of a metabolic profile in plasma and excreta obtained from the animal and first-in-human (FIH) studies followed by administration of a radiolabeled dose of an NME to determine the extent of metabolism and the identity of the metabolites in animals and humans. Various analytical tools such as LC/MS/MS, NMR, and radiochromatography are used to analyze samples from the

in vivo studies. The earlier *in vivo* studies are qualitative, while the later studies are more quantitative. Predictions of human biotransformation pathways are challenging, and no one tool completely predicts the actual *in vivo* metabolism 100% of the time.

5.2 INTRODUCTION

The drug discovery and development process includes many phases, starting with the earliest phase of discovering a target through the registration and approval of the drug with the ultimate goal of treating patients. In the early phases of drug discovery, metabolism data can identify liabilities of a potential drug candidate, whereas later in drug development, determination of the metabolism and disposition of a NME in humans are critical to provide safety and dosing information for the product label. The process to understand the biotransformation of a drug starts early in the discovery phase when *in silico* and *in vitro* methods are utilized and proceeds through development with additional *in vitro* and *in vivo* testing in animals and then humans (Fig. 5.1). Although there is no consensus among pharmaceutical companies on the timing of metabolism studies in humans, it is clear that these studies are a requirement of international regulatory agencies in order to successfully register an NME and are usually desired before the start of phase 3 clinical trials [1,2]. Metabolism is often the major clearance pathway of an NME in humans, and many intrinsic and extrinsic factors such as genotype and coadministered drugs can affect metabolism. In addition, first-pass metabolism that occurs during the absorption process before the NME reaches the systemic circulation can affect the bioavailability. Metabolism can also cause intra- and intersubject variability because of differences in metabolism between occasions and/or individuals, thereby leading to differences in systemic exposure and ultimately in efficacy and/or safety in humans.

Information on the biotransformation pathways in humans is a precursor to many other studies. It is important to assess the potential for alterations in exposure due to either coadministration with other drugs that may inhibit or induce the metabolism of the NME or due to different genotypes and phenotypes. This information is a precursor to the clinical pharmacology plan for an NME, especially regarding the potential for drug–drug interaction studies and the definitive cardiac QT study. In addition, the genotype and phenotype of an individual may affect the degree of metabolism of the NME in that individual compared to others, as many of the enzymes that are responsible for the metabolism of an NME are known to be polymorphic.

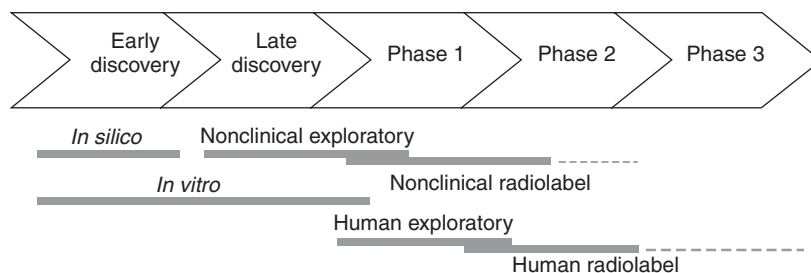


Figure 5.1 Approximate timing of *in silico*, *in vitro*, and *in vivo* assessments over the course of drug discovery and development.

In assessing clearance pathways for NMEs, information on what drives the clearance is obtained first (i.e., metabolism, renal excretion, biliary transport). Once the biotransformation pathways are elucidated, *in vitro* studies are conducted to determine the enzymes that catalyze the reaction, especially if the enzymes are thought to be cytochrome P450 (CYP450) enzymes. On the basis of the *in vitro* studies, appropriate *in vivo* studies with known inhibitors and/or inducers of the specific enzymes will be conducted. If metabolites are formed via enzymes with genetic polymorphisms, additional studies from subpopulations possessing various alleles known to affect the metabolizing enzyme capability may be conducted. Since the definitive clinical QT cardiac safety study is to be conducted with a dose that reaches exposure approximating the “worst case scenario,” an understanding of the factors that may lead to increased exposure is required before conducting this safety study. Without knowledge of the biotransformation pathways in humans, a sponsor would not know which drug–drug interaction studies to conduct or which doses or dose adjustments would be needed under various circumstances.

Much has been written on the issue of metabolites in safety testing (MIST) [3–17]. In both FDA and International Committee on Harmonization (ICH) regulations, sponsors are encouraged to conduct *in vivo* metabolism studies in humans as early as feasible [18,19] since the major metabolites found in humans must have been tested for safety in animals. A metabolite is considered a major human metabolite if it is Insert greater than or equal to sign. 10% of the total of all circulating drug related substances including parent [ICH M3(R2)] [14]. For major human metabolites, the circulating concentrations in animals used in the toxicology studies should approach or be significantly greater than the human exposure. If the human metabolite is not found in the animals in sufficient quantities, then it is considered a “unique or disproportionate” human metabolite, and additional safety testing with the specific metabolite may need to be conducted in animals before initiating large-scale clinical trials. However, in the majority of the cases one or more of the animal species will produce the same metabolites as humans, making additional animal testing unnecessary. Additional safety testing can be time consuming, can take multiple forms, and can require that large amounts of the metabolite be synthesized. Thus, the sooner a sponsor can predict the human biotransformation pathways, the sooner safety coverage can be addressed. The toxicity of metabolites, especially “reactive” metabolites of a given NME, can lead to adverse events in clinical trials and ultimately to the cessation of its development [20]. Circulating metabolite data and demonstration of coverage in animals used in nonclinical safety studies are usually required before exposing large numbers of patients to the drug in phase 3 trials [14].

The liver is usually considered the major organ responsible for metabolism of drugs, as it contains the highest amount of drug-metabolizing enzymes, especially the CYP450 enzymes. Over 75% of the top prescribed drugs are metabolized by either the CYP450 enzymes or the uridine glucuronosyltransferases (UGTs) [21]. In addition to the liver, some of the CYP450 enzymes such as CYP3A are present in high concentrations in the gastrointestinal mucosa, where they metabolize drugs as they enter the body and before reaching the systemic circulation [22,23]. Human carboxyesterases (hCE) and UGTs are also in high concentrations in both the liver and gastrointestinal mucosa where hydrolysis and conjugation can occur [24,25]. Additional extrahepatic sites of metabolism include organs such as the lungs, kidneys, and skin. Other organs such as the brain can also be a source of drug-metabolizing enzymes, but these are less well

studied and the enzymes present in the brain contribute more to metabolism of endogenous compounds and less to exogenous compounds [26,27]. Extrahepatic metabolism can affect both absorption and excretion of an NME and, thus, may be an important factor in its biotransformation. The abundance of drug-metabolizing enzymes in various tissues differs among species, and this may add to difficulties in predicting human metabolism from nonclinical matrices. Many of the studies on distribution of drug-metabolizing enzymes have been conducted in animal tissues because of the difficulty in obtaining human tissues other than liver and gastrointestinal mucosa. Prediction of human biotransformation in extrahepatic tissues relies heavily on calculation of total, hepatic, and nonhepatic clearances and *in vitro* studies using tissues or specific enzyme systems known to be predominantly found in an extrahepatic tissue. *In vivo* studies after administration of both an intravenous and an oral dose of a drug can help in assessing the degree of metabolism due to first-pass metabolism in either the gastrointestinal tract or liver.

In this chapter, tools used to predict human biotransformation pathways are discussed, beginning with *in silico* tools followed by *in vitro* tools, and then *in vivo* tools. This chapter explains the appropriate use of *in silico*, *in vitro*, and *in vivo* tools as well as the pros and cons for each tool in the quest for predicting the human biotransformation pathways.

5.3 IN SILICO TOOLS

Elucidating the biotransformation pathways in humans is an integral part of the discovery and development process, and the earlier this information can be obtained, the better equipped scientists are for making decisions and developing strategies for new compounds. In the discovery phase when no human *in vivo* data are possible and the *in vitro* data require synthesis of a large array of compounds and metabolites, an alternate approach is to utilize *in silico* methods to predict the human biotransformation pathways. *In silico* methods are computer-based techniques that attempt to predict the *in vivo* biotransformation pathways using computational experiments, mathematical calculations, and scientific analyses [28–32]. Some programs predict the most likely sites of metabolism in a molecule and the extent of metabolism, while other programs predict both the site of metabolism and the structure of the predicted metabolites. The backbone of these computational approaches is the knowledge base compiled from experimental data generated from previous *in vitro* and *in vivo* studies and includes biotransformation data, docking, and electronic information about the enzymes and substrates [20]. Often the data are mined from the literature, but they can also include proprietary experimental information. A necessary component of the *in silico* model building is computational power to run algorithms in parallel and then to generate results in a user-friendly manner.

Throughout the development of computational metabolism predictions, various approaches have been utilized. These include the quantitative structure–activity relationship (QSAR) or quantitative structure–metabolism relationship (QSMR), pharmacophore models, and rule-based/knowledge-based expert systems [29,31]. The availability of the X-ray structures of several CYP450 enzymes (i.e., CYP3A4, CYP2C9, and CYP2B6), especially with bound substrates, has been instrumental in developing these models. Although X-ray structures for the crystals of enzymes

involved in glucuronidation and sulfation have not been determined, QSAR models have been developed to predict metabolic interactions with these enzymes from limited data compared to that utilized in the CYP-based models. These QSAR models include general chemical features of the enzyme–molecule interaction such as hydrophobic interaction, hydrogen bonding, and ionization ability [29]. The electronic models include additional calculations of ground-state energies based on molecular orbital calculations, but these have been used less frequently than the QSAR models [29].

There are many commercially available computational programs to predict biotransformation pathways. Some predict the metabolite structures, while others only predict the site of metabolism. Some are based on CYP450 reactions alone, while others are capable of predicting both phase I and phase II metabolism. The accuracy of these programs relies on the amount and quality of the knowledge, information, and data constituting their training sets or knowledge base. The more extensive and diverse the training data set with regard to molecular structure and pathways, the higher the expected accuracy in predicting the true *in vivo* biotransformation pathways [30].

Currently, one program that combines QSAR and rules-based approaches, MetaDrug™ (GeneGo), has been developed with a database of human drug metabolism information including over 10,000 xenobiotic reactions, over 1500 enzyme substrates, and over 1000 enzyme inhibitors [29]. This package first predicts the regions of a molecule most likely to undergo metabolism and then the structures of the most likely metabolites. The program allows for over 66 metabolic pathways using a set of rules to predict the metabolite transformations, both phase I and phase II reactions. In a literature article, MetaDrug was reported to have correctly predicted >73% of phase I metabolites for 28 diverse molecules [29]. A program called MetaSite™ (Molecular Discovery) predicts the site of CYP450-mediated phase I metabolism and the structures of the most likely metabolites.

Another program currently available is StarDrop™ (Optibrium), which predicts the most vulnerable sites for metabolism with CYP3A4, CYP2D6, and CYP2C9. These three enzymes account for the majority of CYP450-mediated metabolism *in vivo*. Molecules can be redesigned with this program to overcome the potential for a metabolism drug–drug interaction involving these three enzymes.

In silico metabolism predictive systems have clear utility, especially in the early phases of drug discovery when a large number of molecules, sometimes virtual, need to be rapidly prioritized in the absence of *in vivo* or *in vitro* animal or human data. In general, the *in silico* programs tend to overpredict the true *in vivo* metabolism, generating a much larger list of metabolites than that actually found *in vivo* [29], probably due to the complex physiological interactions that can occur *in vivo* (e.g., distribution), but are difficult to completely model computationally. The prediction of initial pathways appears to be more accurate than the overall metabolism profile, while the complex metabolic pathways that occur sequentially and involve both phase I and phase II metabolism are more difficult to predict with the current *in silico* models. Scientists need to be aware that excessive “false-positive” predictions may lead to exclusion of potential molecules from consideration for development. One should be particularly mindful of this because the pathways in animals and humans may differ and the *in vivo* physiological processes that affect biotransformation of a molecule may not be completely modeled in the current *in silico* predictive programs. As the field of

prediction advances, it is hoped that newer *in silico* tools and models will predict not only likely pathways and metabolites but also their quantity and rate [20].

5.4 IN VITRO TOOLS TO MAKE PREDICTIONS

5.4.1 Background

The study of xenobiotic metabolism began in the mid-nineteenth century [33] using mainly *in vivo* administration of chemicals to animals and humans, followed by collection of the urine to look for evidence of bioconversion. Amazingly, despite the fact that the analytical techniques of the time were very crude, all the major pathways of drug metabolism were elucidated, including oxidation, reduction, hydrolysis, conjugation (with glycine, sulfuric acid, and glucuronic acid), acetylation, and mercapturic acid synthesis. In the first half of the twentieth century, the terms *metabolism* and *biotransformation* were not used with respect to foreign compounds but instead focused on the “fate” of the chemical after some form of conversion in the body [34]. In 1893, the term *detoxication* was used for the first time in recognition of the fact that some urinary metabolites were less toxic than the compound administered *in vivo*, and this term became a synonym for what we know today as *drug metabolism*.

The laboratory techniques to investigate the involvement of the liver in drug metabolism were developed as early as the 1930s, beginning with the implementation of nondestructive liver homogenization procedures in 1936 [35]. The utilization of differential centrifugation techniques to separate liver organelles from homogenates was pioneered in the laboratory of James and Elizabeth Miller [36] in the 1940s and was followed by the groundbreaking work of Julius Axelrod, which localized the subcellular machinery responsible for the bioconversion activity in the liver to what is today known as the *microsome* [37]. Studies on the rat carcinogen dimethylaminoazobenzene (DAB) by Mueller and Miller [36] led to the important discovery that metabolism of DAB by liver homogenates required oxygen and NADPH as the reducing agent. Beginning in the 1950s, Brodie and his group [38] began to elucidate the molecular basis of drug oxidation and conjugation reactions, as documented in his 1958 review, “Enzymatic metabolism of drugs and other foreign compounds.” Without the collective groundbreaking work of these pioneers, we would not have the tools that are today taken for granted by the drug metabolism scientist. An overview of the predictive power of the most common hepatic subcellular fractions, cell-based systems, and recombinant DNA-expressed systems that are used to conduct contemporary *in vitro* metabolism experiments is presented in this section of the chapter.

5.4.2 Microsomes (Tissue Specific)

The differential centrifugation of a liver homogenate enables the isolation of specific organelles from the rest of the liver cells. The first centrifugation that is traditionally done at 9,000 or 10,000 *g* will remove intact cells, nuclei, and mitochondria. The remaining supernatant is informally known as the *S9 fraction*, which contains the cytosol and fragments of the endoplasmic reticulum (ER). The cytosol contains many of the soluble enzymes (such as aldehyde oxidase and glutathione *S*-transferase), whereas

the ER contains the CYP450 enzyme system responsible for a majority of the oxidations that occur in the liver. At 100,000 g, the ER will sediment out of solution into a pellet, which contains the vesiclelike microsomes. After suspension of the pellet in an appropriate solution, enzyme activity remains relatively stable if the microsomal stock solution is stored in a freezer at -70°C .

It should be noted that microsomes can be prepared from any tissue, but liver microsomes are the most commonly isolated because liver contains the highest concentration of CYP450 enzymes, followed by the upper gastrointestinal tract. For many years, individual drug metabolism laboratories prepared their own liver microsomes and assessed the CYP450 activity in each batch; however, human livers were generally more difficult to acquire than livers from the various animal species. Today, there are several vendors that provide microsomal stock solutions with a known protein concentration and quantified CYP450 enzyme activity from a large number of species. Thus, liver microsomes are the most common *in vitro* system used to predict *in vivo* biotransformation of xenobiotics.

Microsomes are used as a drug disposition tool for three main purposes: (case 1) to determine the rate and extent of intrinsic clearance (or metabolism) of a drug substrate, (case 2) to establish the *in vitro* metabolic profile for a drug substrate, and (case 3) to investigate the CYP450 enzyme inhibition potential of a drug. For all three of these cases, the primary goal is to generate *in vitro* data that can be extrapolated to the whole animal (or human) *in vivo* [39].

In case 1, the microsomal protein concentration is standardized (e.g., 0.5 mg/mL) in a phosphate buffer (pH 7.4), and a compound is incubated for a given period of time (e.g., 15 or 30 min). The use of a standardized microsomal protein concentration in every experiment allows the rank ordering of discovery compounds within a chemical series for “microsomal stability” or “microsomal clearance.” Metabolic stability in human liver microsomes can be effectively classified (e.g., low, moderate, high) with respect to the known human metabolism of a cassette of reference drugs chosen to encompass a wide range of susceptibility to phase 1 (oxidative) metabolism [40].

This *in vitro* system is also ideally designed to help the drug metabolism scientist determine the so-called unbound intrinsic clearance of the compound for a given species, as long as the microsomal protein binding of the compound is known [41,42]. The technique is also frequently used to identify compounds that are prone to hepatic first-pass metabolism in one or more species, including humans. As discussed earlier, extensive first-pass metabolism can significantly reduce the bioavailability of a potential drug and information on this potential can be obtained *in vitro* before the conduct of an *in vivo* study. In general, commercially successful drugs with low first-pass metabolism are preferable unless the drugs are designed to be prodrugs, which are intended to undergo an initial metabolic step (such as hydrolysis) to activate the pharmacological moiety or moieties.

In case 2, the microsomal incubation serves as a vehicle to generate one or more metabolites of a given drug substrate, which can be identified using established liquid chromatography with mass spectrometry (LC-MS) techniques. The wealth of information that is generated from these metabolite profiling and metabolite identification studies can be used to make predictions of biotransformation pathways in animals and humans well before any *in vivo* studies can be conducted. These *in vitro* experiments can frequently over- or underpredict the circulating human metabolites identified via the definitive human ADME (absorption, distribution, metabolism, and excretion)

study with radiolabel [11]. These authors have presented data-driven trends gleaned from a survey of drug development programs, which indicated that with more complex metabolism, the less likely were the *in vitro* data to predict the circulating human metabolites. However, in general, the *in vitro* data were suitable at predicting overall clearance pathways including excreted metabolites, but often failed to correctly predict the levels of the metabolites that circulated in human plasma.

Consistent with the ability of the *in vitro* system to establish clearance pathways, pooled human liver microsomes are also commonly used to identify the specific CYP enzyme(s) responsible for generation of an oxidative metabolite that accounts for at least 25% of the total clearance. This type of assessment is known as *CYP reaction phenotyping* and is an expected component of the clinical pharmacology package at an NDA (New Drug Application) submission [43]. The prediction of CYP enzymes associated with major biotransformation pathways for NMEs is particularly important in the risk assessment of drug interactions. There are some examples of commercial drugs that either have been withdrawn or have undergone significant label changes based on a determination that the drug was heavily dependent on a particular CYP pathway. In the older example of terfenadine, the discovery that this drug caused life-threatening cardiac effects was ultimately related to the fact that terfenadine was metabolized almost exclusively via CYP3A and inhibition of this clearance pathway led to the buildup of cardiotoxic concentrations of the parent drug [44]. In the more recent case of clopidogrel, it was eventually determined that the first major step in bioactivation to the active species was dependent on CYP2C19, which is polymorphic and thus may be absent in a subset of the patient population who would not receive the expected benefit of the drug [45]. In both these examples, these discoveries were made after the drugs were already on the market, but it is important to note in both cases that this information could have been predicted by *in vitro* metabolism experiments.

For case 3, liver microsomes are used to elucidate the drug interaction potential of the NME on other drugs [39], as opposed to the previous case where another drug inhibits a metabolic pathway of the NME. In both these situations, the human liver microsomes are used to probe the impact of one drug on the metabolism of another, and this information is critical for the clinical safety assessment of any drug under development. One of the reasons that liver microsomes are an ideal system for this *in vitro* assessment is that enzyme kinetics can be easily determined. Unlike cell-based systems (similar to hepatocytes, discussed below), the microsomes do not contain a cellular membrane and can immediately equilibrate with the drug substrate without necessitating membrane translocation by either passive or active processes.

More recently, the risk of mechanism-based (also known as *time-dependent*) inhibition has become an area of interest for many pharmaceutical sponsors and regulators since the reduction of metabolic activity of a particular CYP450 is often maintained for a substantial amount of time beyond the cessation of the dosing of the inhibitor. In many cases, the drug irreversibly binds to the enzyme, and full activity is not restored until complete turnover of the enzyme. Once again, human liver microsomes serve as an ideal medium for screening time-dependent inhibitors by allowing preincubation of the potential inhibitor with the microsomes in the presence of the NADPH cofactor. Subsequently, the diluted preincubation microsomal mixture is used to evaluate the extent of biotransformation of a known probe substrate (e.g., midazolam); if the residual activity of the preincubation mixture is reduced compared to the control incubation, it indicates that the drug substrate poses a clinical risk for time-dependent

inhibition. Mibefradil (Posicor) is a well-known example of a commercial drug that was withdrawn from the market when it was determined to be a potent time-dependent inhibitor of CYP3A4 and the sponsor realized that “the complexity of such prescribing information would make it too difficult to implement” [46].

Although the liver microsomes are a very useful tool for a wide variety of predictive purposes, there are some limitations to this system. One downside of this system is that it is optimized for oxidative (phase I) metabolism and does not enable a straightforward assessment of conjugative (phase II) metabolic reactions such as glucuronidations or sulfations. There have been modifications to the standard systems by replacing the NADPH cofactor with uridine diphosphate glucuronic acid (UDPGA) along with some surfactants to facilitate bioconversion via UGTs in the microsomes [47]. However, these incubations can be difficult to reproduce and are not ideal to predict phase II metabolic reactions, especially ones that depend on less well-known cofactors. Another limitation of the liver microsomes is that the optimal activity of the various enzyme systems is dependent on maintenance of a certain pH and temperature. For example, the CYP enzymes are optimized for activity at pH 7.4 and 37°C, whereas the flavin monooxygenase (FMO) family, which catalyzes many heteroatom oxidations such as N-oxidation, exhibits optimal activity at pH 6.8 and is easily deactivated above an incubation temperature of 40°C. Nevertheless, the long-term maintenance of drug-metabolizing activity, availability, ability to compare species-specific metabolism, and ease of use have made liver microsomes the tool of choice for the drug metabolism scientist.

5.4.3 Hepatocytes

The hepatocyte is the main cell in the liver, accounting for approximately 70–80% of the liver mass, and it contains the majority of the ER containing the CYP450 enzymes. Hepatocytes can be separated from the liver via collagenase digestion, followed by plating and culturing for immediate use or cryopreservation (freezing at very low temperatures) for later use. Hepatocytes in primary culture do not maintain their viability or metabolic activity beyond 72 h and often require induction with exogenous chemicals to increase enzyme activity to reasonable levels [48], whereas the cryopreserved hepatocytes (also known as *cryocytes*) can be sensitive to damage during cycles of freezing and thawing. Unlike microsomes, which are ubiquitous in most drug metabolism laboratories, hepatocytes were not as easy to acquire and prepare due to the difficulty in obtaining cells in fresh supply from any species other than rats or mice. More recently, vendors have made cryocytes available in a wide variety of species, and this tool has become much more accepted in drug metabolism laboratories.

In the early days of cryocyte usage, acceptance was limited because of concerns about the impact of the freezing on the activity of the drug-metabolizing enzymes or that cryopreserving would skew selection toward a subgroup of hepatocytes that would not be representative of the native drug-metabolizing activity of hepatocytes obtained from primary culture [49]. However, widespread use of these cryocytes has demonstrated that they are reasonably reliable and reproducible. A major advantage of cryocytes over liver microsomes is that they are capable of biotransforming drug substrates via UGT and sulfotransferase (ST) conjugating enzymes and specific cofactors are not required because the cell operates as its own intact drug metabolism “factory.” The hepatocyte also allows sequential reactions (i.e., oxidation followed by glucuronidation) leading to

an ultimate metabolite excreted in either urine or feces. Thus, hepatocytes obtained from a variety of animal species and humans provide a straightforward means of qualitatively comparing phase I and phase II metabolism of a given drug substrate across species and determining whether the animal species are generating similar metabolic profiles as the human system. This assessment is important to enable the drug metabolism scientist and toxicologist to select the appropriate nonrodent species for nonclinical safety assessment on the basis of relevant human biotransformation pathways.

Another useful role of cryopreserved hepatocytes is their suitability to generate rate of drug clearance within the intact cell. These hepatocyte clearance assessments can be used to rank order compounds in a chemical series, and in some cases, may be more predictive than the microsomal clearance assessment discussed above because the hepatocytes will enable competition between phase I and II metabolic pathways, which is expected to be more physiologically predictive than the microsomes alone.

There have been some reports that hepatocytes are an alternative system to evaluate inhibition enzyme kinetics and inhibition potential of an NME [49,50]. However, there is no universal agreement on the utility of this system for this purpose because of the previously discussed limitations of membrane translocation and potential bioaccumulation of the drug inside the cell.

Finally, human hepatocytes are the tool of choice to predict CYP450 induction (for the inducible isoenzymes CYP1A2, 2B6, and 3A) of an NME before any repeat-dose human *in vivo* data are available. Initially, only fresh hepatocytes were used for this purpose because of long-term stability concerns, but availability issues made it difficult to plan ahead for the experiments. Several detailed investigations of cryopreserved hepatocytes have qualified the use of this *in vitro* system to assess enzyme induction potential with reliable results [49,51]. In some cases, enzyme induction can result in overproduction of a particular CYP450 leading to increased metabolism of a coadministered drug metabolized by the enzyme. In other cases, CYP induction can result in decreased exposure of the NME itself (known as *autoinduction*). In both situations, predicting the risk of CYP450 induction mediated by an NME is important information for the clinical pharmacology package required for registration of the drug. The ability to make these predictions using hepatocytes is an important advantage of this *in vitro* system.

5.4.4 Liver Slices

In 1953, Julius Axelrod demonstrated that amphetamine was completely metabolized in a rabbit liver slice without addition of any cofactors [37]. Liver slices were used quite often as a miniature version of the intact liver long before there was an understanding of the molecular basis of drug metabolism. However, it was determined that the rate and extent of drug metabolism was highly dependent on the size and thickness of the individual slices [52]. This observation led to the advent of precision-cut liver slices, which could be standardized through adjustment of the settings on the mechanical slicers that soon after became commercially available. These uniform slices could be maintained viable in culture for 1 to 10 days and enabled optimal exchange of nutrients, waste, and gases.

Before cryopreserved hepatocytes had gained widespread acceptance and were available from commercial sources, precision-cut liver slices were the principal tool used by drug metabolism scientists to predict phase I and II metabolism of an NME [53].

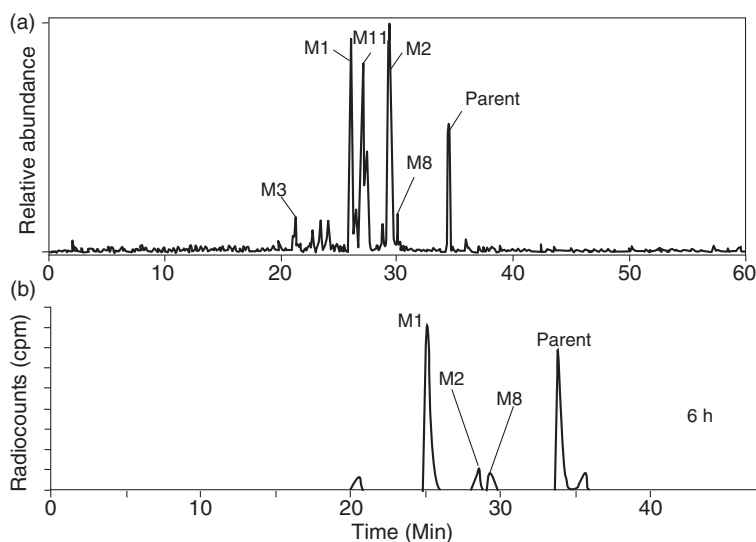


Figure 5.2 (a) LC/MS profile for LY14 from 24-h incubation in human liver slices. (b) Radioprofile for LY14 from human plasma 6 h after an oral dose of 5 mg.

As described above for hepatocytes, the *in vitro* correlation of biotransformation across species was the only opportunity to determine if the animals and humans were generating qualitatively (and perhaps quantitatively) similar metabolic profiles before human *in vivo* data were available. As with any tool, mixed results have been obtained with respect to the predictability of liver slices for *in vivo* metabolism of a given NME. A human liver slice profile for Drug LY14 is shown in Fig. 5.2, along with the radioprofile from human plasma 6 h after an oral dose of 5 mg. These chromatograms illustrate the nice correlation for metabolites M1, M2, and M8 between human liver slice and plasma. Although not shown, the M11 metabolite was found to be significant in human urine, demonstrating the value of *in vitro* systems for prediction of operative metabolic routes. A second example where incubation of an NME with human liver slices was qualitatively (but not quantitatively) predictive of human clinical metabolism has been published [54]. In this case, several *in vitro* and *in vivo* metabolites were generated with a high degree of overlap. However, this example demonstrated that there were also many metabolites generated in the animal liver slices that did not have any clinical relevance at all. Thus, while liver slice profiles can be very useful in providing a baseline for predicting human metabolism, one should not assume that all *in vitro* metabolites will be represented as circulating human metabolites [11].

5.4.5 Expressed Enzyme Systems

Pooled human liver microsomes are the tool of choice to evaluate metabolic turnover, enzyme kinetics, enzyme inhibition, and CYP450 reaction phenotyping. The pooling of different sources of human livers allows for some metabolic diversity to be represented in the incubation mixture. In addition, the use of pooled human liver microsomes for enzyme inhibition experiments enables the assessment of metabolic switching from one metabolic pathway to another when the primary pathway is chemically inhibited

[55]. However, there are occasions when recombinant expressed enzyme systems are useful to determine the individual impact of particular CYP450s on the metabolic clearance of an NME [56]. These commercially available, soluble, purified, recombinant human CYPs are produced in *Escherichia coli* and reconstituted with oxidoreductase, cytochrome *b5*, and the appropriate lipids. When supplemented with NADPH, these specific CYP450 enzymes (e.g., CYP3A4, CYP2D6) are capable of distinguishing which enzyme(s) are responsible for the generation of a particular metabolite.

5.4.6 Intestinal Homogenates

As mentioned earlier, the upper gastrointestinal tract (i.e., duodenum and proximal jejunum) is the organ that exhibits the second highest level of CYP450-mediated metabolism after the liver [57]. First-pass gut metabolism is more prevalent than originally thought and was not well understood until a series of serendipitous events occurred related to cyclosporin drug metabolism. It was discovered that when cyclosporin A was administered with grapefruit juice, the exposure of the immunosuppressant was significantly increased by several fold, resulting in unexpected drug toxicity. This increase in drug exposure initially appeared to result from inhibition of CYP3A-mediated clearance; however, it was not understood how grapefruit juice or any of its components was inhibiting CYP3A-mediated hepatic metabolism. Subsequent studies of grapefruit juice revealed that the furanocoumarins are inhibitors of CYP3A, but this inhibition is manifested locally in the intestine at the gut enterocyte, where the CYP3A expression is maximized [58]. As such, intestinal homogenates have become another *in vitro* tool to investigate extrahepatic CYP3A-mediated metabolism [59] as well as esterase-mediated hydrolysis of various prodrugs [60]. It should be noted, however, that standardization of these preparations can be difficult and more meaningful data regarding extrahepatic metabolism can often be obtained from *in situ* intestinal perfusion experiments in whole animals.

5.5 IN VIVO TOOLS TO MAKE PREDICTIONS—ANIMAL STUDIES

5.5.1 Choice of Species

As discussed previously, early in the drug discovery process *in vitro* studies are conducted to assess the extent of metabolism and to compare the metabolic profiles between animal species and humans. These *in vitro* assessments typically play a critical role in the selection of the rodent and nonrodent species for the toxicity assessment. These predictions are then evaluated to determine whether the *in vivo* profiles are consistent with the *in vitro* data. If the *in vivo* assessments suggest alternate routes of metabolism or species-specific metabolism, the choice of toxicity species may be reevaluated to ensure that anticipated human metabolic routes are covered in the nonclinical species, and thereby the toxicity studies. Ultimately, the definitive metabolism package is conducted using radiolabeled drug and will include metabolism in the relevant animal species used for toxicity testing (generally mouse, rat, and dog or monkey). However, the radiolabeled studies are generally conducted later in drug development and thus are used primarily to assess metabolite coverage and major clearance pathways. These studies occur too late to effectively affect the selection of the animal species to be

used in the initial toxicity studies. No one animal species will routinely predict all the *in vivo* human biotransformation pathways. Sometimes the *in vivo* human metabolism is more similar to the nonrodents (dogs, monkeys), while some other times, the human metabolism is more consistent with rodents (mice, rats).

5.5.2 Studies with Unlabeled Compound

Although *in vitro* assays provide a valuable tool for assessing the metabolic stability of compounds in drug discovery, predicting major metabolic pathways and providing for cross-species comparisons in the selection of the toxicity species, *in vitro* experiments do not always accurately predict the *in vivo* metabolic profile, especially from a quantitative standpoint. Therefore, researchers will examine plasma, urine, and/or bile from nonclinical pharmacokinetic or toxicity studies where animals are dosed with unlabeled drug in an attempt to more thoroughly investigate the operative metabolic pathways before conducting the radiolabeled studies. Such “cold” analyses are the first chance to evaluate the predictive power of the *in vitro* systems. In many cases, these *in vivo* assessments suggest that the major metabolic routes were appropriately captured *in vitro*, thus providing additional confidence around one’s understanding of the extent of metabolism and major clearance pathways (based on urine and/or bile). Not infrequently such *in vivo* assessments suggest more extensive metabolism than predicted by the *in vitro* systems and in many cases suggest additional metabolic pathways not readily predicted by the *in vitro* systems. The more complex the operative metabolic pathways, the more likely that there may be disconnects between the *in vitro* and *in vivo* data (i.e., multiple sequential oxidations, oxidations followed by conjugation, non-CYP450-mediated cleavage reactions, or transformations catalyzed by extrahepatic enzymes). In general, *in vitro* analyses are more effective at predicting clearance pathways than major circulating metabolites, as expected because of all the complexities that contribute to whether a metabolite circulates or is rapidly cleared after its formation [11,61].

The samples from unlabeled studies are typically analyzed using LC/MS/MS techniques. It should be noted that the qualitative identification of metabolites from these early studies is quite reliable, allowing for a reasonably accurate prediction of operative clearance pathways. Quantitative profiling is, however, more challenging, as LC/MS or LC/UV signals do not provide a robust measure of the relative contribution of a metabolite in the absence of a synthetic standard (as relatively small structural modifications can lead to significant changes in the UV absorbance and/or MS ionization of the metabolite relative to parent drug). Therefore, such exploratory assessments can sometimes be misleading in terms of identifying major circulating and/or excreted metabolites, with the possibility of over- or underpredicting the relative contribution of any given metabolite.

High resolution or mass defect filtering (MDF) LC/MS techniques [62–68], as well as noise reduction or isotope-pattern filtering algorithms [69,70], have proven to be valuable in analyzing plasma from unlabeled pharmacokinetic or toxicity studies, as well as early clinical studies. This approach takes advantage of the fact that accurate mass of the parent drug (as well as its anticipated metabolites) can be used as a “fingerprint” to identify drug-related ions. A common trait of all the exact mass filtering algorithms is that they facilitate the extraction of drug-related signals from what is otherwise often a very complex total ion chromatogram, effectively filtering

for metabolites with both anticipated and unanticipated biotransformations. Figure 5.3 illustrates the benefit of MDF for Drug LY15, which was administered as a radio-labeled dose to rats. The MDF chromatogram shows significant correlation with the corresponding radioprofile, whereas most of the larger peaks on the total ion current (TIC) are not drug related. Broader access to more affordable and easy to use high resolution instrumentation have made the analysis of unlabeled samples more effective (and efficient) than with historical nominal mass instrumentation (which relied on less restrictive filtering criteria such as precursor ion scans, neutral loss scans, background subtraction), and thus, this technique is being widely used across the pharmaceutical industry. However, it should be noted that this approach still does not provide quantitative assessments of metabolites in these unlabeled samples.

Exploratory *in vivo* assessments of nonclinical matrices can significantly increase one's understanding of the operative metabolic processes for an NME. Taken together, the *in vitro* data and early *in vivo* nonclinical assessments play a critical role when deciding to bring a molecule forward into drug development, in assessing operative metabolic pathways in animals and thereby predicting human metabolic pathways, and in developing early pharmacogenomic strategies. However, these assessments often miss the mark in terms of predicting *a priori* "major circulating human metabolites." Ultimately, the nonclinical radiolabel metabolism studies will provide the most detailed and accurate assessment of the operative metabolic routes for each animal species. Any decisions regarding major circulating metabolites or metabolic routes that are derived primarily from "cold," exploratory analyses are potentially made at risk.

5.5.3 Studies with Radiolabeled Compound

Definitive data regarding major circulating and/or excreted metabolites and metabolic processes for both the nonclinical species and human will be obtained via the conduct of the corresponding radiolabel studies. The nonclinical studies are typically run at the low end of the toxicology dose range, using the same route of administration as the safety studies. Such studies most commonly use ^{14}C -labeled drug (although the use of a ^3H label is not uncommon, particularly for molecules that are tested earlier in the drug development process or for low dose drugs). Utilization of the radiotracer assists in better defining the operative metabolic processes as well as to obtain the mass balance (the total recovery of administered drug in excreta), the plasma pharmacokinetics of the parent drug and total circulating radioactivity, and the major routes of excretion [71–73].

From a metabolite profiling and identification perspective, radiolabeled drug allows metabolites to be profiled in an effectively quantitative manner (whether via a radio-flow detector, fraction collection and liquid scintillation counting, or solid scintillation plates and a TopCount/MicroBeta counter), allowing for direct comparison of the metabolite exposure relative to the total circulating radioactivity or circulating levels of parent drug in plasma, as well as for the calculation of the percent of dose for excreted metabolites. This enables major circulating metabolites and clearance pathways to be determined in the nonclinical species, including quantification of any direct renal and/or biliary clearance of parent drug. Radiolabeling also enables more complete metabolic profiling, in that most metabolites retain the ^{14}C label, and thus, the radiolabel provides a "flag" for drug-related entities. Figure 5.4 shows radiochromatograms for LY15

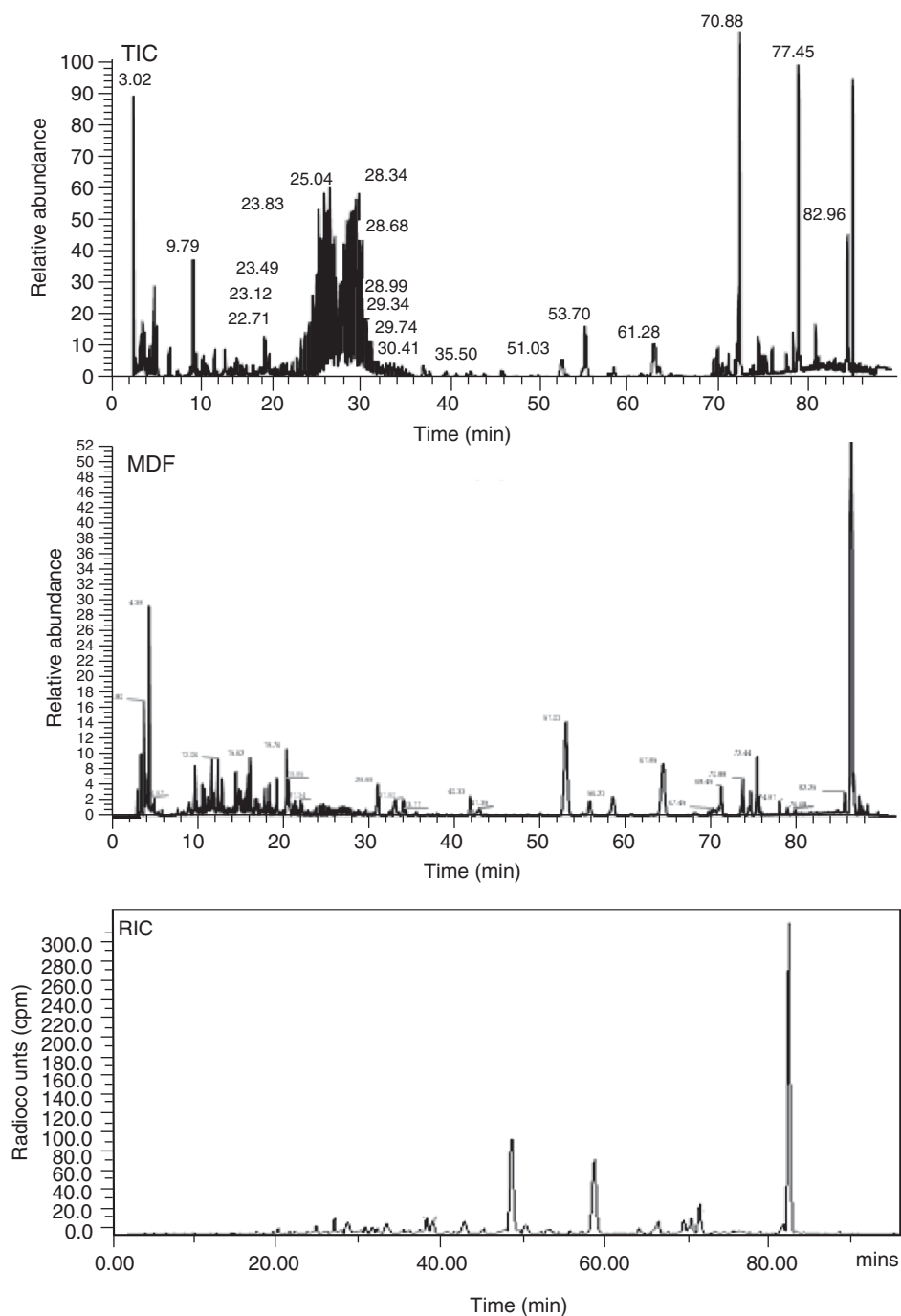


Figure 5.3 The LC/MS total ion chromatogram (TIC), mass defect filtered (MDF) chromatogram, and radioisotope chromatogram (RIC) for LY15 in rat excreta.

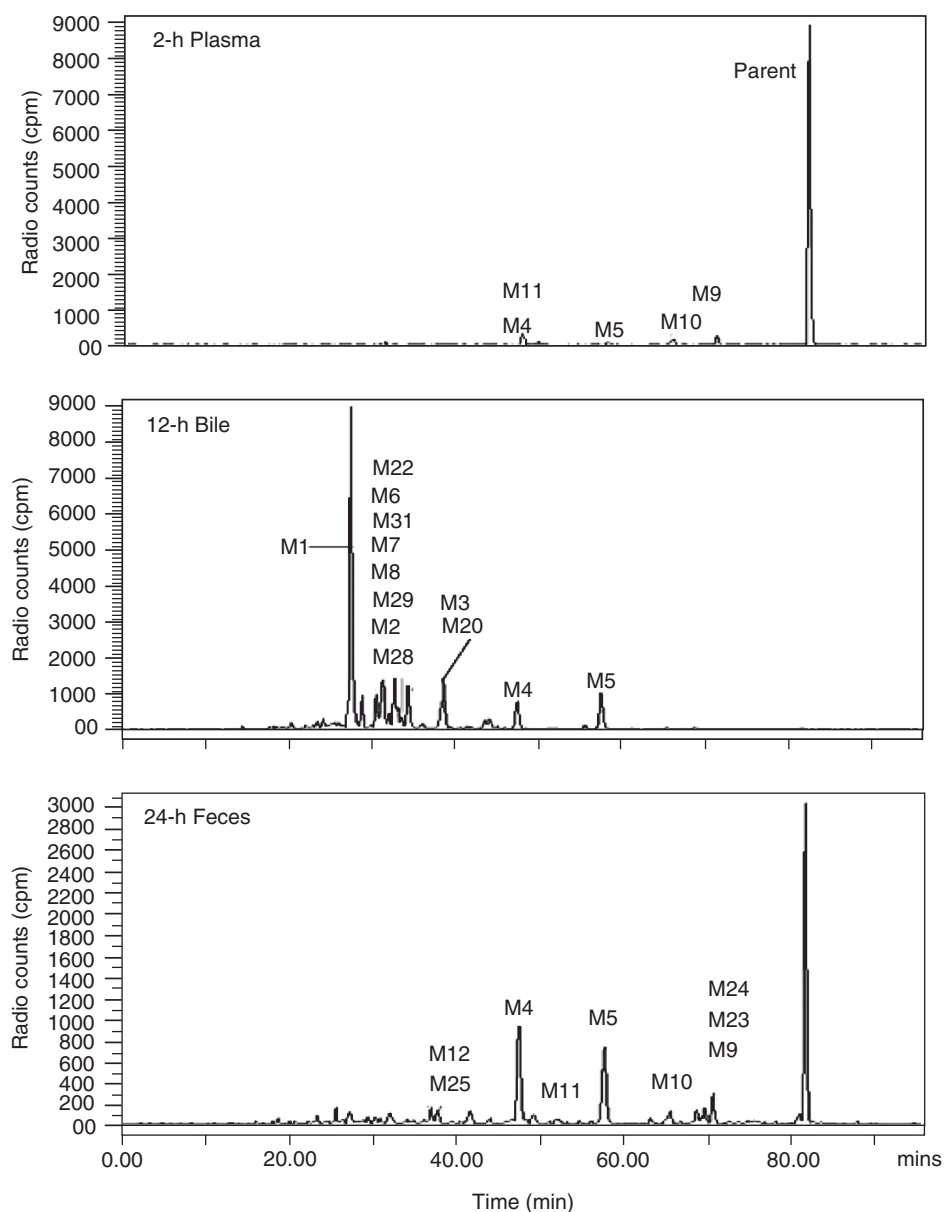


Figure 5.4 Radioprofiles for plasma, feces, and bile after a radiolabeled oral dose of LY15 to rats.

dosed in rat, with profiles for plasma (2 h), feces (0–24 h), and bile (0–12 h). Radiolabeling enables metabolites to be detected that may not otherwise have been identified in unlabeled samples because of poor extraction from the biological matrices, poor ionization, or unanticipated biotransformations (or mass changes).

Like the unlabeled exploratory analyses, the radiolabeled samples (now typically including the feces as well as the plasma, urine, and/or bile) are again analyzed

using LC/MS/MS techniques. The most common metabolic processes include oxidation, reduction, hydrolytic cleavage, or conjugation reactions; thus, to some extent, many metabolic processes can be anticipated based on the chemical structure of the drug. However, only the radiolabeling studies allow one to more or less quantitatively profile all the relevant biotransformation pathways, whether anticipated by mechanism or mass alteration or not.

As for unlabeled studies, high resolution or “exact mass” LC/MS and MS/MS techniques have proven to be incredibly valuable in assigning metabolic structures as part of the radiolabel package. The exact mass component allows one to more effectively filter for drug-related entities, to assign molecular formulas to the observed metabolites, and to assign the relevant MSⁿ fragmentation. Thus, high resolution techniques enable more efficient and effective identification of the relevant transformations and the sites of modification.

However, even with the advantages obtained using high resolution MS data, assigning an identified transformation to an exact position on an NME is structure dependent, and is often not feasible using MS/MS data alone. For example, if oxidation occurs on an aromatic ring (or ring system), an exact site of transformation often cannot be defined due to limited fragmentation of the ring system, and thus, the transformation must be assigned to a region of the drug molecule. Figure 5.5a shows a partial assignment for metabolite M3, a “+16” metabolite of Drug LY06, where mono-oxidation is assigned to the benzazepinone ring (Figure 5.5b). If further structural detail is required (i.e., if a metabolite is a major circulating metabolite, represents a significant primary clearance pathway, or is pharmacologically or toxicologically active), further identification is often accomplished using NMR analysis.

NMR complements LC/MS/MS in the elucidation (or confirmation) of metabolite structures, as it provides data defining structural connectivity. However, NMR typically requires microgram quantity of material and, thus, is much less sensitive than LC/MS. It also requires metabolites to either be isolated/purified or undergo on-line separation (such as LC-NMR). One-dimensional NMR experiments typically analyze for ¹H, ¹³C, or ¹⁹F resonances and provide information on functional groups based on chemical shifts and/or coupling constants. With Two-dimensional NMR (COSY, TOCSY, NOESY/ROESY, HSQC, HMBC) it is possible to look through bond or through space couplings within a molecule, allowing one to establish molecular connectivity and thus provide for more detailed structural elucidation. Figure 5.5c shows

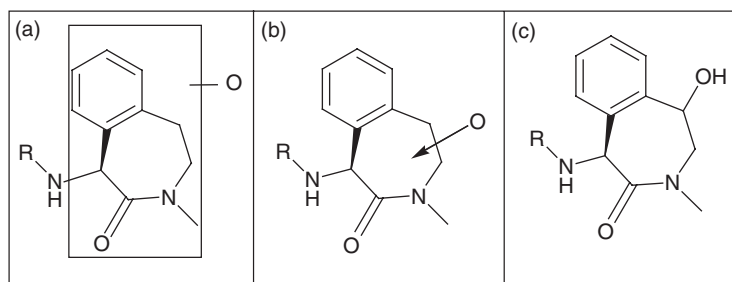


Figure 5.5 Regional assignments for a “+16” metabolite of LY06 (M3) based on LC/MS/MS analysis with (a) and without (b) the loss of water. (c) The definitive assignment for M3 based on NMR analysis.

the definitive assignment for the “+16” metabolite of Drug LY06, where oxidation is assigned definitively to the benzylic carbon based on NMR analysis of a purified sample.

In addition to providing the definitive metabolite profiles for the nonclinical species, the animal radiolabel data are also used to further refine one’s understanding of the relevant human metabolic processes. If *in vitro* data appear to be predictive of *in vivo* and/or the metabolic profiles are similar across the nonclinical species, one is more likely to expect that they have a reasonable understanding of the relevant human pathways. If the *in vitro* data either significantly over- or underpredict the relevant metabolic processes in the nonclinical species and/or the nonclinical species have quite divergent metabolic profiles, one may feel less confident about the anticipated human pathways.

5.6 IN VIVO HUMAN STUDIES

5.6.1 First-in-Human Studies

Exploratory human analyses provide the first direct assessment of *in vivo* human metabolites, including circulating metabolites and excreted metabolites (or clearance pathways) and thus can provide valuable insight into the relevant metabolic processes. These data may be used to trigger additional assessments, including the development of metabolite assays for pharmacologically active or potentially major circulating metabolites, or enzyme identification studies to characterize the enzyme(s) responsible for “major” clearance pathways. As for the nonclinical studies, qualitative identification of metabolites from these studies is quite reliable; however, quantitative profiling can be challenging.

When significant circulating metabolites are noted on the basis of exploratory analyses, a decision is sometimes made to further assess metabolite levels in additional clinical and/or nonclinical trials. There are various levels of bioanalytical assay rigor that can be employed to assess metabolite exposure. The more rigorous approaches (full Good Laboratory Practices (GLP) validation or a qualified research method (QRM)) require definitive identification of the relevant metabolite(s) and synthesis of a synthetic standard(s). Several journal articles have addressed the topic of methodologies to quantitate metabolites [54,74–79] and when either a qualitative or quantitative strategy is appropriate. In addition, the FDA has established guidelines for validation of assays to quantitate compounds, either parent or metabolites, and other regulatory agencies are also writing such guidelines [80,81].

Since one does not usually have a complete set of synthetic standards going into the unlabeled FIH studies, one is typically limited to qualitative or semiquantitative assessments of metabolite levels. For a semiquantitative assessment in the absence of synthetic standards, spectroscopic methods of detection such as NMR (typically either ^1H or ^{19}F) [74,77,79] or UV–vis absorbance [77] have been used to evaluate metabolite exposure. However, both these methods are rather limited due to the poor sensitivity of these techniques relative to LC/MS, and the possibility of significant spectral shifts between parent drug and metabolite(s) for UV analyses.

To overcome the quantitative limitations inherent in the absence of synthetic standards, a series of radiocalibration techniques have been described recently that allow

one to estimate concentrations of metabolites in “cold” samples [75,78]. These techniques create a “calibration standard” by comparing the radioactive and mass spectrometric (MS) signals obtained from a previously generated radiolabeled metabolite (i.e., a metabolite generated in a radiolabeled animal study and/or *in vitro* incubation), enabling one to estimate levels of the same metabolite in unlabeled samples using the acquired MS signal (and the calibration response factor from the radiolabeled sample). This approach allows one to reasonably estimate circulating metabolite levels in early-phase clinical studies before definitive structural assignments and/or synthetic standards are available, enabling an earlier assessment of metabolite coverage and/or possible gaps.

Exploratory analysis of early, unlabeled clinical samples typically provides an improved understanding of the operative human metabolic processes, including major circulating metabolites and clearance pathways. Figure 5.6 illustrates LC/MS chromatograms for the Drug LY14 on the basis of an exploratory analysis of “cold” human plasma and urine samples. The corresponding liver slice profile and human plasma radioprofile were previously shown in Fig. 5.2. In this case, both *in vitro* and exploratory analyses from an early clinical trial were quite predictive of the final human metabolic pathways. However, in some cases (because of the differences in ionization or unanticipated transformations), the exploratory profile can significantly underpredict (or less frequently overpredict) the contribution of a particular metabolite or metabolic pathway, and thus, such data should be considered preliminary. Any decisions regarding safety coverage or major clearance pathways would require further investigation using a more definitive, quantitative assay.

5.6.2 Radiolabeled Studies

Although researchers often have a reasonably good understanding of the major human metabolic pathways before conducting the human [^{14}C] radiolabel ADME study, there are still often gaps, at a minimum, around the quantitative contribution of both circulating and excreted metabolites. Even with newer approaches and technologies such as the use of radiometric calibrants to estimate metabolite exposure in nonradiolabeled studies and high resolution mass filtering to identify unanticipated metabolites, reliable quantitative data concerning important circulating metabolites result from the [^{14}C] human ADME study. No *in silico*, *in vitro*, or exploratory data can definitively address the questions of *in vivo* metabolism.

The human quantitative metabolite data can only be obtained via a radiolabel human ADME study, and for human metabolites that exceed the 10% of total drug-related exposure threshold, one must demonstrate affirmatively that these circulating metabolites are covered, meaning that circulating levels in one or more of the toxicology species must approach (or exceed) circulating human concentrations. If such coverage cannot be established in this manner, direct safety testing of the “disproportionate human metabolites” may be required.

To establish metabolite safety coverage, direct measurement of metabolite exposure based on the single-dose human [^{14}C] study (administered at a pharmacologically relevant dose) has been compared to exposures assessed in the nonclinical [^{14}C] studies (dosed in the low toxicology dose range). Using this approach, plasma, urine, and feces are profiled using LC/radiodetection (using an in-line flow detector or LC fraction collection with subsequent counting) to generate the corresponding radioprofiles.

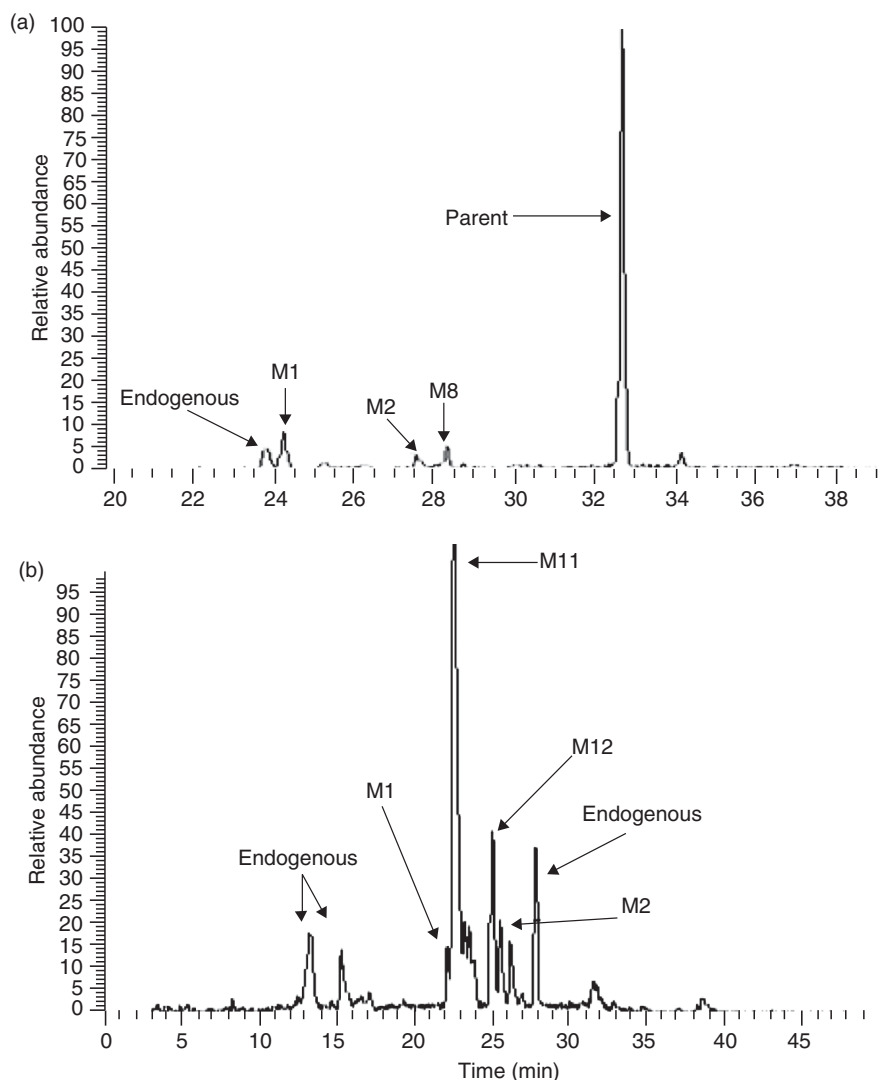


Figure 5.6 (a) LC/MS profile from pooled human plasma 2 h after oral dose of LY14 at 5 mg. (b) LC/MS profile from 0 to 24 h human urine after oral dose of LY14 at 5 mg.

LC/MS/MS data are collected either in parallel with or subsequent to the radioprofiling to identify the corresponding metabolites. The resulting radioactive peaks are integrated to yield the percentage of circulating radioactivity for plasma metabolites or the percentage of dose for excreted metabolites. The relative “exposure” to these metabolites can then be assessed across species, using the quantitation derived from the radioprofiles. Direct comparison between the radiolabel studies allows cross-species assessments to be made even without definitive structural assignments (i.e., regional assignments) based on matched retention times and LC/MS/MS data. This approach tends to be very resource sparing relative to an approach requiring a validated assay, which would require metabolite isolation/purification, NMR assignment, synthesis of

a standard, and the generation of a validated assay in order to establish metabolite coverage. It should be noted, however, that such comparisons are typically limited to single-dose pharmacokinetics, which may not represent steady-state exposure if a metabolite accumulates with multiple dosing.

The final version of the FDA guidance [18] suggests that steady-state exposure of drug-related components is a more appropriate means of evaluating disproportionate human metabolites than single-dose exposure, given that most NMEs are likely to be dosed in a chronic or semichronic manner. The ICH guidance [19] appeared to be purposefully vague in this regard; however, recent presentations and publications by FDA representatives suggest that assessing steady-state metabolite exposure is still preferred [14]. As multiple-dose [^{14}C] human studies present both scientific and operational barriers, the majority of pharmaceutical companies appear to be addressing this concern using more exploratory methods such as the direct comparison of MS signals for metabolites after single and multiple doses for subjects within a single clinical trial, or radiocalibration approaches [16,75,78]. A second approach for estimating steady-state parameters would be to model metabolite accumulation based on the single-dose data. This approach has been endorsed in recent publications [14], but it requires significant assumptions in projecting the half-life.

Although it is clear that the [^{14}C] ADME human study is the industry standard to assess metabolic profiles for healthy subjects, there is no general agreement on the ideal timing of these studies relative to the submission of a NDA. That said, these studies can directly affect resourcing requirements and the viability of a drug and ultimately drive much of the product label and thus are an important milestone in the drug development of an NME.

5.6.3 Microtracer Studies and Accelerator Mass Spectrometry

Microtracer studies and accelerator mass spectrometry (AMS) can provide possible advantages when it comes to defining the broader metabolism package by enabling (i) quantitative profiling of circulating metabolites in phase 1 clinical trials; (ii) profiling for NMEs, where a normal radiolabel dose may present exposure concerns (such as drugs with extended half-lives or unfavorable distribution kinetics); and (iii) multiple-dose human ADME [^{14}C] studies.

Historically, AMS has been used for radiocarbon dating and has more recently found a role in biomedical investigations, including human ADME assessments [82–84]. In this regard, AMS is predominantly used for the detection of ^{14}C in a lightly labeled sample (although other isotopes including ^3H have been utilized). In essence, a sample (such as plasma) containing low levels of ^{14}C is combusted to CO_2 , reduced back to graphite, and analyzed by AMS to determine the $^{14}\text{C}/^{12}\text{C}$ ratio, thus, effectively serving as an ultrasensitive radiocounter.

“Microtracer” studies differentiate themselves from the early “microdose” studies, in that the former administer a low dose of radioactivity (typically 100–200 nCi of ^{14}C) in conjunction with a standard dose of unlabeled drug, whereas the latter administer a low total dose of a drug (typically radiolabeled). The microdose studies have historically been used to evaluate the relative clinical pharmacokinetics for a series of potential drug candidates in the so-called phase 0 [85–87]. The microtracer studies, on the other hand, provide data very comparable to the standard human [^{14}C] studies, enabling the evaluation of pharmacokinetics, mass balance, and metabolism at a pharmacologically

relevant dose [76,88–90]. The main advantage compared to a conventional human [^{14}C] ADME study is that nanocurie doses are administered to humans, and thus, this dose is not considered to be “radioactive,” such that conventional nonclinical dosimetry studies are not required to support human dosing. This enables one to obtain quantitative information about circulating metabolites well before the traditional human radiolabel ADME study could be conducted. The primary disadvantages are that AMS analysis is time consuming and expensive, and conventional LC/MS/MS techniques must be conducted in parallel to enable metabolite identification. These studies are being used in an exploratory mode to address human metabolism, and, in particular, major circulating metabolites, as part of the early clinical package, allowing researchers to better understand and more effectively react to any potential MIST issues very early in drug development.

Additionally, the microtracer study enables a repeat-dose human ADME study to be conducted using multiple [^{14}C]-radiolabeled doses. Multiple nanocurie-level doses of [^{14}C]-radiotracer are dosed in parallel with therapeutic doses of unlabeled drug to obtain steady-state levels for both total radioactivity and parent drug [76]. On the basis of the low radiolabeled doses, radioanalysis would typically require the use of AMS to quantitate the radioactivity in plasma and excreta, adding significant cost and analytical complexity to the human ADME study. Although scientifically quite attractive, this approach has not been widely utilized to date by the pharmaceutical industry, with alternate “unlabeled” approaches typically being used to estimate metabolite exposure at steady state. Further evolution of the AMS technique resulting in higher throughput, reduced cost, and the ability to acquire the radioanalysis and LC/MS/MS for metabolite identification in parallel could result in significant uptake of this approach with time.

5.7 CONCLUSIONS

As discussed in this chapter, the tools used to predict human biotransformation pathways evolve during the drug discovery and development process, usually starting with *in silico* and *in vitro* tools and culminating with the *in vivo* studies in humans. No single tool will be adequate for the determination of the overall metabolism in humans, and no single strategy will work 100% of the time. Predictions of human metabolism are challenging due to the complexity and integration of the physiological organs and systems that comprise the human body. *In silico* and *in vitro* systems tend to rely on isolated systems (i.e., liver microsomes, hepatocytes) that provide the best estimate of what may occur *in vivo*. *In silico* tools will become increasingly better as the knowledge base improves, but they are unlikely to completely replace either *in vitro* or *in vivo* testing. The *in vitro* systems have been shown to predict clearance pathways quite well, but they are less accurate in quantitating the extent or amount of *in vivo* metabolites. One article [11] showed that the *in vitro* data were predictive of the circulating human metabolites 41% of the time, underpredictive 35% of the time, and overpredictive 24% of the time. Another publication [61] showed that *in vitro* systems were capable of predicting >70% of the primary metabolites and metabolic pathways but were less reliable in predicting the secondary pathways. The predictability using *in vitro* tools is likely to improve as analytical techniques for isolation of human hepatocytes and generation of recombinant enzymes improve, as well as the instrumentation to quantitate low levels of metabolites improves. Although the degree

of predictability from the *in vitro* data may vary, there is general agreement that an *in vivo* study in humans using radiolabeled drug is ultimately needed to determine the human biotransformation pathways. Improvements in identification and quantitation of metabolites using technological advances in instrumentation will likely make it easier to detect metabolism pathways *in vivo*. In addition, increasing regulatory scrutiny on the safety qualification of the major human metabolites will necessitate more creative approaches to improve success in predicting and providing adequate animal coverage of the most important metabolites. Thus, the prediction of human biotransformation pathways will certainly continue to evolve with future scientific advances.

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6 Conjugation, Transport, and Elimination of Drugs in Humans

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6.1	Summary	1
6.2	Drug-conjugating (phase II) enzymes in humans	3
6.3	Excretion of drugs in humans and role of transport proteins	14
6.4	Elimination pathways in humans	18
6.5	Enterohepatic recirculation	20
6.6	Impact of hepatic and renal impairment on drug elimination	20
6.7	Nuclear receptors in human drug conjugation and transport	21
6.8	Effect of dietary phytochemicals and herbal supplements on nuclear transporters, drug conjugation, and drug transport	22
6.9	Interspecies differences in drug conjugation, transport, and elimination	23
6.10	Other factors affecting the conjugation, transport, and elimination of drugs in humans	26
6.11	Summary and future perspectives	27
	References	27

6.1 SUMMARY

Drug metabolism is generally described in three phases: phase I (mainly oxidation), phase II (conjugation), and phase III (transport/elimination). Phase I metabolism, mediated by cytochrome P450 (CYP450) and other enzymes has been discussed elsewhere in this series. The major function of phase II and phase III drug metabolism is the detoxification and subsequent transport/elimination of drugs and other xenobiotics. Consistent with this function, conjugation generally produces a metabolite that is more polar, larger in molecular weight, and charged at physiological pH; characteristics that make the metabolite more amenable as a substrate for transport proteins for ultimate excretion into the urine or bile. Most phase II conjugates are inactive and nontoxic; however, there are exceptions in which conjugation results in bioactivation or reactive metabolites.

Phase II conjugation reactions involve the attachment of a glucuronide, sulfate, glutathione (GSH), methyl, acetyl, or an amino acid moiety. These reactions are catalyzed by a group of enzymes known as *UDP-glucuronosyltransferase* (UGT), *sulfonfyl transferase* (SULT), *glutathione S-transferase* (GST), *methyl transferase* (MT), *N-acetyltransferase* (NAT), and *acyl CoA ligase/N-acyltransferases*, respectively. Of these conjugation reactions, glucuronidation is the most common, accounting for roughly 35% of all phase II drug metabolism reactions followed by the SULTs (20%), GSTs (15%), and NATs (10%) [1]. In addition to catalyzing drug metabolism, almost all of these conjugating enzymes are also involved in important biosynthetic and biochemical pathways and hence each also has specific endogenous substrates. On the basis of similarities in their amino acid sequences, each enzyme is divided into families (e.g., UGT1, UGT2) and then further divided into subfamilies (e.g., UGT1A1, UGT1A2), which are typically referred to as *isoenzymes* or *isoforms*. The respective isoforms of the phase II conjugating enzymes are expressed to some degree in virtually all human tissues and have broad and overlapping substrate specificity.

Phase III metabolism involves the rapid excretion/elimination of drug metabolites into bile or urine via transporter proteins. Phase II conjugative metabolites are often quite polar and cannot escape cells (e.g., hepatocytes) via passive diffusion. Therefore, ATP-mediated active transport is often required to enable phase II conjugates to cross biological membranes. Hence, transport proteins play a major role in the excretion of drugs and other xenobiotics into bile and urine. Similar to phase I metabolites, phase II metabolites are also often the substrates for phase III metabolism. There are multiple types of transport proteins that modulate the uptake and excretion of drugs and metabolites into and out of cells. The focus of this chapter is on those transporters that are involved in the excretion of drugs and metabolites out of the kidney proximal tubule epithelia into the urine and out of the hepatocyte into the bile. On the basis of similarities in their amino acid sequences, each transporter is divided into one of two families [the ATP-binding cassette (ABC) or the solute carrier (SLC) family] and then further divided into subfamilies [e.g., multidrug resistance transporter (MDR), organic anion transporter (OAT)].

Regulation of the phase II drug-metabolizing and phase III transporter proteins (in addition to phase I drug-metabolizing enzymes (DMEs)) is governed by nuclear receptors (NRs) [pregnane X receptor (PXR), constitutive androstane receptor (CAR), etc.]. Consequently, many of the phase II and III enzymes are inducible. As a result of being inducible along with having substrate specificity overlap and multiple isoforms, a number of the phase II and III DMEs and transporters have been implicated in clinical drug–drug interactions.

The complexity of how NRs, phase I and II metabolizing enzymes, and transport proteins interact and modulate drug metabolism is not fully understood. Historically, the CYP450 enzymes and other phase I DMEs have been more extensively studied than the phase II DMEs and phase III drug transport proteins. In the last decade, research efforts have become more focused on the phase II conjugation enzymes and the phase III transporter proteins. Though much more is currently understood about the functionality, mechanisms, control, and regulation of phase II and phase III metabolism; research in this area continues to evolve and uncover new findings.

6.2 DRUG-CONJUGATING (PHASE II) ENZYMES IN HUMANS

6.2.1 Glucuronosyltransferases

Glucuronidation is catalyzed by a family of enzymes known as *UGTs*. The reaction catalyzed by UGT enzymes involves the conjugation of an acceptor molecule (aglycone) with glucuronic acid. The source of glucuronic acid is a cosubstrate known as *UDP glucuronic acid* (UDPGA). The acceptor molecule functional group is typically an –OH or –OOH although glucuronidation has been reported on –NH and even –CH and –SH functional groups. The first glucuronide conjugate was reported by Erdmann [2] in 1844 in the urine of cows, which were fed mango leaves. Euxanthic acid (the glucuronide conjugate of euxanthone) was isolated after treatment of the urine with acid. In the early 1870s, Jaffe [3] identified the sugar moiety as a carboxylic derivative of glucose. Later that decade, Schmiedeberg and Meyer [4] identified this sugar as glucuronic acid from experiments they conducted in dogs with camphor. Finally in 1953, Dutton and Storey [5,6] identified UDPGA as the necessary cofactor for glucuronidation.¹

The ultimate objective of glucuronidation is to increase the solubility of xenobiotic compounds so that they are more readily excreted into the bile and urine. Many drugs (Table 6.1) are glucuronidated by the UGT enzymes including acetaminophen, codeine, morphine, *S*-naproxen, oxazepam, and zidovudine [9]. Figure 6.1 shows the glucuronidation of zidovudine (AZT) by UGT2B7, which is responsible for ~60–75% (oral dose) of the elimination of this drug [10–12]. Although this example shows direct O-glucuronidation, often, lipophilic phase I oxidation metabolites are subsequently glucuronidated. Relative to CYP450 metabolism, glucuronidation generally occurs more rapidly. For example for the flavonoid galangin, glucuronidation was found to occur 11–31 times faster (depending on the site of glucuronidation) than CYP450-mediated metabolism [13]. Therefore, in cases where a compound must first be oxidized by CYP450s before conjugation, oxidation becomes rate limiting in the clearance of the compound. Consequently, changes in phase I metabolism can impact the extent of glucuronidation. As with most phase II enzymes, UGTs are also capable of biotransformation of important endogenous substrates such as bilirubin, bile acids, steroids, and glycolipids.

The UGTs consist of four families, UGT1, UGT2, UGT3, and UGT8, of which UGT1 and UGT2 are relatively well characterized. The single enzyme in the UGT8 family, hUGT8 plays a key role in the biosynthesis of galactocerebrosides [15]. UGT3, the last UGT family to be discovered, contains two isoforms hUGT3A1 and hUGT3A2 and until recently little data were available for this family of enzymes [16]. Interestingly as pointed out by Meech and Mackenzie [16], UGT3A1 employs *N*-acetylglucosamine as the sugar donor for conjugation and not UDPGA, which is the primary sugar donor for all other UGTs. All UGTs share a common backbone with specific isoforms differing from each other in the *N*-aminotermini of their respective amino acid sequence. To date, around 19 human UGTs have been characterized in humans. Only the two subfamilies, UGT1 and UGT2, are involved in drug glucuronidation. Of these subfamilies, UGT1A1, UGT1A4, UGT1A9, and UGT2B7 represent the major UGT isoforms and together they are involved in ~87% of hepatic UGT metabolism of drugs [17].

¹This brief history of glucuronidation was summarized from Conti *et al.*, and Dutton [7,8].

TABLE 6.1 Individual UGT Enzymes and Drugs Involved in Drug Metabolism

<i>Individual UGT Enzymes Primarily Involved in Drug Metabolism</i>			
UGT1A1	UGT1A6	UGT1A9	UGT2B15
UGT1A3	UGT1A7	UGT1A10	UGT2B17
UGT1A4	UGT1A8	UGT2B7	UGT2B28
<i>Examples of Drugs that are UGT Substrates (K_m Range 9–9400 μM)</i>			
Acetaminophen	Entacapone	Imipramine	Oxazepam
Carbamazepine	Ethinylestradiol	Ketoprofen	Tamoxifen
Codeine	Ezetimibe	Morphine	Troglitazone
Cyclosporine A	Flurbiprofen	Mycophenolic acid	Valproic Acid
Dichlofenac	Ibuprofen	Naproxen	Zidovudine (AZT)
<i>Examples of Drugs that are in vitro UGT Inhibitors (K_i Range 0.033–200 μM)</i>			
Cyclosporine	Ethinylestradiol	Naproxen	Probenecid
Diazepam	Flurbiprofen	Nortriptyline	Tacrolimus
Dichlofenac	Indomethacin	Oxazepam	Troglitazone
<i>Drugs that are Possible UGT Inducers</i>			
Carbamazepine	Phenobarbital	Phenytoin	Rifampin

Source: Adapted from Ref. 14.

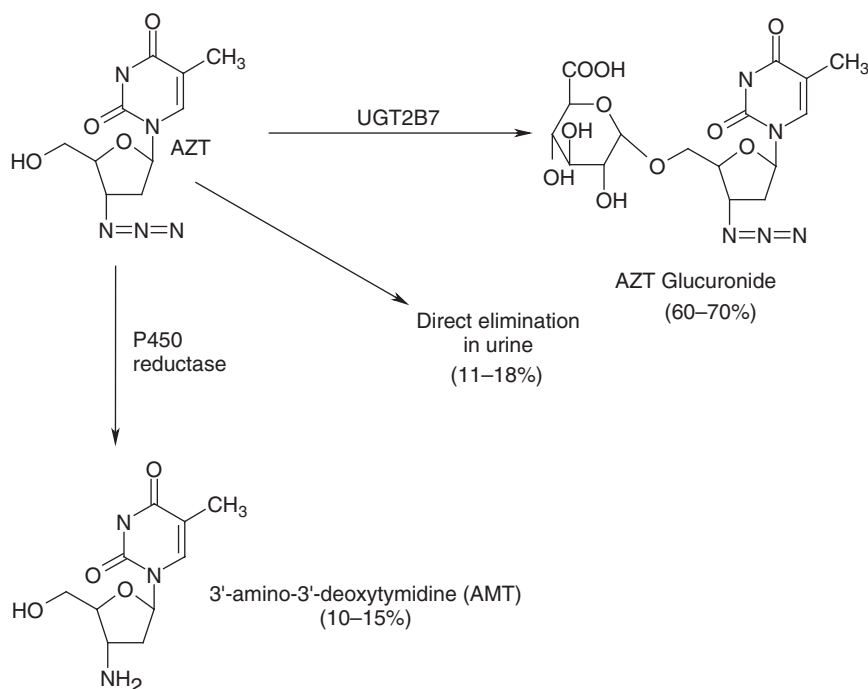


Figure 6.1 Metabolic pathway of zidovudine (also known as AZT) showing glucuronidation. Less than 1% of zidovudine is converted to its active form zidovudine triphosphate *in vivo* (not shown). Source: Adapted from Refs 10–12.

The concentration of UDPGA in human liver ranges from 201 to 349 $\mu\text{mol/kg}$ liver [18]. Hence, glucuronidation is a major phase II metabolic pathway in humans and has the largest capacity of any of the phase II clearance systems. Several commonly used therapeutics have been found to inhibit *in vitro* UGT activity. For example, the drugs tacrolimus, cyclosporine, and diclofenac are among the most potent (K_i values range from 0.033 to 7.9 μM) with probenecid, troglitazone, and naproxen being less potent inhibitors (K_i values range from 20 to 172 μM) [14]. The compound 7,7,7-triphenylheptyl-UDP has been reported to be a mechanism-based inhibitor of UGT [19,20].

UGTs are present in liver, lung, skin, intestine, kidney, brain, and other tissues. They are membrane-bound enzymes with a molecular weight in the range of 50–60 kDa and are located in the endoplasmic reticulum of the cell [21–26]. Unlike the CYP450 enzymes, which have their active sites facing the cytosol, the active site of UGTs face the luminal side of the ER [27]. Hence, UGT conjugation occurs within the ER as opposed to the cytosol [28]. Interestingly, β -glucuronidase is also located in the ER and along with the UGTs may be involved in the futile cycling of hormone and other conjugates within the cell [29]. The first three-dimensional structural information for a mammalian UGT just recently became available with the characterization of the crystal structure of human UGT2B7 C-terminal end [30].

The more common UGT polymorphisms are those associated with the UGT1A1, UGT1A6, and UGT2B7 isoforms [31]. Of these three isoforms, the most prevalent polymorphisms impacting drug metabolism are those involving UGT1A1. Hereditary diseases such as Gilbert's and Crigler–Najjar syndromes occur as a result of UGT1A1 polymorphism and compromise the ability of individuals afflicted with this condition to properly metabolize bilirubin as well as drugs metabolized by UGT1A1 [32]. Both diseases are characterized by hyperbilirubinemia, which is caused by the buildup of bilirubin levels in the blood. Hyperbilirubinemia in Gilbert's syndrome results in only minor jaundice, while in Crigler–Najjar, bilirubin can reach lethal levels unless subjects undergo phototherapy [33]. Crigler–Najjar syndrome is a rare disease (~ 0.6 per million live births or only a few hundred world wide) and is characterized as two types [34]. Type I is more severe as subjects have no expression of UGT1A1 (bilirubin levels $>340 \mu\text{mol/L}$) [35]. Type II is less severe in that patients have some UGT1A1 activity albeit at levels that are 10% of normal (bilirubin levels between 150 and 340 $\mu\text{mol/L}$) [35]. Type II is treatable with phenobarbital, which induces UGT1A1 activity. Gilbert's syndrome occurs in roughly 3–13% of the population and is less severe (expression of UGT1A1 is $\sim 30\%$ of normal) than Crigler–Najjar syndrome [32,36]. Typically, Gilbert's syndrome patients require no treatment. Although UGT1A1 polymorphisms have been fairly well characterized clinically, the clinical significance of UGT1A6 and UGT2B7 polymorphisms is still under investigation. Other polymorphisms of UGTs include UGT1A3, UGT1A4, UGT1A7, UGT1A8, UGT1A9, UGT2A1, UGT2B4, UGT2B15, and UGT2B28; however, the clinical relevance of their associated polymorphisms with respect to drug metabolism is unknown at this time [31].

Drug–drug interactions involving glucuronidation seem to be less prevalent than those identified for CYP450s possibly for the following reasons described by Williams *et al.* [37]. UGTs typically have much higher substrate K_m values (300 μM and often much higher) than those of CYP450s (K_m typically around 3 μM) and are usually metabolized by multiple UGTs. Given that the *in vivo* concentrations of most drugs are

usually below $10\text{ }\mu\text{M}$, UGT-metabolized drugs rarely saturate their own metabolism. This along with the fact that K_i values for most UGT inhibitors² are usually $> 10\text{ }\mu\text{M}$ leads to the conclusion that, in general, the AUC_i/AUC ratio will be relatively low even in situations where the fraction of the drug metabolized by a single UGT is high. Consequently, as further pointed out by Williams *et al.* [37], it is not surprising that clinically, UGT drug–drug interactions typically result in differences in *in vivo* exposures that are twofold or less in the presence of coadministered drugs that inhibit UGT. Drug–drug interactions involving glucuronidation, which result in toxicity are rare but have been observed. For example, lamotrigine coadministered with valproic acid increases the incidence of skin rash, which is a known side effect of lamotrigine [39]. A number of intrinsic and extrinsic factors are known to affect drug glucuronidation in humans including age, cigarette smoking, diet, disease state, ethnicity, genetic factors, hormonal factors, and interaction with other drug therapies [9].

Although glucuronidation is typically associated with detoxification, activation via glucuronidation is known to occur. One of the most well-known pharmacologically active glucuronides is morphine-6-glucuronide, which has been reported to be up to 650 times more potent than morphine and is currently in human clinical trials as a parent drug [40]. In addition, potentially toxic glucuronides may be formed. For example, acyl glucuronides that are formed by conjugation of glucuronic acid with carboxylic acids are prone to nucleophilic attack and acyl migration. *In vitro* studies have suggested that toxicity of acyl glucuronides may occur as a result of covalent binding with microsomal proteins. In some cases, acyl glucuronides have been shown to be toxic *in vivo*; however, there are relatively few examples of *in vivo* toxicity [41].

Lastly, glucuronidation is considered a reversible process. Once glucuronide metabolites enter the intestine, they are exposed to enzymes of the intestinal microflora such as β -glucuronidase and become deconjugated back to the parent drug. Often times, the parent drug is then reabsorbed in the lower intestine by a process known as *entero-hepatic recirculation* (Section 6.5). A number of recent review articles are available on UGTs [16,42–47].

6.2.2 Sulfotransferases

Sulfonation is catalyzed by a large family of enzymes known as *sulfotransferases* (SULTs). The SULTs catalyze the transfer of a sulfonate (SO_3^-) group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a $-\text{COH}$, an $-\text{NOH}$ or to an $-\text{NH}$ group. Sulfonation can either be direct, for example, the sulfonation of troglitazone (Fig. 6.2), or occur after oxidation via phase I metabolism, as for example in the sulfonation of hydroxyphenolbarbitol. Sulfonation was first discovered by Baumann [48] in the 1870s when he administered phenol to a patient and recovered phenyl sulfate in their urine. The involvement of PAPS as a cosubstrate was later identified by Robbins and Lipmann [49,50]. The enzyme responsible for the biosynthesis of PAPS in the cytosol is a single bifunctional protein with a molecular weight of $\sim 70\text{ kDa}$ that exhibits both ATP-sulfurylase and APS-kinase-like activity [51]. The capacity of sulfonation is limited by the quantity of PAPS available *in vivo* for conjugation. Sulfonation occurs more rapidly than glucuronidation but is a saturable

²It should, however, be noted that determination of the K_i and K_m values of UGT2B7 and UGT1A9 have often been over estimated because of inhibition of these isoforms by endogenous fatty acids [38].

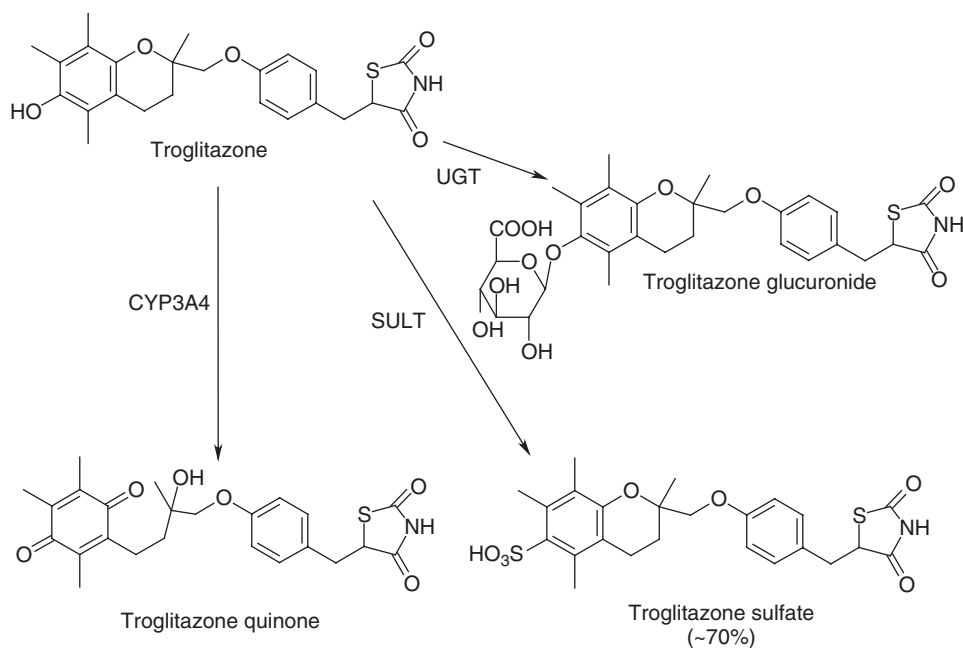


Figure 6.2 Primary metabolic pathway of troglitazone. *Source:* Adapted from Refs 53–55.

process given the relatively low liver levels ($23 \mu\text{mol/kg}$ liver) of the cosubstrate PAPS compared to glucuronic acid [52]. Hence, when sulfonation metabolic pathways become saturated, glucuronidation typically compensates. For this reason, sulfonation and glucuronidation have been considered as complementary metabolic pathways. As with glucuronidation, sulfate conjugation is a reversible process and sulfate conjugates may be desulfated by sulfatases present in the intestinal microflora. Hence, sulfate conjugates can also undergo enterohepatic recirculation.

The SULTs are widely distributed in most tissues including liver, kidney, lung, skin, breast, gastrointestinal, brain, and platelets [56,57]. However, the highest levels are generally found in the small intestine and liver. Interestingly, the enzyme activity of SULT in the human liver with some substrates such as ethinylestradiol has been shown to vary as much as sevenfold [58]. SULTs are either cytosolic or membrane bound. The membrane-bound SULTs are localized in the golgi apparatus of cells and catalyze the conjugation of endogenous macromolecules such as proteins, peptides, and glycosaminoglycans. The membrane-bound SULTs are not involved in drug metabolism. Conversely, the cytosolic SULTs catalyze the conjugation of lower molecular weight endogenous compounds such as neurotransmitters, bile acids, and steroids in addition to drugs and other xenobiotics. Although the main function of SULT enzymes appears to be in the modulation of physiological processes, they are very important in the metabolism of xenobiotics. Drugs sulfonated by the SULT enzymes include ethinylestradiol, ritodrine, minoxidil, salbutamol, acetaminophen, diflunisal, fenoldopam, terbutaline, troglitazone, and hydroxyphenolbarbitol [56]. Some drugs that are reported to inhibit the SULTs include clomifene, testosterone, danazol, spironolactone, cyproterone, ibuprofen, and tamoxifen [56]. Mefenamic acid and salicylic acid potently inhibit SULT1A1 and SULT1A3 [56]. Additional SULT substrates and

TABLE 6.2 Individual SULT Enzymes and Corresponding Drugs Involved in Drug Metabolism

<i>Individual SULT Enzymes Primarily Involved in Drug Metabolism</i>			
SULT1A1	SULT1A3	SULT2A	
<i>Examples of Drugs that are SULT Substrates</i>			
Acetaminophen	Ethinylestradiol	Minoxidil	Terbutaline
Budesonide	Fenoldopam	Ritodrine	Testosterone
Diflunisal	Isoproterenol	Salbutamol	Troglitazone
<i>Examples of Drugs that are In vitro SULT Inhibitors (IC_{50} Range 0.02–74 μM)^a</i>			
Curcumin	Flufenamic Acid	Meclofenamic Acid	Salicylic Acid
Diflunisal	Mefenamic Acid	Niflumic Acid	Tolfenamic Acid
<i>Drugs that are Possible SULT Inducers</i>			
Dexamethasone			

^a2,4-Dichloro-4-nitrophenol and quercetin (a dietary phytochemical) are also potent inhibitors of SULT enzymes.

Source: Adapted from Ref. 56.

inhibitors are listed in Table 6.2. In humans, 13 SULT isoforms have been identified to date and belong to one of the four SULT families: SULT1, SULT2, SULT4, or SULT6 [57]. SULT1 and SULT2 families have been identified as the most important in xenobiotic drug metabolism. The cloned human SULTs contain 284–365 amino acids, have subunit molecular weights ranging from 30 to 35 kDa, and are typically present as dimers in solution [59–61]. The three-dimensional structures for most of the human SULTs have been elucidated and include SULT1A1, 1A3, 1B1, 1C1, 1C2, 1C3, 1E1, 2A1, 2A3, 2B1a, 2B1b, and 4A1³ [62,63]. Data from the crystal structures have revealed a highly conserved PAPS binding pocket with a highly variable substrate binding pocket, which is consistent with their observed overlapping substrate specificity. The recently discovered SULT4A1 is an unusual SULT because of its atypical structure, function, and unique binding properties. In fact, it is inactive and does not catalyze sulfonation reactions. Interestingly, it has been shown to bind well to neurotransmitters, such as epinephrine and norepinephrine, suggesting that it may be involved in brain function [63].

Polymorphisms have been identified in several human SULT genes, including those encoding SULT1A1, SULT1A2, SULT2A1, SULT1C2, and SULT1E1. However, SULT1A1 and 1A2 are the only SULT isoforms for which common genetic polymorphisms are known and have been investigated in epidemiological studies [56]. Polymorphisms of SULT 1A1 have been attributed to carcinogenicity via activation of endogenous substrates. Patients homozygous for SULT1A1*2 have 10-fold lower phenol SULT activity than those homozygous for SULT1A1*1 and have been found to have an increased risk for lung, breast, and other cancers [64–66]. Thus far, despite being polymorphic, few or no clinically relevant drug–drug interactions involving SULTs have been reported [67].

Unstable sulfate conjugates have been shown to be genotoxic. Examples of genotoxic compounds that form unstable sulfate conjugates through binding to DNA are safrole and tamoxifen [68,69]. Additionally, sulfoconjugates are anionic species and

³The human SULT crystal structures and original references are summarized in these two sources.

do not freely permeate cell membranes. This, consequently, may lead to specific sites of SULT-dependent genotoxins. Given sulfate is a good leaving group, cleavage of the C–O bond in sulfoconjugates results in the formation of a carbocation, which is a strong electrophilic species. These reactive species are often hepatotoxic. Examples of hepatotoxic sulfates include phenacetin that binds to microsomal proteins and troglitazone, which is a potent bile salt export pump (BSEP) inhibitor [70]. Inhibition of BSEP causes cholestasis, a condition that results from lower bile salt secretion and reduced bile flow.

6.2.3 Glutathione-S-Transferases

GSH is a tripeptide comprised of the amino acids glycine, cysteine, and glutamic acid and is the cosubstrate for GST. GSH has a very high reduction potential with an E_h of -0.24 V in pH 7.0 phosphate buffer at 40°C (E_h in human liver is -0.26 V) and is very effective in reducing highly reactive electrophilic species [71,72]. Cells often employ GSH to inactivate reactive (toxic) species that are either present as a result of normal cellular metabolism (e.g., H_2O_2 or $\text{O}_2^{\cdot-}$) or are introduced as a xenobiotic. The balance of reduced GSH and oxidized GSH (GSSG) is critical to preventing damaging levels of oxidative species from accumulating within cells. GSH has been shown to be essential in apoptosis and embryonic development [73]. Another indication of its importance is the high intracellular levels of GSH, which can reach 8–10 mM in some tissues [74].

In many cases, oxidative species can be scavenged directly by GSH, however, certain chemical species require enzymatic catalysis for conjugation. Glutathione-S-transferases catalyze the conjugation of GSH with an electrophilic substrate molecule such as an epoxide or quinone. Drugs that are substrates for GSTs include acetaminophen and valproic acid as well as the anticancer drugs cyclophosphamide, busulfan, and melphalan [75–77]. Figure 6.3 shows the GSH conjugation of melphalan [78]. In drug metabolism, GSTs are typically associated with the conjugation of xenobiotics with GSH; however, they are also known to catalyze reduction, thiolysis,

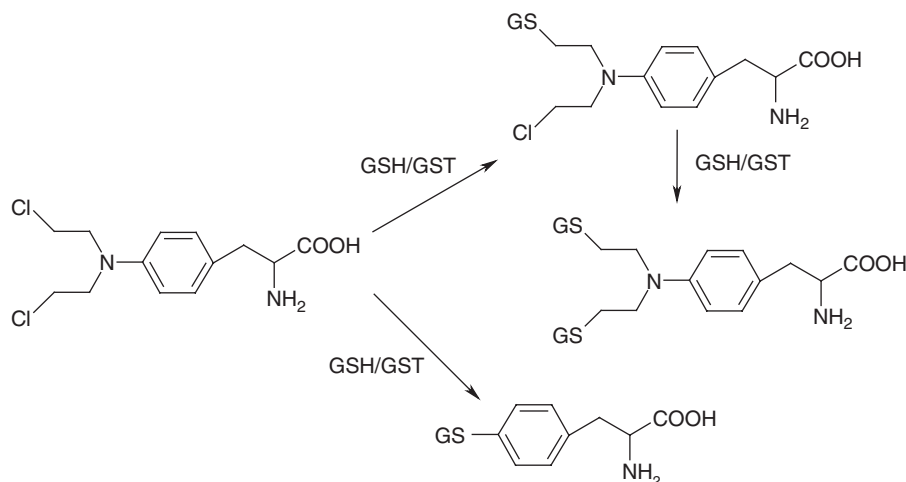


Figure 6.3 Metabolic pathway of melphalan. *Source:* Adapted from Refs 78–80.

and isomerization reactions [77]. Conjugation with GSH usually leads to less reactive products that are readily excreted. The end products of GST conjugation are either excreted by transport proteins into the bile or converted to *N*-acetyl cysteine conjugates and excreted into the urine. In some instances, GSH conjugates can be more reactive than their respective parent compounds. For example, conjugation of GSH with 1,2-dihaloethane results in genotoxic products [77]. Like most other conjugative enzymes, GSTs have endogenous substrates such as prostaglandins.

Most GSTs are cytosolic enzymes, but there are examples of membrane-bound GSTs such as MGST1, MGST2, and MGST3 which are located in the endoplasmic reticulum. A mitochondrial human GST, GSTK1-1 has also been identified. Cytosolic GSTs have a monomeric molecular weight typically around 25 kDa, but generally occur as homo- or heterodimers [81–83]. The cytosolic GSTs have been found in most cells and the crystal structures for many have been resolved. Like UGTs and SULTs, there are different isoforms of GSTs, but the naming convention is slightly different. On the basis of amino acid similarities, there are seven classes of cytosolic GSTs: alpha, mu, pi, sigma, theta, omega, and zeta [77,84]. Polymorphisms are also found within each class of GSTs; however, the focus of this chapter is centered on those that are clinically relevant. The greatest impact of GST polymorphism on drug metabolism arises in the mu and theta classes with the GSTM1*0 and GSTT1*0 genotypes, which are referred to as *null alleles* in which the gene encoding GSTM1 or GSTT1 is absent. These individuals have no active enzyme and are associated with an increased risk for certain cancers [85]. Polymorphisms in the *GSTA1* gene have been shown to cause increased variability of busulfan conjugation, while GSTP1*1, GSTP1*2, and GSTP1*3 polymorphisms have been associated with higher rates of certain cancers [77]. GSTs are induced by increases in the levels of oxidative species that are often produced by electrophiles. The process of induction involves a transcription factor known as *nuclear factor* (erythroid-derived 2)-like 2 (Nrf2), which is released in the presence of electrophiles and binds to the antioxidant response element promoter regions of the genes encoding some of the GSTs (e.g., GSTA1 and GSTA3).

6.2.4 Methyltransferases

MTs catalyze the transfer of a methyl group from a cosubstrate known as *S*-adenosylmethionine (SAM) to an oxygen, sulfur, or nitrogen functional group on the substrate. There are over 100 methyltransferases, however, only a few have been found to catalyze the metabolism of drugs, including catechol methyltransferase (COMT), thiol methyltransferase (TMT), thiopurine methyltransferase (TPMT), and nicotinamide *N*-methyltransferase (NNMT). Nicotinic acid (also known more commonly as *vitamin B₃* or *niacin*) is converted to nicotinamide *in vivo* and subsequently methylated by NNMT [86]. Nicotinic acid at therapeutic doses of 2000 mg has been used to treat atherosclerosis [87]. Histamine *N*-methyltransferase (HNMT) does not catalyze the conjugation of any drugs; however, a number of drugs including promethazine and the antimalarials pyrimethamine and chloroquine have been identified as inhibitors of this enzyme [88].

COMT was first identified as responsible for the methylation of epinephrine and partially characterized by Axelrod [89] in the late 1950s. COMT catalyzes the *O*-methylation of catechols and catecholamines such as dopamine. A single gene containing six exons codes for both cytosolic (*S*-COMT) and membrane-bound COMT

(MB-COMT) [90]. The molecular weights are 24.4 and 30.0 kDa, respectively [91]. Unlike most other small molecule methyltransferases, COMT requires Mg^{2+} for its catalytic activity [90]. The active site of the enzyme contains two binding domains, one for SAM and other for the substrate. The transfer of a methyl group from SAM to the catechol occurs via an S_N2 -like transition state where the hydroxyl group of the catechol attacks the nucleophilic methyl group of SAM. COMT is found in most tissues including brain, lung, and red blood cells but the highest levels are expressed in the liver and kidney. The endogenous substrates of COMT include L-dopa, dopamine, norepinephrine, and epinephrine [92]. Drugs metabolized by COMT include rimeterol, dobutamine, dihydroxyphenylserine, isoprenaline, and carbidopa. Inhibitors of COMT include nitrocatechols such as entacapone, nitecapone, tolcapone, and vinylphenolketones [90]. COMT is polymorphic in humans. One polymorph known as val¹⁵⁸met, has been shown to cause reduced enzyme activity (three- to fourfold) and was originally thought to be linked to an increased risk of breast cancer. However, recent studies have failed to show a correlation of this polymorph to breast cancer [93–96]. Methylation differs from sulfonation, glucuronidation, and GSH conjugation as methyl conjugates are less polar than the parent drug and therefore may not be more easily eliminated [97].

Thiopurine drugs such as azathiopurine, 6-mercaptopurine (6-MP), and 6-thioguanine are S-methylated by TPMT (Fig. 6.4). These drugs are used to treat a wide variety of conditions including leukemia, Crohn's disease, and ulcerative colitis. TPMT was first characterized by Woodson and Weinshilboum in 1983 [98]. It is a cytosolic enzyme of ~36 kDa in molecular weight [98]. TPMT is polymorphic with 18 mutant alleles that contribute to the narrow therapeutic index of thiopurine drugs because of their poor metabolism in the body [99]. Of these alleles, TPMT*2, TPMT*3A, and TPMT*3C have been shown to be associated with most of the intermediate (~11% occurrence) or poor (~0.3% occurrence) enzyme activity in humans [100,101]. Noncompetitive inhibitors of TPMT include naproxen,

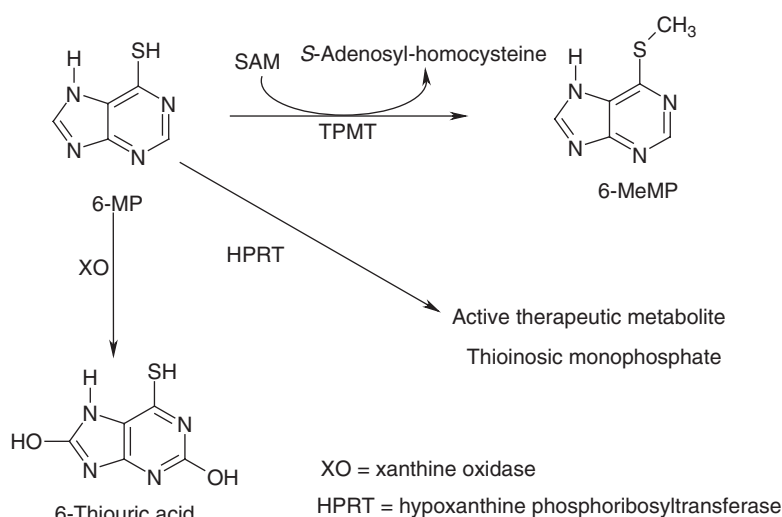


Figure 6.4 Metabolic pathway of 6-mercaptopurine (6-MP). *Source:* Adapted from Refs 103–105.

mefenamic acid, and tolfenamic acid and suggests the potential for clinical drug–drug interactions between NSAIDs and thiopurines [99]. Other inhibitors of TPMT include the diuretics—furosemide, bendroflumethiazide, and trichlormethiazide; and the 5-aminosalicylates—mesalazine, olsalazine, and sulfasalazine [99]. Another S-methyl transferase found in human red blood cell membranes known as TMT also plays a role in drug metabolism and catalyzes the S-methylation of the angiotensin-converting enzyme (ACE) inhibitor captopril as well as other thiol compounds such as N-acetylcysteine and 7 α -thio-spirolactone [102].

6.2.5 N-Acetyltransferases

NATs (also referred to as *arylamine N-acetyltransferases*) catalyze the transfer of an acetyl group from acetyl CoA to aryl amines and N-hydroxyaryl amines. An N-acetylation metabolite of *p*-aminobenzoic acid in humans was first described in 1926 [106]. Similar to methylation, N-acetylation differs from other conjugation reactions, in that it also produces metabolites that are less polar and less water soluble than their parent drug forms. There are two human forms of NAT, NAT1 and NAT2, both of which are comprised of 290 amino acids and are ~33.5 kDa in molecular weight. NAT1 and NAT2 are expressed from separate genes on chromosome 8 with ~81% homology of their amino acid sequences [107]. The crystal structures of human NAT1 and NAT2 were solved in 2007 by Wu *et al.* [108], providing additional insight into the substrate specificity of these two enzymes. The active site of both NATs is highly conserved and exerts its catalytic activity via cysteine, histidine, aspartic acid and amino acid residues. The kinetics of these enzymes proceed via a ping-pong bi-bi reaction mechanism. Therapeutic agents that are NAT substrates include isoniazid, hydralazine, phenelzine, sulphamethazine, endralazine, *p*-aminosalicylic acid, procainamide, nitrazepam, debrisoquine, and dapsone [109,110]. Interestingly, NAT1 has only one proposed endogenous substrate (*p*-aminobenzoyl glutamic acid), while no endogenous substrates have been identified for NAT2 [111–113]. Drugs such as ketoprofen, ibuprofen, paclitaxel, and salicylamide are inhibitors of NATs [114]. Despite the similarities between the two isoforms, they are quite different in their substrate specificity and tissue distribution. NAT1 is widely expressed in many tissues, while NAT2 is found primarily in the liver and gut.

Both forms are polymorphic, however, the polymorphism of NAT2 has been more extensively studied and has been implicated in the variability of the therapeutic activity and toxicity of isoniazid and other hydrazine drugs. According to the most recent update (March 12, 2010) published online by the University of Louisville, 26 NAT1 alleles and 62 NAT2 alleles have been identified (<http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/>). The involvement of NAT2 in the metabolism of isoniazid is presented in Fig. 6.5 [115]. It is estimated that in humans 50–90% of isoniazid is metabolized to acetyl isoniazid [116,117]. NAT2*5B, NAT2*6A, and NAT2*7B have been shown to be the mutant alleles primarily responsible for intermediate/poor metabolism of isoniazid in humans [118–120]. The rate of occurrence of poor/intermediate metabolizers in various ethnic populations has been found to be highly variable, but has been reported to be ~10–30% in Asians and 50–60% in Caucasians [121–123]. Poor metabolizers treated with isoniazid are at a higher risk to develop hepatic disorders and peripheral neuropathy [124–126]. NAT2 polymorphisms have also been attributed

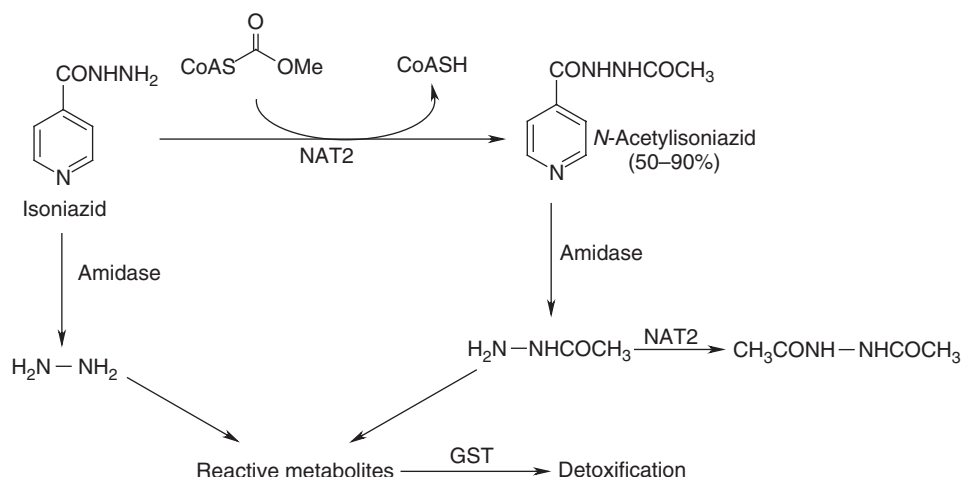


Figure 6.5 Metabolic pathway of isoniazid. *Source:* Adapted from Refs 115 and 134.

to drug–drug interactions when rifampin and doxycycline are used in combination. In rapid acetylators, rifampin levels are higher and doxycycline levels are lower compared to slow acetylators [127]. Additionally, NAT polymorphisms have been associated with an increased risk for cancer. For example, NAT1 polymorphisms have been linked to myeloma, lung, bladder, and other cancers; NAT2 polymorphisms have been linked to liver, colorectal, non-Hodgkin lymphoma, bladder cancer, and other cancers [128]. Although acetylation is generally a detoxification process, it sometimes results in bioactivation. For example, in addition to *N*-acetylation, both NAT1 and NAT2 can catalyze *O*-acetylation. Hence, some aromatic amines are oxidized to form hydroxylamines. Through heterolytic cleavage of the hydroxylamine, an unstable aryl nitrenium can be produced that ultimately can form adducts with DNA and proteins [129]. Similar to other phase II conjugating enzymes, there are a number of acetyltransferases (such as histone acetyl transferase) that are only involved in the acetylation of endogenous substrates. Research on NATs remains very active and a number of reviews have been written that summarize these studies [107,114,123,128, 130–133].

6.2.6 Amino Acid Conjugation

Amino acid conjugation, the first conjugation reaction to be identified in humans, was observed with substrates that have a carboxylic acid functional group. In humans, glycine is typically the conjugating amino acid, but in other species, additional amino acid conjugates such as glutamine, taurine, ornithine, aspartamine, alanine, and histidine have been observed. The enzymes that catalyze amino acid conjugation (acyl CoA ligase and *N*-acyltransferase) are located in the mitochondria of the liver. Hence, amino acid conjugation involves a coupled enzyme system and occurs in a number of steps [135]. First, the xenobiotic carboxylic acid group is activated by ATP. This is followed by the formation of a xenobiotic-CoA-thioester intermediate formed via the acyl CoA ligase (synthetase) catalyzed reaction of the ATP-activated carboxylic

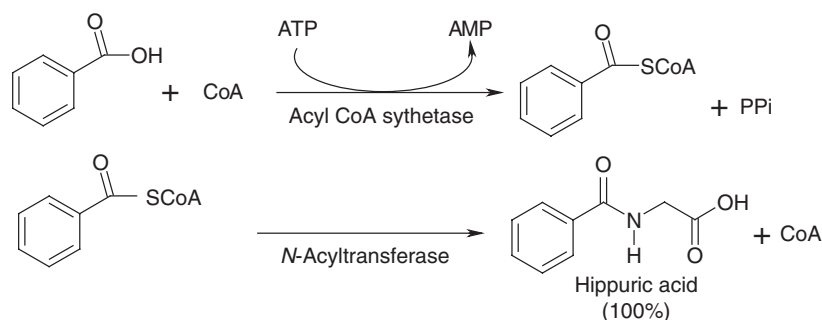


Figure 6.6 Glycine conjugation of benzoic acid. *Source:* Adapted from Ref. 138.

acid with coenzyme A. In the last step, the activated acyl group is conjugated via *N*-acyltransferase with the respective amino acid, and coenzyme A is regenerated. Amino acid conjugation was first reported in humans by Ure in 1841 and confirmed by Keller in 1842 when they independently identified hippuric acid as a metabolite formed from conjugation of benzoic acid with glycine [136,137]. Aspirin is extensively (~75%) conjugated with glycine, and benzoic acid (a food preservative that has been used to treat hyperammonemia) is exclusively conjugated with glycine [135,138]. The glycine conjugation of benzoic acid is depicted in Fig. 6.6. Given that they are generally low molecular weighted and polar, amino acid conjugates are readily eliminated in urine. As a whole, amino acid conjugation is considered a detoxification pathway. However, it is interesting to note that a reactive acyl thioester intermediate is formed in the process that is not always subsequently conjugated. For example, the anticonvulsant drug valproic acid forms the reactive acyl thioester intermediate but not the amino acid conjugate. Though the mechanism of toxicity is not fully defined for valproic acid, it is thought that the valproic acyl thioester is responsible at least in part for the hepatotoxicity observed in some individuals receiving this drug; most likely due to interference with mitochondrial β -oxidation [135,139]. Glycine conjugation (like sulfonation) is readily saturated at high substrate circulating concentrations and is compensated for by glucuronidation. Amino acid conjugation is not a major pathway in the metabolism of most drugs or xenobiotics. Consequently, amino acid conjugation as a route of xenobiotic/drug metabolism is not as well characterized [135].

6.3 EXCRETION OF DRUGS IN HUMANS AND ROLE OF TRANSPORT PROTEINS

6.3.1 Bile versus Urine as a Route of Excretion

The exact mechanism by which compounds are directed for elimination into the urine versus the bile is still not known. It was originally proposed that the main determining factors were molecular weight, polarity, and metabolism [140]. In humans, the molecular weight cutoff for biliary excretion is in the 500–600 Da range. Notable differences in this threshold are observed between species. For example, in rats, guinea pigs, and rabbits, the molecular weight cutoffs are around 325, 400, and 475 Da, respectively [141]. In general, drugs excreted into bile are strongly polar, hence most xenobiotics

and drugs excreted into the bile are in their conjugated form. However, conjugation is not always a prerequisite for biliary excretion. Though the above factors are involved, it is now becoming clear that uptake and excretion in the liver and kidney is largely determined by transport proteins. These proteins dictate to a large degree the elimination pathway of most drugs, xenobiotics, and their respective metabolites. Recent research conducted in rats that are genetically deficient in multidrug resistance-associated protein (Mrp2) has suggested that increased biliary excretion of β -lactam antibiotics by Mrp2 correlates with higher molecular weight [142].

6.3.2 Renal and Hepatic Transport Proteins

Transport proteins are involved in drug absorption, distribution, metabolism, and excretion and are most highly expressed in the intestine, liver, and kidney. The discussion here is primarily focused on transport proteins involved with the excretion of drug conjugates. Membrane transporters belong to one of two superfamilies: the ABC or the SLC. In the human ABC family, there are 49 transporters, which are divided into 7 subfamilies (A to G) [143]. The molecular weight of transporters in the ABC family ranges from 150 to 200 kDa. Each ABC transporter has two transmembrane domains and two ATP-binding domains [144]. The binding and hydrolysis of ATP is required to mediate transport. The SLC family on the other hand, is quite large consisting of ~ 48 subfamilies (SLC1-SLC46, SV2, etc.) with a total of over 380 transport proteins [145]. The SLC proteins consist of 12 putative transmembrane domains and range in molecular weight from 50 to 100 kDa [146].

Some of the transporters involved in biliary and renal uptake and excretion include the BSEP (BSEP, ABC), breast cancer resistance protein (BCRP, ABC), organic cation transporters (OCTs, SLC), organic cation/ergothioneine transporter (OCTN, SLC), OATs (OATs, SLC), organic anion transporting polypeptide (OATP, SLC), multidrug and toxin extrusion protein (MATE, SLC), multidrug resistance proteins (MRPs, ABC), sodium taurocholate cotransporting polypeptide (NTCP, SLC), peptide transporter (PEPT, SLC), P-glycoprotein (P-gp, MDR1, ABC), and urate transporter (URAT, SLC) [147]. Several of the SLC transporters such as a number of the OATs are bidirectional and transport substrates in both directions.

6.3.3 Biliary Excretion

The human liver weighs ~ 1400 – 1600 g and is a highly perfused organ receiving 25–30% of cardiac output [148]. The key component of liver function is the hepatocyte, which performs many tasks including the production of bile. Bile produced in hepatocytes is collected in the bile canaliculi where it flows into the bile ducts. The bile ducts either direct bile into the duodenum to aid in digestion or direct flow into the gall bladder for storage. Once in the intestine, 95% of the bile salts are reabsorbed in the terminal ileum and returned to the liver via the portal vein where they are reused by the hepatocyte. The liver is capable of producing ~ 1 L of bile per day and human bile flow typically ranges from 1.5 to 2.0 mL/min/kg [149].

Once a drug or metabolite is taken up into the hepatocyte via portal vein circulation, there are two paths it can follow. It can either be secreted back into systemic circulation or excreted as unchanged drug and/or as metabolite into the bile. Drug uptake, normally occurs at the sinusoidal membrane while excretion generally occurs

at both the sinusoidal and canicular membranes. Drugs and metabolites excreted into the sinusoidal blood via the basolateral sinusoidal membrane typically end up being eliminated in the urine while those excreted in bile via the canicular membrane end up in the intestine where they are eliminated in the feces or can potentially undergo reabsorption via a process known as *enterohepatic recirculation* (Section 6.5).

6.3.4 Transporters Involved in Hepatic Drug and Metabolite Uptake and Excretion of Phase II Metabolites

In order to be excreted via the hepatobiliary route, parent drug and/or its respective metabolites which are in the systemic circulation and not already in the hepatocyte (e.g., extrahepatic metabolites) must first be taken up into the hepatocyte. Transporter proteins in the OAT, OATP, and OCT families are involved in the ATP-mediated uptake of drugs and metabolites into the hepatocyte. The OATs transport anions that are <500 Da in MW and the OCTs transport hydrophilic low molecular weight (<400 Da MW) monovalent cations [146].

Transporters involved in the biliary excretion of drugs and metabolites out of the hepatocyte into the bile or sinusoidal blood include BCRP, BSEP, P-gp, several of the MRPs and MATE1. While the role of BSEP in the transport of drugs and conjugated metabolites remains unclear, several drugs have been shown to inhibit BSEP (cyclosporine A, bosentan, fluvastatin, glibenclamide, troglitazone, and rifampicin), potentially leading to cholestasis [70]. Most conjugated metabolites are very hydrophilic and cannot passively diffuse out of hepatocytes and must therefore be actively transported. Though still under investigation, the main transport proteins involved in the biliary and basolateral excretion of glucuronide, sulfate, and GSH metabolites are MRP2, BCRP, MRP3, and MRP4 [20,147]. Most glucuronide conjugates that are excreted into the bile are MRP2 substrates, whereas most sulfate conjugates are BCRP substrates [20,147].

There are a number of ATP-mediated transporters responsible for basolateral excretion into the sinusoidal blood. From the MRP family, these include MRP3, MRP4, MRP5, and MRP6 [20]. Of these, MRP3 is efficient in transporting both glucuronide and sulfate conjugates, but its role in transporting GSH conjugates remains unclear [20,147]. The most notable conjugated-drug substrates of this transport protein are morphine-3-glucuronide and morphine-6-glucuronide. MRP4 has been shown to transport both sulfate and glucuronide conjugates in addition to GSH conjugates but appears to have a higher affinity for sulfate conjugates [20,147]. GSH conjugates are also transported by MRP5 and MRP6 [20,147]. However, little is known of MRP5 and MRP6 in their ability to transport conjugated organic anions [20,147].

Biliary transport of sulfate, glucuronide, and GSH metabolites is primarily mediated by MRP2 [150]. The most notable substrates include both the parent drugs and conjugates of ceftriazone, ampicillin, and dibromosulfophthalein. MRP2 is inactive in Dubin–Johnson syndrome leading to impaired secretion of bilirubin in the hepatocytes [151]. Hence, by examining drugs known to be transported by MRP2 in these patients, one can confirm the physiologic role of MRP2. The recently identified canicular drug transport protein BCRP also excretes many sulfate and glucuronide conjugates into bile. Although other canicular transporters, BSEP, MDR2, and P-gp transport a number of drugs, they do not appear to be involved in the biliary transport of sulfate, glucuronide, or GSH conjugates [20].

6.3.5 Renal Excretion

The kidneys receive about 20% of the cardiac output. The nephron in the kidney is where the process of glomerular filtration, tubular secretion, and tubular reabsorption occurs and defines the rate of renal clearance of drugs ($CL_r = R_r/C_p$, where CL_r is the renal clearance, R_r is the renal excretion rate, and C_p is the plasma concentration). When no other factors are involved such as protein binding or secretion/reabsorption, renal clearance of the drug will be equivalent to the glomerular filtration rate (GFR). The glomerulus filters arterial blood and only molecules <5 kDa in molecular weight can pass through the membranes of the glomeruli [152]. Consequently, protein-bound drugs do not pass through the glomeruli. In adults, the GFR is ~120–130 mL/min. The process of reabsorption occurs along the proximal, distal, and collecting tubules with most of the reabsorbed components (e.g., glucose and lipid soluble components) ending up back in the plasma. The net result is that only 1–2 mL/min of the total volume filtered by the glomeruli ends up being eliminated in the urine. The pH of the tubular fluid ranges from 4.5 to 8 and is important in determining if drugs are excreted into the urine or reabsorbed. While uncharged nonionic molecules can diffuse passively through the cell membrane, the renal secretion of drugs (which are usually ionic) occurs against a concentration gradient and therefore requires active transport. As mentioned above, if a drug is not protein bound, secreted, or reabsorbed (e.g., digoxin and kanamycin), then its renal clearance will be equivalent to the GFR. Although some drugs are reabsorbed by the proximal tubules via active transport such as β -lactam antibiotics (e.g., dicloxacillin), reabsorption of most lipid soluble organic ions occurs passively [153,154]. A second mechanism of reabsorption in the kidney is endocytosis. Endocytosis in the kidney occurs by the formation of vesicles along the brush border membrane, which can take up fluids and compounds [154]. Although drugs are primarily metabolized in the liver, the kidney is also capable of oxidative, reductive, and conjugative (glucuronide, sulfate, and GSH) metabolism of drugs [155].

In summary, renal excretion of drugs occurs via a combination of three processes: (i) glomerular filtration, (ii) renal tubule secretion (the basolateral uptake and apical efflux transporters are involved in this process), and (iii) renal tubular reabsorption (apical transporters are involved in this process). Transporter proteins mediate uptake and excretion as well as secretion processes. For example, MDR1/P-gp is localized at the apical brush border membrane of the proximal renal tubule and is involved in secretion processes in the kidney.

6.3.6 Transporters Involved in Renal Uptake and Excretion

In the kidney, efflux transporters are located on the luminal (apical) brush border membrane and can transport an unchanged drug or its metabolites into urine. The primary site of transport of drugs and metabolites from blood into urine is the proximal tubule [154]. The uptake of drugs from systemic circulation by the basolateral membrane of proximal renal epithelial cells is mediated by several transporter families including OCT, OAT, and OATP [156,157]. There are also bidirectional transporters that, in addition to excretion into the urine, can transport endogenous substrates and potentially drugs from the urine back into the renal epithelial cells. These include OAT4, URAT1, and PEPT1/2. For example, PEPT2 is involved in the reabsorption of peptidic drugs, while the bidirectional transporters URAT1 and OAT4 thus far have only been shown

to be involved in the reabsorption of endogenous substrates such as urate [146,158]. Transporters involved in the renal excretion of drugs and metabolites out of the kidney proximal tubule epithelia into the urine include OAT4, MRP2, MRP4, MDR1/P-gp, MATE1, and OCTN1 [146,147]. As in hepatobiliary excretion, MRP2 is primarily involved in the renal excretion of glucuronide, sulfate, and GSH conjugates. GSH S-conjugates of polyhaloalkanes and hydroquinones have shown nephrotoxic potential as a consequence of active secretory transport and ultimate accumulation of conjugates in high concentration in the proximal renal tubules [159].

6.3.7 Drug Interactions and Clinical Relevance

Given the impact of transporters on the pharmacokinetics (PK) of drugs and the potential for drug–drug interactions, an international transporter consortium was formed and discussed the issues surrounding drug transporters relating to drug–drug interactions. A recent review article summarizes their findings and provides suggested decision trees for handling potential transporter interactions [147]. As pointed out in this review by Giacomini *et al.*, a number of transporters including OATP, OAT, OCT, P-gp, and BCRP have been implicated in drug–drug interactions. Some of the drug pairs and corresponding transporters involved in these interactions are pravastatin/cyclosporine (OATP), bosentan/rifampicin (OATP), acyclovir/probenecid (OAT), pilsicainide/cimetidine (OAT), digoxin/ritonavir (P-gp), and topotecan/GF120918 (BCRP) [147].

Not all drug–drug interactions involving transporters are adverse and some have been exploited to extend the desired therapeutic effect. For example, an inhibitor of OAT-mediated tubular secretion, probenecid, has been used clinically to extend the pharmacological activity of cephalosporins and penicillins in patients [160,161]. Probenecid coadministered with cephadrine resulted in a 2.4-fold increase in AUC [162]. Similarly, coadministration with dicloxacillin resulted in a 1.9-fold increase in AUC [163]. In both cases, renal clearance was reduced by inhibiting tubular secretion (1.8-fold for cephadrine and 3.6-fold for dichloxacillin). In fact, this property of probenecid was used during World War II to increase the length of efficacy of penicillin, which was in short supply [154].

6.4 ELIMINATION PATHWAYS IN HUMANS

6.4.1 Feces, Urine, and Other Routes of Drug Elimination in Humans

The most common routes of drug and metabolite elimination in humans are via the urine and feces. As discussed above, drugs and metabolites, which are substrates for the efflux transporters in the proximal tubule end up being excreted out of the kidney into the urine. Drugs and metabolites, which are substrates for efflux transporters in the liver, end up being excreted via the bile into the intestine where they are eliminated in feces.

Excretion of drugs into other body fluids (saliva, sweat, tears, and breast milk) and tissues (hair and skin) generally represent minor elimination pathways in humans. Volatile drugs such as the anesthetic halothane may be primarily eliminated unchanged in expired air, whereas other nonvolatile drugs, for example, aminopyrine may generate CO₂ as a metabolite, which is eliminated via the breath [164,165].

6.4.2 Mass Balance Studies in Humans

The most comprehensive way to quantify drug and metabolite elimination in feces and urine is through a human mass balance study. In mass balance studies, radiolabeled (usually ^{14}C) drug is dosed via the therapeutic administration route. The position of the radiolabel in the compound is chosen such that it is metabolically stable to potential biotransformation pathways. Samples of blood, plasma, urine, and feces are collected, and the amount of radioactivity in the samples is quantitated using a variety of analytical techniques. Human mass balance studies are required by the FDA for a New Drug Application (NDA) approval. Complete mass balance (100% recovery of radioactive material) is rarely achieved because of sampling or analytical issues. However, recoveries of greater than 80% are normally achievable in human mass balance studies, and no less than 90% recovery has been recommended in animal studies as a minimum cut-off for acceptability [166]. Recovery in human mass balance studies is generally lower than that obtained from rat or dog studies, most likely due to the difficulty in collecting every last drop of excreta samples from human subjects. For compounds and metabolites with a very long half-life, it is more difficult to achieve mass balance, and sample collection times and study objectives should be adjusted accordingly. Low recovery (e.g., lower than 80% in human studies) does not necessarily invalidate the study if the objectives of the mass balance study have been largely met. However, significantly lower recovery could indicate covalent binding or noncovalent tissue sequestration and raise safety concerns regarding the drug. In addition to providing information on the quantities of unchanged drug and its metabolites that are eliminated via the excreta, mass balance studies can also provide important data regarding the following [167,168]:

1. Clearance mechanism of the drug
2. Levels of circulating metabolites versus levels of circulating parent drug
3. Ratio of blood/plasma levels of the drug and metabolites
4. Appropriateness of the species used in the preclinical safety studies (e.g., no unique human metabolites are observed)
5. Pharmacologically active or toxic metabolites
6. Proportion of unchanged drug eliminated.

Comparison of metabolite profiles from human mass balance studies to those obtained from preclinical safety studies can divulge differing levels of active/toxic metabolites or the presence of unique human metabolites that may help explain why human toxicity differs from that observed in the animal species used for preclinical safety studies. Given that some drugs are extensively metabolized and the resulting metabolites were not routinely identified or quantified, guidelines were developed in order to reduce clinical toxicity/safety issues arising from the above scenario. The guidelines were first presented entitled as “Metabolites in Safety Testing,” which is often referred to as the *MIST document*⁴ [170,171]. They ultimately served as a basis for the FDA guidance document entitled “Safety Testing of Drug Metabolites,” which was implemented in February, 2008.

A number of excellent reviews have recently been published on this topic [166,168,169].

⁴Also see Volume 22, Issue 2 (February 2009) of *Chemical Research in Toxicology*.

6.5 ENTEROHEPATIC RECIRCULATION

Enterohepatic recirculation is the process by which a drug or its conjugated metabolites (e.g., indomethacin glucuronide) undergoes biliary excretion followed by reabsorption in the intestine [172]. It is usually observed in the drug concentration–time PK profiles as one or more peaks that occur after the initial $C_{\max}$ and generally increases the half-life and systemic exposure (AUC) of the drug. In some cases (e.g., the drug ezetimibe), the appearance of these peaks in the concentration–time profiles coincide with gall bladder emptying via the Sphincter of Oddi into the small intestine [173]. Enterohepatic recirculation can occur with either the parent drug or a conjugated metabolite. Conjugated metabolites, such as sulfates or glucuronides, are generally converted back to parent drug by the β -glucuronidases or sulfatases present in the microflora of the intestine. Enterohepatic recirculation also occurs for some endogenous compounds such as bilirubin and bile acids. In addition, GSH conjugates excreted into the bile have also been known to undergo enterohepatic recirculation [174]. Given that biliary excretion of conjugates is mediated by transport proteins such as MRP2, drug and metabolite transport is an important component of enterohepatic recirculation. Clinically, enterohepatic recirculation is impacted by various inhibitors and inducers of drug transport and disease states that affect transporter activity. Hence, overall characterization of this recycling process requires determination of the rate of biliary excretion and re-excretion, absorption in the intestine, and elimination of the parent compound and metabolites in the feces and urine [175]. Recycling ends when the endogenous or exogenous compound is ultimately eliminated in the feces or urine. Many drugs undergo enterohepatic recirculation, including digoxin, morphine, indomethacin, and erythromycin. Other drugs that undergo enterohepatic recirculation are summarized in Table 6.3 [174]. The extent of enterohepatic recirculation can be impacted by factors that mediate the absorption, biotransformation, and excretion of the drug and its metabolites. In humans, these factors include genetics, age, gender, diet, disease states, and coadministration of other drugs or xenobiotics [174].

6.6 IMPACT OF HEPATIC AND RENAL IMPAIRMENT ON DRUG ELIMINATION

When liver function is impaired, the hepatic clearance of most drugs cleared via this pathway will be compromised leading to drug accumulation and toxicity. Ultimately, a dose reduction is typically required. The phase I CYP450 enzymes are generally impacted to a greater degree by liver impairment than the phase II conjugation enzymes. For example, hepatic impairment has been shown to impact the CYP450 enzymes

TABLE 6.3 Examples of Drugs Excreted in Human Bile

Ampicillin	Erythromycin	Irinotecan	Sulfamethoxazole
Cephazolin	Estradiol Ezetimibe	Lorazepam	Testosterone
Diazepam Digoxin	Gentamycin	Methotrexate	Tetracycline
Doxycycline	Indomethacin	Morphine	Troglitazone
		Phenytoin	Valproic acid

Source: Adapted from Ref. 174.

involved in the clearance of antiarrhythmic drugs but not phase II metabolism [176]. In another study, the extent of formation of dabigatran glucuronides, as well as glucuronidation capacity, was found to be unchanged in moderate liver disease [177].

Renal impairment has led to implementation of modified dosing regimens for elderly patients with impaired kidney function and has resulted in FDA guidelines for when and how to conduct renal impairment studies [178,179]. Interestingly, data suggests that kidney disease can impact transporters and metabolic enzymes in the liver and gastrointestinal tract, potentially because of the accumulation of uremic toxins. Currently, it is postulated that uremic toxins such as urea, parathyroid hormone, indoxyl sulfate, and cytokines might cause transcriptional/translational modifications or inhibition of DMEs and transporters and data collected thus far seem to support this theory [180]. Assessment of the impact of renal impairment on the systemic exposure of new molecular entities from recently submitted investigational new drug applications (INDs) provided evidence that renal impairment can affect the PKs of drugs that are predominately eliminated by nonrenal processes such as metabolism (CYP and non-CYP) and/or active transport [181].

6.7 NUCLEAR RECEPTORS IN HUMAN DRUG CONJUGATION AND TRANSPORT

6.7.1 Nuclear Receptors

NRs are proteins that control the transcriptional genes that modulate the levels of numerous proteins including the phase II conjugating enzymes and phase III transport proteins. Induction of a particular protein occurs when the transcriptional gene is activated via the NR. Transcriptional activation results in the expression of higher levels of the corresponding protein in tissues. When agonist binding results in conformational changes in the ligand binding domain of NRs, they are translocated to the nucleus, and subsequent involvement of additional transcription factors (such as RNA polymerase) allow transcription of mRNA and ultimately expression of the protein/enzyme [182]. Similar to the phase I, II, and III DME systems, NRs are widely expressed in many tissues, but the highest levels are found in liver and intestine. The NRs comprise a superfamily of receptors and include the aromatic hydrocarbon receptor (AhR), CAR, farnesoid X receptor (FXR), glucocorticoid receptor (GR), hepatocyte nuclear factor (HNF), nuclear factor erythroid 2-related factor 2 (Nrf2), peroxisome proliferator-activated receptor (PPAR), PXR, and the vitamin D receptor (VDR).

6.7.2 Transcriptional Regulation of Drug Conjugation and Transport in Humans

The phase II drug-conjugating enzymes are regulated by PXR, CAR, FXR, and VDR with some involvement of AhR, PPAR, HNF, and Nrf2 [183,184]. PXR and CAR are the primary regulators of the phase II enzymes. Through experiments conducted in human cell lines such as human hepatoma Hep G2 cells, UGT1A1 and UGT1A3 expression was shown to be increased by activation of PXR with rifampicin [185]. Similar experiments on CAR utilizing human hepatocytes demonstrated that the expression of GSTA2, UGT1A1, and SULT1A1 was increased by activation

with 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITGO) [186]. Overall, UGTs are regulated by the NRs—AhR, CAR, PPAR, and PXR [42]. The NRs involved in the regulation of all human SULTs have not yet been fully delineated, but thus far AhR, CAR, FXR, GR, PPAR α , and PXR have been shown to be potentially involved in the induction of the SULT enzymes [61,187]. SULTs do not appear to be impacted by induction to the same degree as the UGTs [188,189]. In fact, those that appear to be inducible are only weakly so. Steroids and glucocorticoids have been shown to induce SULTs in rats and humans [57]. For example, human SULT2A has been shown to be inducible by dexamethasone [190]. Transgenic, knockout, and humanized mice with a variety of NRs such as PXR, CAR, PPAR, AhR, and Nrf2 have been employed to study the function and regulation of UGTs. For example, experiments using PXR transgenic mice have helped to corroborate the regulation of UGT1A1 and UGT1A6 by PXR [44].

Given the potential for induction of the phase II DMEs as a result of their association with PXR and CAR, it is reasonable to assume their involvement in potential clinical drug–drug interactions. Indeed, induction of CAR (which regulates UGT1A1) by phenobarbital has been employed clinically to alleviate hyperbilirubimemia caused by the genetic disease Crigler–Najjar syndrome [191]. As discussed previously, these patients express very low levels of UGT1A1, an enzyme required for the metabolism of the endogenous compound bilirubin. However, drug–drug interactions due to induction are rare, and fewer than 20 drugs have been cited to date [192]. Examples of induction type drug–drug interactions include the interaction between rifampicin and zidovudine and rifampicin and lamotrigine [193,194].

Similar to drug-conjugating enzymes, transport proteins are regulated through PXR, CAR, FXR, and VDR [183]. Of these, PXR and CAR are the primary regulators of drug transport and are involved in the regulation of many of the human transport proteins including MDR1, MRP2, MRP3, MRP4, MRP5, BCRP, OATP1A2, OATP1B1, and OATP1B3 [195]. Research on the regulation of transport proteins continues to progress and the associated clinical implications have not been fully investigated. However, there are a few examples where clinical relevance of regulation of transport proteins has been demonstrated. For example, elimination of digoxin into bile has been increased by induction of MDR1 via PXR with rifampin, and the renal clearance of talinolol was increased after induction with carbamazepine [196,197]. These particular drugs were studied because they were not complicated by induction of DMEs and show that induction directly regulates the transport of drugs.

6.8 EFFECT OF DIETARY PHYTOCHEMICALS AND HERBAL SUPPLEMENTS ON NUCLEAR TRANSPORTERS, DRUG CONJUGATION, AND DRUG TRANSPORT

There are a number of dietary phytochemicals present in foods and herbal supplements, which have been shown to affect NRs, drug-conjugating enzymes, and transporters and result in food–drug or herb–drug interactions. In fact, clinically significant herb–drug interactions are becoming more common as more people are taking both herbal supplements and prescription drugs [198]. Herbal supplements that have been shown to affect the metabolism and transport of drugs include St. John’s wort, garlic, ginseng, milk thistle, and ginkgo [199]. St. John’s wort, which is used to treat depression, is one

of the most widely studied herbal medications. An extract of St. John's wort, *Hypericum perforatum*, has been shown to reduce the plasma exposure (AUC) of a number of drugs including midazolam, amitriptyline, nortriptyline, cyclosporine, and digoxin [199]. These drugs are primarily metabolized by CYP450 enzymes including CYP3A4 or in the case of digoxin, a substrate for P-gp. Protein levels of CYP3A4 and P-gp are modulated by PXR, hence St. John's wort appears to primarily exert its effect through PXR [200,201]. Similarly, the protein levels of the MRP2 transporter and GST-P 1 have been shown to be increased by St. John's wort. However, studies with St John's wort and examining its effect on NAT2 and UGTs have shown no interaction.

Garlic contains organosulfur compounds (e.g., diallyl sulfide, diallyl disulfide, and diallyl trisulfide) and has been shown to inhibit GST and induce both GST and UGT [202,203]. Ginseng contains seven ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) as its active components and has been found to moderately inhibit P-gp [204]. Milk thistle, the active component of which is mostly silybin (a flavonolignan), has been shown *in vitro* to inhibit UGT1 and the transporters P-gp, BCRP, and OATP1B1 [199]. Clinically, however, the PK of most drugs studied thus far (indinavir, digoxin, rosuvastatin, etc.) have been shown to be unaffected by milk thistle, most likely because of the poor bioavailability (~1%) of its active components. [199]. Ginkgo, which has been widely studied for potential induction and inhibitory effects on the CYP450 enzymes, has not been as extensively studied in this regard for the phase II enzymes and transporters.

Dietary phytochemicals such as flavonoids and isothiocyanates present in food have demonstrated biological activity and have the potential to impact the NRs, DMEs, and transporters. Interest in phytochemicals present in cruciferous vegetables (e.g., broccoli and cauliflower) was piqued when researchers observed a connection between diets high in these vegetables and lower cancer rates in patients with liver, ovarian, lung, and cancers of the gastrointestinal tract [205]. One of the most active isothiocyanates is sulforaphane, which is found in high levels in cruciferous vegetables. Recent studies have demonstrated that sulforaphane exerts this effect as an antagonist of PXR, resulting in inhibition of PXR-mediated CYP3A4 expression and ultimately lower levels of CYP3A4 [206]. Given that it is believed that reactive oxidation metabolites formed by CYP3A4 may be the cause of some of these cancers, it is reasonable to assume that reduction of CYP3A4 enzyme levels would reduce the rate of cancer incidence [207]. The flavonoid quercetin that is present in high levels in foods such as fried onions has been shown to induce ABC transporters, such BCRP, as well as inhibit phase II metabolism of resveratrol [208]. Similarly, other dietary flavonoids and isothiocyanates have been shown to have inductive/inhibitory effects on PXR and BCRP as well as AhR, UGTs, GSTs, MRP-1, P-gp, and MRP-2 [199,209–211].

6.9 INTERSPECIES DIFFERENCES IN DRUG CONJUGATION, TRANSPORT, AND ELIMINATION

As a requirement for first time in man (FTIM) clinical studies, new drug entities (NDEs) must first undergo preclinical safety evaluation in other mammalian species (e.g., rats, dogs, and monkeys). In these studies, dose levels are chosen so that target organ toxicity can be determined and the plasma levels associated with the onset of toxicity at a given dose level are defined. Employing scaling factors, the results from these

studies are then extrapolated to select a safe starting dose for FTIM phase I clinical studies. Given the interspecies differences in physiology, anatomy, endocrinology, drug absorption, protein binding, expression levels of drug target, DMEs (phase I to III), and so on, the extrapolation from preclinical species to humans is one of the most challenging aspects of drug development. Lin [212] has shown that, in terms of describing differences that impact drug metabolism and elimination, it is appropriate to first consider the allometric relationship of blood circulation time (s) and total body weight (kg) between rats and humans. The allometric equation used for this calculation is $Y = aW^b$ (Y = physiological parameter, a = allometric coefficient, W = body weight, and b = the allometric exponent), which becomes $Y(\text{blood circulation time}) = 21 W^{0.21}$ [213]. Hence, if weights of 0.25 kg (rat) and 70 kg (human) are entered into the equation, the blood circulation times become 15 and 50 s, respectively, which means it takes roughly four times longer for blood to circulate in humans than in rats [212]. Similar calculations can be performed for hepatic and renal blood flow. Overall, this example demonstrates that for smaller species drug will circulate through organs more frequently and thereby is eliminated more quickly than in larger species. If this were the only factor implicated in interspecies differences then extrapolation between species would be relatively straightforward.

Walton *et al.* [214] has examined the magnitude of interspecies differences in exposure of drugs for which glucuronidation was the major metabolic pathway and demonstrated the necessity for risk assessments focused on fundamental processes that influence exposure. Species differences in glucuronidation, elimination, and metabolic pathways and the corresponding impact on drug exposure are clearly delineated. For example, for zomepirac the variation in $C_{\text{max}}/\text{dose}$ is 3200, 5700, 1700, and 2900 (ng/mL)/(mg/kg), respectively for human, rabbit, rat, and mouse. For lamotrigine, clearance ranges from 0.55 in humans to 12 mL/min/kg in dog and 14 mL/min/kg in rabbit (factors of 22 and 25, respectively). Species differences in metabolic and elimination pathways are quite profound. For example, in humans lamotrigine is cleared primarily in urine (85–94% of the dose) as the *N*-2- and *N*-5-glucuronides (86%). In dogs, lamotrigine is also cleared primarily in urine (53–67% of the dose) but as the *N*-2-methyl conjugate (80%). Finally, in rats 59–69% of the lamotrigine dose is cleared via the urine but mostly as the unchanged drug (57%). Species differences in the metabolism and elimination of AZT, oxazepam, morphine, lamotrigine, and paracetamol are summarized in Table 6.4. The predictability of other species as a model of biotransformation in man has been reported previously. For example, correct correlations to man have been found ~92% of the time in the rhesus monkey, 59% of the time in other nonprimate species, and only 41% of the time in the rat [215].

Species differences have also been noted for transport proteins. The canicular multispecific organic anion transporter (cMOAT) showed differences in *in vivo* clearance where transport values were highest in rat, followed by mouse, guinea pig, rabbit, and dog [217]. *In vitro* experiments using hepatocytes (fresh and cryopreserved) and fluorescent dye efflux assays have shown species differences between rats and humans for both MRP/Mrp function (rat elimination rate was higher) and BCRP/Bcrp (human elimination was higher). However, similar experiments on P-gp showed that although the elimination rate was functionally low for both species; no species differences in elimination rate due to P-gp were observed [218]. In a subsequent study, in which the levels of MRP2/Mrp2 were directly measured by LC/MS/MS in liver tissue, Li and coworkers found that the absolute amounts of MRP2/Mrp2 were highest in rat

TABLE 6.4 Species Differences in Drug Glucuronidation

Drug, OBA, Elimination Route	Human	Dog	Rat	Rabbit	Mouse
<i>AZT (Oral)</i>					
%OBA	65	NA	80–90	NA	86
% of TD in urine	70–99	56	NA	NA	NA
% of TD in feces	NA	NA	~10–20	NA	NA
% Glucuronide in urine	80–87	15	6	NA	NA
% Parent in urine	14–20	71	88	NA	NA
% Parent in feces	NA	NA	NA	NA	NA
<i>Oxazepam (oral)</i>					
OBA	~100	70	40	NA	NA
% of TD in urine	70–80	60–80	13–19	NA	22–25
% of TD in feces	NA	17–36	52–71	NA	62–63
% Glucuronide in urine and/or feces	70–80	NA	NA	NA	5–13
% Sulfate feces and/or urine	NA	NA	22	NA	NA
% Oxidative metabolites feces and urine	NA	NA	30	NA	16–20
<i>Morphine (IV)</i>					
% of TD in urine	80–90	63–83	23	68	NA
% of TD in feces	NA	14–35	65	NA	NA
% Glucuronide in urine	70–80	76–88	NA	89	NA
% Glucuronide in bile/feces	NA	NA	50	NA	NA
<i>Lamotrigine (oral)</i>					
OBA	100	>98	NA	NA	NA
% of TD in urine	85–94	53–67	59–69	46–63	62–68
% of TD in feces	NA	25–41	NA	7–36	23–31
% Glucuronide in urine	86	NA	11	76	15
% Parent in urine	NA	10	57	NA	47*
% Parent in feces	NA	NA	NA	NA	47*
% <i>N</i> -2-Methyl conjugate in urine	NA	80%	NA	NA	9
<i>Paracetamol (oral)</i>					
OBA	100	55–79	NA	NA	NA
% of TD in urine	83–100	96	72–98	96 (IV)	83–94
% of TD in feces	NA	NA	NA	NA	NA
% Glucuronides in urine	65	75	NA	78 (IV)	60
% Sulfates in urine	30	15	90	NA	15

Abbreviations: TD, total dose; NA, not available; *feces + urine.

Source: Adapted from Refs 10,214, and 216.

followed by monkey, human, and dog. Additionally, the tendency of rodent species to preferentially excrete compounds of lower molecular weight compared to humans has been discussed [219]. Species differences in biliary excretion are a primary factor accounting for differences in enterohepatic recirculation observed across species [174]. Additionally, interspecies variations in the intestinal microflora and their respective enzymes (e.g., β -glucuronidase and sulfatase) can cause significant differences in the rate and the extent of hydrolysis of drug conjugates [220]. Consequently, species differences in enterohepatic recirculation can impact the levels of metabolites observed [214,221].

It is clear that differences in metabolism, transport, and elimination can result in dramatically different exposures of drugs and their metabolites across species. Given that ADME and toxicity studies are first conducted in animals to gain an understanding of how a given NDE will behave in humans, great care should be taken in the planning, execution, and interpretation of data for these studies.

6.10 OTHER FACTORS AFFECTING THE CONJUGATION, TRANSPORT, AND ELIMINATION OF DRUGS IN HUMANS

Both intrinsic factors such as genetics, gender, age, race, organ impairment; and extrinsic factors such as diet, smoking, alcohol consumption, and concomitantly administered drugs can impact drug metabolism and transport and ultimately drug exposure and response.

Genetic factors and genetic diseases involving polymorphisms of drug-conjugating enzymes have been discussed and can clearly have a profound clinical impact. For example, genetic polymorphisms are estimated to influence the clinical outcome of 20–25% of all drug therapies [222,223]. Age has been shown to be more of a factor in the extent of UGT drug conjugation in children than adults [224]. For example, in neonates, the expression level of UGT1A6 is low but increases rapidly within the first month of birth, ultimately reaching adult levels after age of 10 [225,226]. Activity of UGT1A1 is low in neonates but increases to adult levels within three months of birth [227]. Similarly, UGT2B7 expression levels are low in infants but expression levels increase steadily and at 12–17 years of age are at 50% of adult levels [228,229]. In elderly patients, the conjugation of oxazepam, lorazepam, morphine, and lamotrigine have been reported as being reduced resulting in an apparent age-related reduction in clearance [230–233]. Overall, most studies have shown that UGT activity or the activity of other drug-conjugating enzymes is not extensively impacted in older individuals who are over 65 years old [229,234–236]. Recent data obtained from human liver banks have supported these findings for UGT enzymes [229]. In terms of other conjugation enzymes, *N*-methyltransferase and most SULTs are at adult levels in newborns [236]. In fact, sulfonation compensates for glucuronidation in neonates and children as evidenced by the higher levels of sulfate conjugates observed for some drugs (e.g., salicylamide) [225]. Expression levels of GSTs are higher in neonates compared to adults while glycine conjugation is deficient in neonates [236].

Gender differences have been implicated in the variation of phase II metabolism for several drugs [223,237]. One of the most notable involves conjugation of the common over-the-counter (OTC) drugs aspirin and acetaminophen (paracetamol). Men produce higher levels of glycine and glucuronic acid conjugates of aspirin than women, which may explain its higher bioavailability in women [238]. Similarly, acetaminophen clearance rates in men were found to be 22% higher than in women because of higher levels of UGT activity in men [239]. Additionally, glucuronidation of *S*-oxazepam was 65% higher in livers from male donors versus female donors [231,240]. In recent NDA applications, it has been shown that in around 7% of these filings, there is at least a 40% difference in PK between men and women [241].

There have not been extensive investigations on alcohol consumption and drug conjugation. From data collected from human liver banks, alcohol consumption has been associated with high levels of glucuronidation for UGT1A probes but did not appear to

affect other UGT isoenzymes (e.g., UGT2B7 or UGT2B15) [229]. Studies conducted, which investigate the effect of alcohol on glucuronidation, have been inconsistent with some studies showing an effect and others showing no effect [229]. The findings from studies conducted on cigarette smoking yielded similar conflicting findings [229]. Little or no data are available on the effects of alcohol consumption or smoking on other drug-conjugating enzymes. Although smoking does not appear to have much of an impact on the extent of drug conjugation, recent studies have been conducted that correlate polymorphisms of NAT1 with an increased risk of pancreatic cancer [242]. The results have shown that women who are heavy smokers and carry the NAT1*10 allele have a fourfold higher risk of developing pancreatic cancer presumably because of the decreased ability to metabolize the suspected pancreatic carcinogens, *N*-nitroso and aromatic amines found in cigarette smoke.

6.11 SUMMARY AND FUTURE PERSPECTIVES

Over the last two decades of research, significant progress has been made in our understanding of drug conjugation and transport. During this time, it has become clear that the coordination of conjugation metabolism, conjugate transport via transport proteins, and elimination pathways are inexorably linked and complex. Therefore, changes in one could dramatically impact drug exposure and elimination, and hence the efficacy/toxicity of a drug. One also has to consider the role of NRs in the regulation of drug-conjugating enzymes and transport proteins as chemical inducers and genetic variations in NRs as having a potential effect on drug exposure. Additionally, intrinsic and extrinsic factors have an impact on the conjugation enzymes, transport proteins, and NRs. The human body has a remarkable ability to protect itself from potentially harmful chemicals. As such, these protection mechanisms have greatly impeded our ability to make safe and efficacious drugs. Hence, in order to improve our ability to optimize drug development, a deeper understanding of the mechanisms of drug conjugation, transport, and NRs and their interdependency is required.

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7 Role and Clinical Consequences of Human UDP-Glucuronosyltransferases

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7.1	Introduction	1
7.2	Classification	2
7.3	Substrate specificity	3
7.4	Tissue distribution	9
7.5	Role of UGTs in absorption	10
7.6	Bioactivation by UGTs	12
7.7	Prediction of human clearance for compounds cleared VIA glucuronidation	20
7.8	Factors affecting UGT activity and its clinical significance	22
7.9	Clinical implications of UGT-based drug–drug interactions	27
7.10	Summary	28
	References	28

7.1 INTRODUCTION

Glucuronidation is one of the most common pathways of conjugative metabolism. It is responsible for the elimination of ~40–70% of all drugs that are metabolized via phase II conjugation [1–6]. The reaction is catalyzed by a family of uridine 5'-diphosphate-glucuronosyltransferases (UGTs) and involves condensation of a pharmacologically active drug molecule (or its phase I product) or endogenous substances with the activated form of glucuronic acid, uridine-5'-diphospho- α -D-glucuronic acid (UDPGA) (Fig. 7.1). The UGT enzymes recognize a multitude of functional groups including aromatic and aliphatic alcohols, carboxylic acids, amines, and the free sulfhydryl moiety. The outcome is the formation of an *O*-, an *N*-, a *C*-, or a *S*-glucuronide [3]. Examples of endogenous substrates for UGT include bilirubin, steroid hormones, thyroid hormones, bile acids, and fat-soluble vitamins. Similarly, numerous exogenous therapeutic agents belonging to various classes including opioids, analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs), and anticonvulsants are also glucuronidated (Fig. 7.2). Molecules that possess both the hydroxyl and carboxyl groups such as mycophenolic acid (MPA) or diflunisal can be transformed to both types of glucuronide conjugates.

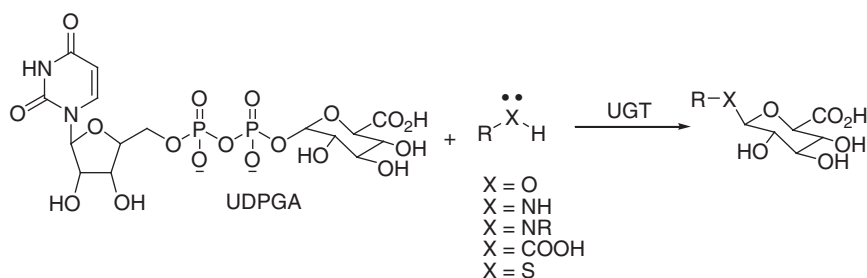


Figure 7.1 Scheme showing glucuronidation reaction. UDPGA, uridine-5'-diphospho- α -D-glucuronic acid; UGT, uridine 5'-diphosphate-glucuronosyltransferase.

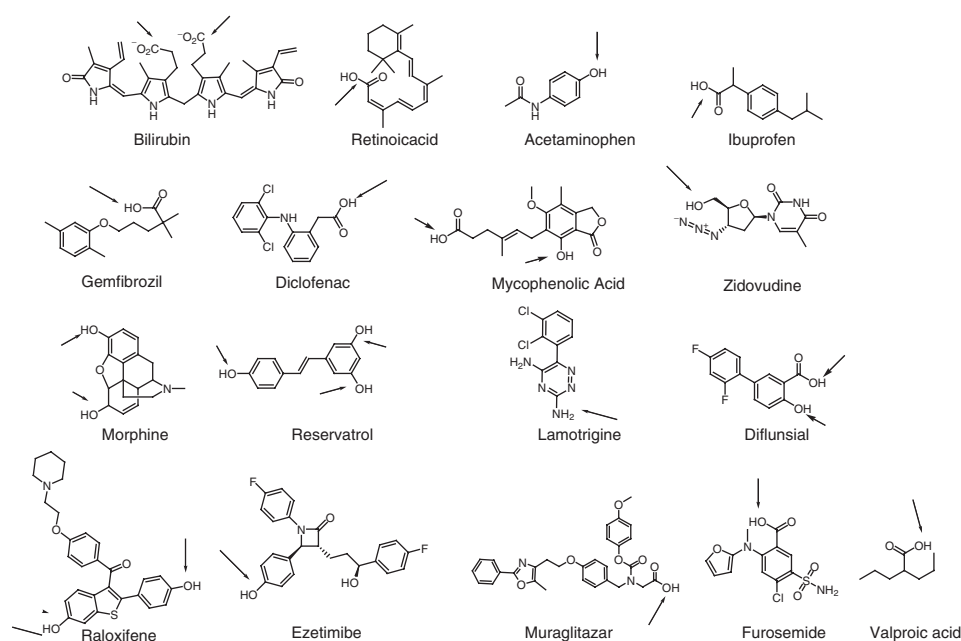


Figure 7.2 Some examples of endogenous compounds and drugs that undergo glucuronidation.

The introduction of a carbohydrate moiety containing a readily ionizable carboxy group ($\text{pK}_a = 3\text{--}4$) renders the corresponding conjugates water soluble, thus enhancing their excretion renally. In some cases, the polar conjugates exhibit good substrate properties for efflux proteins/transporters that result in the excretion of these conjugates into the bile [6].

7.2 CLASSIFICATION

Like cytochrome P450s (CYPs), human UGTs are a superfamily of enzymes. The mammalian UGT gene superfamily currently has 117 members that are divided into four families, UGT1, UGT2, UGT3, and UGT8 [7,8]. Of these four families, UGT1 and

UGT2 use UDP glucuronic acid to glucuronidate endogenous and exogenous chemicals. UGT8, in contrast, uses uridine 5'-diphosphate (UDP) galactose to galactosidate ceramide, an important step in the synthesis of glycosphingolipids and cerebroside. The function of the fourth family, UGT3, is unknown at this time. However, studies show that this enzyme catalyzes the transfer of *N*-acetylglucosamine from UDP *N*-acetylglucosamine to ursodeoxycholic acid (3 α , 7 β -dihydroxy-5 β -cholanoic acid). Of all the families, the UGT1 and UGT2 families are more relevant to humans owing to their role in metabolizing several xenobiotics that include drugs, environmental toxicants, carcinogens, and more. The UGT1 family consists of nine isoforms: UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10 whose catalytic functions have been well characterized (Table 7.1). Eight of these isoforms are clinically relevant and are involved in the metabolism of several drugs. The isoforms in the UGT1 family are encoded by human *UGT1 gene* that is located on chromosome 2 at locus 2q37 with common exons 2–5 and at least 13 unique varieties of exon 1 [7]. Thus, all isoforms have the same carboxy termini but different amino-terminal domains. The human UGT2 family is subdivided into two subfamilies: UGT2A and UGT2B and are clustered on chromosome 4p13 [7]. As the UGT1A family, exon-sharing is also seen with the 6-exon UGT2A1 and UGT2A2 gene families. However, UGT2A3 and the UGT2B subfamilies are encoded by separate genes. The UGT2B family is composed of the following functional proteins: UGT2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28 (Table 7.1). Even though all UGT isoforms in this family are involved in the metabolism of compounds, isoforms UGT2B7 and 2B15 are more important clinically since they are involved in catalyzing the conjugation of some important drugs such as morphine, diclofenac, ezetimibe, entacapone, or oxazepam (Table 7.2). Overall, the UGT enzymes in each family share at least 50% homology in their DNA sequences, whereas enzymes in each subfamily share at least 60% homology in their DNA sequences.

7.3 SUBSTRATE SPECIFICITY

Unlike the CYPs, the determination of substrate specificity for different UGTs is complicated by the overlapping substrate activities (i.e., one substrate is metabolized by several isoforms) and broad substrate specificity where one isoform glucuronidates a wide range of substrates [9–11]. It is important to note that even though one compound may be metabolized by several UGTs, the efficiencies and turnover rates may vary among the isoforms. For instance, 4-nitrophenol and 1-naphthol or the coumarin derivative, 4-methylumbelliferone (4MU), is glucuronidated by most UGT proteins, but these compounds are catalyzed most efficiently by the UGT1 proteins, with the exception of UGT1A4. Tables 7.2 and 7.3 summarize the substrates that are metabolized by UGT1A and UGT2B families, respectively.

A comprehensive list of substrates for various UGT isoforms can also be found in the review by Kiang *et al.* [12]. Despite the overlapping substrate selectivity, some substrates have been identified that are more selective toward a particular UGT isoform. For example, the endogenous substance bilirubin is predominantly (if not solely) glucuronidated by UGT1A1. However, given the technical difficulties such as the light-sensitive nature of the compound and difficulty in assaying owing to high protein binding and poor recovery, it is not recommended for use as a probe for UGT1A1. Other probes for UGT1A1 include etoposide (almost exclusively glucuronidated by

TABLE 7.1 List of Individual Isozymes in the Human UGT1 and UGT2 Family and Their Tissue Distribution

UGT1 Family	Tissues											
1A1	Liver	Biliary tract	Stomach	Small intestine	Colon	—	—	—	—	Kidney	—	—
1A3	Liver	Biliary tract	Stomach	Small intestine	Colon	—	—	Prostrate, testes	—	Kidney	—	—
1A4	Liver	Biliary tract		Small intestine	Colon	—	—	—	—	—	—	—
1A5	—	—	—	—	—	—	—	—	—	—	—	—
1A6	Liver	Biliary tract	Stomach	Small intestine	Colon	Esophagus	—	—	—	Kidney	Skin	Brain
1A7	—	—	Stomach	—	—	Esophagus	Lung	—	—	—	—	—
1A8	—	—	—	Small intestine	Colon	Esophagus	—	—	—	Kidney		
1A9	Liver	—	Stomach	—	Colon	Esophagus	—	Testes, ovaries, prostrate	—	Kidney	Skin	—
1A10	—	—	Stomach	Small intestine	Colon	—	—	—	—	Kidney	—	—
UGT2 Family	—	—		—	—	—	—	—	—	—	—	—
2A1	—	—	—	—	—	—	Nasal epithelium	—	—	—	—	—

2B4	Liver	—	—	Small intestine	—	—	—	—	—	—	—	—
2B7	Liver	—	—	Small intestine	Colon	Esophagus	—	—	—	Kidney	—	Brain
2B10	Liver	—	—	Small intestine	—	Esophagus	—	Prostrate	—	—	—	—
2B11	Liver	—	—	—	—	—	—	Prostrate	Mammary gland	—	—	—
2B15	Liver	—	—	Small intestine	—	Esophagus	—	Prostrate	—	—	—	—
2B17	Liver	—	—	—	—	—	—	Prostrate	Uterus, placenta, testes	Kidney	Skin	Adrenal gland
2B28	Liver	—	—	—	—	—	—	—	Mammary gland	—	—	

TABLE 7.2 List of Substrates Metabolized by UGT2 Family

UGT2B4	UGT2B7	UGT2B15	UGT2B17	UGT2B28
1-Naphthol	Almokalant	6-Hydroxyquinoline	Androsterone	Eugenol
4-Methylumbelliferone	Androsterone	8-Hydroxyquinoline	Dihydrotestosterone	Etiocolanolone
	Buprenorphine	Androstane-3 α -diol		Androstenediol
5- β -Pregnanane-3 α ,20 β -one	Chloramphenicol	Diethylstilbesterol	Testosterone	4-Methylumbelliferone
	<i>cis</i> -4-Hydroxytamoxifen	Dihydrotestosterone		Testosterone
Cathecol estrogens	Clofibrate	Acetaminophen		Lithocholic acid
Eugenol	Codeine	Ezetimibe		
Hyodeoxycholic acid	Diflusal	Quercetin		
	Diclofenac	Hydroxyphenylhydantoin		
Propranolol	Endoxifen	Dienestrol		
Naloxone	Estrogens			
Codeine	(4-hydroxyestrone and 4-hydroxyestradiol)			
Morphine	Fenofibric acid			
	Fenoprofen			
	Flurbiprofen			
	Hydromorphone			
	Hyodeoxycholic acid			
	Ibuprofen			
	Indomethacin			
	Ketoprofen			
	Losartan			
	Morphine			
	Nalmefene			
	Naloxone			
	Naltrexone			
	Oxazepam			
	Oxycodone			
	Oxymorphone			
	Propranolol			
	<i>S</i> -naproxen			
	Steroids			
	Sulindac			
	Temazepam			
	Tiaprofenic acid			
	<i>trans</i> -4-Hydroxytamoxifen			
	Valproic acid			
	Zidovudine			
	Zomepirac			
	Acetaminophen			
	Etodolac			
	Mycophenolic acid			
	Quercetin			
	Gemfibrozil			
	Niflumic acid			

TABLE 7.3 List of Substrates Metabolized by UGT1 Family

UGT1A1	UGT1A3	UGT1A4	UGT1A6	UGT1A7	UGT1A8	UGT1A9	UGT1A10
Bilirubin	Amitriptyline	Amitriptyline	Acetaminophen	7-Ethyl-10-	Morphine	Entacapone	Troglitazone
Acetaminophen	Buprenorphine	Chlorpromazine	7-Ethyl-10-	hydroxycam-	Mycophenolic acid	Troglitazone	Thyroxine
Buprenorphine	Ciprofibrate	mazine	hydroxycam-	ptothecin (SN38)	Ciprofibrate	Acetaminophen	<i>trans</i> -4-
Carvedilol	Clonixin	Clozapine	ptothecin (SN38)	(SN38)	Clofibrate	Almokalant	Hydroxyt-
Ethinylestradiol	Clofibrate	Cotinine	Morphine	Mycophenolic acid	Valproic acid	Cotinine	amoxifen <i>cis</i> -4-
Entacapone	Clozapine	Posaconazole	Valproic acid		Furosemide	7-Ethyl-10-	Hydroxyt-
7-Ethyl-10-	Cyproheptadine	Cyproheptadine	5-Hydroxytryptophan		Diffusinal 17-	hydroxycam-	amoxifen
hydroxycam-	Diclofenac	Diphenhydramine	<i>N</i> -Hydroxynapthylamine		Ethinylestradiol	ptothecin (SN38)	Endoxifen
ptothecin (SN38)	Ezetimibe	Doxepin	Naproxen		All	Flavopiridol	Niflumic acid
Etoposide	Fenoprofen	Desmethylclozapine	Lasofloxifene		<i>trans</i> -retinoic acid Naloxone	Flurbiprofen	Raloxifene
Ezetimibe <i>cis</i> -4-Hydroxytamoxifen	Ibuprofen	Imipramine	Serotonin		Naltrexone	Gemfibrozil	Mycophenolic acid
Morphine	Ketoprofen <i>R</i> - and <i>S</i> -Ketotifen	Lamotrigine			Niflumic acid	<i>cis</i> -Hydroxytamoxifen	Lasofloxifene
Mycophenolic acid	Morphine	Loxapine			Propofol	<i>trans</i> -	
Nalorphine	Naproxen Norbuprenorphine	Olanzapine			Raloxifene	Hydroxytamoxifen	
Naltrexone	Pitavastatin	Promethazine			Troglitazone	Ibuprofen	
Retigabine	Valproic acid	Retigabine			Genistein	Ketoprofen	
Thiocoraline	Chlorpromazine	7-Ethyl-10-			Naringenin	Morphine	
Troglitazone	Hydroxymorphone Loxapine	hydroxycamptothecin			Quercetin	Mycophenolic acid	
All	Naloxone 2-	Hydroxytamoxifen Ketotifen			Fisetin	Nicotine	
<i>trans</i> -retinoic acid	Hydroxyestrone	Nicotine			Apigenin	Niflumic acid	
Thyroxine	2-	Trifluoperazine			Chrysin 7-Hydroxyflavone	(<i>R</i>)-Oxazepam	
	Hydroxyestradiol	Tamoxifen			Alizarin	Propofol	
		Mosetinib			Anthraflavic acid	Raloxifene	
						Thiocoraline	

(continued overleaf)

TABLE 7.3 (*Continued*)

UGT1A1	UGT1A3	UGT1A4	UGT1A6	UGT1A7	UGT1A8	UGT1A9	UGT1A10
Gemfibrozil	Doxepin				Quinalizarin	Tolcapone	
Lasofloxifene	Promethazine				Emodin	Valproic acid	
Raltegravir	Valproic acid				Hydroxyestrone	Fenoprofen	
	Thyroxine				Diethylstilbestrol	Furosemide	
	Sulindac				Eugenol	Naproxen	
	Indomethacin				<i>p</i> -Nitrophenol	Mefenamic	
	Sulindac sulfone				1-Naphthol	acid 4-Catechol	
	Gemfibrozil				Carvacrol	estrogen Estriol	
	Lasofloxifene				Scopoletin	Estradiol	
	Losartan				4-Hydroxy-	2-Catechol	
	Candesartan				coumarin	estrogen	
	Zolersartan				Umbelliferone	Lasofloxifene	
	Fulvestrant				Esculetin	Dapsone	
					4-Hydroxyt-	Ethinylestradiol	
					amoxifen	Estrone 4-	
					Phenolphthalein	Hydroxyestrone	
					Thyroxine	Labetalol	
					<i>trans</i> -4-Hydroxyt-	3,3',5'-Triodo-	
					amoxifen	L-thyronine	
					Endoxifen	Propranolol	
					Lasofloxifene		
					Androgens		
					(17-OH)		

UGT1A1) and β -estradiol. It should be noted that UGT1A3 also contributes to glucuronidation of β -estradiol [11]. Similarly, trifluoperazine has been used as a probe substrate for UGT1A4. Other compounds that have been regarded as probe substrates for different UGT isoforms are listed in Table 7.4 [10,11,13]. Some of these compounds have been considered as possible probes for individual UGT isoforms because they are the major isoforms responsible for the glucuronidation of these compounds in tissue fractions *in vitro*. Since the isoform selective substrates for UGT are limited, reaction phenotyping of UGT substrates is most commonly done using recombinant isoforms. Sometimes, a correlation analysis in human liver microsomes (HLMs) has been used to facilitate the identification of UGT isoforms [11].

7.4 TISSUE DISTRIBUTION

In the cell, the UGT enzymes are located in the lipid bilayer of the endoplasmic reticulum along with the CYP isoforms. This unique location of UGTs next to CYPs helps in converting the CYP-mediated oxidative products to more water soluble compounds, in addition to parent drugs, and helps in modulating concentrations of the parent drug and its phase I metabolites in circulation and in various tissues. To understand the clinical implications mediated by the UGT enzymes, it is important to know the tissue distribution and the content of these enzymes in specific tissues, in addition to the drug/metabolite concentrations. The reported information on tissue distribution of the most clinically relevant isoforms of UGT partially parallels that described for CYP in that the activities are highest in organs/tissues that are responsible in excreting the chemicals out of the body such as the liver and kidney. Table 7.1 also summarizes the tissue distribution of all known isoforms of UGT. The UGT isoforms primarily expressed in the liver include UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28 [14], while mRNAs of UGTs 1A3, 1A6, 1A8, 1A9, 1A10, 2B7, and 2B17 have been detected in the kidney tissues [3,5,15,16]. Studies in the past two decades have shown that UGTs are also present in the gastrointestinal tract including gastric tissue, small intestine, and colon where they can also affect the absorption of drugs [17]. The following UGT mRNAs have been detected in the small intestine:

TABLE 7.4 Potentially Useful Probe Substrates of Human UGTs [10,11,14]

UGT Isoforms	Substrates
UGT1A1	Bilirubin; etoposide, β -estradiol
UGT1A3	<i>R</i> -Lorazepam
UGT1A4	Trifluoperazine; lamotrigine
UGT1A6	Serotonin
UGT1A8	Raloxifene
UGT1A9	Profolol; mycophenolic acid
UGT2A1	(-)-Citronellol
UGT2B4	Hydrodeoxycholic acid
UGT2B7	Zidovudine, morphine
UGT2B15	<i>S</i> -Oxazepam; <i>S</i> -lorazepam
UGT2B17	Dihydrotestosterone

UGT1A1, 1A3, 1A4, 1A6, 1A8, 1A10, 2B4, 2B7, 2B10, and 2B15, and the colon contains 1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, and 2B7. In addition, UGT1A and 2B isoforms are also differentially expressed in various tissues including the olfactory epithelium, ovary, lung, mammary gland, adipose tissue, testis, and prostate. For example, UGT1A8 and 1A10 are exclusively expressed in the GI tract but not detected in the liver. Likewise, UGT1A5, 1A7, 1A10, and 2B28 are expressed in high levels in the GI tract relative to the liver [18,19]. Similarly, current literature evidence indicates that UGT2A1 is mainly expressed in the nasal epithelium [20]. Likewise, the UGTs from the UGT2B family are abundantly expressed in steroid-sensitive tissues such as the prostate (UGT2B10, 2B15 and 2B17) or mammary tissue (UGT2B11 and 2B28) [12]. Other tissues where glucuronidation can occur include the brain and placenta [12]. The factors that govern this tissue specificity of UGT expression remain largely unknown.

7.5 ROLE OF UGTs IN ABSORPTION

Glucuronidation is one of the important enzymatic processes that can contribute to presystemic or “first-pass” drug metabolism. For many years, hepatic metabolism has been recognized as the primary organ that impacts oral bioavailability of drugs. However, the intestine is now recognized as an important contributor in impacting oral bioavailability/absorption of drugs via glucuronidation, thereby altering their systemic exposure [17]. Drugs that fall in this category include gemfibrozil, diclofenac, MPA, raloxifene, ezetimibe, zidovudine, resveratrol, morphine, and lamotrigine (Fig. 7.2). Following oral administration, these drugs are rapidly absorbed. However, the plasma concentrations of the parent are typically found to be lower compared to their corresponding phase II metabolites such as glucuronide or sulfate conjugates. Although the liver and intestine are thought to be responsible for this extensive first pass, mechanistic *in vivo* and *in vitro* studies have indicated that contribution of the intestine in the glucuronidation for most of these drugs is greater than that by the liver. For instance, in humans, the absolute oral bioavailability of raloxifene, a selective estrogen receptor modulator indicated for the treatment of osteoporosis and breast cancer prevention, is only 2% at a dose of 60 mg once daily [21]. This drug is extensively metabolized via glucuronidation with <1% of the parent being excreted in human urine. Two conjugates have been detected in plasma following oral administration of raloxifene (Fig. 7.3). Recent *in vitro* studies suggest that this drug is primarily metabolized in the small intestine by UGT1A8 and 1A10, indicating that presystemic intestinal glucuronidation has a significant impact on the oral bioavailability of raloxifene [22]. Furthermore, kinetic evaluation of intestinal glucuronidation on oral bioavailability (F) was assessed using reported human data of raloxifene by determining the fraction absorbed (F_f), presystemic intestinal availability (F_{pg}), and presystemic hepatic availability (F_h) [23]. This analysis revealed that F_{pg} was 5.4%, whereas F_f and F_h were 63% and 59.3%, respectively. Lower F_{pg} value compared to F_h suggested that intestinal glucuronidation exerts a greater impact on oral bioavailability than hepatic glucuronidation.

Similarly, studies with ezetimibe, a new class of cholesterol-lowering agent, have also shown that its poor exposure in systemic circulation is primarily owing to extensive intestinal first-pass metabolism of the compound [24]. The main circulating metabolite of this drug in human plasma is the phenolic glucuronide conjugate of

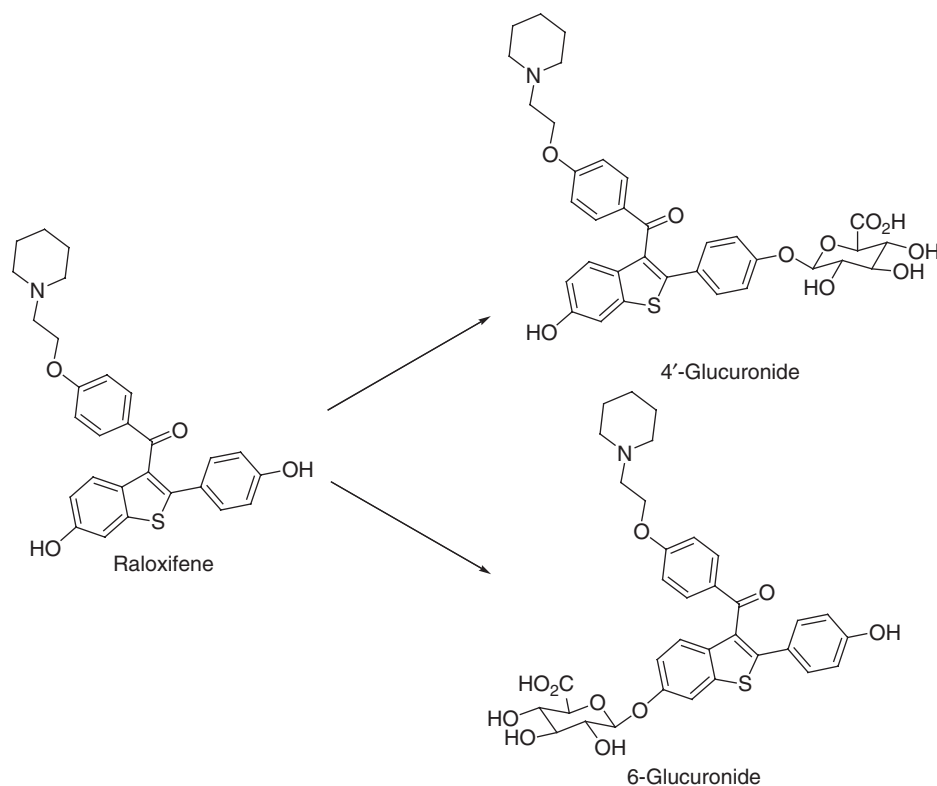


Figure 7.3 Glucuronidation of raloxifene to 4'-glucuronide and 6-glucuronide.

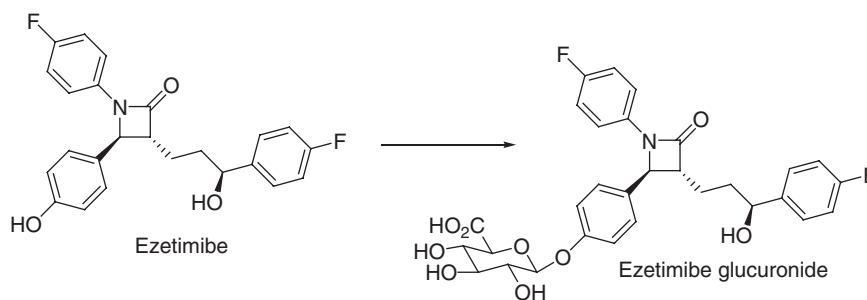


Figure 7.4 Glucuronidation of ezetimibe.

ezetimibe (Fig. 7.4) [25]. The primary UGTs involved in the metabolism of ezetimibe were UGT1A1, 1A3, and 2B15. However, intrinsic clearance values ($V_{\max}/K_m$ ratio) for glucuronide formation were similar between liver and the intestine, indicating that the contribution of intestine in clearing the compound was significant. This was confirmed when 1-min intraduodenal delivery of ezetimibe resulted in glucuronide as the major metabolite in portal plasma of rats [26].

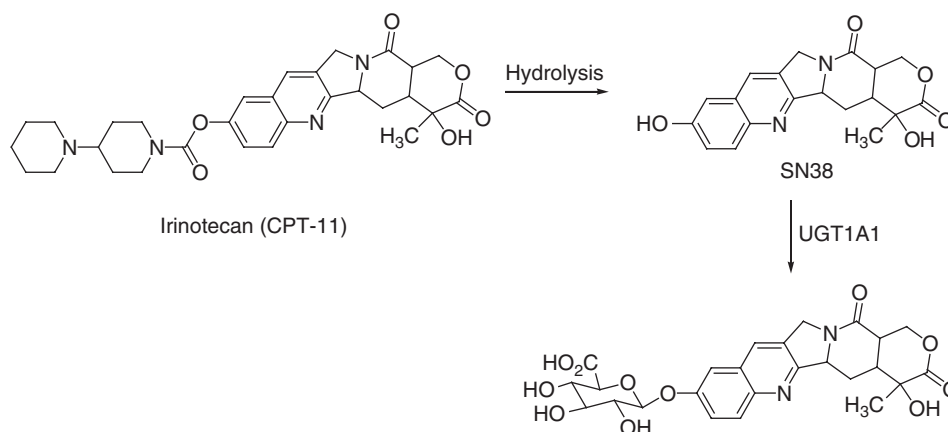


Figure 7.5 Metabolic scheme of irinotecan.

Intestinal UGTs may affect the biological and adverse effects of drugs on the enterocytes by facilitating the inactivation of drugs and by lowering their intracellular concentrations. For instance, the effect of MPA on intestinal cell proliferation is potentially reduced as a result of its glucuronidation, thereby reducing its concentration in the target cells [27,28]. Similar phenomenon has also been observed on the antiproliferative effect for the antineoplastic agent irinotecan (Fig. 7.5) [29,30]. Following an intravenous (IV) dose of irinotecan, this drug undergoes hydrolysis by carboxyesterases to form the active metabolite SN38, a potent topoisomerase inhibitor that causes disruption of DNA replication. SN38 is then eliminated primarily by UGT1A1-mediated glucuronidation (Fig. 7.5). *In vitro*, an inverse correlation has been observed between SN38 concentration and its antiproliferative effect in colorectal cancer cells [29,30]. It is possible that glucuronidation of SN38 may reduce the intracellular concentration of this compound, thus attenuating its antiproliferative effect.

7.6 BIOACTIVATION BY UGTS

7.6.1 Toxicological Implications

While glucuronidation is a process that facilitates inactivation, detoxification, and elimination of compounds from the body, there are instances where this pathway could potentially produce metabolites that elicit adverse drug reactions by damaging intracellular proteins. On the other hand, glucuronide conjugates may retain or have enhanced or different pharmacological activity compared to the parent drug such as the metabolites formed by CYPs [31,32].

From a toxicological point of view, glucuronide conjugates can elicit their toxicity in two ways. The first involves a covalent interaction of a glucuronide conjugate with macromolecules such as the proteins or nucleic acids owing to the inherent chemical reactivity of some glucuronides. Such an interaction can result in a macromolecular adduct that could interfere with the normal functioning of the cell systems or, alternatively, induce an immune response against the drug protein adduct. Another

“indirect” way by which the glucuronides can educe toxicity is via the interaction of the conjugates with transporters. Most glucuronide conjugates are substrates for MRP2 efflux transporters due to the presence of the anionic glucuronic acid moiety. Thus, glucuronidation can act as a pathway that helps to shuttle proximate or ultimate carcinogens and/or toxins to the biliary-intestinal tract, kidney, and bladder and facilitate organ specific toxicity [33].

Although most glucuronide conjugates are chemically stable, the conjugation of a glucuronic acid moiety with hydroxamate or carboxylate groups is of toxicological importance owing to their inherent chemical reactivity. Of greater clinical significance among the two are the acyl glucuronides, which are formed via esterification of compounds containing carboxylic acid groups and glucuronic acid (Fig. 7.1). This is because several carboxylic acid containing compounds are primarily metabolized by UGTs. Some substrates with a carboxylic acid group include endogenous compounds such as bilirubin, leukotrienes bile acids, fatty acids, and retinoic acid and drugs such as nonsteroidal anti-inflammatory (NSAIDs), hypolipidemic (“fibrates”), hypoglycemic (“glitazars”) agents, immunosuppressants (MPA), diuretics (furosemide), and anticonvulsants (valproic acid) (Fig. 7.2). In some cases, even if a compound is not a carboxylic acid, it may be metabolized to a carboxylic acid through phase I metabolism, which may then undergo glucuronidation, as observed for celecoxib (Fig. 7.6).

The chemical reactivity and, therefore, the covalent bond formation between these conjugates and the proteins are attributed to the ester functionality in acyl glucuronides. These molecules can react with macromolecules by two possible ways. The first one involves a simple displacement of the glucuronosyl group by nucleophilic moieties in the macromolecules that results in acylation of the protein with the aglycone (Fig. 7.7a). This is known as the *transacylation reaction* [31,34]. Proteins with nucleophilic groups on the amino acids such as the ϵ -amino of the lysine, the phenolic hydroxyl group of tyrosine, and the thiol of cysteine are more prone to these kinds of reaction and result in the acylation of the proteins. An alternate mechanism involves an intramolecular rearrangement as depicted in Fig. 7.7b. This so-called glycation reaction differs from the transacylation reaction in that both the aglycone and the sugar group are present in the final adduct. This mechanism involves an acyl migration in which the acyl group rearranges from its initial C-1 position on the glucuronic acid to position C-2 and subsequently to C-3 or C-4 (Fig. 7.7b). The mechanism is proposed to involve the nucleophilic attack by hydroxyl on the adjacent carbon resulting in the formation of β -glucuronidase resistant glucuronides. This intramolecular rearrangement exposes the hemiacetal group of the sugar moiety and leads to the transient ring opening to form

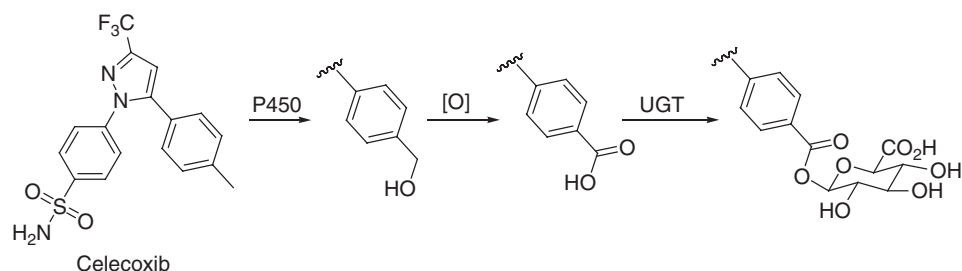


Figure 7.6 Metabolism of celecoxib to acyl glucuronide via oxidation by P450.

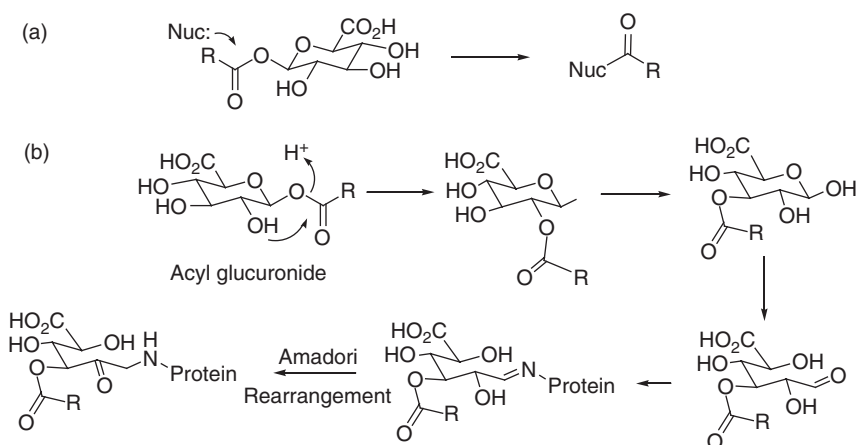
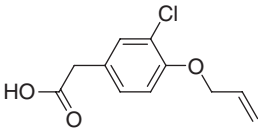
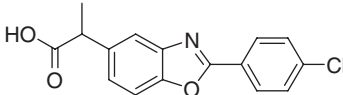
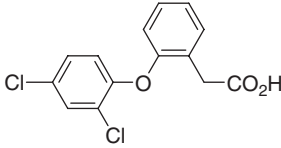
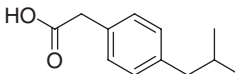
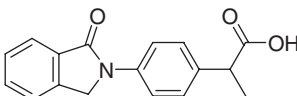
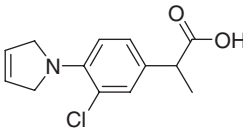
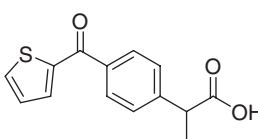
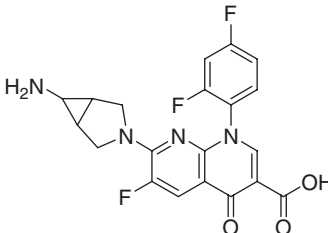


Figure 7.7 (a) Transacylation of proteins by acyl glucuronides. (b) Migration of acyl glucuronides and covalent binding to proteins (glycation).

the open chain aldehyde intermediate. The corresponding aldehyde group can then condense with amino groups on the lysines or nucleic acids to form imines that undergo a rearrangement (amadori rearrangement) to yield the final adduct, a more stable 1-amino-1-deoxyketose structure, which retains both the sugar and the aglycone. Both these mechanisms have been discussed in several reviews over the years [31,32,35–43].

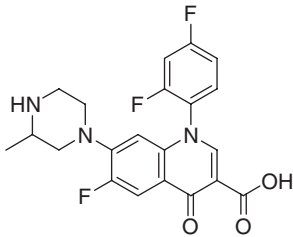
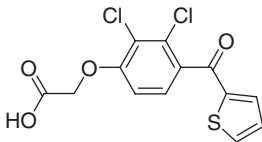
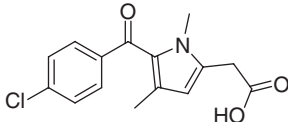
Although no definitive relationship has been established, it is now accepted that covalent binding to amino acid residues of macromolecules and bases of nucleic acids often can result in hypersensitivity reactions, organ toxicities (hepatic or renal toxicities), mutagenesis, or carcinogenesis. An unacceptably high incidence of significant adverse drug reactions has been reported for drugs containing carboxylic acid. Several drugs with this group have been withdrawn after introduction to the market or have otherwise been associated with liver toxicity (Table 7.5). One of the earliest documented examples of a carboxylic acid compound involved in severe toxic reactions is that of the NSAID ibufenac. Ibufenac is a structural analogue of ibuprofen and was marketed in the late 1960s. Within a few years, ibufenac was withdrawn from the market. The compound is primarily metabolized to an acyl glucuronide and is shown to covalently bind to proteins at least, *in vitro* [44]. Similarly, Smith *et al.* [45] have shown that zomepirac acyl glucuronide reacts with human serum albumin (HSA) *in vitro*. An *in vivo* study in human volunteers showed a linear correlation between the area under the plasma concentration-time curve (AUC) of zomepirac acyl glucuronide (but not zomepirac itself) in plasma and the amount of zomepirac covalently bound to plasma proteins [45]. This suggests that zomepirac acyl glucuronide is responsible for forming covalent protein adducts. The mechanism for binding of zomepirac to HSA is mediated by both transacylation and Schiff-base formation with lysine residues in the protein, which undergoes an amadori rearrangement leading to a stable adduct described above [46,47]. Similar *in vivo* results have been reported for other structurally related NSAIDs such as tolmetin, diclofenac, benoxaprofen, and ticrynafen [32,37,43,48]. The formation of acyl glucuronide is a primary metabolic pathway for all the above-mentioned compounds. Furthermore, all these acyl glucuronides have been shown to undergo acyl migration and amadori rearrangement described above to some degree. In addition to

TABLE 7.5 Carboxylic-Acid-Containing Drugs Withdrawn from the Market Because of Toxic Effects

Drug	Structure	Toxicity
Alcofenac		Skin rash
Benoxaprofen		Hepatotoxicity
Fenclofenac		Skin rash
Ibufenac		Drug-induced meningitis
Indoprofen		Aplastic anemia
Pirprofen		Hepatotoxicity
Suprofen		Renal toxicity
Trovaflaxacin		Hepatotoxicity

(continued overleaf)

TABLE 7.5 (Continued)

Drug	Structure	Toxicity
Temafloracin		Severe anaphylaxis
Ticrynafen		Hepatotoxicity
Zomepirac		Pulmonary toxicity

HSA, the acyl glucuronides have also been shown to react with other proteins. For instance, diclofenac, which is still in use in most of the world today, has been associated with hepatotoxicity as well as immune mediated toxicity. Work by Kretz-Rommel and Boelsterli as well as others has shown that the *in vitro* covalent binding of diclofenac to hepatocellular proteins including dipeptidyl peptidase IV (DPP IV) was dependent on diclofenac acyl glucuronide formation [49,50]. Similar covalent binding to DPP IV and tubulin has been shown for zomepirac as well [51,52]. In another instance, DNA is a target for the acyl glucuronide conjugate of gemfibrozil. Incubations of gemfibrozil acyl glucuronide conjugate with DNA resulted in the formation of covalent adducts and mutagenesis [53].

As mentioned earlier, transporter-mediated excretion of glucuronide conjugates is another means by which these metabolites can mediate organ or intestinal toxicity. In this process, efficient canalicular transporter(s) mediate extrusion of toxic or reactive glucuronide conjugates from liver into the biliary tract and the gastrointestinal lumen [32,38]. This results in high concentration of these toxic conjugates at the distal sites and subsequently may elicit toxicity. For instance, several NSAIDs are known to cause acyl-glucuronide-mediated damage to the small intestine. Similarly, MRP-2-mediated efflux transporter of glucuronide conjugates of carcinogens from the liver (where they are formed) can result in high exposure of the toxins and carcinogens via their local regeneration by β -glucuronidase catalyzed hydrolysis of the β -glucuronides. Thus, the combined effects of UGTs and MRP2 may have toxicological consequences by intracellular detoxification of the compounds, efflux of these compounds via the MRP2-dependent translocation of the conjugate to the distal site, and regeneration of

the aglycone [54,55]. Coupling of glucuronidation with the transporter-mediated effect of the conjugate has been implicated in the intestinal toxicity of irinotecan [32,56,57]. Major dose-limiting toxicities of irinotecan (Fig. 7.5) are severe diarrhea and myelosuppression. The major route of excretion of the aglycone and its glucuronide conjugate (SN38 glucuronide) is the bile and feces following an IV administration. Although the concentrations of SN38 glucuronide in the bile are equal or greater than the aglycone, a significant amount of SN38 is found in the feces compared to its glucuronide conjugate. This is consistent with the β -glucuronidase-mediated hydrolysis of the metabolite in the gut. Several studies have shown that SN38 glucuronide is a substrate of MRP2 and that its excretion into the bile is mediated via efflux of the glucuronide by this transporter. Taken together, although glucuronidation acts as a detoxification pathway of SN38 *in vivo*, its toxicity (delayed diarrhea) is mediated via its transport of the glucuronide (via the bile) by MRP2 to the GI tract coupled with regeneration of the aglycone at this site by β -glucuronidase.

Another way that the glucuronide conjugates can elicit toxicity is by indirectly increasing the concentrations of the parent drug in systemic circulation. This has been commonly observed in NSAIDs where the acyl glucuronides are the major metabolites (as described previously). Since the ester linkage in these glucuronide conjugates is prone to a nonenzymatic hydrolytic cleavage back to the aglycone under mild alkaline conditions, a situation where there is an impairment of the elimination pathway of acyl glucuronides could result in “futile cycling” of the parent drug [37]. This “reversible metabolism of acyl glucuronide” can have potential clinical implications especially in patients with decreased renal clearance. When an acyl glucuronide is a primary metabolite of a drug, renal impairment will affect the elimination of the glucuronide conjugate via the urine. The decreased renal elimination of the glucuronide conjugate can result in its hydrolysis back to the parent drug and, therefore, decrease the apparent metabolic clearance of the aglycone and increase its systemic exposure. For instance, benoxaprofen was withdrawn from the market because of reports of renal impairment and cholestatic liver toxicity and a high fatality rate. The drug has a relatively long half-life in the human body (33 h), with the majority of the dose eliminated by renal excretion as the acyl glucuronide. Although the exact reason for the observed toxicity still remains uncertain, it has been speculated that impaired renal excretion could lead to increased systemic and tissue drug load, causing cholestasis and renal injury, which would further decrease excretion of the drug and eventually cause toxicity. This has been observed for other drugs including clofibrilic acid, diflunisal, indoprofen, and ketoprofen.

Although uncommon, in recent years, reports of inhibition of CYPs by acyl glucuronides have also appeared in the literature. A classic case where a glucuronide conjugate has led to inhibition of a CYP enzyme and, therefore, has been implicated in drug–drug interactions (DDIs) is that of gemfibrozil [58,59]. Although gemfibrozil has been shown to be a weak inhibitor of CYP2C8, gemfibrozil-1-*O*- β -glucuronide (GEM-1-*O*-gluc) metabolite was found to be a more potent inhibitor of CYP2C8 activity. Furthermore, Ogilvie and coworkers found that preincubation of the inhibitor lowered the IC_{50} for inhibition of CYP2C8-mediated paclitaxel metabolism from 24 to 1.8 μ M *in vitro*. Additional studies conducted to understand the mechanism of inactivation of the CYP enzyme by glucuronide conjugate suggested that it was covalently linked to the prosthetic heme group during catalysis. The mechanism involved oxidation of the GEM-1-*O*-gluc to a benzylic radical intermediate that partitioned between oxygen

rebound and addition to the γ -meso carbon of heme [59]. Formation of the GEM-1-*O*-gluc-heme adduct most assuredly would block additional substrates from accessing the catalytic heme center of CYP2C8 and result in the observed mechanism-based inhibition.

7.6.2 Pharmacological Implications

Although many pharmacologically active metabolites are known to be formed through oxidative or reductive processes (phase I reactions), glucuronide conjugates can also interact with receptors and impart biological effects through noncovalent interactions with the receptor. Active glucuronide conjugates may have equivalent, and in some cases, superior pharmacological activity and can, therefore, contribute to the overall pharmacological effect of the parent drug. Since the overall efficacy of a drug can be augmented by the formation of active metabolite(s), characterization of the isozymes that catalyze the formation of these conjugates is extremely important.

Morphine is a classic example where a glucuronide conjugate contributes to the pharmacological activity of the parent [60]. Morphine is primarily eliminated by glucuronidation, a process that results in phenolic 3-OH glucuronide and the alcoholic 6-OH glucuronide (Fig. 7.8). Only the 6-OH glucuronide is active and displays full agonist properties at the μ -opioid receptors. Humans convert 10%–20% of an administered morphine dose to the 6-OH glucuronide and this reaction is catalyzed by UGT2B7. Since the conjugate is more potent than the parent and accumulates in plasma at concentrations greater than that of morphine during chronic treatments, it is considered to contribute significantly to the clinical efficacy during chronic morphine therapy.

Besides morphine, several other drugs also have pharmacologically active glucuronide conjugates. For instance, the phenolic glucuronide conjugate of ezetimibe has been shown to be equally potent toward the intended pharmacological target

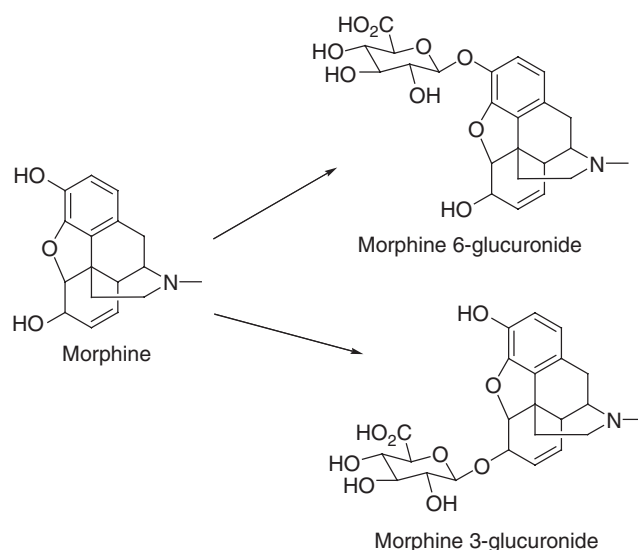


Figure 7.8 Metabolic schemes of morphine and codeine.

(Fig. 7.4) [26,61,62]. Similarly, the hypocholesterolemic effect of S-8921, a novel inhibitor of an ileal apical sodium-dependent bile acid transporter (ASBT/SLC10A2), is attributed to its glucuronide conjugate (S-8921G) that is formed in the intestine (Fig. 7.9) [62,63]. Conversion of S-8921 to its glucuronide conjugate is mediated by several UGTs, particularly UGT1A1, 1A3, 1A8, 1A9, and 1A10. This glucuronide conjugate is a 6000-fold more potent inhibitor of an ileal apical sodium-dependent bile acid transporter (SLC10A2) than the parent compound. Similarly, the glucuronide conjugates of retinoic acid and retinol have demonstrated enhanced retinoid activity relative to the parent compound [31].

7.6.3 Prevention of Toxicity by Glucuronidation

Even though some glucuronide conjugates have been implicated to be associated with eliciting the toxicity of a drug, studies have shown that glucuronidation mostly play an important role in detoxication of carcinogens and hepatotoxicants [64]. Differential expression of UGTs in specific tissues can change the metabolic ratio between bioactivation and detoxification. A change favoring bioactivation can render increased susceptibility to toxic effects and vice versa. Thus, the knowledge of glucuronidation capacity of the UGTs and the type of UGTs involved in the catalysis can provide an increased understanding of the bioactivation/detoxification balance for these toxicants. For instance, many phenolic compounds are prone to oxidative bioactivation via CYP or prostaglandin H synthase to highly toxic electrophilic and/or free radical reactive intermediates that can damage cellular macromolecules (e.g., DNA, protein, and lipids) and/or enhance oxidative stress. This is also true for many compounds where aromatic hydroxylation is the primary pathway of metabolism. However, most often, high efficiency of the glucuronidation process in eliminating these compounds deters them from undergoing metabolic oxidation. Thus, glucuronidation and elimination of the xenobiotic and/or its stable metabolite serves as a toxicological gatekeeper, directing metabolism away from bioactivation. Generally, genetic predisposition of subjects, which can result in low expression of UGTs that are primarily involved in the metabolism of these compounds, drives the metabolism of these compounds toward bioactivation and generation of reactive metabolites [65]. A classic case illustrating this is that of acetaminophen, which is hepatotoxic and nephrotoxic at high doses.

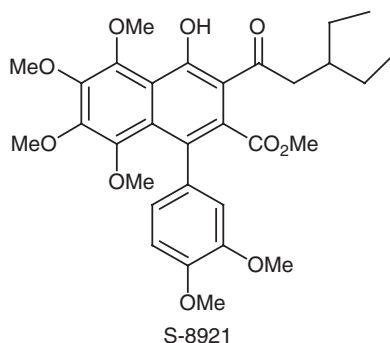


Figure 7.9 Structure of S-8921.

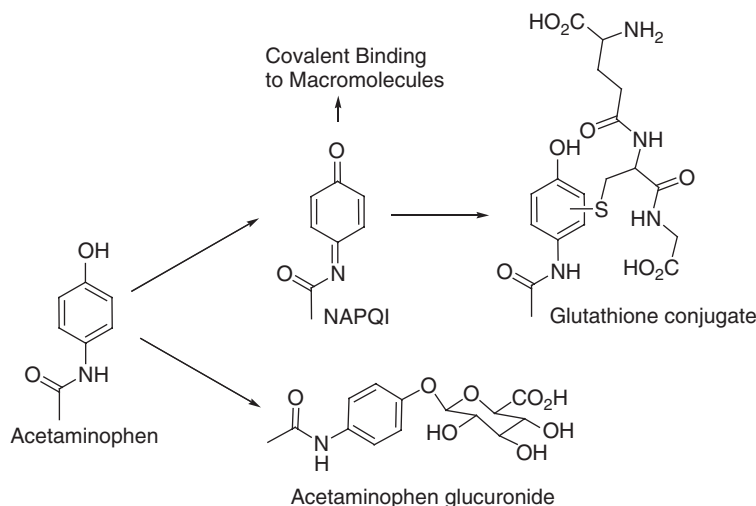


Figure 7.10 Metabolic activation and glucuronidation of acetaminophen.

This widely used analgesic undergoes metabolism primarily via UGT1A1-mediated glucuronidation of the phenolic group and CYP-mediated oxidation to a quinone imine (NAPQI) (Fig. 7.10). The latter has been shown to covalently bind to proteins and this has been implicated in the hepatotoxicity and renal toxicity observed in patients. Studies using intravenous drug administration in humans with a hereditary UGT1A1 deficiency showed reduced glucuronidation of acetaminophen and, conversely, enhanced bioactivation as determined by the formation of glutathione conjugates of acetaminophen, which was a marker for the formation of NAPQI intermediate [66,67]. Similar results were observed in mutant Gunn and RHA rats, which lack all UGT1A enzymes. These animals had reduced acetaminophen glucuronidation, enhanced CYP-catalyzed bioactivation of acetaminophen and covalent binding to hepatocellular proteins, and enhanced hepatocellular centrilobular and renal cellular necrosis.

A similar conclusion can be made in the case of raloxifene that is readily bioactivated to electrophilic intermediates and is a potent inactivator of CYP3A4. Despite these *in vitro* findings, in humans, no major raloxifene-related toxicities have been linked to bioactivation of this drug. It is hypothesized that this disconnect between safety of raloxifene and its *in vitro* bioactivation is attributed to its presystemic metabolism via glucuronidation. The effect of glucuronidation on the covalent binding of raloxifene to liver microsomal proteins has been demonstrated [68]. These *in vitro* studies with [^{14}C]raloxifene suggest that the covalent binding to microsomal protein is significantly decreased in the presence of glucuronidating processes. These results support the notion that intestinal glucuronidation of raloxifene limits its bioactivation by liver in humans.

7.7 PREDICTION OF HUMAN CLEARANCE FOR COMPOUNDS CLEARED VIA GLUCURONIDATION

Since a number of drugs and early candidates are primarily cleared via glucuronidation, the ability to predict human metabolic clearance for compounds that are primarily

cleared by this route can be an asset in early discovery and development of drugs. This not only assists in the selection of a candidate with good PK properties but also gives an early assessment of the dosing regimens for clinical studies. The ready availability of HLMs, isolated hepatocytes, and recombinant enzymes has made it possible to conduct kinetic studies that enable determination of microsomal intrinsic clearance (CL_{int}), which can be further extrapolated to determine the CL_{int} in the whole liver and eventually human clearance (CL_h) and extraction ratio [69–72]. Although this approach has proved useful for many CYP-catalyzed reactions, drug clearance predictions using microsomal data have tended to under-predict *in vivo* clearance for compounds that are primarily eliminated via glucuronidation [11,73–79]. Methods for studying glucuronidation in microsomes have varied considerably, which has led to questions about the suitability of this *in vitro* system to accurately predict the glucuronidation clearance [79].

Although reasons for this underestimation are unclear, many experimental variables have been shown to influence the scaling of *in vitro* glucuronidation clearance to an *in vivo* setting. First and foremost among these factors is the influence of localization of the UGT in comparison to CYPs. The UGT is localized on the luminal side of the microsomal membrane and this may give rise to “diffusional” barriers. Several methods have been introduced to circumvent this problem, including the use of the pore-forming agent alamethicin or a detergent or sonication treatment [80,81]. The use of alamethicin has proved very useful and recently become a standard practice to assess glucuronidation reactions and kinetics. More recently, the general implications on the suitability of alamethicin-activated microsomes for predicting the clearance for compounds with parallel CYP and UGT pathways have been described [75]. The results of this study suggested that alamethicin did not affect CYP activity since the sum of the individual CL_{int} values from the individual cofactor studies were approximately equal to the combined cofactor CL_{int} for all of the drugs investigated. In addition, experimental conditions can also be another reason for the failure of the microsomal procedures to predict glucuronidation clearance. Incubation components are known to modulate microsomal UGT activity, and therefore the kinetics of drug glucuronide formation can vary with experimental conditions such as buffer type, pH, and ionic strength, the presence of $MgCl_2$, and detergent used [11].

An additional factor that may contribute to the observed underprediction is the influence of nonhepatic UGTs to the metabolism of the evaluated drug, which typically is not included in the current methods of *in vitro* to *in vivo* scaling. As discussed previously, some UGTs, notably UGT1A7, 1A8, and 1A10 are expressed in the gastrointestinal tract [17]. Similarly several UGTs are highly expressed in the kidney [82]. Several well-known drugs are metabolized by the intestinal or renal UGTs (e.g., pro-folol, raloxifene, or troglitazone) [22,83,84]. Hence, the *in vivo* CL_{int} values scaled from incubations with HLMs may be underestimates or erroneous for compounds that are primarily metabolized by nonhepatic UGT enzymes. Comparison of CL_{int} values derived using liver, kidney, and intestinal microsomes may provide important insights into the relative contribution of these organs to the overall CL and/or first-pass extraction [85]. For instance, scaled intestinal CL_{int} , UGT (per g tissue) was six- and ninefold higher than hepatic for raloxifene and troglitazone, respectively [71,86]. Hence, it may be necessary to include this factor in the equation to obtain a more accurate prediction. Nonspecific microsomal binding can present another confounding factor in the

calculation of kinetic constants for human liver microsomal drug metabolism reactions, leading to overestimation of K_m and, hence, underprediction of CL_{int} . Finally, glucuronidation reactions are usually more complicated than CYP-mediated oxidation reactions. Although the Michaelis–Menten plots are generally used to fit the kinetic data, the glucuronidation reactions have a tendency to exhibit nonlinear kinetic behavior [11,78]. Hence, unless a proper non-Michaelis–Menten model is applied, the CL prediction obtained from these data will not be predictive of the *in vivo* clearance observed in humans. Thus, the interpretation and analysis of drug glucuronidation kinetic data can further impact the reliability and predictive value of *in vitro* CL_{int} .

Miners *et al.* [76] have shown that underprediction persists even when the above confounders (nonspecific binding, scaling factors, inappropriate kinetic modeling, and failure to account for extrahepatic metabolism) are added into the equation and experimental conditions are optimized. Most often, such discrepancies have been ascribed to the high K_m values that are observed following kinetic experiments, which employ HLM or recombinant enzymes. For example, K_m values from HLM for the UGT2B7 substrates, morphine and zidovudine, range from ~ 1100 to $4300 \mu M$ following incubation with HLM or recombinant UGTs [87]. The high K_m values, in turn, result in an underestimation of *in vitro* and extrapolated *in vivo* CL_{int} . This overestimation of the K_m values has been attributed to the presence of the long chain unsaturated fatty acids, notably arachidonic, linoleic, and oleic acids, released from membranes during the course of an incubation. These fatty acids act as potent competitive inhibitors of some of the UGT enzymes, rendering the estimation of K_m to be inaccurate. The addition of bovine serum albumin (BSA) or fatty-acid-free human serum albumin (HSA-FAF), which sequester the inhibitory fatty acids, to incubations of recombinant UGT or HLM results in a 5- to 10-fold reduction in K_m values with HLM and recombinant UGT as the respective enzyme sources, thereby increasing the CL_{int} of these compounds [88,89]. This effect appears to be more pronounced with UGT1A9 and UGT2B7. For example, extrapolation of the microsomal CL_{int} for the glucuronidation of the UGT2B7 substrate, zidovudine, generated in the presence of BSA, HSA-FAF, or human intestinal fatty acid binding protein (IFABP), resulted in a threefold underprediction of CL_H [89,90], compared to an up to 23-fold underprediction for data generated without fatty-acid sequestration [87,90]. Similar results have been reported for several other drugs glucuronidated predominantly by UGT2B7, using the substrate-depletion approach [75].

In contrast to UGT1A9 and UGT2B7, fatty acids do not appear to affect the *in vitro* activities of UGT1A1, UGT1A4, and UGT1A6 [88,91]. This is illustrated by the clearance predictions of profolol. The K_m (and therefore CL_{int}) values are essentially unaltered by the presence of albumin. Thus, the CL prediction with BSA supplemented HLMs and recombinant UGT 1A9 enzyme were within range compared to the clearance values obtained from incubations that were conducted in the absence of BSA.

7.8 FACTORS AFFECTING UGT ACTIVITY AND ITS CLINICAL SIGNIFICANCE

Numerous factors such as age, diet, disease states, ethnicity, genetic polymorphisms, DDIs, and hormonal effects are known to alter human UGT activity *in vivo* [14,78,92–94]. Knowledge of these factors that are responsible for this variation is of

considerable importance, since they can affect both drug efficacy and toxicity. Only the impact of genetic polymorphisms and DDI on UGT activity *in vivo* is discussed here.

7.8.1 Genetic Polymorphism

Genetic variations and single nucleotide polymorphisms within the UGT family of enzymes is relatively common [95]. In the UGT1 family, genetic polymorphisms have been detected for UGT1A1, 1A6, 1A7, 1A8, 1A9, and 1A10, while in the UGT2 family, genetic polymorphisms of UGT2B4, 2B7, and 2B15 have been reported [96,97]. Genetic polymorphism of UGT1A1 has been extensively studied because of its role in well-known hereditary jaundices. Mutation in UGT1A1 leads to Gilbert syndrome, a mild hereditary unconjugated hyperbilirubinemia. In some cases, certain mutations in UGT1A1 can result in a severe condition called *Crigler–Najjar syndrome*, which may lead to brain damage in infants. Besides its association with hereditary disease, mutations in genes encoding UGT enzymes may also have clinical relevance to a drug's pharmacokinetics and clinical outcomes, as illustrated by the examples of irinotecan and MPA. As mentioned earlier, the active metabolite (SN38) of irinotecan is inactivated primarily by UGT1A1. One of the polymorphisms of UGT1A1, the UGT1A1*28 allele, which has 7 TA repeats in a TATA box of the promoter of *UGT1A1* gene, shows reduced expression of the enzyme with resultant decreased glucuronidation activity compared to its wild-type allele (UGT1A1*1), which has 6 TA repeats. This genetic change is associated with a condition called *Gilbert's syndrome*. Patients homozygous for UGT1A1*28 have reduced glucuronidation of SN38 and an elevated risk of adverse effects from this metabolite, including diarrhea and bone marrow suppression compared with patients with one or two wild-type alleles [98,99]. Besides UGT1A1*28, emerging data suggest that polymorphisms in UGT1A7*3/*3 genotype has also been shown to predict hematologic toxicity in the first cycle of irinotecan treatment, while UGT1A1*60 was a better predictor of SN38 glucuronidation [100]. Thus, genotyping with haplotype rather than a single polymorphism may provide better prediction of those patients who are at the highest risk of toxicity [101]. Ethnic differences may also play an important role in determining the appropriate genotype for assessment. For instance, in East Asians, the frequency of UGT1A1*28 is only one-third of that in the Caucasians or African-Americans. However, another low activity allele, UGT1A1*6, shows the same frequency as UGT1A1*28 in East Asians but is not detected in the Caucasians and African-Americans. A pharmacokinetics/pharmacodynamics study in Japanese cancer patients showed that the haplotypes significantly associated with reduced area under the concentration curve ratio (SN38 glucuronide to SN38) and neutropenia contained UGT1A1*6 or UGT1A1*28 [102]. A retrospective analysis in Japanese cancer patients revealed a significant association of neutropenia with UGT1A1*6 [103], illustrating the importance of taking ethnic differences in pharmacogenetic traits into consideration.

Another example of the potential impact of UGT polymorphisms on drug metabolism and interindividual variation in pharmacokinetics is demonstrated using mycophenolate mofetil (MMF). MPA, an active metabolite of MMF, is a selective inhibitor of inosine monophosphate dehydrogenase. The pharmacokinetics of this drug shows substantial interindividual variability in humans [104,105]. Because MPA has a narrow therapeutic index, variations in its concentrations can result in acute toxicity or transplant rejection. Following administration to humans, MMF, which is a prodrug,

is converted to its active metabolite MPA, which then undergoes further metabolism by glucuronidation of the phenolic hydroxyl group (major metabolite) and, to a lesser extent, formation of an acyl glucuronide. The glucuronide conjugates are subsequently excreted in urine [106,107]. *In vitro* studies indicate that glucuronidation of MPA to its hydroxyphenyl glucuronide conjugate is mediated mainly by UGT1A9 in the liver and by UGT1A7, 1A8, and 1A10 extrahepatically [108,109]. The formation of the acyl glucuronide is mediated by UGT2B7 [109].

Since UGT1A9 plays a major role in the glucuronidation of MPA, several studies have been conducted to evaluate the impact of genetic polymorphisms of this enzyme on MPA pharmacokinetics. Results suggest that in healthy volunteers and in renal transplant patients, carriers of the UGT1A9 promoter single nucleotide polymorphism (SNPs) –2152 C>T and –275 T>A were associated with lower systemic exposure to MPA [110,111] and predicted the risk of developing acute rejection in renal transplant patients [111]. The less frequent UGT1A9*3 SNP was associated with a higher MPA systemic exposure. In addition to these SNPs, Baldelli *et al.* [112] have also shown that –440 C>T and –331 T>C SNPs of the UGT1A9 promoter region were associated with an increased systemic exposure to MPA in renal transplant patients. Besides UGT1A9, the role of genetic polymorphisms in other UGTs on MPA pharmacokinetics is less clear. For example, UGT1A8*2/*2 has been associated with a slight increase in MPA AUC (18%) only in patients receiving concomitant cyclosporine (CsA), but not in patients receiving concomitant tacrolimus [111]. However, Johnson *et al.* [113] have reported that MPA dose-adjusted trough concentration was 60% higher in subjects heterozygous or homozygous for UGT1A8*2 than in those with wild type; this effect is apparent only in patients receiving concomitant tacrolimus. The reason for this discrepancy is unknown.

Recent investigations evaluating the potential contribution of UGT polymorphisms to adverse events of MPA revealed that polymorphism in the UGT2B7 promoter region –840G>A was found not to be associated with the incidence of leucopenia or diarrhea [114]. However, in a pilot study in pediatric kidney transplant recipients, Prausa *et al.* [115] showed that carriers of the polymorphisms in the UGT1A9 -331 and, possibly, of UGT2B7 -900 SNPs appeared to be associated with an increased risk for developing an adverse event such as leucopenia. If confirmed, dosing recommendations for MMF may need to be reevaluated based on the genotype to ensure safe use of this drug while maintaining its therapeutic benefits [115].

7.8.2 Drug–Drug Interactions

Management of many diseases requires the use of multiple drug therapy, which predisposes the patients to an increased risk of experiencing DDIs through alteration of the clearance mechanisms by the interacting drugs. The most common mechanisms involved in UGT-mediated DDIs include inhibition or induction of the UGT enzymes. Other proposed mechanisms may involve alterations of availability of UDPGA and/or inhibition of the excretion of glucuronides, with subsequent hydrolysis of the glucuronides to parent substrate aglycone [116]. Several classes of drugs have shown to inhibit UGTs [12]. Moreover, no specific inhibitors of individual UGT isoforms have been identified that may be safely administered clinically. Although not completely selective, more recently erlotinib, a reversible inhibitor of the epidermal growth factor

receptor, exhibited selective potent competitive inhibition against 4-MU and bilirubin glucuronidation by UGT1A1 [117]. Similarly, atazanavir is shown to be a potent inhibitor of UGT1A1 [118], while fluconazole inhibits UGT2B7 [119]. As the CYPs, several enzymes in UGT family are subject to induction on the basis of *in vitro* experiments conducted with primary cultures of human hepatocytes or cultured human cell lines. Inducible human UGT enzymes include UGT1A1 [120,121], UGT1A3, UGT1A4 [122], UGT1A6 [122–124], UGT1A9 [124], and UGT2B7 [122,124]. Many common inducers of CYP enzymes such as oral contraceptives, rifampin, phenobarbital, phenytoin, and carbamazepine can increase the expression of UGTs [125].

Since clinical studies on DDIs involving inhibition or induction of UGT enzymes have been extensively reviewed [12,116,126], this section focuses on the DDI with drugs that are of narrow therapeutic index with potentially important clinical implications resulting from DDI such as zidovudine, MPA, and lamotrigine.

7.8.3 Inhibition of Glucuronidation

7.8.3.1 Zidovudine. Among the drugs that undergo glucuronidation as a major clearance mechanism, zidovudine has been one of the most extensively evaluated drugs for UGT-mediated DDI [12,127]. In humans, zidovudine is renally eliminated primarily as a glucuronide conjugate of the parent drug [128]. *In vitro*, glucuronidation of zidovudine is predominantly mediated by UGT2B7 [87,129–131]. Several DDIs involving zidovudine and other agents such as atovaquone, fluconazole, and probenecid have been reported [132–134]. For instance, coadministration of atovaquone 750 mg Q12h and zidovudine 200 mg Q8h for 12 days was associated with a 33% increase in zidovudine AUC compared to zidovudine alone [132]. A concomitant decrease in zidovudine glucuronide levels was observed, suggesting that the mechanism of interaction may be related to inhibition of zidovudine glucuronidation. Similarly, patients who received concomitant administration of fluconazole (400 mg daily) and zidovudine (200 mg Q8h) for 7 days exhibited an increase in the AUC and $C_{\max}$ of zidovudine by 74% and 84%, respectively [133]. The apparent oral formation clearance to zidovudine glucuronide decreased by 48%, which suggests that a likely mechanism of this interaction is attributable to inhibition of glucuronidation. Likewise, concurrent administration of probenecid to patients with AIDS resulted in a twofold increase in the mean plasma AUC of zidovudine and its glucuronide conjugate compared to administration of zidovudine alone [134]. Probenecid administration also significantly reduced the renal clearance of zidovudine glucuronide as well as the zidovudine glucuronide to zidovudine urinary excretion ratio. A mechanism of this interaction may involve, at least in part, inhibition of glucuronidation by probenecid. However, this was confounded by the observation that the plasma concentration of probenecid achieved (200–400 μM) was considerably lower than the *in vitro* K_i value for probenecid to inhibit zidovudine glucuronidation in HLMs (700–900 μM). A proposed alternative explanation for the increase in plasma AUC of zidovudine involves probenecid inhibiting the renal tubular secretion of zidovudine glucuronide, with subsequent hydrolysis of the glucuronide conjugate back to the parent drug [116]. Further evidence is needed to support this alternate hypothesis. Other DDI studies of zidovudine with drugs such as efavirenz (Sustiva prescribing label), methadone, and valproic acid have also been reported [135–137]. These studies showed changes in zidovudine systemic exposure ranging from no increase (efavirenz) to a twofold (valproic acid) increase.

7.8.3.2 Mycophenolate Motevil (MMF). Assessment of MPA pharmacokinetics in renal transplant patients indicates that patients receiving tacrolimus and MMF displayed a 56% increase in AUC of MPA compared to those who received MMF and CsA [138]. Conversely, the AUC of MPA glucuronide was ~40% lower in these patients. A subsequent *in vitro* study demonstrates that tacrolimus inhibits the conversion of MPA to its hydroxyphenyl glucuronide in a concentration dependent manner, providing evidence to support that the interaction between tacrolimus and MPA may be mediated by inhibition of UGT [139].

7.8.3.3 Lamotrigine. Lamotrigine is an antiepileptic drug indicated for the treatment of partial seizures, primary generalized tonic-clonic seizures, and bipolar disorder. In humans, lamotrigine is primarily metabolized to form a 2*N*-glucuronide conjugate, which was excreted in urine [140]. *In vitro*, glucuronidation of lamotrigine is mediated by UGT1A3 and UGT1A4, and possibly UGT2B7 [91,141]. Several DDI studies with lamotrigine have been reported, which likely involve inhibition or induction of the UGT(s) mediating the glucuronidation of this drug. A study conducted in six healthy male subjects revealed that concomitant administration of valproic acid (200 mg Q8h for two days) increased the plasma lamotrigine AUC by ~30% and prolonged its elimination $t_{1/2}$ from 37 to 48 h [142]. Renal clearance of lamotrigine remained unchanged. The authors postulated that the explanation for this observation likely was related to valproic acid impairing lamotrigine glucuronide formation in the liver, although assessment of the pharmacokinetics for this metabolite was not performed in this study.

7.8.3.4 Induction of Glucuronidation.

7.8.3.4.1 Zidovudine. *In vitro*, rifampin is a known inducer of selected isoforms of UGT enzymes [125,143]. In humans, rifampin coadministration decreased plasma zidovudine C_{max} and AUC by 43% and 47%, respectively, and urinary recovery of zidovudine by 37% [144]. In contrast, rifampin minimally altered the plasma C_{max} and AUC of zidovudine glucuronide, while urinary recovery of this metabolite increased by 11%. Ratio of plasma AUC of zidovudine glucuronide to zidovudine increased 99% by rifampin, while the urinary ratio of metabolite to parent increased by 75%. Since rifampin increased the oral formation clearance of its glucuronide metabolite by twofold while renal clearance of zidovudine and its glucuronide conjugate was unchanged, this supports the hypothesis that the mechanism of this interaction likely results from the induction of the relevant UGT enzyme(s) by rifampin. Similarly, coadministration of ritonavir along with zidovudine has also been shown to affect the systemic exposure of zidovudine in human [145]. Ritonavir administration reduced plasma zidovudine C_{max} and AUC by about 26% while there was little change in the systemic exposure to zidovudine glucuronide. Since the urinary recovery of parent and metabolite was not reported, it could not be confirmed if the apparent change was related to induction of UGT enzymes.

7.8.3.4.2 Lamotrigine. A few case reports indicate that concomitant use of oral contraceptives and lamotrigine increased seizure frequency or recurrence of seizures, which could be attributed to altered levels of lamotrigine in these women [146]. A subsequent study showed that in the presence of the oral contraceptives, steady-state lamotrigine C_{max} and AUC values decreased by 39% and 52%, respectively [147]. This magnitude of change likely is clinically relevant with direct implication of dosing

of lamotrigine in women who may initiate or stop a combined oral contraceptive with this drug. Similarly, administration of multiple doses of a combination of atazanavir (300 mg) and ritonavir (100 mg) has also been shown to affect the pharmacokinetics of lamotrigine in healthy male volunteers, although atazanavir alone did not appear to have a significant effect [148]. Lamotrigine plasma AUC and elimination half-life decreased by 32% and 27%, respectively. Likewise, a combination of lopinavir and ritonavir reduced the plasma AUC and elimination half-life of lamotrigine by ~50% [149]. The median ratios of AUC for the 2*N*-glucuronide conjugate to parent increased from 0.57 to 1.12, suggesting that the likely mechanism of this interaction may be related to an induction of UGT enzyme(s) responsible for the glucuronidation of lamotrigine.

7.8.3.4.3 Mycophenolate Mofetil. In eight stable renal allograft recipients, all treated with MMF without concomitant use of calcineurin inhibitors, administration of rifampin for eight days led to a decrease in plasma exposure of MPA with most reduction occurring at 6–12 h postdose [107]. This suggested that rifampin alters the enterohepatic recirculation of MPA. Rifampin also increased plasma AUC for MPA glucuronide and mycophenolic acyl glucuronide by 34% and 193%, respectively. Urinary recovery of these two glucuronide conjugates also increased by 49% and 138%, respectively, but renal clearance did not change with rifampin treatment. An increase in oral formation clearance of these glucuronide conjugates is consistent with the hypothesis that rifampin induces MPA glucuronidation. In addition, rifampin may also alter the excretion/transport of MPA glucuronide conjugates, thus impacting the systemic exposure and enterohepatic recirculation of this drug.

Telmisartan, an angiotensin receptor blocker, has been shown to reduce the exposure of MPA in Japanese renal transplant recipients having urine protein or hypertension [150]. Telmisartan coadministration was associated with a 29% decrease in mean dose-adjusted plasma AUC of MPA in patients receiving combination immunosuppressive therapy of tacrolimus, MMF, and corticosteroids. Conversely, coadministration of valsartan or candesartan did not alter the pharmacokinetics of MPA. This disconnect was attributed to the fact that telmisartan, but not valsartan or candesartan, is a peroxisome proliferator-activator receptor gamma (PPAR- γ) activator, which enhances UGT1A9 expression *in vitro* [151]. Thus, telmisartan may stimulate the metabolism of MPA to its glucuronide conjugate and decreased the systemic exposure to MPA. Interestingly, telmisartan coadministration did not alter the plasma pharmacokinetics of MPA glucuronide compared to the other three groups. Thus, further work will be needed to confirm the mechanism of this interaction.

7.9 CLINICAL IMPLICATIONS OF UGT-BASED DRUG-DRUG INTERACTIONS

The examples presented in this chapter as well as those from other reviews illustrate that with a few exceptions, DDI involving inhibition of UGT typically results in a modest (twofold or less) increase in the systemic exposure of the substrate. This contrasts sharply with those observed with CYP DDI, which could result in upward of 20- to 30-fold increase in AUC of the victim drug. Williams *et al.* [126] and Lin and Wong [116] have postulated several reasons for this observation: low substrate affinity to the UGT enzymes (or high K_m values), low inhibitor affinity to the UGT enzymes relative to the inhibitor concentration (or relatively low I/K_i ratio), and presence of multiple

enzymes (both CYPs and UGTs) that mediate the metabolism of the substrates. To date, evidence of increased toxicity arising from DDI involving UGT inhibition is rare. Nevertheless, for any given drug, the clinical consequence of such an interaction should be considered in light of its exposure-toxicity relationship. Likewise, induction of UGT enzymes was associated with a reduction of the AUC for the affected drug by ~50%, which is considerably less than those observed with CYP induction. However, despite this relatively modest change, clinical consequences may still arise as exemplified by the increased seizure frequency from the lamotrigine–oral contraceptive combination. Thus, patients should be monitored closely while dose adjustment should be considered for those drugs that display a narrow therapeutic index.

7.10 SUMMARY

This chapter has provided an overview of the role and clinical consequences of glucuronidation of drugs by UGTs. Glucuronidation via UGT enzymes not only contributes to multiple important physiologic functions in the body involving regulation of the homeostasis of endogenous substances, but is an important detoxification pathway and a major clearance mechanism for certain drugs. Because of their ubiquitous nature, UGTs can affect the pharmacokinetics and the disposition of drugs. Intestinal UGTs can contribute to the presystemic “first-pass” metabolism and therefore the drug’s bioavailability. This can indirectly influence the biological responses to drugs. In addition to detoxification, glucuronidation of certain drugs can lead to bioactivation and therefore induce drug toxicity. As the CYPs, genetic polymorphisms have been observed in the UGT family. These abnormalities have been associated with diseases such as hyperbilirubinemia and/or an increased risk of certain cancers. Genetic polymorphisms of this diverse super family of enzymes also contribute to wide interindividual variation in pharmacokinetics of drugs that are metabolized by these enzymes, which may predispose patients to an increased risk of untoward effects. For drugs with narrow therapeutic index and severe toxicity (e.g., irinotecan), genetic testing to guide dose selection may be warranted. In general, the magnitude of DDI as a result of modulation of UGT enzyme expression or activity is considerably less than those observed with CYP enzymes. However, appropriate monitoring of the potential clinical outcome of such changes is still prudent. At this time, prediction of *in vivo* clearance from *in vitro* systems appears to be feasible for selected drugs/UGT enzyme pairs; however, this remains an area of scientific uncertainty.

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8 Herb–Drug and Food–Drug Interactions

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8.1	Summary	1
8.2	Introduction	2
8.3	Drug interactions	2
8.4	Drug–herb interactions	3
8.5	Drug–food/food constituent interactions	11
8.6	Conclusions	18
	References	19

8.1 SUMMARY

Medication with plants and herbs has been practised for thousands of years, and a substantial proportion of the world's population is thought to use herbal medicines. As a result of their widespread use, herbal medicines are likely to be taken with prescribed drugs, leading to the risk of herb–drug interactions. Herbal consumption can diminish the therapeutic effect of drugs as well as giving rise to adverse reactions and toxicity. These clinical effects are caused by herb–induced changes in (i) pharmacokinetics, particularly through inhibition or induction of the cytochrome P450 drug-metabolizing enzymes (CYPs) and (ii) drug receptor sensitivity. St. John's wort has been the subject of much research on herb–drug interactions. It induces the activity of human cytochrome P450s, particularly, CYP3A4, which has led to the characterization of interactions involving more than 100 drugs. Decreased or loss of therapeutic effect has been observed for drugs such as immunosuppressants, oral contraceptives, and methadone. Other herbs that have been shown to cause pharmacokinetic interactions, the significance of which requires confirmation in many cases, include Echinacea, garlic, ginkgo, ginseng, kava, and milk thistle.

Because of the regularity in food intake, therapeutic drugs may be taken before, at the same time as, or after a meal. There are many instances whereby food itself as well as its constituents has been shown to influence the pharmacokinetics of drugs and response to drugs, sometimes causing substantially diminished therapeutic effects or adverse reactions. The most notable and widely studied example of a food product interacting with drugs is that of grapefruit juice. The juice is an inhibitor of CYP3A4,

and has been shown to impair the metabolism of a large number of drug substrates of this enzyme, in some cases leading to clinically significant interactions.

It should be said that much of the published evidence on herb-drug and food-drug interactions is based on case reports or on pharmacokinetic data alone, and these require substantiation by studies measuring the clinical effects of drugs. Because herbal and food products are not regulated to the same extent as therapeutic drugs, and because of the variability in the clinical effects produced, herb and food interactions are more difficult to document, characterize, and predict. Caution should be exercised in the use of pharmaceutical drugs with herbal remedies and food.

8.2 INTRODUCTION

Humans have used plants and herbs as medicines for thousands of years. It has been estimated that at least 20% of the general population in the United States use herbal medicines [1,2], and that about 20–26% of people in the United Kingdom had bought over-the-counter herbal medicines over the last one to two years [3]. Because they occur naturally, many users take the view that herbal medicines are harmless, and many do not reveal their use to their health-care provider. However, it is established that some herbal medicines, which are often a mixture of active ingredients, can cause adverse effects [4]. As a result of their widespread use, herbal medicines are likely to be taken with therapeutic drugs leading to the risk of herb-drug interactions, occurring both from changes in pharmacokinetics and at the level of the receptor, that is, pharmacodynamics.

Because of the regularity in food intake, therapeutic drugs may be taken before, at the same time as, or soon after a meal. There is a large literature on how food itself as well as its constituents can influence the pharmacokinetics of and pharmacodynamic response to drugs, sometimes causing substantially diminished therapeutic effects or adverse reactions [5].

The aim of this chapter is to review the relevant literature on herb-drug and food/food constituent-drug interactions and their clinical importance. The greatest coverage is devoted to drug interactions involving St. John's wort and grapefruit juice, because they have been the subject of most research. The chapter begins with a description of the mechanisms by which drug interactions occur.

8.3 DRUG INTERACTIONS

When two or more drugs are administered to a patient at the same time, there are several possible consequences. First, one drug could enhance the therapeutic effect of another, and there are a number of examples of these beneficial drug-drug interactions. Second, one drug could diminish or abolish the therapeutic effect of another, an unwanted effect. Third, two drugs might interact to cause an adverse drug reaction or toxicity.

The question of how common drug-drug interactions are, is difficult to answer. The results of a relatively recent and large prospective study suggested that drug-drug interactions were responsible for about 1% of admissions to two UK hospitals over a period of six months [6]. There is also good evidence that the more the number of drugs taken by a patient, the greater the risk of an interaction [7].

Drug–drug interactions occur through a variety of mechanisms: (i) pharmaceutical incompatibility, where there is a chemical reaction between two drugs (in some cases, leading to precipitation from solution), precluding their use in the same formulation; (ii) pharmacodynamics, where the action of one drug is altered by another at the site of pharmacological action, that is, the receptor or by a physiological mechanism; (iii) pharmacokinetics, where the absorption, distribution, metabolism, or excretion of one drug is affected by the coadministration of another [5]. In recent years, many important drug–drug interactions have been identified that involve inhibition or induction of the drug-metabolizing enzymes, notably the cytochrome P450s (CYPs). Drug–drug interactions mediated by CYPs will form a substantial part of this chapter.

8.4 DRUG–HERB INTERACTIONS

This chapter is restricted to a discussion of herb–drug interactions involving commonly used drugs and to those interactions where there is at least some evidence of a clinically significant effect. For more detailed and comprehensive coverage of drug–herb interactions, the reader is referred to reviews by Izzo and Ernst [1], Hu *et al.* [8], Shord *et al.* [2], Williamson *et al.* [9], Kennedy and Seely [10].

8.4.1 St. John's Wort

St. John's wort or *Hypericum perforatum* is a plant with yellow flowers. It appears to be named after John, the Baptist, since it flowers in late June at the time of the feast of St. John [11]. Its most common use is for the treatment of depression [12]. The major active components of St. John's wort are hypericin and hyperforin, although it contains many other chemicals [8]. The inhibition of 5-hydroxytryptamine, dopamine, and noradrenalin by St. John's wort [13] may form the basis of its antidepressant effect. Izzo [14] cites a number of cases of the serotonin syndrome (characterized by increased heart rate, shivering, sweating, agitation, or more seriously hyperthermia, high blood pressure, coma, and/or rhabdomyolysis) occurring when St. John's wort is taken with serotonin–reuptake inhibitors. However, existing evidence suggests that St. John's wort has relatively few unwanted effects [15].

Many *in vivo* studies have shown that a range of interactions, mainly pharmacokinetic and some clinically important, occur through the coadministration of St. John's wort with prescribed drugs. The major mechanism for these interactions is the induction of CYP activity as well as P-glycoprotein (PgP) by St. John's wort, leading to decreased and sometimes subtherapeutic plasma drug concentrations. The most important group of interactions involving St. John's wort, with respect to both number and clinical significance, occur through the induction of CYP3A4 [2]. St. John's wort can also induce CYP2C19 [16] and CYP2E1 [17], enzymes that are generally less important than CYP3A4 in human drug metabolism. Three other significant drug-metabolizing CYPs, CYP1A2, CYP2C9, and CYP2D6, do not appear to be induced by St. John's wort [18].

Shord *et al.* [2] review the ability of St. John's wort to inhibit rather than induce the activity of a range of human CYPs *in vitro*, but the relationship between the inhibition and induction of CYP by St. John's wort *in vivo* is unclear.

There is evidence that St. John's wort can induce the transport protein PgP [19,20]. The considerable overlap among substrates, inducers, and inhibitors of PgP and CYP3A4 indicates that both proteins may contribute to the many interactions originally attributed to CYP3A4 alone.

8.4.1.1 Immunosuppressive Drugs. There are numerous case reports of interactions between St. John's wort and drugs used to prevent rejection of transplanted organs [21], two of which, cyclosporin and tacrolimus are well-established substrates of CYP3A4 and PgP. Coadministration of St. John's wort resulted in decreased plasma concentrations of the immunosuppressives in some cases to subtherapeutic values, leading to instances of organ rejection. Organ function improved in the majority of patients on withdrawal of St. John's wort. In one substantial report, 30 transplant patients taking St. John's wort experienced a 47% average decrease in plasma cyclosporin concentrations [22]. Titrating the dose of cyclosporin upward brought the plasma concentrations back to the preinteraction levels. When St. John's wort was stopped, the patients were stabilized on the same dose of cyclosporin that they were taking before the interaction.

Another case report involved a renal transplant patient receiving tacrolimus and mycophenolate to prevent rejection [23] (Fig. 8.1). Following self-medication with St. John's wort, tacrolimus concentrations decreased by about fourfold, which was

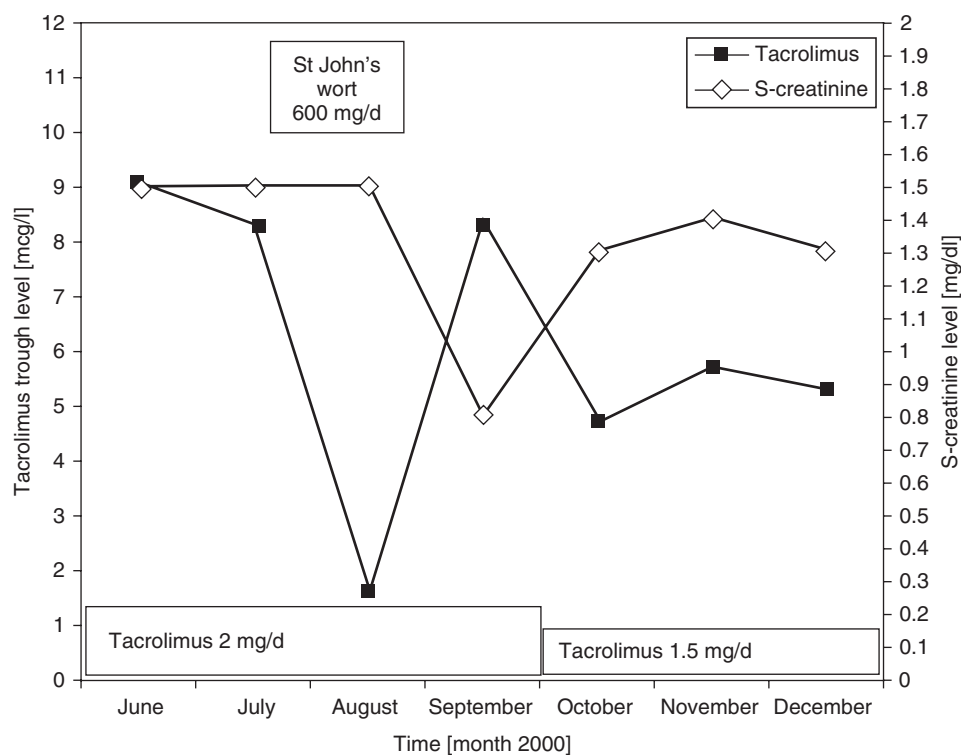


Figure 8.1 Tacrolimus whole blood trough concentrations, serum creatinine, and daily tacrolimus dosage in relation to St. John's wort intake in a renal transplant patient. *Source:* From Bolley *et al.*, 2002; with permission.

accompanied by a decreasing renal function. Withdrawal of St. John's wort led to the return of tacrolimus concentrations to the preinteraction range, and to an improvement in renal function.

The effect of St. John's wort on the pharmacokinetics of cyclosporin and tacrolimus has been corroborated in several prospective clinical trials [1]. In one study of renal transplant patients receiving 600 mg of St. John's wort extract for 14 days, trough plasma cyclosporin concentrations decreased by a mean of 41%, requiring a near doubling of the dose of the latter to maintain an adequate degree of immunosuppression [24].

Bell *et al.* [25] showed that concurrent administration of St. John's wort had no significant effect on the single-dose pharmacokinetics of prednisone and prednisolone in healthy males.

8.4.1.2 Oral Contraceptives. There is substantial *in vivo* evidence that St. John's wort enhances the elimination of the active constituents of the contraceptive pill. Hormonal components such as ethinylestradiol [26], 3-ketodesogestrel [27], and norethindrone [28] are all metabolized by CYP3A4, and St. John's wort has been demonstrated to hasten their clearance [1]. In this context, a number of reports of unwanted pregnancies in women taking oral contraceptives and St. John's wort have been reported to the European drug regulatory authorities [29]. In a more detailed report, Schwarz *et al.* [30] describe a 36-year-old depressed woman attending a gynaecology clinic with an unexpected pregnancy. She was a regular user of an oral contraceptive containing ethinylestradiol and dienogestrol, and then began taking over-the-counter hypericum extract three months before conception. Use of the oral contraceptive was terminated, resulting in a healthy foetus. Breakthrough bleeding has also been reported in women taking oral contraceptives and St. John's wort [1]. For example, in a randomized controlled trial of 18 healthy females taking oral contraceptives [31], the authors found that St. John's wort (300 mg twice or three times daily) was associated with an increase in intracyclic bleeding episodes, but that there was no evidence of ovulation. Furthermore, St. John's wort was associated with a decrease in the plasma concentration of 3-ketodesogestrel but not that of ethinylestradiol. The induction of the metabolism of the active constituents of the contraceptive pill by St. John's wort leading to subtherapeutic concentrations probably caused these pregnancies and episodes of breakthrough bleeding. Because of the risk of a loss of therapeutic effect, the Committee on the Safety of Medicines UK [32] has advised that St. John's wort should not be used with oral contraceptives.

8.4.1.3 Anticancer Drugs. Yang *et al.* [33] have reviewed herbal interactions with anticancer drugs, and some may be clinically significant. For example, St. John's wort decreased the plasma concentrations of the active metabolite of irinotecan by 42%, which was accompanied by reduced myelosuppression [34]. Similar effects on the clearance of imatinib during treatment with St. John's wort have been observed [35]. Both of these drugs are metabolized by CYP3A4. Avoidance of St. John's wort should reduce the risk of subtherapeutic concentrations of these drugs.

8.4.1.4 Anti-HIV Drugs. Although St. John's wort has been shown to decrease the concentrations of the antiretroviral drugs, indinavir [36] and nevirapine [37], the clinical significance of this reaction remains to be established. Both Izzo and Ernst

[1] and Hu *et al.* [8] cite a case of an increased HIV RNA viral load in a patient being treated with indinavir and lamivudine, and who was also taking St. John's wort. Although this appears to be the only report of diminished therapeutic effect of anti-HIV treatment in patients taking St. John's wort, The European Medicine Evaluation Agency [38] has advised against the use of protease inhibitors with St. John's wort.

8.4.1.5 Drugs Affecting the CNS. St. John's wort has been shown to interact with antidepressant drugs, such as paroxetine and sertraline, that block serotonin reuptake in the brain, leading to the "serotonin syndrome" [39]. Symptoms can include increased heart rate, shivering, sweating, agitation, or more seriously hyperthermia, high blood pressure, coma, and rhabdomyolysis. A complex case of a patient exhibiting mania when taking St. John's wort and sertraline is described by Barbenel *et al.* [40]. This patient was advised to stop taking St. John's wort before starting sertraline but declined. His symptoms included overarousal, distractibility, and delusions of grandeur. Use of St. John's wort taken alone has also been associated with the serotonin syndrome [41]. Because the clinical significance of this interaction is not fully understood, Hu *et al.* [8] advise that the combined use of St. John's wort and the selective serotonin reuptake inhibitor (SSRI) antidepressants should be avoided.

Chronically administered St. John's wort has been shown to decrease the concentrations of several benzodiazepines [1], notably midazolam, which is an important substrate of CYP3A4 [42]. Wang *et al.* [18] have performed one of the most comprehensive studies on the effect of St. John's wort on the pharmacokinetics of midazolam. Administration of St. John's wort to healthy subjects for two weeks (900 mg/day) caused a >50% decrease in the plasma concentration of midazolam, but the effect on the pharmacodynamics was not assessed. Decreased efficacy of the benzodiazepines when they are given with St. John's wort over the long term should be anticipated, although more research is needed to define the effects of the herb on the sedative properties of these drugs.

Interactions between St. John's wort and treatments for opiate dependence have been identified. Eic-Hochli *et al.* [43] reported that St. John's wort (900 mg/day for 14–47 days) was associated with a 19–60% decrease in methadone plasma concentration-to-dose ratios in four patients taking methadone. Two of these patients reported symptoms of withdrawal. Single cases of mania [44] and orofacial dystonia [45] have been described in patients taking bupropion, a drug used to aid smoking cessation. In a recent study in Chinese healthy males, St. John's wort (975 mg/day for two weeks) decreased the AUC of bupropion by 14% [46].

8.4.1.6 Cardiovascular Drugs. The Swedish Medical Products Agency have described seven case reports between 1998 and 1999 of patients who were stabilized on the anticoagulant, warfarin [47]. (Table 8.1). On taking St. John's wort, their international normalized ratio (INR) decreased from the normal range 2–4 to about 1.5. A dosage increase was required in two patients. When St. John's wort was withdrawn, the INR values returned to the normal range in four of the patients. The likely mechanism for this interaction is the induction of the activities of CYP2C9, although other studies have shown that CYP2C9 is not inducible by St. John's wort [18], and CYP3A4 both of which catalyze the metabolism of warfarin. In a randomized crossover study in healthy subjects, administration of St. John's wort for 14 days led to a relatively small decrease in the AUC of both *R*- and *S*-warfarin [48].

TABLE 8.1 Seven Cases of Decreased Effect of Warfarin During Concomitant Treatment with St. John's Wort

Patients	INR Before	INR During	Duration of Warfarin Treatment	Warfarin Dose once St. John's Wort Treatment Started
79 years, female	2.5–3.8	1.7	2.5 years	Increased from 18.5 to 21.25 mg/week
65 years, male	2.4–3.6	2.0–2.1	4 years	Increased from 37.5 to 40 mg/week. INR returned to 2.7 on the ordinary dose of 37.5 mg/week once St. John's wort was stopped
76 years, male	2.3	1.1	10 days	Increased
61 years, female	NA	1.2	Years	INR decreased and then increased after St. John's wort was stopped
84 years, female	2.9–3.6	1.5	Stable ≥ 6 mo	Stable for ≥ 6 mo. INR returned to target range once St. John's wort stopped
56 years, female	2.6	1.5	Unknown	INR returned to previous value after St. John's wort stopped
85 years, male	2.1–4.1	1.5	Long time	INR decreased so dose increased while St. John's wort was continued

Abbreviation: NA, not available.

Source: From Yu *et al.*, 2000; with permission.

St. John's wort (900 mg/day for 14 days) given to eight healthy subjects who had been administered racemic verapamil jejunally, decreased the AUC of its R- and S-isomers by 78% and 80%, respectively [49]. Similarly, St. John's wort caused a 40% decrease in the AUC of nifedipine, administered to healthy subjects given a 10-mg oral dose of the drug [50]. The pharmacodynamic consequences of the effect of St. John's wort were not assessed in either of these studies.

St. John's wort (900 mg/day for two weeks) decreased the AUC of simvastatin (10 mg), a widely used cholesterol lowering drug, by 62% but had no effect on the pharmacokinetics of pravastatin (20 mg) in healthy subjects [51]. The clinical significance of this decrease was not established. St. John's wort (600 mg/day for four weeks) caused a small (12%) but significant increase in serum concentrations of low density lipoprotein (LDL) in 16 patients with hypercholesterolemia treated with a stable dose of atorvastatin (10–40 mg/day) for at least three months [52]. The authors suggest that there may be a need to increase the dose of atorvastatin in patients taking St. John's wort. Simvastatin and atorvastatin but not pravastatin are substrates of CYP3A4 and PgP [51].

Digoxin, which is used to treat heart failure, is a substrate of PgP [53], which is induced by St. John's wort [20]. The herb (900 mg for 10 days) decreased the AUC and trough concentration of digoxin given to healthy subjects for five days, by 28% and 37%, respectively [54]. In a further study in healthy subjects, St. John's wort caused similar changes in the pharmacokinetics of digoxin [55]. Lower dose preparations of St. John's wort do not appear to affect the pharmacokinetics of digoxin [56]. Because

of the risk of toxicity the UK drug regulator has recommended that patients taking digoxin should not self-medicate with St. John's wort [32].

8.4.1.7 Other Drugs. The oral clearance of fexofenadine, a nonsedating antihistamine drug and a well-established substrate of PgP, is decreased by 12–14 days treatment with St. John's wort [57,58]. This interaction is unlikely to be of clinical significance, but it should be suspected if any loss of antihistamine effect is observed.

A case report documents an increased dose requirement for the asthma drug theophylline, a CYP1A2 substrate, in a patient who had started taking St. John's wort (300 mg/day) two months previously [59]. On stopping the herb, the serum theophylline concentrations doubled within one week, and the dose was reduced accordingly. Interpretation of this suspected interaction is confounded by the patient taking a range of other drugs and her being a smoker, in whom CYP1A2 is induced. A subsequent study in healthy subjects found that St. John's wort (900 mg/day for 15 days) did not influence the pharmacokinetics of theophylline [60].

St. John's wort (900 mg for 14 days) decreased the AUC of the proton pump inhibitor omeprazole (20 mg, single dose) by about 40%, probably by inducing CYP2C19 and CYP3A4 [61]. A dose increase in omeprazole or any of the other proton pump inhibitors, that are metabolised by CYP2C19 and CYP3A4, should be considered where there is loss of effect in patients taking this combination.

8.4.2 Echinacea

Echinacea extracts are made from the purple coneflower species of plants, which are reported to have anti-inflammatory, antiviral, anticancer, and immunostimulating effects, and are widely used for the prevention and treatment of upper respiratory tract infections [62].

There is conflicting evidence that Echinacea preparations influence the activities of the CYPs [1,63–65]. Izzo and Ernst [1] comment that these variant findings could be due to products containing different species or parts of the plant, as well as to different dosages and duration of treatment.

Gorski *et al.* [63] found that the AUC of single-dose caffeine (200 mg), a CYP1A2 substrate, was increased by about 30% in healthy subjects given *Echinacea purpurea* root (1600 mg for eight days). However, a second, similarly designed study did not confirm these findings [64]. The oral bioavailability of midazolam, a CYP3A4 substrate, given orally and intravenously to healthy subjects who had ingested *E. purpurea* root (1600 mg for 28 days) was shown to increase by 50% [63]. The pharmacodynamic consequences of this interaction were not assessed. A second study did not show any effect of Echinacea on the pharmacokinetics of oral midazolam [64]. Echinacea was found to cause a small decrease in the plasma concentrations of (*S*)-warfarin in healthy subjects, but did not affect the pharmacodynamics of this anticoagulant [66].

8.4.3 Garlic (*Allium sativum*)

Garlic is a herb belonging to the onion family. In addition to its culinary use, it is reputed to have beneficial effects on the immune and cardiovascular systems as well as antibacterial, antifungal, and antiviral properties [67]. Garlic extracts have been

shown to inhibit the activities of CYP3A4, CYP3A5, CYP2C9, and CYP2C19 *in vitro* [68] but only CYP2E1 activity appears to be inhibited *in vivo* [69,70].

There is some evidence that garlic interacts with HIV-protease inhibitors. Garlic taken in the form of a dietary supplement was associated with a 50% decrease in the plasma concentrations of saquinavir, suggesting induction of metabolism [71]. However, the results of another study with garlic extract did not support this finding [72]. Severe gastrointestinal toxicity has been reported in two patients taking concomitant garlic and ritonavir, but when these subjects were rechallenged with low dose ritonavir after they had stopped taking garlic, the symptoms reappeared [73]. No changes in the pharmacokinetics of single-dose ritonavir were observed when healthy subjects were administered garlic extract twice daily for four days [74].

Garlic has been linked to decreased platelet aggregation, but probably does not cause an important interaction with warfarin and other coumarin anticoagulants. Ingestion of garlic extract for eight weeks was associated with more than a twofold increase in the INR of a patient stabilised on warfarin [75]. When the patient stopped taking garlic, the INR decreased to its previous value. However, subsequent trials have not confirmed such an interaction, showing no effect of garlic on the pharmacokinetics and pharmacodynamics of warfarin [76,77].

McCoubrie [78] reports a patient developing low blood pressure and becoming faint when taking lisinopril and after self-administering garlic capsules. Blood pressure returned to its previous value when the garlic was stopped. No subsequent studies have been performed to explore this putative interaction.

8.4.4 Ginkgo (*Ginkgo biloba*)

The leaves of the *Ginkgo biloba* tree are used to treat circulatory disorders, such as intermittent claudication and dementia, and to enhance memory. A recent review has assessed the clinical relevance of interactions between ginkgo and drugs [79]. Izzo and Ernst [1] report some inconsistency in the results of studies investigating the effects of ginkgo on the activities of individual forms of CYP, and it appears that in most cases any effects are unlikely to be clinically significant. The influence of ginkgo on the actions of a number of drugs has been investigated.

Ginkgo has been linked to bleeding and platelet disorders [80], and thus patients taking this drug in combination with anticoagulant and antiplatelet drugs may at particular risk of bleeding. A case of intracerebral hemorrhage has been reported in a patient stabilized on warfarin for five years occurring about two months after starting ginkgo [81]. However, three reports showed that ginkgo had no effect on the pharmacokinetics, anticoagulant action, or dosage requirement of warfarin [82–84]. Meisel *et al.* [85] describe a fatal case of intracerebral bleeding in a patient taking ginkgo occurring four weeks after starting ibuprofen (600 mg/day). In another case, a subdural haematoma after a head injury was reported in a patient taking ginkgo and rofecoxib [86]. Finally, spontaneous bleeding from the iris into the anterior chamber of the eye one week after starting ginkgo was described in a patient who had been taking aspirin daily for three years without problems [87]. Clearly, these case reports do not prove cause and effect, and Izzo and Ernst [1] cite clinical trials that do not substantiate these observations. Isolated cases of unwanted effects in patients taking ginkgo and trazodone [88] and phenytoin and valproic acid [89] have been reported but have not been confirmed by further research.

8.4.5 Ginseng

Ginseng is the root of the *Panax* genus of plant and has been used particularly in the Far East for a variety of ailments and medical conditions including cancer prevention, the enhancement of cognition, cardiovascular disease, and hepatotoxicity [90]. Ginseng appears to have little or no effect on the activity of individual CYP enzymes [69].

In two case reports, self-medication with ginseng was associated with a decrease in INR in patients stabilized on warfarin [91,92]. In a placebo-controlled study in healthy subjects taking warfarin (5 mg/day for five days), a small decrease in the INR of 0.16 was observed, compared with a nonsignificant reduction of 0.02 in the placebo group [93]. A small decrease in the AUC of warfarin was also reported. However, in another study ginseng (3 g/day for two weeks) did not affect the pharmacokinetics or pharmacodynamics of a single 25-mg dose of warfarin in healthy subjects [48].

Two patients taking phenelzine for depression experienced symptoms such as headache, insomnia, and irritability after self-medicating with ginseng [94,95]. One of these patients began taking ginseng again three years later and suffered similar symptoms [96], providing stronger evidence that ginseng was the cause of these CNS effects.

8.4.6 Kava

Kava (*Piper methysticum*) is a plant of the Western Pacific. Preparations of its root are used to treat anxiety and insomnia [97]. Despite many reports that kava causes hepatotoxicity [98], it is still a widely used herbal remedy.

Kava has been shown to affect the *in vivo* activity of CYP2E1 but not those of CYP1A2, CYP2D6, or CYP3A [99] or PgP [100].

There is unconfirmed evidence that kava may enhance the CNS effects of alcohol [101]. In a single case report, a patient taking alprazolam, cimetidine, and terazosin was admitted to hospital in an agitated and disorientated state [102]. He admitted taking kava for the previous three days. The patient showed increased alertness after several hours. The authors suggested that kava may have supplemented the sedative effects of alprazolam. The pharmacokinetics of midazolam, a CYP3A4 substrate, appear to be unaffected by kava [99].

8.4.7 Milk Thistle (*Silybum marianum*)

Milk thistle in the form of tinctures and capsules has been used to prevent and treat liver damage, and may have anticancer, antidiabetic, and cardioprotective effects [103]. Milk thistle was shown not to affect the activities of CYP1A2, CYP2D6, CYP2E1, CYP3A4 [64], or PgP [104]. Two of the constituents of milk thistle, silymarin and silibinin, have been found to inhibit the *in vitro* activity of CYP2C9 using warfarin as a substrate, at concentrations that are believed to be clinically achievable [105]. The authors recommend a clinical evaluation of a potential milk thistle/warfarin interaction. The effect of milk thistle on the pharmacokinetics and pharmacodynamics of a number of other drugs has been studied, but no clinically significant effects have been identified [2,8].

8.5 DRUG–FOOD/FOOD CONSTITUENT INTERACTIONS

8.5.1 Food

A large number of interactions have been attributed to the effect of food through changes in drug absorption [5,106]. Interactions may be influenced by the amount and nature of the food eaten, as well as the time of drug intake in relation to the meal. Effects can occur through changes in gastric acidity, gastrointestinal motility, bile secretion, or chelation of a drug by a food constituent. Food high in fat can increase the bioavailability of lipophilic drugs, and in contrast food with high fiber content can decrease bioavailability in some cases.

A pivotal set of interactions involving nonselective monoamine oxidase inhibitor antidepressants and tyramine-rich foods, such as cheese, caviar, and pickled herring, was identified in the 1960s [107]. Patients suffered with potentially life threatening increase in blood pressure, which was sometimes accompanied by headache, flushing, sweating, and nausea and vomiting. In a series of early cases, tranylcypromine and phenelzine were most frequently implicated [107]. Under normal conditions, tyramine is metabolized in the gut and liver by monoamine oxidase. However, nonselective monoamine oxidase inhibitors block this activity, allowing absorption of tyramine, which causes vasoconstriction leading to increased blood pressure.

Another group of well-established and clinically important drug interactions occur through the consumption of dairy products and other foods along with the tetracycline antibacterials. Calcium ions present in milk, for example, chelate with tetracyclines forming complexes that are much less well absorbed from the gastrointestinal tract than the parent drugs. In one representative study, the consumption of homogenized milk decreased the absorption of tetracycline by 65% in healthy subjects, leading to a corresponding reduction on plasma drug concentrations [108]. The normal recommendation is that tetracyclines should be taken at least 1 h before or 2 h after food.

A comprehensive discussion and extensive lists of individual food–drug interactions can found in reviews by Schmidt and Dalhoff [106] and Baxter [5].

8.5.2 Grapefruit Juice

What has been commonly termed the *grapefruit juice effect* was discovered through a serendipitous finding, from a study investigating the interaction between the calcium channel antagonist felodipine and ethanol [109]. From a range of fruit juices, white double-strength grapefruit juice was chosen to mask the taste of ethanol. The combination of grapefruit juice alone and felodipine was associated with higher plasma felodipine concentrations, a lower standing blood pressure and a higher frequency of orthostatic hypertension compared to previous studies with felodipine alone. In a subsequent pilot study of a single subject, plasma concentrations of felodipine were more than five times higher with grapefruit juice than with water [110] (Fig. 8.2). A further study of six subjects with borderline hypertension, who had taken a single 5 mg oral dose of felodipine with water, grapefruit juice, or orange juice, demonstrated that grapefruit juice increased the bioavailability of the drug by 284% compared with water [111]. This was accompanied by a lower blood pressure and higher heart rate. No change in the elimination half-life of nifedipine was observed. Orange juice did not affect the pharmacokinetics or pharmacodynamics of nifedipine.

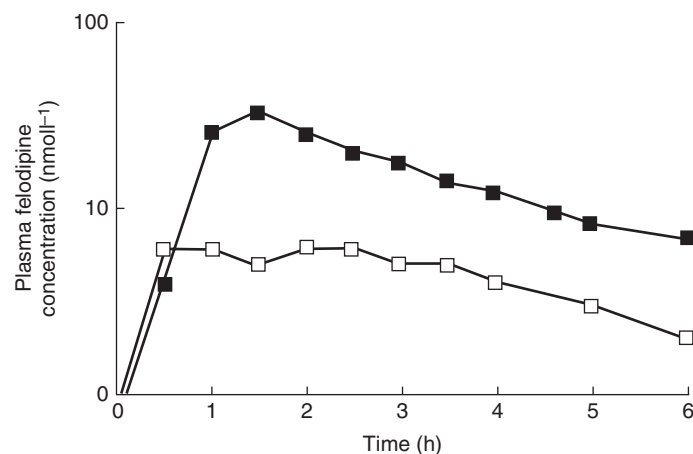


Figure 8.2 Plasma felodipine concentration–time profile from the pilot study in one subject. Felodipine as 5 mg regular tablet was administered with 350 mL double-strength grapefruit juice (■) or water (□). *Source:* From Bailey *et al.*, 1998; with permission.

Drug interactions with grapefruit juice occur after a single ingestion [112], and there is evidence that the effect is dose dependent [113]. An investigation of the duration of effect of grapefruit juice showed that the extent of the interaction declined slowly with time with a half-life of 12 h [114]. In addition, the magnitude of the interaction may vary according to the source and preparation of the juice [110].

A lack of effect of grapefruit juice on the pharmacokinetics of intravenous nifedipine suggested that the interaction occurs as a result of inhibition of the presystemic metabolism of nifedipine in the small intestine [112]. This hypothesis was supported by other studies investigating the effect of grapefruit juice on the intravenous and oral pharmacokinetics of midazolam [115], nifedipine [116], and nicardipine [117]. Nifedipine is a substrate of CYP3A4/5 [110], indicating that this enzyme is inhibited by grapefruit juice. Overall, grapefruit juice has been found to interact only with those drugs exhibiting high intestinal presystemic elimination.

Lown *et al.* [113] reported that administration of grapefruit juice to healthy subjects resulted in a 62% decrease in immunoreactive intestinal CYP3A4 and CYP3A5, without affecting CYP3A4 mRNA concentrations. However, grapefruit juice did not alter hepatic CYP3A4 activity, measured by the erythromycin breath test; colon concentrations of CYP3A5; and small bowel concentrations of PgP, villin, CYP1A1, or CYP2D6. It was concluded that grapefruit juice causes a downregulation of intestinal CYP3A but not hepatic CYP3A. The unchanged mRNA concentrations indicate that the decrease in CYP3A expression is unlikely to involve a reduced gene transcription or reduced stability of mRNA. Grapefruit juice is believed to exert its effects on intestinal CYP3A through mechanism-based inactivation by one or more components of the juice [118]. The activity of CYP3A has been shown to recover to preinteraction values after three days, which is compatible with enzyme regeneration after mechanism-based inhibition [119].

Early evidence suggested that grapefruit juice has a stimulatory effect on the transport of PgP substrates [120], possible counteracting its effect on intestinal CYP3A4. However, most evidence points to an inhibitory effect of grapefruit juice

on PgP activity [121]. Furthermore, the *in vitro* inhibition of a number of organic anion transporter polypeptides by grapefruit and other juices has been reported [121]. This suggests that drug bioavailability is decreased by inhibition of intestinal uptake transporters by fruit juices.

Much research has been directed to the identification of the constituents of grapefruit juice that cause this interaction [110], which was first attributed to the action of naringin, a flavonoid present in high concentrations in grapefruit juice [122]. However, subsequent studies in which pure naringin was administered to healthy subjects did not reproduce the effects seen with grapefruit juice [123]. It now seems more likely that other flavonoids, particularly, derivatives of the furanocoumarin bergamottin are responsible for the interaction [110]. For example, a range of dimers of 6',7'-dihydroxybergamottin have been shown to be highly potent inhibitors of gut CYP3A4 *in vitro* [124,125].

In addition to their effects on CYP3A activity, furanocoumarins have been found to inhibit CYP1A2, CYP1B1, CYP2A6, CYP2C9, CYP2C19, and CYP2D6, but were generally much less potent inhibitors of these enzymes than of CYP3A [124,126,127]. Grapefruit juice interactions mediated by CYP isoforms other than CYP3A are probably unlikely because they are not expressed or are expressed at low levels in the small intestine [128].

The effect of other fruit juices on drug disposition has been comprehensively reviewed by Farkas and Greenblatt [129]. Of the juices studied, which include orange, pomelo, lime, lemon, apple, cranberry, pomegranate, and grape, only grapefruit juice appears to show a consistent effect on the human pharmacokinetics of CYP3A substrates. However, Seville orange juice increased the AUC of felodipine to about the same extent as grapefruit juice [130]. Resveratrol, a compound found in high concentrations in red wine [131], was shown to inhibit human recombinant CYP1A1, CYP1B1, and CYP1A2 [132]. Furthermore, evaporated red wine (but not white wine) reconstituted in buffer caused inhibition of CYP3A4 and CYP2E1 in human liver microsomes [133]. Had these findings been confirmed *in vivo*, interactions with red wine could be of important clinical significance given the widespread consumption of this alcoholic beverage. However, subsequent studies showed that red wine does not affect the AUC of cisapride and felodipine [123,134]. On the other hand, red wine produced “dose dumping” in half the subjects in the felodipine study, causing a rapid increase in the plasma concentrations (but no change in the AUC) of the drug given as an extended release tablet [123]. It was suggested that the alcohol in red wine may have rapidly dissolved the tablet coating, leading to accelerated absorption of felodipine.

The following is a summary of the important interactions with grapefruit juice (and where relevant other juices). Emphasis is placed on those interactions that are clinically significant or potentially clinically significant, or involving drugs with a low therapeutic index.

8.5.2.1 Cardiovascular Drugs. Orange juice (200 ml) given three times daily to healthy subjects decreased the AUC of single-dose atenolol, a widely used β -blocker, by 40% [135] (Fig. 8.3). The authors suggested that impairment of transporters in the intestinal wall and/or of paracellular permeation of atenolol might contribute to this interaction, which they believed to be clinically significant. There are no published studies investigating the effect of grapefruit juice on the disposition of atenolol. The

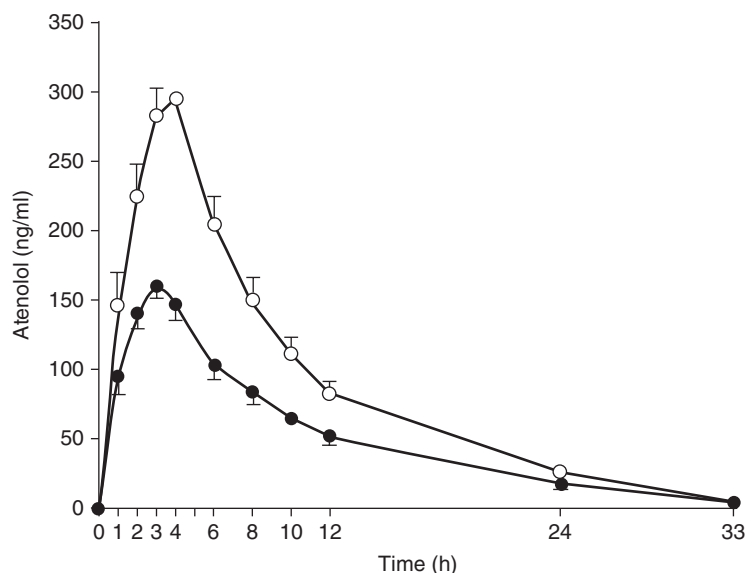


Figure 8.3 Plasma concentrations (mean values $\pm$ SEM) of atenolol in 10 healthy subjects after a single oral dose of 50 mg atenolol; after ingestion of 200 mL orange juice (*solid circles*) or water (*open circles*) three times a day for three days; and on day 3 1 h before, with atenolol and 4 h and 10 h after atenolol administration. In addition, 200 mL orange juice or water was ingested at 08:00 and 13:00 h on day 4. Source: From Lilja *et al.*, 2005; with permission.

effect of grapefruit juice on the pharmacokinetics of other much less widely used β -blockers has been reviewed by Baxter [5].

The bioavailabilities of the calcium channel antagonists, felodipine, nicardipine, nifedipine, nimodipine, and nitrendipine, are all increased by drinking grapefruit juice [110]. A two- to threefold increase was observed for felodipine [111], and was associated with a decrease in diastolic blood pressure and an increase in heart rate. The severity of adverse effects such as headaches, facial flushing, and lightheadedness was also enhanced. Although increased bioavailabilities of the other dihydropyridines were observed, grapefruit juice caused only minor changes in hemodynamics. Probably, because it possesses high bioavailability, the pharmacokinetics of amlodipine was affected to a much smaller extent by grapefruit juice [136]. The effects of grapefruit juice on the pharmacodynamics of the dihydropyridine calcium channel antagonists vary, but it is probably advisable to avoid the combination, particularly, in the case of felodipine.

Grapefruit juice was shown to have no [137] or little effect (a 20% increase in the AUC) on the pharmacokinetics of the calcium channel antagonists diltiazem or on blood pressure or heart rate [138].

The AUC of the calcium channel antagonist, verapamil, was increased by a mean of 45% in subjects who drank grapefruit juice daily for three days [139]. A small increase, which was of borderline statistical significance, in the PR interval was observed. In a study of healthy subjects, grapefruit juice consumed two to four times daily for three to four days increased the AUC of verapamil by about 30%, but had no effect on its pharmacodynamics [140]. A recent case of severe toxicity in a grapefruit juice

consumer who had accidentally taken three tablets of verapamil (120 mg each) [141] further suggests that this interaction may be clinically significant.

Overall, grapefruit juice appears to have little effect on the pharmacokinetics of digoxin, a substrate of PgP. However, in one study, the plasma concentrations of the drug increased by 50% in two subjects, and this was associated with asymptomatic changes in the ECG (electrocardiography) [142]. Such an interaction should be considered if unexpected responses to digoxin are observed.

Elevated plasma concentrations of the statins increase the risk of adverse effects (muscular pain and more seriously rhabdomyolysis). Grapefruit juice has a substantial effect on the pharmacokinetics of atorvastatin, lovastatin, and simvastatin, which are all substrates of CYP3A4 [143]. A 2.5- and 1.5-fold increase in the AUC of atorvastatin acid and total HMG-CoA reductase inhibitors, respectively, were observed in healthy subjects given double-strength grapefruit juice for five days and a single 40-mg dose of atorvastatin on day 3 [144]. Two further studies confirmed these findings [145,146]. Double-strength grapefruit juice given for three days and then at the same time as a 80-mg single dose of lovastatin increased the AUC of the latter and its active metabolite, lovastatin acid, by 15- and 5-fold, respectively [147]. Pretreatment with as little as 200 mL of normal strength grapefruit juice increased the AUCs of simvastatin, given as a single 40 mg oral dose, and simvastatin acid by 3.6- and 3.3-fold, respectively [148] (Fig. 8.4). Rhabdomyolysis was reported in a single patient taking simvastatin 80 mg/day, and who had begun eating grapefruit daily for the previous four days [149]. On the other hand, grapefruit juice does not appear to affect the pharmacokinetics of pravastatin, which is not metabolized by CYP3A4 [144]. These data, particularly for lovastatin and simvastatin, suggest that patients should be made aware of the risks of consuming grapefruit juice while taking statins.

Grapefruit juice and pomegranate juice may increase the INR of warfarin [150,151], but further research is needed to confirm these findings. Baxter [152] has reviewed the controversial interaction between warfarin and cranberry juice, citing a number of cases of elevated INR values, one of which was fatal. However, based on a systematic review of the published literature, Zikria *et al.* [153] have questioned the importance of this interaction, based on the paucity of scientific evidence. Lilja *et al.* [154] have shown that daily ingestion of cranberry juice barely affected the pharmacokinetics of warfarin, indicating that a pharmacokinetic mechanism for this interaction seems unlikely.

8.5.2.2 Drugs Affecting the CNS. The effects of grapefruit juice on the plasma concentrations of the tricyclic antidepressants, amitriptyline and clomipramine, appear to be minimal [155].

Grapefruit juice has been shown to increase the plasma concentrations of the SSRI antidepressants, fluvoxamine and sertraline, by 50% and 33%, respectively [156,157]. In a single case report, it was suggested that grapefruit eaten in excessive amounts by a patient taking fluoxetine may have caused serotonin syndromelike symptoms [158].

In epileptic patients taking daily carbamazepine, which is metabolized mainly by CYP3A4, a single 300 mL drink of grapefruit juice increased the AUC of the drug by 40% [159]. The authors suggest that grapefruit juice should be avoided in patients taking carbamazepine, a recommendation supported by a single case report of a patient experiencing CNS toxicity accompanied by elevated drug concentrations when taking carbamazepine and eating one grapefruit per day [160].

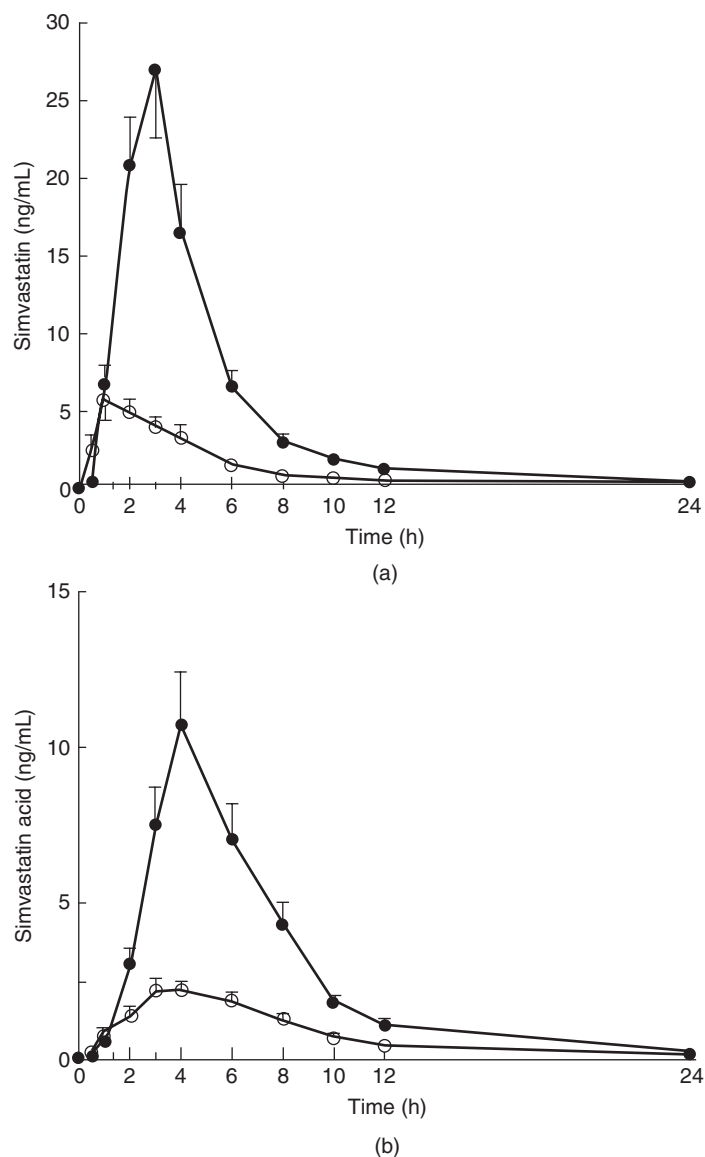


Figure 8.4 Mean plasma concentrations of (a) simvastatin and (b) simvastatin acid after administration of a single 40-mg dose of simvastatin with 200 mL water (control) (○) or grapefruit juice (●) ingested once daily for three days. *Source:* From Lilja *et al.*, 2004; with permission.

Grapefruit does not affect the pharmacokinetics of the antipsychotic drug clozapine, a substrate of CYP1A2 [161], or of halofantrine [162].

The AUC of a single dose of the benzodiazepine diazepam was increased by three-fold in healthy subjects who drank 250 mL of grapefruit juice [163]. The effect on the pharmacodynamics of diazepam was not evaluated. A single 250 mL drink of grapefruit juice increased the AUC of oral midazolam by 52% and enhanced its pharmacodynamic effects [115]. In contrast, the clearance of oral or intravenous midazolam was

not impaired by pomegranate juice [164]. A 50% increase in the AUC of oral triazolam was observed in subjects who drank 250 mL of grapefruit juice, and this was accompanied by a small increase in drowsiness and sedation [165].

Grapefruit juice has been shown not to enhance the pharmacodynamic effects of caffeine, although a slight decrease in its clearance has been observed [166,167].

Only small changes in the pharmacokinetics of opiates have been observed when taken with grapefruit juice. For example, a 17% increase in the AUC of methadone, a CYP3A substrate, was reported when 200 mL of grapefruit was drunk 30 min before and at the same time as the drug was taken [168].

8.5.2.3 Immunosuppressive Drugs. Cyclosporin, which is predominantly metabolized by CYP3A, has a narrow therapeutic range, and elevated plasma drug concentrations may lead to nephrotoxicity. There has been considerable research on the influence of grapefruit juice on the pharmacokinetics of this drug [1,169], and trials have shown that the bioavailability of cyclosporin is substantially increased by the juice. In a representative study, 10 medically stable transplant patients were given 175 mL of grapefruit juice or water for one week at the same time as their daily cyclosporin therapy [170]. Grapefruit juice increased the AUC and trough plasma concentration of cyclosporin by 34% and 77%, respectively. This established interaction is likely to be clinically significant, and grapefruit juice and cyclosporine should not be taken together. Because of the high cost of cyclosporin, it has been suggested that grapefruit juice should be coingested on economic grounds to reduce the dose of the former [171]. However, this view has been challenged [172]. Because of the considerable variability in the interaction, caused by variability in cyclosporin pharmacokinetics and in grapefruit juice batches, consistent plasma cyclosporine concentrations may be difficult if not impossible to achieve.

Similar findings have been reported for tacrolimus, another drug widely used to prevent organ rejection [169]. In one study, the whole blood trough concentrations of tacrolimus were doubled by ingestion of grapefruit juice daily for seven days in liver transplant patients [173]. Grapefruit juice has been shown to increase (by 75%) the plasma concentrations of methylprednisolone [174] but not those of prednisone or prednisolone [175].

8.5.2.4 HIV-Protease Inhibitors. The AUC of single-dose saquinavir, a substrate of CYP3A, was shown to be increased by 50% following ingestion of 400 mL of grapefruit juice [176], but this increase was not believed to be clinically significant. Grapefruit juice does not appear to affect the pharmacokinetics of indinavir [177].

8.5.2.5 Other Drugs. Ingestion of grapefruit juice is associated with small, clinically insignificant increases in the plasma concentrations of estradiol and ethinylestradiol, components of the oral contraceptive pill [178,179].

Tasseneeyakul *et al.* [180] reported a lack of effect of grapefruit juice on the AUC of the proton pump inhibitor omeprazole in healthy subjects.

Grapefruit juice was found to increase the AUC of single dose of the antibiotic erythromycin (which is mainly metabolized by CYP3A4) by 49% in healthy subjects [181]. It has been suggested that the combination might lead to even larger increases in plasma concentration in patients who regularly consume grapefruit juice, resulting from greater inhibition of CYP3A4 [182].

Grapefruit juice was found to increase the AUC of single-dose sildenafil, a drug used to treat erectile dysfunction, by 23% in healthy subjects [183]. The authors of this study advise that, although the risks of adverse effects are small, patients should avoid this combination.

8.5.3 Green Tea

Green tea is made from the leaves of the *Camellia sinensis* plant. There is equivocal evidence that it can reduce the risks of cancer and cardiovascular disease and there are claims for other health benefits [184]. There is conflicting evidence that green tea can inhibit the activities of CYP enzymes [2], and no clinical interactions have been identified based on this mechanism.

8.5.4 Licorice

Licorice is an extract of the root *Glycyrrhiza glabra* and is used as a sweetening and flavoring agent. It has been taken to treat a range of medical conditions including gastritis and upper respiratory tract infections [185]. The main active constituent of licorice is glycyrrhizic acid. There is contradictory evidence that licorice can inhibit the activities of the CYP enzymes [2]. The lack of effect of aqueous licorice extract on the pharmacokinetics and sedative effects of midazolam, a probe substrate for CYP3A4, indicates that licorice did not influence the activity of this enzyme [186]. On the other hand, oral dosing with glycyrrhizin has been shown to increase the AUC of prednisolone [187], which is a substrate of CYP3A4 [188]. In a more recent study, the AUC of omeprazole was slightly decreased by glycyrrhizin, possibly through induction of CYP3A4 [189].

8.6 CONCLUSIONS

There is a large body of research on herb–drug and food/food constituent–drug interactions, particularly, those involving St. John’s wort and grapefruit juice, and knowledge is increasing rapidly. Many of these interactions occur as a result of changes in the activities of the CYP enzymes and transporter proteins such as PgP. A number of clinically significant interactions have been identified, which can have serious consequences. However, much of the published evidence is based on case reports or on pharmacokinetic data alone, and these require substantiation by studies measuring the clinical effects, of drugs. It is clear that the extent of herb–drug and food/food constituent–drug interaction show considerable interindividual variability, due in part to the wide variation in the activities of the CYP enzymes and transporter proteins. Research into interactions of this type is further limited by the characteristics of herbal and food products themselves. Many are composed of an intricate mix of bioactive compounds, which is likely to vary from batch to batch, and each may play a role in precipitating an interaction. Because herbal and food products are not regulated to the same extent and not subject to the same rigorous evaluation as therapeutic drugs, and because of the variability in the clinical effects produced, herb and food interactions are more difficult to document, characterize, and predict. It is essential that future research should be directed to the further characterization of the active constituents of

herb and food constituents and to the elucidation of their pharmacokinetics in humans. In conclusion, caution should be exercised in the consumption of herbal remedies and food with prescribed drugs, and health-care practitioners and patients should be made more aware of the potential risks of their concomitant use.

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9 Effects of Liver Disease on Drug Metabolism in Humans

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9.1	Summary	1
9.2	The human liver and drug metabolism	2
9.3	Liver disease and drug metabolism	14
9.4	Cirrhosis and drug metabolism	19
9.5	Liver surgery and regeneration	25
9.6	Liver transplantation and drug metabolism	26
9.7	The use of herbal preparations in patients with liver disease	32
9.8	Conclusions	33
	References	33

9.1 SUMMARY

Drug disposition in humans is influenced by its absorption, volume of distribution, metabolism, and, finally, its elimination. Drug metabolism involves phase I and phase II biotransformations, most of which occur in the human liver. Various physiological and pathological states may affect the hepatic metabolism of drugs. This chapter focuses on how physiological states may affect hepatic metabolism and the impact of liver disease on drug handling. The liver is vulnerable to a wide variety of insults, but the repertoire of its response to injury is limited. Likewise, the functional consequences of liver disease on drug disposition are determined by the extent of the injury, as opposed to its etiology. Although the liver's enormous functional reserve can mask the clinical impact of early liver damage, liver disease can ultimately produce multiple pathological consequences. Predicting drug metabolism in patients with liver disease is particularly challenging. No universal rule can be applied to the modification of drug dosages in patients with liver disease. Cirrhosis is the final common pathway for chronic liver disease. It has the propensity to affect drug metabolism more than any other form of liver disease. Unfortunately, there is little data available for patients with

severe liver disease, in whom pharmacokinetic and pharmacodynamic changes are most pronounced. Liver transplantation (LT) is the only curative option in patients with severe hepatic dysfunction or hepatic failure. Drug metabolism may also be altered in the liver transplant recipient. Herbal and alternative medicines are used as “liver remedies” in many patients with liver diseases, and their interactions with drugs and their potential to influence drug metabolism should also be considered in this patient group.

9.2 THE HUMAN LIVER AND DRUG METABOLISM

The liver is the largest internal organ in the body and is responsible for a wide variety of functions, including drug metabolism.

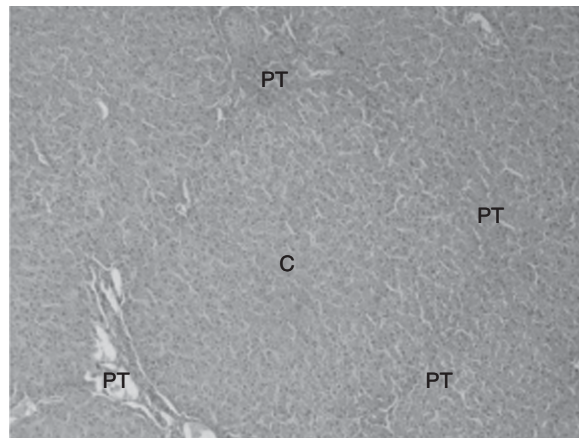
9.2.1 Liver Anatomy and Blood Supply

Blood flow to the human liver is unique, in that it receives a dual supply. The hepatic artery, a branch of the celiac artery, and the portal vein enter the liver via the porta hepatis. Both these vessels convey blood to the liver. The hepatic artery brings oxygenated blood to the liver, and the portal vein brings venous blood rich in the products of digestion, which have been absorbed from the gastrointestinal tract [1]. About 1100 mL of blood flows from the portal vein into the liver sinusoids each minute, and approximately an additional 350 mL flows into the sinusoids from the hepatic artery. This represents about 25% of resting cardiac output. Under normal circumstances, the portal vein supplies ~70% of the liver's blood supply and the hepatic artery is responsible for the remaining 30%. Blood from the hepatic artery and the portal vein perfuses the liver sinusoids and is conducted to the central vein of each liver lobule (Fig. 9.1). The pressure in the portal vein leading into the liver averages to about 9 mm Hg, and the pressure in the hepatic vein leading from the liver into the vena cava averages to 0 mm Hg. Hence, resistance to blood flow in the liver sinusoids is low, allowing for about 1.45 L of blood flow per minute under normal circumstances [2]. The central veins drain into the hepatic veins, which drain directly into the inferior vena cava.

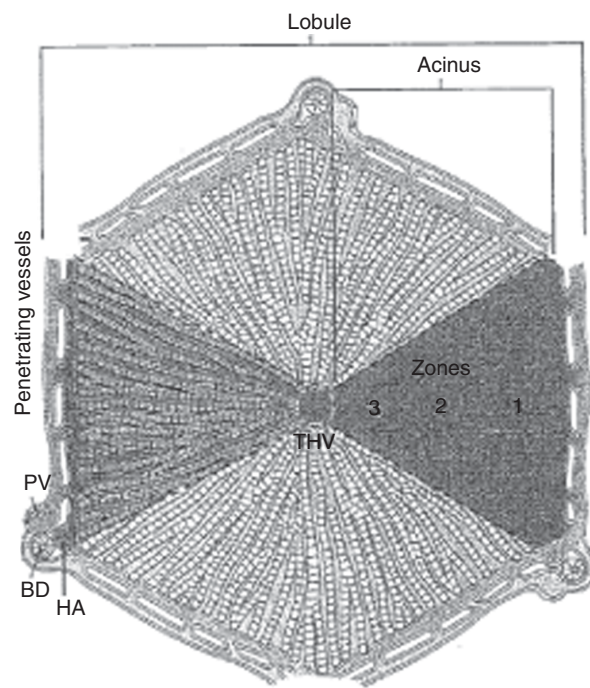
The liver is made up of many liver lobules, which are the basic functional units of the liver. On average, an adult human liver contains 50,000–100,000 individual lobules.

Each liver lobule is centered around a central vein that empties into the hepatic veins (Fig. 9.2). The central vein (or terminal hepatic venule) of each lobule is a tributary of the hepatic veins. Functionally, however, hepatic “acini” are roughly a triangular portion of the liver lobule where the portal tracts form their bases and the central veins their apices [3]. The parenchyma of the hepatic acinus is divided into three zones (Fig. 9.1). Zone 1 is closest to the portal tract, while zone 3 is closest to the central vein and farthest from the portal tract. Zone 2 lies between zones 1 and 3. A lobular gradient of activity exists for many hepatic enzymes, which follow a zonal distribution [4]. As a result, many forms of hepatic injury also exhibit a zonal distribution.

The lobule itself is composed of many hepatic cellular “plates” seen microscopically as cords of cells that radiate from the central vein like spokes of a wheel. Each hepatic plate is one to two cells thick. The portal tracts, which lie between the hepatic lobules, contain the portal triad: branches of the hepatic artery, portal vein, and bile duct (Fig. 9.2). Between the “plates” or cords of the hepatocytes are vascular sinusoids.

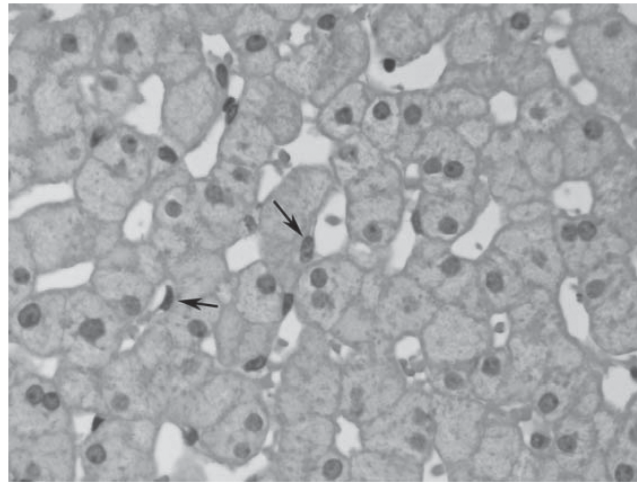


(a)

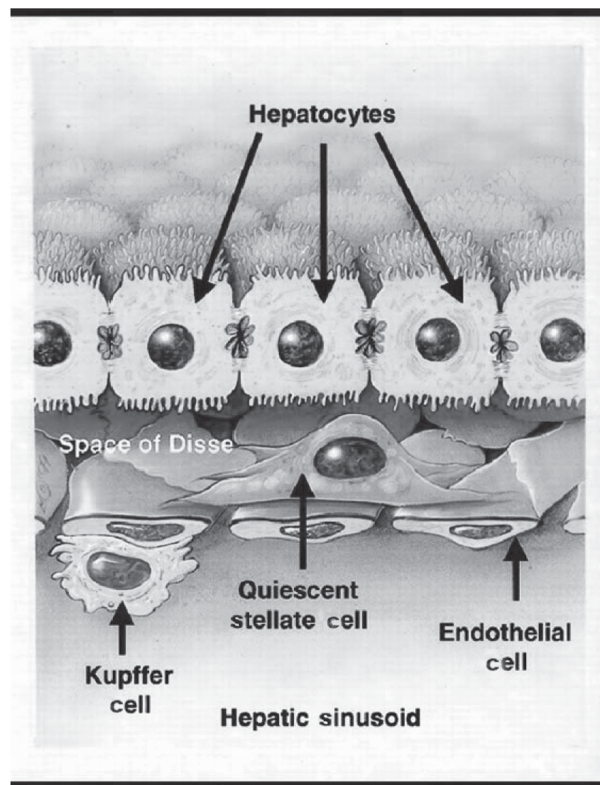


(b)

Figure 9.1 Liver architecture. (a) Human liver architecture. *Source:* Histomicrograph courtesy of Professor M. Isabel Fiel, MD, Mount Sinai School of Medicine, New York, NY. (b) Microscopic liver architecture depicted schematically [2]. *Source:* Reprinted with permission. Copyright Elsevier 1999. PT, portal tract; C, central vein; BD, bile duct; HA, hepatic artery; PV, portal vein; THV, terminal hepatic venule. (See color insert.)



(a)



(b)

Figure 9.3 Space of Disse. (a) Space of Disse (human liver). Arrows point to the space of Disse. *Source:* Histomicrograph courtesy of Professor M. Isabel Fiel, MD, Mount Sinai School of Medicine, New York, New York. (b) Schema diagram of the space of Disse. *Source:* Friedman SL. *J Biol Chem* 2000. Reprinted with permission. Copyright 2000 The American Society for Biochemistry and Molecular Biology. (See color insert.)

even large plasma proteins can diffuse freely into the space of Disse. This also allows lipophilic drug molecules and its metabolites to readily diffuse into and out of the hepatocytes. However, polar compounds do not cross the lipid domain of the hepatic sinusoidal membrane readily, and carrier-mediated transport processes are required for this [6]. The spaces of Disse connect with lymphatic vessels in the interlobular septa. Excess fluid in these spaces is removed via the lymphatics.

9.2.2 Drug Metabolism in the Liver

Drug disposition in humans is influenced by its absorption, volume of distribution, metabolism, and, finally, its elimination.

Drug metabolism involves phase I and phase II biotransformations. Most phase I and phase II reactions occur in the human liver.

Phase I biotransformation consists of oxidation, reduction, or hydrolysis reactions. The microsomal mixed-function oxidase system is the main system responsible for oxidation and some reduction reactions. This membrane-bound system of the endoplasmic reticulum consists of two enzymes (cytochrome P450 and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytochrome P450 reductase) and requires NADPH and molecular oxygen to function. The liver is the major site of cytochrome P450-mediated drug metabolism [7], although CYP450 can also be found in smaller amounts in extrahepatic tissue. In humans, numerous cytochrome P450 genes have been identified, but only a small number of encoded proteins contribute to drug metabolism, namely, the CYP1, CYP2, and CYP3 families. Together, they are involved in 80% of oxidative drug metabolism and account for close to 50% of overall elimination of commonly used drugs [7].

Phase II biotransformation conjugates the parent drug or its phase I metabolite to endogenous substrates such as glucuronic acid, sulfate, or glycine to render the compounds more hydrophilic for excretion. Methylation and acetylation are other phase II conjugation reactions. Phase II biotransformations mostly occur in the liver cytosol, with the exception of the endoplasmic reticulum membrane-bound uridinediphosphoglucuronate glucuronosyltransferases (UGTs).

Phase III reactions lead to the transport of drugs or their metabolites into bile. Biliary excretion is mediated by ATP-dependent transporters located in the bile canaliculi.

Further details on the enzyme systems involved in drug metabolism can be found in the volume titled *Review of Drug Metabolism and Interactions. Enzyme Systems Involved in Drug Metabolism and Interactions*, part titled *Enzyme Systems Involved in Drug Metabolism and Interactions in Animals and Humans* and on the human cytochrome P450 system in the chapter titled *CYP450 Enzymes in Drug Discovery and Development: An Overview* in this encyclopedia.

9.2.3 Hepatic Clearance

Hepatic clearance is the volume of blood perfusing into the liver that is cleared of drug per unit time. Factors that affect hepatic clearance include hepatic blood flow, the fraction of unbound drug, drug-metabolizing enzymes and their activity, and intrinsic clearance that would be observed in the absence of blood flow, biliary excretion, or enterohepatic circulation and protein-binding restrictions. Hepatic clearance is usually, although not always, a first-order process (e.g., phenytoin). In the single-compartment

hepatic clearance model, the entire liver is perceived to be one compartment. Drug concentrations reach the liver via hepatic artery and portal vein supply. By this theory, at steady state, intrahepatic drug concentrations are in equilibrium with drug concentrations in emerging hepatic venous blood. Intrinsic clearance is the ability of the liver to eliminate the fraction of unbound drug in the absence of hepatic blood flow limitations. Unlike in the assessment of renal function where the measurement of creatinine clearance can be used, there is no similar tool or gold standard in the assessment of hepatic clearance.

Hepatic clearance is restrictive for drugs that have a low extraction ratio ($E_H < 0.3$), as it is limited by protein binding and enzymatic activity rather than by hepatic blood flow. Hepatic metabolism of such drugs (e.g., chlordiazepoxide) is often the principal pathway of elimination, resulting in long elimination-phase half-lives. Hepatic clearance of these drugs can be broadly influenced by changes in their plasma protein bound fractions or by changes in hepatic drug-metabolizing enzymes (e.g., induction or inhibition) (Table 9.1). Any altered physiological or pathological factors that could influence these two factors would also alter hepatic clearance. For example, pregnancy reduces the concentration of plasma proteins and may alter protein binding of drugs or the concomitant use of other drugs may inhibit or induce metabolizing enzymes, which

TABLE 9.1 Common CYP450 Enzyme Inducers and Inhibitors

Examples of CYP450 Inducers	Examples of CYP450 Inhibitors
CYP1A	
• Antimicrobials – Rifampin – Griseofulvin • Anticonvulsants – Carbamazepine • Anti-HIV agent – Ritonavir • Proton pump inhibitors – Omeprazole, esomeprazole – Lansoprazole • Others – Insulin – Charbroiled meat – Cigarette smoke	• Antimicrobials – Acyclovir – Ciprofloxacin – Norfloxacin – Ofloxacin • Antiarrhythmic agents – Amiodarone – Lidocaine – Verapamil – Mexiletine – Propafenone • Antidepressants – Fluvoxamine – Moclobemide • H₂-antagonists – Cimetidine – Famotidine • Others – Ticlopidine – Oral contraceptives – Flutamide – Caffeine – Grapefruit juice – Echinacea

(continued overleaf)

TABLE 9.1 (*continued*)

Examples of CYP450 Inducers	Examples of CYP450 Inhibitors
CYP2B	
• Antimicrobials – Rifampin • Anticonvulsants – Phenobarbital – Phenytoin • Anti-HIV agents – Lopinavir – Ritonavir	• Antimicrobials – Ketoconazole • Anti-HIV agents – Efavirenz – Nelfinavir – Ritonavir • Antidepressants – Fluvoxamine – Fluoxetine – Paroxetine • Others – Clopidogrel – Ticlopidine – Oral contraceptives
CYP2C	
• Antimicrobials – Rifampin • Anticonvulsants – Barbiturates – Carbamazepine • Anti-HIV agents – Ritonavir • Others – St John's wort (chronic use)	• Antimicrobials – Trimethoprim – Sulfamethoxazole – Chloramphenicol – Anastrozole – Fluconazole – Ketoconazole – Voriconazole – Isoniazid • Anticonvulsants – Valproic acid • Anti-HIV agents – Efavirenz • Antiarrhythmic agents – Amiodarone • Glitazones • Antidepressants – Fluoxetine • H₂-antagonists/proton pump inhibitors – Omeprazole/esomeprazole – Cimetidine • Lipid-lowering agents – Gemfibrozil – Fenofibrate – Fluvastatin • Others – Oral contraceptives – Tamoxifen – Ticlopidine – 5-Fluorouracil

TABLE 9.1 (*continued*)

Examples of CYP450 Inducers	Examples of CYP450 Inhibitors
CYP2D	
• Antimicrobials – Rifampin	• Anti-HIV agent – Ritonavir • Antiarrhythmic agents – Quinidine – Amiodarone • Antidepressants/antipsychotic agents – Clomipramine – Fluoxetine – Fluvoxamine – Haloperidol – Paroxetine – Citalopram • Others – Ticlopidine – Metoclopramide – Celecoxib – Chlorpheniramine – Methadone
CYP3A	
• Antimicrobials – Rifabutin – Rifampin – Rifapentine – Nafcillin • Anticonvulsants – Carbamazepine – Phenobarbital – Phenytoin • Anti-HIV agents – Efavirenz – Nevirapine • Others – Glucocorticoids – Pioglitazone – St John's wort	• Antimicrobials – Clarithromycin – Erythromycin – Doxycycline – Ciprofloxacin – Troleandomycin – Telithromycin – Fluconazole – Itraconazole – Miconazole – Ketoconazole – Voriconazole • Antiarrhythmic agents – Amiodarone – Diltiazem – Verapamil • Anti-HIV agents – Delavirdine – Indinavir – Ritonovir – Saquinavir – Nelfinavir • Others – Grapefruit juice – Fluvoxamine – Mifepristone – Nefazodone – Echinacea

would in turn influence the metabolism of such drugs. Hepatic clearance for restrictively metabolized drugs that have low protein binding would be less affected by changes in protein binding. If the drug is not highly bound, the hepatic clearance after drug administration depends primarily on the hepatic intrinsic clearance of unbound drug, regardless of the route of administration. Conversely, hepatic clearance is significantly increased in highly protein-bound drugs, which are restrictively metabolized when they are displaced from their protein binding sites. Hepatic clearance then depends on the extent of protein binding and the intrinsic clearance of the unbound drug. However, free drug concentrations at steady state will remain unchanged if there is no change in hepatic intrinsic clearance. As drug effects are related to their unbound concentrations, patients are at toxicity risk for only a brief period in pure displacement-type drug interactions and dose adjustments are not needed. Although alterations in protein binding would change total drug concentrations, unbound drug concentrations should remain constant if hepatic clearance of unbound drug is unchanged. Drug levels should be measured as unbound concentrations, rather than as total concentrations, in order to avoid inappropriate dose adjustments or increases in the clinical setting. If unbound drug concentrations remain unchanged, dosage adjustment may not be warranted. Hepatic disease and drug interactions that affect drug-metabolizing enzymes (e.g., by inhibition or induction) would alter the intrinsic clearance of restrictively eliminated drugs. Dosage adjustments for low extraction ratio drugs administered either intravenously or orally would be necessary only when hepatic disease or drug interactions alter the hepatic intrinsic clearance of unbound drug. Expectedly, changes in liver blood flow are not expected to significantly affect total or unbound concentrations of a low extraction drug. The hepatic clearance of these compounds is not rate limited by liver blood flow.

Conversely, hepatic clearance is nonrestrictive in drugs that have high extraction ratios ($E_H > 0.7$). As the liver can extract these drugs as rapidly as they are presented to the organ, hepatic clearance is rate limited by liver blood flow. These drugs (e.g., propranolol) that are efficiently extracted by the liver have short elimination-phase half-lives and are thus sensitive to changes in hepatic blood flow, which has a major effect on their hepatic clearance. Intravenous administration of drugs with high extraction ratios may require dosage adjustments when disease or drug-induced alterations in hepatic blood flow occur. Alterations in protein binding will not change total hepatic clearance for drugs that exhibit a high extraction ratio, but they rather change the hepatic clearance of unbound drug and alter unbound drug concentrations. Dosage adjustment may be necessary to maintain the same unbound drug concentrations, even though total drug concentrations remain constant. As mentioned previously, drug levels in the clinical setting should be measured as unbound concentrations rather than as total concentrations, and dose adjustments should be made based on unbound concentrations. Changes in the hepatic intrinsic clearance of unbound drug do not alter total or unbound concentrations of a high extraction ratio drug significantly after intravenous administration because their hepatic clearance is rate limited by liver blood flow. Orally administered nonrestrictively metabolized drugs have extensive first-pass metabolism, which reduces their bioavailability, where only a small fraction of the absorbed dose reaches the systemic circulation (e.g., propranolol). Theoretically, a small change in the first pass of efficiently extracted drugs may alter systemic bioavailability markedly. In general, oral doses of such drugs do not need adjustments in response to changes in hepatic blood flow.

Differential effects of reduced liver blood flow and decreased enzyme activity make it theoretically possible for the elimination of one drug to be abnormal, while another drug is handled virtually normally in liver disease.

The activity of several drug-metabolizing enzymes (e.g., cytochrome P450, *N*-acetyltransferase, thiopurine methyltransferase) is influenced by genetic polymorphisms or mutations, which may result in more rapid or slower drug metabolism than the average population. For details on the genetic influence on drug metabolism and disposition, refer to the chapter titled *Clinical Implications of CYP Induction-Mediated Drug–Drug Interactions* in this encyclopedia.

9.2.4 Bile Formation and Biliary Excretion

Hepatocytes secrete bile at a constant rate into the bile canaliculi, which drain into the interlobular bile ducts. Few drugs are excreted, unchanged, into bile. Bile excretion requires the substance to (i) cross the hepatic sinusoidal endothelium and (ii) cross both the luminal and canalicular membrane surfaces of the hepatocyte (Fig. 9.2). Efficacy of the active transport mechanisms that are required for passage across these two hepatocyte membrane surfaces is determined by the chemical structure, polarity, and molecular weight of the substance. Bile, an aqueous solution, favors excretion of more water-soluble polar compounds [8]. Larger polar compounds with a molecular weight of 500–600 Da are excreted in bile, whereas smaller compounds with a lower molecular weight are eliminated by the kidneys. Compounds that enhance bile production would stimulate the excretion of drugs eliminated by this route, while compounds that inhibit bile flow or production will decrease the excretion of such drugs [9]. Orally administered drugs can be extracted by the liver and excreted into bile to a greater degree than with intravenous administration. Interlobular bile ducts join together to form progressively larger ducts, the right and left hepatic ducts and, eventually, the common hepatic duct. The common hepatic duct and the cystic duct from the gallbladder form the common bile duct, which delivers bile to the duodenum. Drugs that are excreted via bile enter this enterohepatic circulation where they are excreted into the small intestine and are reabsorbed back into the portal system either as the parent drug or as its metabolite [8]. As the intestinal mucosa also possesses metabolizing enzymes, the compound that is excreted into bile may or may not be the same as that reabsorbed via the enterohepatic circulation. In other words, a drug's metabolite or conjugate may be excreted into bile but should be hydrolyzed or converted by gut enzymes before systemic reabsorption. This increases a drug's bioavailability and prolongs its elimination phase (e.g., ursodeoxycholic acid, benzodiazepines, digoxin). The enterohepatic circulation also increases the total exposure of the gut mucosa to drugs with potential gastrointestinal toxicity (e.g., NSAIDs).

9.2.5 Physiological Changes in Hepatic Drug Metabolism

9.2.5.1 The Young. Fetal and newborn livers generally have reduced xenobiotic metabolism capacity, even though detectable smooth endoplasmic reticulum and monooxygenase activity have been found as early as the week 6 of gestation [10–13]. Multiple studies have been performed to evaluate P450 content of the liver, but there have been no direct studies evaluating the effects of hepatic blood flow on infants.

Although cytochrome P450 enzymes have been isolated from fetal liver [14], antibody [15–17] and mRNA [18] analyses confirm that it lacks most adult forms of P450. CYP3A7, and to a lesser extent, CYP1A1 predominate in fetal liver but are mostly absent in adult liver [19,20]. Other phase I enzymes present in fetal liver include flavin-containing monooxygenases that increase rapidly during fetal development [21]. Human fetal liver also has phase II GSH transferase activity [22,23], which is present mainly as an acidic isoform that declines with fetal development and is only weakly expressed at birth. The adult enzyme exists in several basic forms instead [24]. However, a rapid turnover results in low GSH levels in the fetal liver [25].

Newborns have ~30% of the adult levels of cytochrome P450. The activities of these enzymes rise two to three times over adult levels the first month after birth and remain high for several years [11,12]. Although there is interindividual and interenzyme variability in the development of hepatic enzymes, they approach at least adult levels by the first year of life. The capacity to metabolize CYP3A4 substrates is reduced below the age of 15 years, and this is especially pronounced in infants below 3 months of age [26]. Flavin-containing monooxygenases are present in the neonatal liver, mainly in the form of FMO1 and, to a lesser extent, FMO4, unlike adult human liver that contains the forms FMO3, FMO4, and FMO5 [27]. The capacity of some phase II reactions is also lower at birth. For example, delayed development of glucuronyl transferase may play a role in the pathogenesis of physiological neonatal jaundice [28,29]. Acetaminophen is predominantly cleared by glucuronidation in adults but instead by sulfation in neonates and young children.

Ontogeny and drug metabolism in the young are covered in greater detail in the chapters titled *Idiosyncratic Drug Reactions* and *Drug Metabolism and Interactions in Pediatric Patients* in this encyclopedia.

9.2.5.2 The Elderly. Elderly patients are also more likely to be on polypharmacy, increasing their risk of drug–drug interactions or drug–enzyme interactions.

The plasma half-lives of drugs are generally increased in old age [30]. Possible explanations include changes in the volume of distribution, decreased hepatic blood flow, decreased rates of drug metabolism, and decreased renal clearance [30]. The result of this decrease in drug biotransformation is decreased metabolic clearance and increased exposure of the individual to the drug at a given dose. This may predispose the elderly to medication toxicity and even hepatotoxicity with hepatotoxins.

Phase I biotransformations catalyzed by CYP3A in the elderly have most consistently been shown to be decreased, by up to 40%. Prototype CYP3A substrates that exemplify this are midazolam and triazolam [31,32]. Hence, older patients treated with a given dose of triazolam will experience increased sedative effects [33]. It is unknown if CYP3A activity in the gastrointestinal mucosa is affected by aging as well [34]. However, some studies of human tissue have not demonstrated age-related reductions in the levels of NADPH P450 reductase, cytochrome P450 isoforms 3A2 and 2C8, or glutathione [35,36], suggesting that P450 declines with age may be restricted to only certain forms. A decline in P450 forms 1A2 and 2C with advancing age has been reported in humans, regardless of gender [37]. Modest decreases in clearance of warfarin (CYP2C9), phenytoin (CYP2C19), theophylline (CYP1A2), and caffeine (CYP1A2) have been reported in older individuals [38–41].

Age does not affect the frequency of genetic polymorphism in hepatic enzymes.

Enzyme induction is generally considered to be normal in the elderly [30]. However, the enzyme induction by rifampicin and cigarette smoking that is seen in younger adults is not seen in the elderly. This suggests that enzyme induction may be impaired in the elderly, although this has not been a consistent finding.

Age has not been shown to alter phase II biotransformations. Prototype substrates studied include lorazepam (glucuronidation), oxazepam (glucuronidation), isoniazid (acetylation), procainamide (acetylation), and acetaminophen (sulfation) [42].

9.2.5.3 Nutritional Status. Nutritional deficiencies [43] and severe malnutrition can have major effects on drug metabolism [44]. Short fasting periods of up to seven days have not been shown to affect the metabolic clearance of most drugs [45]. However, theophylline clearance, which is dependent on CYP1A2, is reduced by a protein-deficient diet [43]. Another exception is glutathione *S*-transferase reactions that may be decreased with fasting, as demonstrated in rat studies [46] and human subjects with anorexia nervosa [47]. Malnutrition is also known to deplete glutathione stores and this may have important clinical implications. For example, such patients would be at greater risk of acetaminophen hepatotoxicity from metabolism via the CYP2E1 pathway, forming the toxic metabolite *N*-acetyl-*p*-benzoquinoneimine.

9.2.5.4 Pregnancy. Hormonal changes during pregnancy involve marked increases in a woman's estradiol and progesterone levels [48–50]. These changes can affect hepatic enzymatic activity and, consequently, drug elimination clearance.

Some phase I (i.e., CYP1A2) and II activities (i.e., *N*-acetyltransferase) have been found to be reduced in late pregnancy [51,52]. Caffeine elimination via CYP1A2 is decreased by half by midgestation and by three times by the third trimester as compared to the postpartum period [53]. Intrinsic hepatic clearance of theophylline via CYP1A2 is also reduced during pregnancy. However, its associated decrease in drug binding to plasma proteins during pregnancy results in less of a change in its hepatic clearance [54]. Furthermore, as a result of the changes in both the renal and hepatic clearance, the total elimination clearance of theophylline is unchanged in the third trimester of pregnancy. A population-based study has demonstrated that CYP2C19-dependent clearance of proguanil (an antimalarial) decreased by 50% [55]. The ratio of proguanil to cycloguanil (active metabolite of proguanil) is increased by as much as 60% during pregnancy due to this decreased metabolism [56]. Phase II caffeine metabolism via *N*-acetyltransferase has been demonstrated to decrease with pregnancy, as compared to the normal healthy nonpregnant state [52,57].

In contrast, other forms of P450 (i.e., CYP3A4, CYP2D6) may be induced [58,59]. Both estradiol and progestins have been found to activate the human orphan nuclear pregnane X receptor (PXR), which forms a heterodimer complex with the 9-*cis*-retinoic acid receptor (RXR). This heterodimer complex binds to the promoter region of the CYP3A4 gene and mediates its transcription [60,61]. The hepatic clearance of phenytoin (via CYP2C9) increases during pregnancy, resulting in correspondingly lower total plasma concentrations [62]. Decreased drug protein binding results in constant free plasma concentrations of phenytoin until late pregnancy when its intrinsic clearance increases [63,64]. Multiple studies have confirmed that CYP3A4 activity increases in pregnancy. Midazolam is exclusively eliminated by CYP3A4 metabolism [65,66] and is a recognized marker of CYP3A4 activity [67,68]. At term, the clearance of midazolam has been shown to be 2.9-fold greater in pregnant women [69]. The metabolic

clearances of cortisol [70], nifedipine [71], extended-release metronidazole [72], and methadone [73], all CYP3A4 substrates, have also been shown to be increased during pregnancy.

Lamotrigine (metabolized by glucuronidation) clearance has been shown to increase by >50% in pregnancy, necessitating dose adjustment [74]. Betamethasone (metabolized by glucuronidation) clearance has also been shown to be increased in pregnancy, and this increase has been demonstrated to be greater in twin than in singleton gestations [75].

CYP2D6 isoforms may be increased or decreased in pregnancy. CYP2D6 activity is increased during pregnancy in individuals who are homozygous and heterozygous extensive metabolizers. However, enzyme activity is decreased in homozygous poor metabolizers [76].

Serum concentration of some proteins (though not all) are altered during pregnancy. There is a decrease in albumin concentration, which is likely due to reduced albumin synthesis or increased albumin clearance. The reduction in albumin concentration can potentially alter the binding of drugs (e.g., theophylline) commonly bound to serum albumin, whereas total protein and α_1 -acid glycoprotein concentrations remain unchanged in pregnancy [77,78].

After delivery, pharmacokinetic values return to near normal values within days to weeks for most drugs.

Further details on metabolism and drug–drug interactions in pregnancy are covered in the chapter titled *Influence of Changes in Physiology, Transporters, and Enzyme Expression on Disposition and Metabolism of Drugs during Pregnancy and Clinical Implications* in this encyclopedia.

9.3 LIVER DISEASE AND DRUG METABOLISM

The liver is vulnerable to a wide variety of insults, but the repertoire of its response to injury is limited. Regardless of cause, five general responses are seen: hepatocyte degeneration, necrosis or apoptosis, inflammation, regeneration, and fibrosis. Likewise, the functional consequences of liver disease on drug disposition are determined by the extent of the injury, as opposed to its etiology. Although the liver's enormous functional reserve can mask the clinical impact of early liver damage, liver disease can ultimately produce multiple pathological consequences.

Liver diseases may alter drug absorption, disposition, and pharmacology. Drug dosing in patients with liver disease requires the consideration of the nature and severity of the liver disease, hemodynamic factors, and the drug's pharmacokinetics. Predicting drug metabolism in patients with liver disease is challenging for a number of reasons. First, there is no test available that can correlate changes in drug metabolism with the degree of hepatic impairment. Second, pharmacokinetic or pharmacodynamic consequences of a particular disease may exhibit interindividual or even intraindividual variability. Third, various hepatic factors that influence drug metabolism may be independently or even codependently altered in liver disease. Thus, no universal rule can be applied to the modification of drug dosages in patients with liver disease. This is why it can be difficult to ascertain whether certain drugs in clinical trials have inherent hepatotoxicity and why this is discovered after some drugs have come into the market.

Biochemical "liver function" tests, which are routinely employed in the clinical setting, are surrogate markers of specific hepatic functions (e.g., serum albumin reflects

protein synthesis) or of pathologic conditions (e.g., serum aminotransferase elevation reflects hepatocyte damage). These tests indicate the potential for qualitative pharmacokinetic changes, although they lack sufficient discrimination to be consistent predictors of impaired drug metabolism [79].

9.3.1 Parenchymal Disease

The etiology of acute hepatitis is myriad, ranging from viruses to hepatotoxins to autoimmune or metabolic causes. The plasma half-lives of many drugs are prolonged in patients with parenchymal liver disease [80] as a result of reduced hepatic drug metabolism, which may be due to various causes apart from altered enzyme activity alone. For example, decreased availability of NADPH may be caused by altered cellular energy metabolism, cholestasis, altered hepatic blood flow, loss of hepatocyte volume, and so on. Directly assaying enzyme activity from biopsy specimens may help in identifying the primary contributing factors [81]. Studies have found the concentration levels of P450 in moderate degrees of hepatitis and compensated cirrhosis to be normal [82].

Studies of adult patients with acute hepatitis indicate that the changes in drug disposition are variable and related to the extent of damage incurred. Mild disease may have no alterations, while severe disease may have significant alterations in drug disposition.

In acute viral hepatitis, drug elimination is usually normal or only modestly impaired. Observed changes tend to be variable and related to the extent of hepatocellular damage incurred [83]. If the acute episode of hepatitis is short-lived, drug disposition can return to normal. As the acute disease resolves, drug disposition returns to normal and may do so before normalization of the biochemical liver function tests [84].

In acute drug-induced liver injury, however, altered drug metabolism can precipitate further injury as the elimination of other potentially toxic compounds is impaired. For example, the elimination phase of acetaminophen is significantly prolonged in patients with hepatotoxicity (mean, 5.8 h; range, 4.3–7.7 h) and in patients with hepatorenal toxicity (mean, 7.7 h; range, 4.3–13.9 h), as compared to patients without evidence of liver impairment (mean, 2.7 h) [85]. Fatal outcome has been associated with acetaminophen elimination half-lives of more than 10 h.

Chronic liver disease may be caused by chronic viral hepatitis, autoimmune or metabolic causes, or chronic alcohol abuse. In alcoholic liver disease, fat accumulation within the hepatocyte leads to steatosis and overall hepatomegaly. Although cytochrome P450 content per weight tissue is decreased, drug metabolism is not impaired in early disease as this is compensated for by the increased liver size [86]. A study conducted in liver grafts discarded for transplantation because of >40% macrosteatosis demonstrated a reduction in microsomal P450 activities [87]. In chronic viral hepatitis, drug elimination may be impaired, although this usually occurs much later in the disease [88]. Patients with chronic viral hepatitis may also have extrahepatic involvement of disease, including glomerulonephritis with renal impairment [89–91]. In this case, drugs that depend on the kidneys for elimination will have a prolonged elimination half-life, increasing the body's exposure to the drug. Such drugs (e.g., nucleos[t]ide analogs used for chronic hepatitis B treatment) will need to be dose adjusted to the appropriate renal function.

Induction of P450 enzymes may be affected by liver disease. However, in mild hepatic disease and inactive cirrhosis, normal induction of P450 by drugs such as

phenobarbital, phenytoin, and glutethimide has been observed. Overall, changes in drug disposition tend to be less in acute hepatitis than in chronic liver disease [92].

9.3.2 Alcohol and Drug Metabolism

Chronic alcoholism is a common cause of liver disease, which may lead to abnormal drug disposition. Alcohol may alter drug absorption by changing its solubility, increasing intestinal blood flow, inhibiting gastric emptying, producing conformational changes in intestinal membranes, and affecting drug-metabolizing enzymes in the liver. In general, acute alcohol ingestion depresses, while chronic alcohol ingestion enhances, the rate of drug metabolism. The potential for pharmacokinetic drug interactions with alcohol is great because the same cytochrome P450 enzymes also metabolize many other drugs.

9.3.2.1 Acute Alcohol Ingestion. Acute alcohol ingestion may inhibit the activity of drug-metabolizing enzymes through different mechanisms.

First, alcohol may compete with the drug for the same microsomal system required for metabolism [93]. Drugs and alcohol may share a similar microsomal ethanol-oxidizing system. Acute alcohol ingestion inhibits this system, whereas chronic alcohol ingestion increases it [94,95]. Alcohol also inhibits cytochrome P450 reductase, a rate-limiting step in the oxidative biotransformation of drugs [96]. The breakdown and excretion of affected drugs are delayed because the drug must compete with alcohol for breakdown by cytochrome P450. This interaction has been described mostly for CYP2E1, but it may also involve CYP3A4 and CYP1A2 [97]. Second, alcohol may inhibit drug metabolism by competing with or displacing the other compound from its cytochrome P450 binding site [98], or indirectly decreases the availability of NADPH by inhibiting the citric acid cycle [99]. Third, alcohol induces the release of corticosterone that may also serve as an alternative substrate for the microsomal enzyme system, resulting in indirect inhibition of the drug-metabolizing system [100]. Fourth, alcohol may disturb the lipid bilayer membrane of the drug-metabolizing system, increasing the fluidity of hepatic microsomal membranes, causing a conformational change in the enzyme [101–103].

Alcohol also has an influence on phase II reactions. Acute alcohol ingestion impairs glucuronidation [104–107]. Intracellular nicotinamide adenine dinucleotide (NAD^+) and reduced NAD^+ (NADH) are needed for the function of many enzymes. Oxidative alcohol metabolism by alcohol dehydrogenase results in the conversion of NAD^+ into NADH, increasing the hepatocyte's NADH levels. Elevated NADH levels may stimulate steatosis and prevent the liver from generating UDP-glucuronic acid for conjugation. Heavy alcohol consumption reduces the amount of glutathione in liver cells, particularly in the mitochondria. Acute alcohol ingestion enhances acetylation by 20% [108].

9.3.2.2 Chronic Alcohol Ingestion. Chronic alcohol ingestion increases both microsomal mass [109] and activity of the monooxygenase system [110–113]. When alcohol is not present to simultaneously compete for the cytochromes, increased cytochrome activity results in an increased elimination rate for drugs metabolized by these enzymes. This may predispose a chronic alcoholic with acetaminophen overdose to hepatotoxicity via the formation of *N*-acetyl-*p*-benzoquinoneimine by the CYP2E1 pathway.

Chronic alcohol ingestion induces glucuronyl transferase [114] and its action of glucuronidation. The effect of chronic alcohol ingestion on acetylation has not been evaluated.

9.3.2.3 Pharmacodynamic Effects with Alcohol. The concomitant acute ingestion of ethanol and other agents with sedative action may result in greater psychomotor impairment than that produced by each agent independently. First, the more profound effect may simply reflect the combined action of both sedative drugs on the central nervous system. Second, the sedative compound may itself alter the metabolism of ethanol. For instance, chloral hydrate, which is metabolized to trichlorethanol by alcohol dehydrogenase, vies with ethanol for the same pathway and results in higher plasma concentrations of ethanol [115–117]. However, a number of other sedatives [118], including pentobarbital, meprobamate, and the benzodiazepines clobazam [119] and clorazepate [120], do not disturb the metabolism of ethanol. Third, ethanol may impair the degradation of other sedative compounds (e.g., lorazepam, diazepam).

9.3.2.4 Drug Disposition and Elimination with Alcohol

9.3.2.4.1 Benzodiazepines. Short-term alcohol ingestion diminishes the hepatic clearance of a number of widely used benzodiazepines [121–123], without an effect on drug binding or distribution. With impaired biotransformation of benzodiazepines, more drug may reach the cerebral receptor sites, resulting in a greater sedative effect. The effect of chronic alcohol ingestion on benzodiazepine metabolism is less well established. After chronic ethanol intake and during subsequent ethanol withdrawal, plasma concentrations on day 2 of withdrawal were higher than those on day 6 [124], which suggests decreased (not increased) drug elimination. Sellman *et al.* [125] showed that the plasma concentrations of diazepam after an oral dose were lower than those in control; this implies decreased absorption or increased clearance.

9.3.2.4.2 Chloral Hydrate. Chloral hydrate shares a common metabolic pathway with ethanol and is rapidly converted by alcohol dehydrogenase to trichlorethanol. Acute ethanol ingestion results in significantly higher levels of trichlorethanol and lower levels of its resultant glucuronide metabolite in the plasma and urine. In turn, the conversion of ethanol to acetylaldehyde is diminished and results in higher plasma ethanol concentrations [115]. These result in the greater sedative effect seen following combined alcohol and chloral hydrate administration.

9.3.3 Cholestatic Disease

Cholestasis may occur as a result of cellular transporter defects (such as mutations of transporter genes or acquired dysfunction of transport systems) or from biliary obstruction [126]. Bile salts not able to exit the hepatocyte in cholestasis are effectively removed across the basolateral membrane [127]. Studies have demonstrated up to 70% reduction of cytochrome P450 levels in liver biopsy specimens of subjects with obstructive liver disease, correlating with altered antipyrine clearance [128]. CYP1A2 and 2E1 have specifically been found to be reduced in cholestatic disease [129,130]. The findings in the setting of extrahepatic cholestasis have been conflicting. The elimination of drugs that are excreted mainly via bile is decreased in any cause of cholestasis

[9]. The bioavailability of such drugs is reduced due to the disruption of enterohepatic cycling.

Furthermore, drugs such as cholestyramine (frequently used in patients with liver disease such as primary biliary cirrhosis) that are bile sequestrants would interfere with the pharmacokinetics of drugs that undergo significant enterohepatic circulation. Conversely, the discontinuation of cholestyramine would potentiate drug toxicity (e.g., digitalis) if it had been titrated to a maintenance dose while the patient was taking cholestyramine. Cholestyramine also acts as an ion-exchange resin and reduces the absorption of concomitant oral medication such as warfarin, thiazide diuretics, propranolol, penicillin, phenobarbital, and digitalis.

9.3.4 Gilbert's Syndrome

Gilbert's syndrome is the most common inherited disorder of bilirubin glucuronidation. It has also been called *constitutional hepatic dysfunction* or *familial nonhemolytic jaundice* [131]. UGTs mediate the glucuronidation of various compounds. A mutation in the promoter region of the gene encoding bilirubin-UGT that conjugates bilirubin to glucuronic acid (UGT1A1) results in reduced production of bilirubin-UGT [132]. The inheritance of Gilbert's syndrome is most similar to that of an autosomal recessive trait.

As UGT1A1 is involved in the glucuronidation of estrogen, drugs, and carcinogens, individuals with Gilbert's syndrome may be more susceptible to the toxic effect of substances (e.g., acetaminophen, tolbutamide) that require UGT1A1-mediated hepatic glucuronidation before excretion [133,134]. Diminished drug excretion may cause their accumulation and increase toxicity. However, consequences of clinical significance have not been reported for most drugs. For example, intravenous, but not oral, administration of acetaminophen demonstrated reduced glucuronidation of the drug in subjects with Gilbert's syndrome [133,135,136]. While, the anticytotoxic agent irinotecan is glucuronidated in the liver mainly by bilirubin-UGT and excreted in bile. In subjects with UGT1A1 polymorphisms (e.g., Gilbert's and Crigler–Najjar syndrome), reduced glucuronidation of its active metabolite results in increased toxicity (e.g., diarrhea, severe neutropenia) [137–140].

Drugs that increase the hepatic uptake of bilirubin (e.g., corticosteroids), or hepatic enzyme inducers (e.g., phenobarbital, clofibrate), may reduce plasma bilirubin concentrations in patients with Gilbert's syndrome [141–144].

Unlike in Gilbert's syndrome where a defect in the promoter region results in reduced amounts of the normal protein produced, in Crigler–Najjar syndrome (autosomal recessive inheritance), mutations in UGT1A1 gene itself result in abnormal protein production, resulting in complete loss or severely low levels of UGT1A1 activity [145]. Crigler–Najjar syndrome type I was first described in 1952 [146]. It is usually diagnosed in infancy, and its clinical course is characterized by the development of progressive, permanent neurologic sequelae resembling kernicterus. Death usually occurs in the first two years of life. Patients who survive longer often have severe neurologic impairment [147,148]. Necropsy studies of such patients demonstrate massive bilirubin deposition in various organs, including the cerebral cortex and other structures of the central nervous system, and neuronal loss [146,149]. Total plasma bilirubin levels range from 15 to over 50 mg/dL. Type II disease is diagnosed later in childhood, and patients may survive into adulthood without permanent neurological impairment.

Further details and clinical relevance of the UGT enzymes are covered in the chapters titled *UDP-Glucuronosyltransferases: Pharmacogenetics, Functional Characterization, and Clinical Relevance* and *Role and Clinical Consequences of Human UDP-Glucuronosyltransferases* in this encyclopedia.

9.4 CIRRHOSIS AND DRUG METABOLISM

Cirrhosis is the final common pathway for chronic liver disease. It is characterized by the activation of fibroblasts and collagen deposition with nodular formation and resultant portal hypertension as the disease progresses.

There are several tools to stage the severity of liver cirrhosis, the most commonly used in clinical practice being the Child-Turcotte-Pugh (CTP) scoring [150,151], which ranges from a minimum score of 5 to a maximum score of 15 (Table 9.2). The Child-Pugh classification of disease severity is often used to make drug dosing recommendations for patients with cirrhosis [152]. However, it has several limitations, including poorer discrimination in early cirrhosis, a maximum cutoff score which scores all severe cirrhotic patients equally, and the fact that other factors apart from those included in the CTP scoring system may help to stratify the degree of hepatic impairment or its prognosis. The Model for End-Stage Liver Disease (MELD) [153] is a validated chronic liver disease severity scoring system [154,155], which incorporates the patient's serum bilirubin, serum creatinine, and the international normalized ratio for prothrombin time to predict survival. MELD has been modified slightly since its original development, eliminating negative scores and disease etiology. An increasing MELD score is associated with increasing severity of hepatic dysfunction and risk of death [156]. This model is also currently used by the United Network for Organ Sharing (UNOS) and in many transplant centers around the world in prioritizing allocation of deceased donor organs for LT. Several online calculators are available for ease of calculating the MELD score (e.g., <http://www.mayoclinic.org/meld/mayomodel6.html>. Accessed 5 May, 2010).

Unfortunately, most clinical trials only enroll patients with mild or moderate liver disease. Caution should be taken in extrapolating these findings to patients with liver diseases of different etiology or degrees of severity. Unfortunately, there is little data

TABLE 9.2 Child-Turcotte-Pugh Scoring in Liver Cirrhosis

Parameters	Points Assigned		
	1	2	3
Serum albumin (g/dL)	>3.5	2.8–3.5	<2.8
Serum bilirubin (mg/dL)	1–2	2–3	<3
Prothrombin time (seconds above control)	1–4	4–10	>10
Ascites	Absent	Slight	Moderate
Encephalopathy grade	0	1–2	3–4
Child-Turcotte-Pugh classification	A (mild)	B (moderate)	C (severe)
Total number of points	5–6	7–9	>9

Source: Adapted from Pugh RN *et al.* [151]. Reprinted with permission. Copyright John Wiley and Sons 1973.

available for patients with severe liver disease, in whom pharmacokinetic and pharmacodynamic changes would be the most pronounced.

Cirrhosis affects drug metabolism more than any other form of liver disease by various mechanisms, some of which are detailed below.

9.4.1 Vascular Architecture and Hepatic Blood Supply in Cirrhosis

Fibrosis is, generally, an irreversible consequence of hepatic damage. In the initial stages, fibrosis may develop around portal tracts or central venules, or it may be deposited directly within the space of Disse (Fig. 9.3). In the normal liver, interstitial collagens (types I and III) are concentrated in the portal tracts and around the central veins, while type IV collagen courses alongside the hepatocytes in the space of Disse. The central pathogenic process in cirrhosis is progressive fibrosis. In cirrhosis, types I and III collagen are deposited in the lobule, creating delicate or broad septal tracts. Activated hepatic stellate cells, which lie in the space of Disse, transform into myofibroblast-like cells and are the major source of excess collagen in cirrhosis. When collagen is deposited in the hepatic sinusoids [86], it forms a basement membrane devoid of microvilli along the sinusoidal surface of the hepatocyte. New vascular channels in the septa connecting the portal veins with the central veins, the altered sinusoidal membrane, and the collagen barrier between the hepatocyte and sinusoid all result in functional shunting of blood past the hepatocyte. Intrahepatic shunting thus results from intrahepatic vascular anastomoses that bypass hepatic sinusoids and from the functional sinusoidal barrier caused by collagen deposition. This shunting interferes significantly with the hepatic uptake of oxygen, plasma constituents, drugs, and metabolites. Anatomic and functional intrahepatic shunting averages 25% of total liver blood flow in normal subjects but is increased to 33% in patients with chronic viral hepatitis and to 52% in cirrhotic patients [157].

The deposition of fibrous bands also disrupts the normal hepatic vascular architecture, increasing vascular resistance and portal venous pressure. Transformation of stellate cells into myofibroblasts also increases vascular resistance within the liver parenchyma by tonic contraction, which constricts the sinusoidal vascular channels. The net outcome is a fibrotic, nodular liver in which delivery of blood to hepatocytes is severely compromised.

When extreme amounts of fibrous tissue develop within the liver structure, it causes destruction of the parenchymal cells and it eventually contracts around the blood vessels, impeding the flow of portal blood through the liver. Portal venous flow that accounts for 70% of the normal total liver blood flow is reduced [158], but in the early stages, this decrease is compensated for by an increase in hepatic arterial supply to maintain a normal hepatic blood supply despite chronic viral hepatitis or cirrhosis [157]. This highlights the unique and important feature of the liver's dual blood supply. This increased resistance to portal flow at the level of the sinusoids and compression of the central veins by perivenular fibrosis and expansile parenchymal nodules result in the portal hypertension of cirrhosis. Anastomoses between the arterial and portal systems in the fibrous bands also contribute to portal hypertension by imposing arterial pressure on the low pressure portal venous system [5]. The increased portal pressure results in the compensatory formation of portosystemic shunts. Common sites of portosystemic collaterals include, but are not limited to, the gastroesophageal junction, splenic hilum, and umbilicus. In a study of cirrhotic patients with bleeding esophageal

varices, an average of 70% of mesenteric and 95% of splenic blood flow was found to be diverted through extrahepatic shunts [159]. Portocaval shunting can impair the efficiency of hepatic extraction and reduce the extraction ratio of drugs.

9.4.2 First Pass and Bioavailability in Cirrhosis

The bioavailability of orally administered high extraction drugs in patients with cirrhosis may be increased significantly because of a decreased first-pass effect, especially in patients who exhibit significant portosystemic shunting with decreased intrinsic clearance [160]. Oral dosing of high extraction drugs will result in high plasma concentrations. Coupled with abnormal clearance from hepatic dysfunction, this may produce prolonged pharmacologic effects with potential toxic consequences. This may be dangerous, especially for drugs with a low therapeutic index. Propranolol [161,162], labetalol [163], lidocaine [164], salicylamide [165], meperidine [166], pentazocine [165,166], and nicardipine [160] are examples of drugs having markedly increased bioavailabilities when administered to cirrhotic patients.

The clearance of highly extracted drugs is more greatly affected in patients having a high degree of portosystemic shunting, whereas low extraction drugs are more sensitive to changes in intrinsic clearance [80,167]. As hepatic blood flow and perfusion are reduced in patients with advanced cirrhosis, hepatic clearance is reduced and the bioavailability is increased for highly extracted drugs to the extent that it no longer approximates hepatic blood flow but is influenced by hepatic intrinsic clearance instead [168]. This increased bioavailability, which is accompanied by decreased elimination for some drugs (e.g., propranolol [161,162]), further increases the body's exposure to the drug. Hence, portosystemic shunting and hepatocellular damage significantly increase the bioavailability of drugs that would normally have an extensive first pass. The clearance of high extraction drugs is more consistently decreased in chronic liver disease than that of low extraction drugs [167]. Portocaval shunting has less impact on drug bioavailability of restrictively metabolized drugs that have minimal first-pass metabolism even in normal subjects.

9.4.3 Protein Binding and Volume of Distribution in Cirrhosis

Cirrhotic patients have a hypoproteinemic and hypoalbuminemic state that may reduce drug binding to plasma proteins [169]. Also, bilirubin and bile acids accumulate in cirrhosis and may displace drugs from their protein binding sites. The volume of drugs, especially those that are highly protein bound (>90%), may be increased in patients with chronic liver disease who exhibit hypoalbuminemia or ascites [80,170,171].

For restrictively metabolized drugs, hepatic clearance is increased with reduced protein binding. The effect of hepatic clearance or metabolism of a drug in cirrhosis depends on the net result of changes for various factors. For example, liver disease may decrease the intrinsic clearance of drugs with low intrinsic clearance, which are highly protein bound, yet increase the fraction of unbound drug by reducing protein binding. However, unbound drug concentrations will not be affected by decreases in the protein binding of restrictively metabolized drugs. Therefore, no dosage alterations are required for these drugs if protein binding is the only parameter that is unchanged. Liver disease generally produces no change in warfarin clearance, decreases diazepam clearance, and increases tolbutamide clearance.

Conversely, for nonrestrictively metabolized drugs, reduced protein binding does not affect the clearance or total plasma concentration, but will increase the drug's free concentration. This potentially increases the pharmacological effects observed at a given total drug concentration [169]. For such drugs, reduced protein binding increases their distribution volume and, hence, their elimination-phase half-life and the time required to reach steady state. For example, cirrhosis reduces verapamil clearance by 50% yet increases the drug's volume of distribution, resulting in a significant increase in its half-life (from 3.7 to 14.2 h) [172]. This extends both the time to reach steady state and the time to eliminate the drug from 12 to 45 h. Such alterations in pharmacokinetics have also been described with lorazepam [173] and chloramphenicol [174] in cirrhotic patients. Therefore, in nonrestrictively metabolized drugs, a reduction in plasma protein binding necessitates a corresponding reduction in drug dosage.

9.4.4 Hepatic Enzymes in Cirrhosis

As cirrhosis progresses, extensive fibrosis causes a reduction in liver size, resulting in a marked reduction of total P450 concentrations [82,175,176]. This limits and decreases drug metabolism and hepatic clearance of most drugs. However, not all P450 activities are decreased uniformly in severe liver disease; for example, ethylmorphine demethylase activity is unaltered [177,178]. In the cirrhotic liver, the levels of CYP isoforms 1A2, 2E1, and 3A4 are reduced [130,179]. The clearance of *S*-mephenytoin, a CYP2C19 probe, was shown to be decreased by 63% in patients with mild cirrhosis and by 96% in patients with moderate cirrhosis, whereas debrisoquine clearance (CYP2D6 probe) was normal [180]. Although CYP2C activity is barely altered with hepatocellular disease, it has been shown to be markedly reduced in patients with cirrhosis due to cholestatic disease [130]. Commonly used drugs which may have altered metabolism in this setting include theophylline (CYP1A2), alcohol (CYP2E1), acetaminophen (CYP2E1), calcineurin inhibitors (CYP3A4), HMG-CoA reductase inhibitors (CYP3A4), and warfarin (CYP2C9). NADPH-cytochrome P450 reductase activity is normal even in severe liver disease [130].

However, P450 levels do not always correlate well with half-lives for the clearance of some drugs [177] and some commonly used drugs in cirrhotic patients should have their doses reduced by half (Table 9.3). Most of these drugs have high first-pass metabolism, which is markedly reduced with impaired liver function. Decreased elimination half-lives further increase drug exposure to standard doses. Active metabolite formation also complicates drug response and dose adjustments. For example, losartan has an active metabolite that is primarily responsible for the extent and duration of its pharmacological effects [181]. Standard doses of losartan in cirrhotic patients increase plasma concentrations of the parent drug by four to five times but increase the plasma concentration of its active metabolite by only 1.5–2 times [182,183].

Generally, the clearance of drugs that undergo only conjugation is not altered significantly in patients with severe liver disease, but this remains controversial [184,185]. Analysis of human liver biopsy specimens reveals minimal change in bilirubin-glucuronidating capacity in hepatitis or cirrhosis [186–188]. Although there is evidence that this pathway is compromised in severe liver disease [189], increased enzyme activity in remaining viable hepatocytes preserves overall glucuronidating capacity in cirrhotic patients [190]. Hence, drugs that only undergo glucuronidation for elimination (e.g., oxazepam and lorazepam) are unaffected by liver disease

TABLE 9.3 Common Drugs with Reduced Clearance in Cirrhotic Patients

β -Blockers	Anesthetics/sedatives
• Propranolol	• Diazepam
• Metoprolol	• Hexobarbital
Calcium channel blockers	• Chlordiazepoxide
• Verapamil	• Lidocaine
• Nifedipine	• Encainide
Antibiotics	• Morphine
• Chloramphenicol	• Tocainide
• Erythromycin	• Pentazocine
	• Meperidine
	Others
	• Acetaminophen
	• Caffeine/Theophylline
	• Omeprazole
	• Losartan
	• Antipyrine

Source: Adapted from Susla GM, Atkinson Jr AJ. Effect of liver disease on pharmacokinetics. In: Atkinson Jr AJ, Abernethy DR, Daniels CE, *et al.*, editors. Principles of clinical pharmacology. 2nd ed. MA: Academic Press, Elsevier; Copyright Elsevier 2007 & Brouwer KLR, Dukes GE, Powell JR [79]. Copyright Lippincott, Williams & Wilkins 1992.

[173,191,192]. However, studies have described well-preserved glucuronide conjugation of morphine in patients with compensated cirrhosis but a 59% reduction in decompensated cirrhotic patients with a history of hepatic encephalopathy [193,194].

9.4.5 Renal Impairment in Cirrhosis

Renal impairment is often underrecognized in the setting of cirrhosis. The fluid status in the clinical setting of cirrhosis is notoriously difficult to assess. Portal hypertension results in ascites and peripheral edema, akin to a hypervolemic state. However, this results in intravascular fluid depletion, resulting in an intravascular hypovolemic state instead. Elevated intra-abdominal pressure, as seen in ascitic patients, may influence central venous pressure measurements [195], which have also been shown to be of little value in assessing fluid responsiveness in critically ill patients [196–198]. Serum creatinine, which is the most commonly used marker of kidney injury, has diagnostic limitations. It lags behind renal injury and is a delayed marker of decreased renal function [199], it may be normal or only minimally elevated because of renal reserve or enhanced tubular secretion [200,201], and it is unable to distinguish between the various causes of kidney injury. It is also influenced by several nonrenal factors such as gender, age, race, body weight, drugs, muscle metabolism, and protein intake [200]. In the cirrhotic patient with low muscle mass, serum creatinine levels may be falsely low. Laboratories using the chemical (instead of enzymatic) method to measure serum creatinine levels result in falsely low readings in the presence of severe hyperbilirubinemia [202].

Cirrhotic patients are at risk of developing significant kidney injury for a number of reasons, including, but not limited to, prerenal azotemia and acute tubular necrosis from septicemia [203]. The hepatorenal syndrome occurs when vasodilatation of

the splanchnic circulation results in a relative “hypovolemic” state that activates pressor responses and causes marked vasoconstriction of the renal circulation [204]. This reduces renal perfusion and glomerular filtration rate significantly in these patients. The functional nature of this syndrome is reflected by its reversal following successful LT and the absence of significant renal damage on histology. The risk of developing hepatorenal syndrome in cirrhotic patients with ascites is 18% in one year and 39% in five years [205]. Drug metabolism is altered in such patients as the elimination-phase half-life would be prolonged for drugs excreted primarily by the kidneys [206–208]. Hence, drug dosages need to be adjusted correspondingly.

Cirrhotic patients may also be at increased risk of developing acute renal failure with potentially nephrotoxic drugs [209] (i.e., aminoglycosides and nonsteroidal anti-inflammatory drugs). The use of nephrotoxic drugs should be avoided or, at the very least, appropriately dose adjusted in cirrhotic patients.

9.4.6 Drug Response in Cirrhosis

Altered physiology in the state of cirrhosis may cause the body to respond differently to standard doses of drugs. Unfortunately, little is known about the pharmacodynamics of drugs in patients with hepatic dysfunction.

For example, standard doses of sedatives are known to precipitate hepatic encephalopathy in the cirrhotic patient. Studies have demonstrated both impaired drug metabolism and increased γ -aminobutyric acid-mediated inhibitory neurotransmission in hepatic encephalopathy, providing the basis for brain hypersensitivity and this exaggerated response to sedatives [210,211]. Encephalopathy has also been found to reverse with the administration of flumazenil, a benzodiazepine antagonist [211]. Central nervous system sensitivity is also increased with morphine [194,212], chlorpromazine, diazepam, and other sedatives, analgesics, and tranquilizers [210,213–215].

An empirical and cautious approach to medication use in liver patients should be guided by the clinical response and side effects and in some cases, plasma drug monitoring is recommended. As an initial dosing guideline, the dose of drugs eliminated by oxidative metabolism should be halved. In hepatic decompensation (e.g., ascites, encephalopathy), even lower doses may be used. Dose adjustments should be made based on therapeutic response or adverse effects. Drugs that are extensively metabolized before reaching the systemic circulation may be completely absorbed as parent drug in cirrhotic patients. Similarly, the first-pass effect may not exist in patients with a portocaval shunt [79].

More studies are required to evaluate the effects of liver disease on the pharmacokinetics and pharmacodynamic effects of drugs, especially those likely to be used in patients with hepatic dysfunction.

9.4.7 Anesthesia in Cirrhosis

Apart from increased perioperative risks arising from the hyperdynamic circulatory, altered fluid and coagulopathic status of the patient, the cirrhotic liver is also particularly susceptible to the effects of anesthetic drugs administered. Patients with liver disease may have altered drug pharmacokinetics because of increased volume of distribution, decreased cytochrome P450 enzyme metabolism, decreased serum drug binding due to low protein/albumin levels, and sometimes decreased biliary drug excretion.

The autoregulation of blood and oxygen supply to the liver between the portal vein and hepatic artery can also be disrupted by hepatic disease or volatile anesthetic agents. Halothane and enflurane decrease both portal venous and hepatic arterial flow as a result of systemic vasodilatation and a slightly negative inotropic effect [216–219]. Isoflurane, desflurane, and sevoflurane increase hepatic artery flow but decrease portal venous flow [220], making them the preferred anesthetic agents in patients with liver disease [221]. Isoflurane, desflurane, and sevoflurane undergo less hepatic metabolism (0.2% of isoflurane) than either halothane (20%) or enflurane (2–4%) and have a lower risk of drug-induced hepatitis [216,222,223]. Halothane hepatitis may involve immune sensitization to trifluoroacetylated proteins formed by CYP2E1 metabolism of halothane in genetically predisposed individuals [222]. Its metabolism can be inhibited by prior administration of disulfiram.

The actions of neuromuscular blocking agents (e.g., mivacurium) may be prolonged in patients with liver disease because of reduced plasma pseudocholinesterase activity, decreased biliary excretion, and an increase in the volume of distribution [224]. Atracurium and cisatracurium are metabolized independent of the liver, making them the preferred agents in patients with liver disease or biliary obstruction [221]. The long-acting doxacurium can be used for prolonged surgical procedures including LT [225]. Other neuromuscular blocking agents such as vecuronium and rocuronium are degraded by the hepatic system exclusively [226].

Because of reduced hepatic blood flow, the metabolism of morphine, meperidine, and oxycodone may be decreased in patients with liver disease and portal hypertension, resulting in a prolonged half-life and increased bioavailability; in contrast, fentanyl, sufentanyl, and remifentanyl clearance is unaffected and these are the preferred narcotic agents [227]. If the use of longer-acting opioids is desired, lower doses at less frequent intervals should be administered in cirrhotic patients.

Slower metabolism of benzodiazepines such as diazepam, midazolam, and chlor-diazepoxide can lead to accumulation and prolonged clinical effects. Hence, oxanepam and lorazepam, which are eliminated by glucuronidation without hepatic metabolism, are preferred [228].

Barbiturates, which do not affect hepatic blood flow significantly, bind to γ -aminobutyric acid receptors in the brain and can precipitate hepatic encephalopathy. They should be used with caution in patients with liver disease. Although the metabolism of thiopental is decreased in patients with cirrhosis, plasma protein binding of thiopental is also decreased, so that total body clearance of the drug is unaltered in cirrhosis [229]. Although drug elimination is largely unchanged after a single induction dose of intravenous sodium thiopental or propofol, multiple doses or a continuous infusion may lead to prolonged clinical effects [230].

9.5 LIVER SURGERY AND REGENERATION

The human liver has the unique ability to regenerate after injury, restoring its original mass and function. Hepatic resection is safely accomplished for malignant and benign diseases because of this unique ability. Regeneration is a highly regulated process involving intra- and extrahepatic cellular signals and pathways. Animal studies show that cytokines responsible for hepatic regeneration include hepatocyte growth factor, transforming growth factor (TGF) α , insulin, and glucagon [231–238]. Hepatocellular

regeneration is influenced by age. Older livers show delayed and slower regeneration after acute injury than younger livers. In steatosis, abnormalities in induction of cytochrome P450 play a role in the pathophysiology of mitochondrial damage and may contribute to poor liver regeneration [239–241].

Follow up of adult living liver donors shows that a large amount of regeneration occurs in the first one to two weeks after resection for donation, but most remnant livers do not return to original size even after a year [242,243]. Numerous influences may interfere with the balance between maintaining differentiated hepatocyte function and cellular replication for survival [244]. After hepatic resection or transplantation, hepatocytes recover and regenerate at the expense of normal hepatic metabolism. The success of restoring lost liver mass and repairing tissue injury determines the ability of the liver to recover and support normal metabolic function. An inability to maintain this process after injury or liver resection may lead to hepatic decompensation, liver failure, and “small-for-size” syndrome [245].

9.6 LIVER TRANSPLANTATION AND DRUG METABOLISM

LT is the only curative option in patients with severe hepatic dysfunction or hepatic failure. It is also a means of cure for some liver tumors, most commonly hepatocellular carcinoma. In children, it is performed primarily for biliary atresia and inborn errors of metabolism.

The first step in LT is harvesting or procurement of the donor organ. Following this, the graft is preserved in solution until the time of implantation in the recipient. Preservative solutions can now extend this preservation period for close to 24 h, and this has changed procurement practices and the availability of organs for the better. The recipient operation involves the removal of the recipient liver (resulting in the anhepatic phase) and then graft implantation, which requires revascularization and biliary reconstruction.

Regeneration is crucial in LT. In cadaveric whole graft LT, ischemia-perfusion injury occurs during graft preservation. Regeneration occurs immediately after transplant, according to the extent of preservation injury [246,247]. Similarly, hepatic regeneration is important in transplanting a small graft into a larger recipient, as is the case in adult-to-adult live donor transplantation [248].

LT also involves removal of the gallbladder with a consequential disruption of bile flow. In cases where a temporary T-tube is required to drain the bile duct for a longer period, the absorption of fat-soluble drugs may be further affected.

9.6.1 Graft Versus Recipient Size

Advancement in surgical techniques has enabled living donor LT to increase the availability of liver grafts available in the organ donor pool. In pediatric LT, deceased donor size-matched allografts are rarely available. To overcome this, reduced size LT where the donor liver is surgically reduced in size to meet the needs of the pediatric recipient is used in split-LT. With this technique, a potential deceased donor graft benefits two potential recipients [249]. Similarly, in pediatric living-related donor operations, the left lobe of the liver, or a portion of it, is taken as the donor graft

from the parent [250]. The same is applied in adult-to-adult live donor transplantation, in which the right hepatic lobe is used as the donor graft, unless the size of the donor's left hepatic lobe per se suffices. In general, a donor liver allograft has to be within 20% of the recipient's size. A large-for-size graft can impair breathing or closure of the abdomen, whereas a small-for-size graft may be inadequate for survival. The amount of liver graft implanted is important in post-LT regeneration. The safety limits of selecting graft size/volume in living donors vary from 30–50% of “standard liver volume” to 0.8–1.0% of recipient body weight [251–255]. Small-for-size grafts [256] (graft weight/standard liver volume ratio <40% or graft-to-recipient weight ratio <0.8%) are associated with poor survival and prolonged hyperbilirubinemia [251,257]. Although small grafts can regenerate, there is significant functional impairment of the grafts resulting in prolonged cholestasis and histology consistent with ischemic injury. This functional impairment may interfere with drug pharmacokinetics. Other clinical features of this syndrome include delayed protein synthesis and increased surgical complications, massive ascites, gastrointestinal bleeding, increased infection, and renal dysfunction [251,255,258].

Studies have shown that adult-to-adult live donor transplant recipients require lower doses of immunosuppression in the early postoperative period than patients receiving whole grafts [259], even if the graft received is of adequate weight for the recipient.

9.6.2 Drug Metabolism in the Post-LT Patient

The post-liver transplant patient often has fluid, electrolyte, and nutritional abnormalities, as well as biliary tract dysfunction. These multiple physiologic changes can result in pharmacokinetic alterations of drugs administered in the post-LT setting [260].

9.6.2.1 Absorption. Post-LT, the absorption of lipophilic drugs may be dramatically improved. This has been demonstrated for the absorption of lipid-soluble drugs (e.g., cyclosporine A) and lipid-soluble vitamins (e.g., vitamin A, vitamin E), which improves after successful LT and reestablishment of bile flow and normal excretion of bile salts. In children, vitamin E deficiency and its neurologic complications in liver failure patients are reversed after LT.

9.6.2.2 Protein Binding. Drug's protein binding is affected by the synthetic capacity of the liver and the pathophysiologic changes associated post-LT.

The serum concentration of albumin in liver transplant recipients remains low for months following surgery. This results in a lower protein-bound fraction for drugs, which are albumin bound. However, when compared to patients with chronic liver disease, the protein binding of diazepam and salicylic acid is greater in post-liver transplant patients because of the removal of endogenous binding inhibitors.

Conversely, the concentration of α_1 -acid glycoprotein increases post-LT and remains elevated beyond a month. Accordingly, drugs that bind to this protein will have lower free concentrations. The unbound fraction of lidocaine in the plasma of post-liver transplant patients is lower than that in normal volunteers.

9.6.2.3 Hepatic Clearance. Drug metabolism in the post-liver transplant patient may be altered by a multitude of factors, including preservation injury, initial decreased

hepatic blood flow, and induction or inhibition of microsomal enzymes by immunosuppressant agents or other commonly used drugs in the post-LT period. Physiologic changes as a result of improved organ function include improved liver metabolism and improved bile salt and protein production, although the organ function is not considered to be entirely normal. In the early postoperative period, all the above changes may affect the pharmacokinetics of administered medications.

First-pass metabolism is also altered in the transition to normal hepatic function. Altered biliary function affects the absorption of lipophilic compounds and the elimination of drugs and their metabolites as well. For example, biliary dysfunction in liver transplant patients results in a high concentration of cyclosporine metabolites in the blood.

Generally, cytochrome P450 activity is depressed in the early posttransplantation period and recovers over the first few months. Hepatic mRNA gene expression for CYP3A4, 3A5, 2E1, and 1A2 increase significantly over time from LT [261]. However, studies in liver transplant recipients have shown that CYP2E1 has enhanced activity in the first month [262], while CYP2D6 appears to be unaffected by the procedure [263,264]. Drugs that are substrates for CYP2E1 may require a dosage adjustment during this early period. Recipient age does not appear to influence the changes in major CYP450 enzyme capacity following LT. Donor gene polymorphisms of CYP450 may be more important in alterations of drug metabolism than recipient polymorphisms [265]. In the late LT period, stable liver transplant patients have demonstrated, by antipyrine kinetics, similar hepatic oxidative metabolizing capacity as compared to normal subjects.

Phase II metabolism has not demonstrated alterations with LT. Although sulfation and glucuronidation of acetaminophen is minimally altered in post-liver transplant patients, renal elimination of the conjugates/metabolites is impaired [266,267]. For numerous reasons, renal clearance of drugs in liver transplant patients is usually impaired in the immediate and early postoperative period.

9.6.3 Commonly Used Immunosuppressants in LT

The standard immunosuppression regime after LT is composed of corticosteroids plus a calcineurin inhibitor (tacrolimus (TAC) or cyclosporine A). An antiproliferative agent may be added for patients at higher risk of rejection. More recent studies have also used various agents (e.g., IL-2 receptor monoclonal antibodies) either as steroid-sparing agents or to delay the use of calcineurin inhibitors until renal function recovers (Table 9.4).

Most immunosuppressants have narrow therapeutic windows and are extensively metabolized by the liver. Fluctuating hepatic function from hepatic regeneration and the need for polypharmacy, especially in the early postoperative period, puts the patient at increased risk of drug–drug interactions or adverse drug reactions. In the period of hepatic regeneration, immunosuppressive drugs require intensive drug monitoring, in view of their narrow therapeutic window, adverse reactions, drug–drug interactions, and changing pharmacokinetics.

9.6.3.1 Steroids. Prednisone is well absorbed orally and has a long half-life, allowing for once daily dosing. It is converted to active prednisolone in the body and has multiple suppressive effects on the immune system. Barbiturates, phenytoin, and

TABLE 9.4 Immunosuppressants Used in Liver Transplantation

Steroids
• Methylprednisolone
• Prednisolone
• Prednisone
Immunophilin-binding agents
• Tacrolimus (Prograf®)
• Cyclosporine (Sandimmune®, Neoral®)
• Sirolimus (Rapamune®)
Antiproliferative agents
• Mycophenolate mofetil (CellCept®, Myfortic®)
• Azathioprine (Imuran®)
Anti-T-cell antibodies
• Muromonab-CD3 monoclonal antibody (OKT3)
• ALG/ATG globulin (Atgam® or Thymoglobulin®)
Interleukin-2 receptor monoclonal antibodies
• Daclizumab (Zenapax®)
• Basiliximab (Simulect®)

rifampicin decrease the effectiveness of prednisone, whereas prednisone will decrease the effectiveness of vaccines and toxoids.

The steroid dose varies from center to center but is highest during the anhepatic phase and immediately after LT, with a variable period of dose taper. Administration of high dose IV methylprednisolone or high dose oral prednisone (200 mg) is the first-line therapy for the treatment of acute graft rejection.

Common adverse effects of steroids include increased appetite, insomnia, and mood changes. Side effects seen with high doses or prolonged therapy include cataracts, hyperglycemia, hypertension, hirsutism, acne, sodium and water retention, and growth suppression.

9.6.3.2 Immunophilin-Binding Agents

9.6.3.2.1 Tacrolimus. TAC (Prograf®) binds to the cytoplasmic immunophilin, FK binding protein (FKBP-12), with subsequent inhibition of the activity of calcineurin. Calcineurin is an enzyme that stimulates cytokine production (e.g., IL-2) by activated T cells.

Oral TAC has incomplete and variable absorption resulting in poor bioavailability in liver transplant patients (~29% bioavailable). This requires the use of higher oral than intravenous doses to obtain similar blood concentrations. TAC decomposes in alkaline media, and medications that increase the pH of the gastrointestinal tract (e.g., antacids) will further decrease its oral absorption. Such drugs' administration should be spaced 2 h apart from TAC administration. TAC is 99% protein bound (mainly to albumin or α_1 -acid glycoprotein).

TAC is mainly metabolized by CYP3A4 to eight other metabolites, some of which are active. Expectedly, other drugs or pathophysiological processes affecting CYP3A4 activity can alter TAC concentrations. Macrolide antibiotics (e.g., erythromycin) and antifungal medications (e.g., itraconazole, fluconazole) can increase TAC levels, whereas anticonvulsants (e.g., phenytoin, phenobarbital) and rifampin can lower TAC

levels. Patients with hepatic dysfunction may require a reduction in dose. Less than 2% of the drug is excreted unchanged in the urine. At times, such drug interactions are taken advantage of clinically. For example, in patients who cannot afford the cost of TAC or who may have genetic polymorphisms that result in rapid TAC metabolism, enzyme inhibitors such as verapamil may be used to allow for a lower dosing of TAC while achieving therapeutic levels of TAC. The same principle is true for cyclosporine A as well.

The main adverse effects of TAC are neurologic. These range from mild effects such as insomnia, tremors, headaches, nightmares, and hyperesthesia to major effects such as seizures, confusion, psychosis, and coma, which may necessitate a withdrawal of the drug. TAC is nephrotoxic, and caution should be exercised with the concurrent use of other nephrotoxic drugs.

Intravenous TAC is dosed at 0.03–0.05 mg/kg/day by continuous infusion in the initial postoperative period. Oral TAC is dosed at 0.1 to 0.15 mg/kg/day as two divided doses. Dose is then adjusted according to trough blood levels and clinical findings. Pediatric patients clear the drug more rapidly and require two to four times higher doses than adults to maintain equivalent therapeutic considerations

9.6.3.2.2 Cyclosporine. Cyclosporine binds to the cytoplasmic immunophilin cyclophilin, which also blocks the action of calcineurin.

Cyclosporine is highly lipophilic and is dependent on bile for intestinal absorption. Following oral administration, the absorption of cyclosporine is incomplete (~30%) and erratic, especially in liver recipients with T-tube diversion of bile. The absorption of cyclosporine can be enhanced by a moderate fatty meal, so the patient should be advised to take cyclosporine with meals. Cyclosporine is available as standard formulation (Sandimmune®) or as a microemulsion (Neoral®). The microemulsion formulation has better and more reliable oral absorption, as it is less dependent on bile for its absorption. In a study of liver recipients with T-tube diversion, the bioavailability of the microemulsion was 6.5 times greater than that of the standard formulation [268]. Hence, the two formulations are not bioequivalent and cannot be used interchangeably. Pharmacologically, the microemulsion formulation has also been associated with a lower incidence of rejection compared to standard formulation [269]. Once absorbed, cyclosporine is highly protein bound (>90% to lipoproteins).

Cyclosporine is also mainly metabolized by CYP3A4 and shares the same drug interactions as TAC. Inhibitors of CYP3A will reduce the metabolism of cyclosporine and increase its blood concentrations. Substances that induce CYP3A activity will decrease its metabolism and elimination, decreasing its blood concentration and resulting in subtherapeutic levels with a risk of graft rejection. For example, grapefruit juice (enzyme inhibitor) decreases the metabolism of cyclosporine, while St John's Wort (enzyme inducer) increases its metabolism. More drug interactions have been reported with cyclosporine, although this may be a consequence of greater and longer experience with cyclosporine use. All the metabolites of cyclosporine A have less biological activity or toxicity than the parent compound. Dosage adjustments for cyclosporine are required in hepatic dysfunction.

Adverse reactions to cyclosporine include hypertension, nephrotoxicity, gingival hypertrophy, hirsutism, and tremor. Less common side effects may occur including seizures, headache, psychosis, paresthesias, and pancreatitis. Cyclosporine is also nephrotoxic. Caution should be exercised in using other nephrotoxic drugs concurrently.

The adult dose for intravenous cyclosporine is 2–5 mg/kg/day as a continuous intravenous infusion. Initial oral dose is 15 mg/kg/day and is tapered to a long-term dose of 5 mg/kg/day divided into one or two doses per day. As with TAC, children require higher doses of cyclosporine to maintain therapeutic drug concentrations. In large part, cyclosporine has been supplanted by TAC as the primary immunosuppressive agent post LT.

9.6.3.2.3 Sirolimus. The sirolimus–immunophilin complex binds to the mammalian target of rapamycin (mTOR), which inhibits the signal necessary for IL-2-induced T-cell proliferation [270].

Sirolimus is a lipophilic drug with poor oral bioavailability and extensive tissue distribution [271]. Sirolimus is predominantly metabolized by CYP3A4, and coadministration of enzyme inducers or inhibitors will affect the rate and extent of its metabolism. Examples of these are detailed above, as with TAC and cyclosporine.

Early studies showed that sirolimus was both effective when used in combination with cyclosporine in a steroid-sparing protocol [272], or in combination with prednisone and low dose TAC, which resulted in less TAC-associated nephrotoxicity [273]. However, recent findings have now prompted the US boxed warning that sirolimus is not recommended for use in de novo LT patients due to increased risk of hepatic artery thrombosis, graft failure, and the increased mortality risk when used in combination with TAC and/or cyclosporine.

9.6.3.3 Antiproliferative Agents

9.6.3.3.1 Mycophenolate Mofetil. Mycophenolate mofetil has a cytostatic effect on both the B and T lymphocytes.

Oral mycophenolate mofetil is available in a standard form (CellCept®) or as a delayed-release preparation (Myfortic®). Both forms should not be used interchangeably due to their differences in absorption. Antacids decrease the absorption of mycophenolate mofetil, and administration of the two drugs should be separated by at least 2 h. Food delays the absorption of mycophenolate mofetil, and when possible, the drug should be taken on an empty stomach. However, in some cases, mycophenolate mofetil is taken with food to minimize adverse gastrointestinal effects. After oral absorption, mycophenolate mofetil is hydrolyzed by the liver and gastrointestinal tract to the active metabolite, mycophenolic acid. Mycophenolic acid is conjugated with glucuronide to form an inactive compound. However, this metabolite may be converted back to the active parent drug in the blood. Mycophenolic acid enters the enterohepatic recirculation. The drug is highly albumin bound (97%). Total plasma concentrations will vary as protein synthetic capacity of the liver increases with recovery of the liver. Patients should be monitored closely when inhibitors or inducers of CYP3A4 are used. Plasma concentration monitoring has been suggested for mycophenolate mofetil, but a good correlation with efficacy and adverse effects has not been demonstrated.

Adverse effects with mycophenolate mofetil include gastrointestinal disturbances (nausea, vomiting, diarrhea), pure red cell aplasia, neutropenia, thrombocytopenia, headache, weakness, dizziness, and insomnia. Drugs with adverse effects similar to immunosuppressive agents, such as nephrotoxic drugs, should be used cautiously.

In adults, mycophenolate mofetil is dosed 1.5 g twice daily. In children, it is dosed at 40 mg/kg/day, in two divided doses.

9.6.3.3.2 Azathioprine. Azathioprine is an antiproliferative agent against both the B and T lymphocytes. It is metabolized to its active metabolites mercaptopurine, 6-thionosinic acid, and 6-thioguanine. Azathioprine is commonly used to treat autoimmune hepatitis. It can be used in LT to prevent graft rejection, but not as a treatment for established rejection. It is recommended that its dose be reduced by at least two-thirds during concurrent therapy with xanthine oxidase inhibitor allopurinol and that both drugs are not used together unless absolutely necessary. Xanthine oxidase converts the active metabolite of azathioprine into an inactive compound. Hence, drug interactions have been reported to cause significant drug toxicity, including fatalities [274–278].

9.7 THE USE OF HERBAL PREPARATIONS IN PATIENTS WITH LIVER DISEASE

Herbal medicine and its potential drug interactions are particularly challenging for the hepatologist. Although herbal medications are exempted from premarketing safety standards, many herb–drug interactions are known to occur. Surveys have demonstrated that up to 65% of patients with liver disease take some form of herbal preparation [279–282]. Herbal therapy usually consists of a mixture of ingredients, rather than a single pharmacologic agent. Plants can also vary based on the geographic location of their growth and the time of the harvest [283]. Preparations often contain constituents that can compete with the action of the principal agent. Botanical misidentification, product contamination or adulteration, mislabeling and variability in the collection and extraction processes adopted, and lack of active compound stability may all have an effect on purity of the final product. For example, an aqueous extract will not contain the same amount of an herb as an alcohol extract, or as the grounded leaves of the herb without extraction. Many herbs and plants may also be marketed under different names. For example, Echinacea is also known as *cone flower*, *black Samson*, *black susan*, *comb flower*, *scurvyroot*, *hedgehog*, *Missouri snakeroot*, and *Indian head* [284]. It is also challenging for the hepatologist to demonstrate hepatotoxicity or drug–herb interactions because patients usually omit mentioning their use, even upon direct questioning, because of their perceived safety [285].

Induction of cytochrome P450 enzymes by other drugs or alcohol may increase the production of toxic metabolites that mediate liver injury. For example, pyrrolizidine alkaloids and diterpenoid constituents of *Scutellaria* (found in skullcap, germander, and other *Teucrium* spp.) are well-known hepatotoxins metabolized via CYP3A4, and pharmacologic induction of CYP3A4 has been associated with increased toxicity from these herbal agents [286–289]. Pennyroyal, used as a general herbal tonic, is metabolized via CYP2E1 to the toxic metabolites pulegone and methofuran. Chronic alcohol ingestion that induces CYP2E1 activity may increase the risk of hepatotoxicity from this herb [283].

Conversely, herbs may also have a direct effect on the induction or inhibition of CYP450 enzymes, affecting their metabolism of other drugs (Table 9.5). Transplant

TABLE 9.5 Common Drug–Herb Interactions in Liver Disease

Herb	Interacting Medication
Danshen (<i>Salvia miltiorrhiza</i>)	Corticosteroids
Devil’s claw (<i>Harpagophytum procumbens</i>)	Anticoagulants, aspirin
Dong quai (<i>Angelica sinensis</i>)	Corticosteroids
Grapefruit juice	Cyclosporine, calcium channel blockers
Glycyrrhizin (licorice root)	Prednisolone, spironolactone
St John’s wort (<i>Hypericum perforatum</i> , goatweed)	Cyclosporin, indinavir, other protease inhibitors
Garlic, ginger, papaya extract, or tamarind (<i>Tamarindus indica</i>)	Aspirin, anticoagulants

Source: Adapted from Schiano TD [285]. Copyright Elsevier 2003.

recipients require immunosuppressants and other drugs that may have narrow therapeutic indices. St John’s Wort (*Hypericum perforatum*) is a widely used herbal product with many reported drug–herb interactions. The herb is known to induce CYP3A4 and P-glycoprotein expression. The results of a systemic review indicate that St John’s Wort lowers the blood concentration of multiple drugs, including cyclosporine, digoxin, warfarin, and theophylline [290]. Graft rejection related to subtherapeutic levels of cyclosporine caused by St John’s Wort has been reported in transplant patients [291].

Herbal or alternative medicine hepatotoxicity superimposed on preexisting liver disease must always be considered, especially because many herbs are used as “liver remedies” in patients with viral hepatitis and other chronic liver diseases. Alternative medicine-induced liver injury, such as drug hepatotoxicity, can clinically and histologically mimic any type of liver disease [285].

Other relevant chapters on drug–herb interactions and metabolism in this encyclopedia include *Enzyme Kinetics of Drug-Metabolizing Reactions and Drug–Drug Interactions*, *ADME of Herbal Dietary Supplements*, *Animal Models of Idiosyncratic, Drug-Induced Liver Injury: Emphasis on the Inflammatory Stress Hypothesis*, *Herb–Drug and Food–Drug Interactions*.

9.8 CONCLUSIONS

In humans, the liver is a major site of drug metabolism. The multiple functions this organ is responsible for to maintain homeostasis is the reason why liver disease can sometimes result in several altered states in the body (e.g., coagulopathy, altered protein levels, altered fluid balance). Hence, any liver disease has the potential to alter drug disposition and metabolism. Dosing in patients with cirrhosis should be undertaken with caution, and the physician or pharmacist needs to be mindful that many common drugs may require dose adjustments while prescribing for such patients.

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10 Role of Phase I Metabolism in Drug Activation and Clinical Treatment Outcomes

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10.1	Summary	1
10.2	Background	2
10.3	Drugs with active metabolites	5
10.4	Carrier-linked prodrugs	8
10.5	Bioprecursors	11
10.6	Metabolic drug–drug interactions: the case of anti-HIV protease inhibitors (PIs)	16
10.7	Conclusion	16
	References	17

10.1 SUMMARY

This chapter explores the relation between drug biotransformation and pharmacological activity. More precisely, it surveys the formation of active metabolites and exemplifies cases of metabolites as or more active than the parent drug. Because very few drug conjugates are pharmacologically active, the focus is on metabolic reactions of functionalization (i.e., phase I), namely, oxidations, reductions, or hydrolyses.

The first section sets the scene by explaining the *pharmacokinetic/pharmacodynamic (PK/PD) interplay* that underlies drug action, and the existing continuum ranging from drugs devoid of active metabolites, to drugs yielding some active metabolite(s), to prodrugs whose activity is due solely to the metabolite. *Soft drugs* are briefly exemplified since they nicely illustrate the concept of drugs designed to be rapidly metabolized to inactive, atoxic metabolites.

The second section is dedicated to drugs whose activity depends in part on the presence of one or more active metabolite(s). Thus, the drug's action can be prolonged, as exemplified with *diazepam* and some other benzodiazepines. Alternatively, a drug's

pharmacological spectrum can be broadened by a metabolite; witness the antinociceptive activity of *codeine*, which is essentially due to its metabolite morphine. In another scenario, both the drug and its metabolite contribute comparatively to the clinical effect (e.g., *tramadol*). Finally, a drug such as *tamoxifen* is metabolized to one or more highly active metabolite(s).

The third section covers carrier-linked prodrugs, the examples presented therein having been selected to illustrate significant objectives in prodrug design. Thus, *fospropofol* was developed with the aim of overcoming a pharmaceutical hurdle, namely the low solubility of propofol. The lack of oral absorption of the anti-influenza agent Ro-64-0802 was overcome using simple physicochemical principles, namely, by masking its highly hydrophilic carboxylate group with a bioreversible ester function to yield *oseltamivir*. Another strategy to improve oral absorption is by targeting intestinal transporters, as illustrated with *valacyclovir*. The last example, *capecitabine*, exemplifies a more ambitious objective, namely, tissue targeting via organ-selective activation.

The fourth section deals with another class of prodrugs known as *bioprecursors*. Here, metabolic activation is by redox modification of a functional group, without cleavage of a promoiety. Four examples are presented, namely, *nabumetone*, a long-acting non-steroidal anti-inflammatory (NSAI) prodrug with low gastric toxicity, the two anti-aggregating agents *clopidogrel* and *prasugrel*, whose bioactivation unmasks a highly reactive sulfenic acid group, and *tirapazamine*, an antitumor bioprecursor activated by reduction to a DNA-damaging radical. Clopidogrel is treated in some detail to illustrate the influence of genetic factors on clinical outcomes.

The fifth section briefly illustrates drug–drug interactions (DDIs) at the metabolic level, taking the antiretroviral protease inhibitors (PIs) as a case in point. A concluding section then completes the chapter.

10.2 BACKGROUND

To open this chapter, we note that the continuously increasing significance of drug metabolism investigations in drug discovery and development cannot be fortuitous [1–9]. The pharmacological and toxicological consequences of drug metabolism account for this phenomenon, which simultaneously drives, and is driven by, the many methodological, factual, and conceptual advances in this discipline. In a perspective of drug discovery, a number of metabolites of established drugs were found to have comparable or improved therapeutic properties compared to their parent and have become useful drugs in their own right. In addition to the examples discussed in the text, one can single out desloratadine (from loratadine), cetirizine (from hydroxyzine), fexofenadine (from terfenadine), and oxazepam (from diazepam). Even more significant is the discovery of paracetamol, which has replaced phenacetin, its more toxic parent. As a result, the pharmacological activity of metabolites even plays a role in bioequivalence studies [10].

10.2.1 The PK–PD interplay

Basically, there are two types of interactions between bioactive compounds and biological systems [6,11]. A drug acting on a biological system elicits a pharmacological and/or a toxic response, in other words a *pharmacodynamic (PD) event*. In the present

context, we discriminate between pharmacological activities (in effect, wanted and/or favorable PD effects) and toxic effects (in fact, all adverse effects a drug or chemical can elicit). In concert with PD events, the biological system “acts” on the xenobiotic by absorbing, distributing, metabolizing, and excreting it. These are the *pharmacokinetic (PK) events*. Most of these processes are mediated by enzymes or transporters and are active in the sense that they consume energy produced by the biosystem; others in contrast are passive.

Significantly, there is a mutual interdependence between PD and PK events, since it is well known that PK events such as metabolic activation or inactivation influence PD responses. This is the main focus of this chapter. Symmetrically, the PK behavior of a xenobiotic can be markedly influenced by the overexpression of metabolizing enzymes (a typical PD response) as mediated by substrates or other xenobiotics binding to nuclear receptors [12,13]. Such a situation is exemplified in Section 10.6.

10.2.2 Drugs, Metabolites, and Bioactivities—A Continuum

An important information in any drug’s dossier is the activity (or lack thereof) of its metabolites. What should be realized, however, is that “activity” is usually understood to imply the same pharmacological target as the parent molecule [1], an implicit meaning we also adopt. Here, we outline the various combinations of activities to be illustrated, (i) from intrinsically active drugs having no active metabolite to (ii) intrinsically active drugs having one or more active metabolites to (iii) intrinsically inactive “drugs” (i.e., prodrugs), whose therapeutic effects necessitate metabolism to an active metabolite (i.e., bioactivation). For example, sedative-hypnotic benzodiazepines fall in two categories [14]. Some have no active metabolite, for example, the 3-hydroxylated benzodiazepines such as lorazepam, oxazepam, and temazepam, which undergo O-glucuronidation and cleavage reactions. Other intrinsically active benzodiazepines such as diazepam have one or more active metabolite(s), sometimes long-acting ones. In a quantitative perspective, some drugs have a metabolite of comparable activity (e.g., tramadol), while some others have one or more highly active ones (e.g., tamoxifen). The extreme case of prodrugs will be treated separately.

10.2.3 Bioactivation and Metabolic Conjugation

This chapter is dedicated to drug activation by phase I reactions. As a reminder, these imply the creation or modification of a functional group in a substrate molecule by reactions of oxidation or reduction (redox reactions catalyzed by oxidoreductases, EC 1) or reactions of hydrolysis catalyzed by hydrolases (EC 3) [4,5,15].

The reason for not including phase II reactions (i.e., conjugation reactions catalyzed mainly by transferases EC 2) is that only a very small number of drug conjugates are pharmacologically active (i.e., having a wanted and/or favorable PD effect). A frequently cited example is that of *morphine*, which is conjugated on its phenolic and secondary alcohol groups to form the 3-*O*-glucuronide (a weak opiate antagonist) and the 6-*O*-glucuronide (a strong opiate agonist), respectively [16].

An intriguing and very rare reaction of conjugation occurs for *minoxidil*, an hypotensive agent that also stimulates hair growth. This drug is an *N*-oxide, and the actual active form responsible for the different therapeutic effects is the stable *N*-*O*-sulfate ester [17].

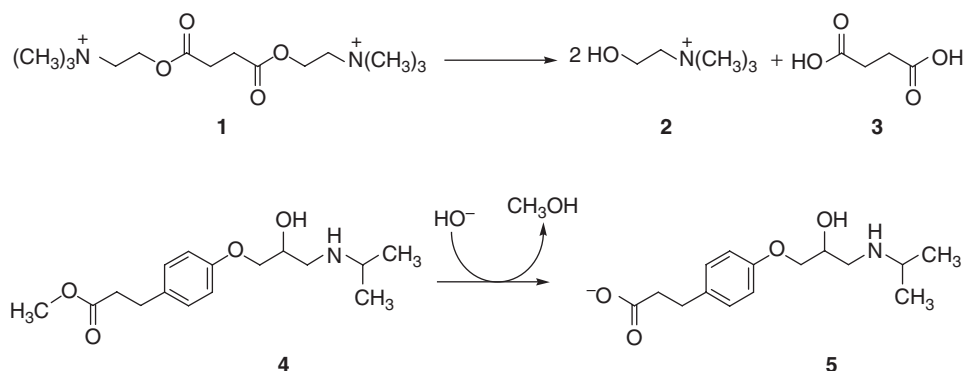


Figure 10.1 Examples of soft drugs, namely, the curarimimetic succinylcholine (1), which is hydrolyzed to choline (2) and succinic acid (3), and the β -blocker esmolol (4), whose metabolite is inactive due to the unmasking of a hydrophilic carboxylate group.

10.2.4 Drugs Having No Active Metabolite

An example of drugs designed to have neither active nor toxic metabolites is that of *soft drugs*, a concept pioneered and extensively developed by Bodor and Buchwald [18]. In practical terms, soft drugs (i) are bioisosteres of known drugs, (ii) contain a labile bridge (often an ester group), and (iii) are cleaved to metabolites known to lack activity and toxicity. In other words, soft drugs bear a close stereoelectronic analogy with the target drugs, but a rapid breakdown to inactive metabolites is programmed into their chemical structure. A typical example of a soft drug is *succinylcholine* (1 in Fig. 10.1), although the discovery of this agent predates by decades the concept and term of soft drugs. In most individuals, this curarimimetic agent is very rapidly hydrolyzed to choline (2) and succinic acid (3) by plasma cholinesterase with a half-life of about 4 min [19,20].

Soft drugs may be found in a variety of therapeutic classes. Thus, a valuable class of soft β -blockers are aryloxypropanolamines, which possess an ester group in the para-position, as illustrated by the clinically useful *esmolol* (4) [21]. In this compound, as in others, the para-substituent has an intermediate polarity compatible with good receptor affinity, but hydrolysis to its metabolite 5 unmasks a carboxylate group, whose high polarity is incompatible with receptor affinity. The *in vitro* half-life of esmolol in whole blood was about 2, 13, and 23–27 min in rats, dogs, and humans, respectively. In patients, recovery from β -blockade began 2 min after discontinuation of infusion and was complete at about 18 min.

Paracetamol (6 in Fig. 10.2; also known as *acetaminophen*) is another example of a drug having no active metabolite, but we mention it here to highlight how unexpected findings remain possible even with highly popular drugs. Recently indeed, evidence has begun to challenge the general belief by suggesting that part of the clinical effect of paracetamol might in fact be contributed by an unusual metabolite [22]. One of the pain pathways and its inhibition involve vanilloid receptors, including the capsaicin receptor (transient receptor potential cation channel, subfamily V, member 1; TRPV1). This receptor shows a marked affinity for metabolites of arachidonic acid (the so-called endovanilloids), the best known of which is anandamide. Intriguingly,

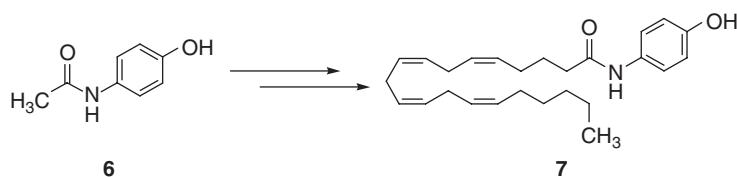


Figure 10.2 Paracetamol (**6**) and its recently discovered metabolite *N*-arachidonoyl-4-hydroxyaniline (**7**).

N-arachidonoyl-4-hydroxyaniline (**7**), a known metabolite of paracetamol and an analog of anandamide, was shown to have a potent inhibitory effect on TRPV1. However, the clinical contribution of this metabolite in patients administered paracetamol remains to be understood.

10.3 DRUGS WITH ACTIVE METABOLITES

Drugs having both intrinsic activity and active metabolite(s) may be more numerous than generally believed, but information is not always available. In this section, we examine a number of cases chosen for the different perspectives they allow on this aspect of pharmacotherapy.

10.3.1 Benzodiazepines, Drugs With or Without Active Metabolites

As mentioned above, benzodiazepine drugs can be classified into two groups, those having one or more active metabolite(s) and those having none. Criteria of half-life and duration of action allow further discrimination among these drugs [23–27]. Thus, *midazolam* and *triazolam* both have a short half-life in humans, as have their active metabolites *1*-hydroxymidazolam and *1*-hydroxytriazolam, respectively. As a result, these two drugs have a short duration of action, which makes them good short-acting hypnotics.

Benzodiazepines of longer duration of action are useful as sedatives or antiepileptics. Three examples are shown here, each of which has a different story to tell. *Clorazepate* is unusual among benzodiazepines due to the presence of its carboxylate group. Its metabolism is by decarboxylation to the long-acting *desmethyldiazepam*, followed by 3-hydroxylation to *oxazepam*. The drug does have affinity for the benzodiazepine receptor [26], but it appears incapable of crossing the blood–brain barrier and is rapidly metabolized to these two active metabolites [28]. In other words, it is a prodrug not because of lack of target affinity but for its incapacity to reach the target organ. *Diazepam* is also a sedative with a long, biphasic half-life, but one that acts both directly and via its three active metabolites, *desmethyldiazepam*, 3-hydroxydiazepam (*temazepam*), and *oxazepam*. *Flurazepam* is marketed mainly as a hypnotic, and indeed, its half-life is very short, the drug having disappeared from plasma 3 h after an oral dose to human volunteers [29]. An active metabolite is *N*¹-(2-hydroxyethyl)flurazepam, which is eliminated within a few hours. However, its *N*(1)-dealkylation produces *desalkylflurazepam*, a close analogue of *desmethyldiazepam* with an even longer half-life. As a result, flurazepam is unsuitable as a hypnotic in individuals who produce high levels of *desalkylflurazepam*.

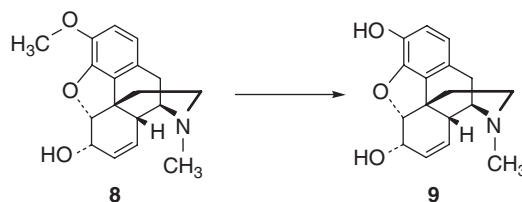


Figure 10.3 Codeine (**8**) and its active metabolite (**9**) produced by a CYP2D6-catalyzed reaction of O-demethylation.

10.3.2 Codeine, a Drug With a Differently Acting Metabolite

There are two main reasons why *codeine* (**8** in Fig. 10.3) is of significance in our context. First because the production of *morphine* (**9**), its active metabolite, is under strong genetic control [30]. And second, because there is a qualitative difference in the activity of drug and metabolite, in contrast with many known cases of active metabolites.

While codeine *per se* is an antitussive agent, it is often prescribed as an analgesic of medium strength. About two decades ago, reports began to appear on the lack of analgesic response in some patients administered codeine [31]. This fact was correlated with barely detectable plasma levels of morphine in some patients subsequently phenotyped as “poor metabolizers,” an obsolete terminology to describe individuals lacking a given drug-metabolizing activity. The culprit enzyme, in this case, is cytochrome P450 2D6 (CYP2D6), the affected patients (ca. 5–8% of the population) having nonfunctional *CYP2D6* alleles [12].

The story does not end here, however, since there is now recent evidence that a few individuals suffered from morphine intoxication following codeine intake [32–34]. The postoperative death of a healthy two-year-old boy was also traced to morphine intoxication following normal dosage of codeine [35]. The cause of overproduction of morphine in these patients is also genetic, in this case, gene duplication or multiplication resulting in three or more functional *CYP2D6* alleles.

10.3.3 Tramadol, a Drug With a Metabolite of Comparable Activity

Tramadol (**10** in Fig. 10.4) is a frequently used centrally acting analgesic characterized by a high efficacy, low potential for dependence or abuse, and low level of side effects (no relevant respiratory depression or effects on blood pressure and heart rate) [36–39]. This drug is particularly worthy of note for its dual mechanism of action, being both a weak opioid agonist and an inhibitor of monoamine neurotransmitter reuptake. For good reasons, the drug is used as the racemic mixture of two of its four stereoisomers, both its (–)-(1*S*,2*S*)- and (+)-(1*R*,2*R*)-enantiomers contributing synergistically to its *in vivo* activity.

In experimental pharmacology, (+)-tramadol proved a more active μ -receptor agonist than its (–)-enantiomer, and it also inhibited serotonin reuptake. In contrast, the (–)-enantiomer is an inhibitor of noradrenaline reuptake in addition to its weaker opioid effects. Furthermore, both enantiomers are metabolized by CYP2D6 to the corresponding *O*-desmethyltramadol enantiomers (**11**) with little or no substrate enantioselectivity. Both enantiomers of *O*-desmethyltramadol are more active than (+)-tramadol as μ -receptor agonists, but their blood–brain barrier permeation is lower. It is noteworthy

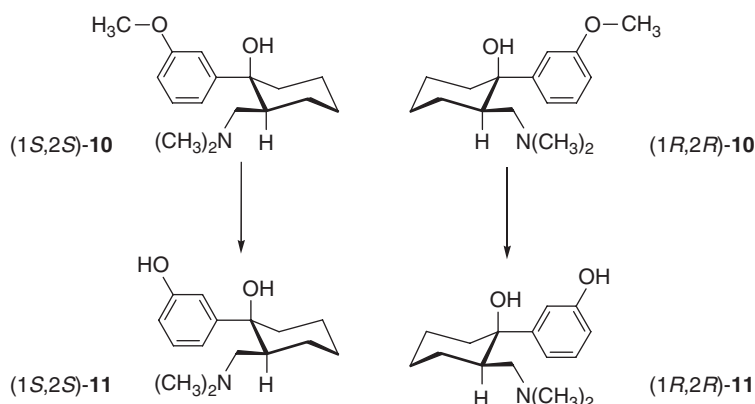


Figure 10.4 The two (active) enantiomers of *trans*-tramadol (**10**) and their even more active *O*-desmethyl metabolites.

that to the best of our knowledge, no effect on monoamine neurotransmitter reuptake has been reported for the metabolites. In clinical pharmacology, tramadol-induced analgesia is thus found to result from the combined and synergetic effects of four active entities. And while CYP2D6-deficient patients produce very little *O*-desmethyltramadol, tramadol activity is weaker but not absent in such patients [30,34].

10.3.4 Tamoxifen, a Drug Having Highly Active Metabolites

The complex and clinically highly relevant case of *tamoxifen* (**12** in Fig. 10.5) is summarized here. This estrogen receptor antagonist is extensively used for endocrine

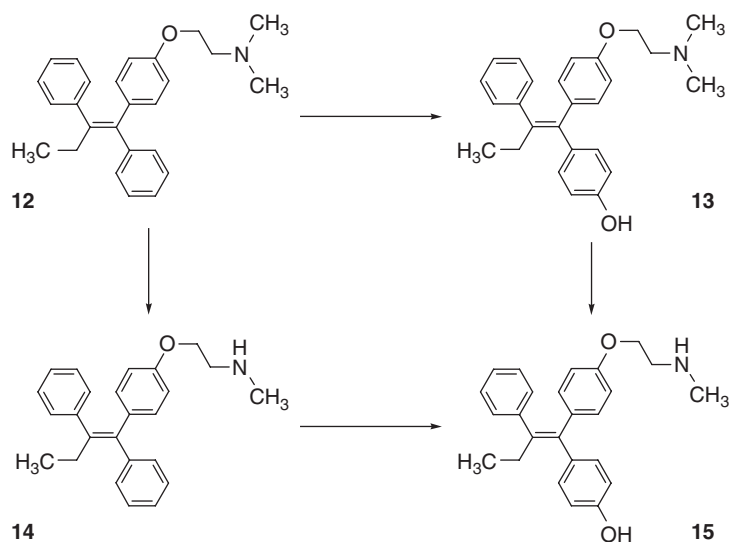


Figure 10.5 Tamoxifen (**12**) and its highly active metabolites 4-hydroxytamoxifen (**13**) and *N*-desmethyl-4-hydroxytamoxifen (**15**; endoxifen).

treatment of breast cancer [40]. Its metabolism is quite complex and leads to at least 13 oxidative metabolites [41,42], not to mention conjugation reactions. A pharmacologically significant route is aromatic oxidation to the active *4-hydroxytamoxifen* (**13**). However, the most important metabolic pathway in qualitative and quantitative terms is N-demethylation to the secondary amine *N*-desmethyltamoxifen (**14**), a reaction catalyzed mainly by CYP3A4. This metabolite, while reaching high plasma levels, is not as active as 4-hydroxytamoxifen and the second-generation metabolite *N*-desmethyl-4-hydroxytamoxifen (**15**) known as *endoxifen*.

A number of investigations have demonstrated that 4-hydroxytamoxifen and endoxifen are equipotent and many times more active as antiestrogens than the parent drug [43,44]. When taking plasma levels in consideration, it seems that the main contributor to therapeutic activity in patients is endoxifen, and to a lesser extent tamoxifen and 4-hydroxytamoxifen [45–47]. This has led to concerns that genotypic differences in CYP2D6 and the coadministration of drugs inhibiting this enzyme may have an impact on clinical outcomes in women treated with tamoxifen [48].

10.4 CARRIER-LINKED PRODRUGS

10.4.1 Introduction

What makes prodrugs different from other drugs is the fact that they are devoid of intrinsic pharmacological activity, as clearly defined by Adrien Albert [49], who coined the term. As such, prodrugs are the opposite of soft drugs (see above). *Intentional prodrugs* are by far the most frequent ones in drug research and development and are obtained by rational derivatization or modification of a known active agent. A historically important example is that of the *fortuitous prodrug* *prontosil*, whose *in vivo* antibacterial properties were discovered in 1932 by Gerhard Domagk, winning him the 1939 Nobel Prize in Medicine [50]. But the prodrug nature of *prontosil* remained unknown until 1935, when the discovery of its active metabolite sulfanilamide was the milestone that led to the creation of all antibacterial sulfonamides [51].

The prodrug concept aims at helping medicinal chemists and pharmaceutical scientists to overcome a number of development problems unsolvable by analog design [52–56]. Overcoming such hurdles translates into a number of *objectives in prodrug design*. Pharmaceutical objectives imply solving serious formulation problems caused by poor solubility, insufficient chemical stability, or poor organoleptic properties. PK objectives are currently the most important ones in prodrug research. Foremost among these is a need to improve oral bioavailability by enhancing the oral absorption of the drug and/or decreasing its presystemic metabolism; other objectives are to improve parenteral absorption, to lengthen the duration of action of the drug by slow metabolic release, and finally to achieve organ/tissue-selective delivery.

PD objectives often imply decreasing systemic toxicity, including the masking of a reactive agent, the *in situ* activation of a cytotoxic agent, and more generally improving a drug's therapeutic index. As a note of caution, it should be clear from the onset that prodrug objectives are often strongly intertwined. As demonstrated by the examples below, the prodrug concept may be a valuable strategy to disentangle PK and PD optimization in the drug discovery phases.

This section deals with prodrugs composed of the drug molecule coupled to a promoiety (also known as a *carrier*). Here, bioactivation occurs by cleavage of the

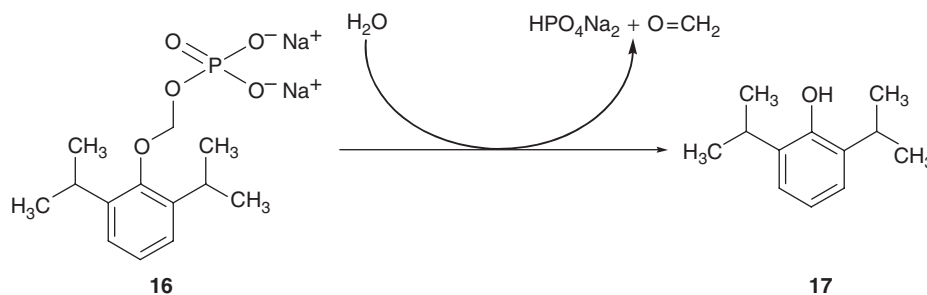


Figure 10.6 Fospropofol (**16**), a water-soluble, marketed prodrug of the sedative-hypnotic drug propofol (**17**).

link between drug and carrier, in most cases due to hydrolysis. Section 10.5 considers redox-activated prodrugs (bioprecursors).

10.4.2 Fospropofol, a Product for Improved Solubility

Propofol (**17** in Fig. 10.6) is a sedative-hypnotic, whose mechanism of action is based on an allosteric modulation of GABA_A receptors, causing increased binding of γ -aminobutyric acid, its physiological ligand [57–59]. propofol provides sedation to patients undergoing uncomfortable interventions such as bronchoscopy or colonoscopy. It is also used for induction and maintenance of general anesthesia and in intensive care units for short or longer term sedation of medically ventilated adults.

Despite its advantages and extensive use, propofol suffers from a number of disadvantages such as pain at the injection site, a narrow therapeutic window, and a steep concentration–response curve bridging moderate sedation to general anesthesia. More relevant in the present context is its very low water solubility. As a result, propofol is administered intravenously as a lipid emulsion, which calls for absolute sterility. The lipid-based emulsions of propofol are fairly stable but tend to separate with the addition of other chemicals and changes in temperature or pH [59]. A most worrisome complication is propofol infusion syndrome, a rare but life-threatening event resulting from the long-term administration of high doses of the drug [60]. The syndrome is characterized by metabolic acidosis, acute renal failure, rhabdomyolysis, hyperlipidemia, and cardiac dysfunction.

The *pharmaceutical problems* associated with the poor solubility of propofol have provided a worthy justification to the development of water-soluble prodrugs. *Fospropofol* (**16**; propofol *O*-methyl phosphate disodium) has emerged as the lead candidate for a water-soluble prodrug of propofol and has recently been approved for monitored anesthesia in adults undergoing diagnostic or therapeutic procedures [61–63]. The prodrug is very stable toward chemical hydrolysis [64] and is very rapidly hydrolyzed by hepatic and endothelial alkaline phosphatases. The products of the reaction are propofol, formaldehyde (which is rapidly converted to formate), and phosphate.

Our understanding of the PK/PD relations of fospropofol underwent severe regression when it was found that the bioanalytical assay used in all PK/PD studies published up to 2009 was inadequate and yielded unreliable propofol plasma concentrations [65]. As a result, six papers were withdrawn [66], yet some unaware readers might continue to rely on conclusions found in previously published reviews.



19

10.4.3 Oseltamivir, a Prodrug for Oral Absorption

Achieving improved oral absorption and generally oral bioavailability is a frequent rationale in marketed prodrugs [67,68]. Inhibitors of angiotensin-converting enzyme (ACE) such as benazepril and enalapril offer much-reviewed examples [69,70]. Here, we focus on two neuraminidase inhibitors of value against type A and B influenza in humans [71–73]. Target-oriented rational design led to highly hydrophilic agents that are not absorbed orally. One drug in clinical use is *zanamivir*, a highly hydrophilic drug administered in aerosol form.

Another active agent is Ro-64-0802 (**19** in Fig. 10.7; now known as *oseltamivir carboxylate*), which also shows high *in vitro* inhibitory efficacy but practically no oral bioavailability due to its high polarity [74,75]. In contrast, its ethyl ester pro-drug *oseltamivir* (**18**) is well absorbed orally and undergoes rapid carboxylesterase 1 (CES1)-catalyzed hydrolysis *in vivo* to produce high and sustained plasma levels of the active agent [76–80]. In this example, the lack of oral absorption of the anti-influenza agent Ro-64-0802 was overcome with the help of a simple physicochemical principle, namely, the masking of a highly hydrophilic carboxylate group with a reversible ester function.

10.4.4 Valacyclovir, a Prodrug Targeting an Intestinal Transporter

Acyclovir (**21** in Fig. 10.8) is a nucleoside analogue used systemically and locally to treat herpes simplex virus (HSV-1 and -2) infections. It is also used in higher doses to treat infections from the varicella zoster virus (VZV). Acyclovir is a valuable drug, yet its oral absorption is modest (15–30%) [81]. This has led to many potential prodrugs being prepared and examined, with the (*S*)-valyl ester being selected and now marketed as *valacyclovir* (**20**) [82,83].

This prodrug is of particular interest as its intestinal absorption is an active one mediated by the di-/tripeptide transporter PEPT1 [84], the resulting bioavailability of the active agent being ~50–60%. This bioavailability is clearly less than expected, given the good affinity of valacyclovir for the transporter. And indeed stability studies have shown valacyclovir to undergo partial degradation in the upper lumen of the small intestine [85].

Practically, the totality of absorbed valacyclovir is activated to acyclovir by a serine hydrolase initially known as *biphenyl hydrolase-like protein* (BPHL). This enzyme, now characterized as human valacyclovirase, is specific for α -amino acid esters and

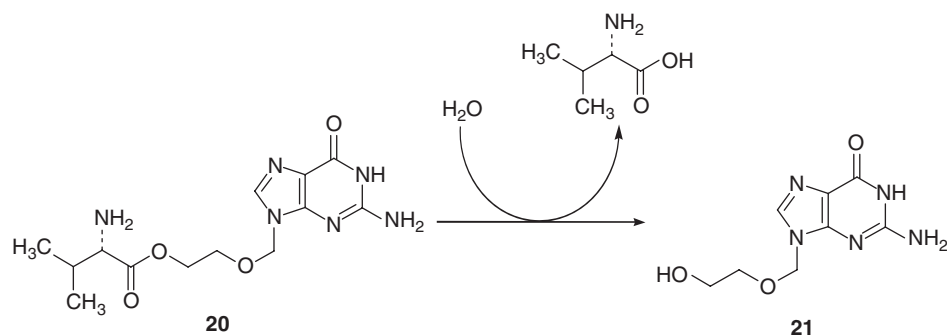


Figure 10.8 Acyclovir (**21**), a poorly absorbed antiherpes virus drug, and its (*S*)-valyl ester prodrug valacyclovir (**20**).

has been shown to hydrolyze the prodrugs of a broad range of antiviral and anticancer nucleoside analogs such as zidovudine and gemcitabine [86,87].

10.4.5 Capecitabine, a Prodrug for Targeted Delivery

The objective of organ- or tissue-selective delivery, also known as the search for the *magic bullet*, is receiving ever increasing interest in the tumor-selective delivery of anti-cancer agents. A clinically significant example is that of *capecitabine* (**22** in Fig. 10.9), a multistep, orally active prodrug of 5-fluorouracil (**25**; 5-FU) [82,88]. Capecitabine is well absorbed orally and undergoes three activation steps resulting in high tumor concentrations of the active drug. It is first hydrolyzed by liver carboxylesterase (CES), the resulting metabolite being a carbamic acid that spontaneously decarboxylates to 5'-deoxy-5-fluorocytidine (**23**). The enzyme cytidine deaminase (EC 3.5.4.5), which is present in the liver and tumors, then transforms 5'-deoxy-5-fluorocytidine into 5'-deoxy-5-fluorouridine (**24**). Transformation into 5-FU (**25**) is catalyzed by thymidine phosphorylase (EC 2.4.2.4) and occurs selectively in tumor cells [89–93].

Capecitabine was first approved for the cotreatment of refractory metastatic breast cancer. Its therapeutic spectrum now includes metastatic colorectal cancer, and there are hopes that it might broaden further as positive results of new clinical trials become available. Capecitabine thus affords an impressive gain in therapeutic benefit compared to 5-FU because of its oral bioavailability and a relatively selective activation in (and thus delivery to) tumors.

10.5 BIOPRECURSORS

In contrast to the more traditional, carrier-linked prodrugs, bioprecursors do not contain a promoiety (i.e., a carrier moiety) but are activated enzymatically either by reduction or by oxidation, hence, their alternative designation as *redox-activated prodrugs* [94]. The bioactivation reaction creates either a *pharmacophoric group* (e.g., nabumetone) or a *chemically reactive group* (e.g., clopidogrel and tirapazamine).

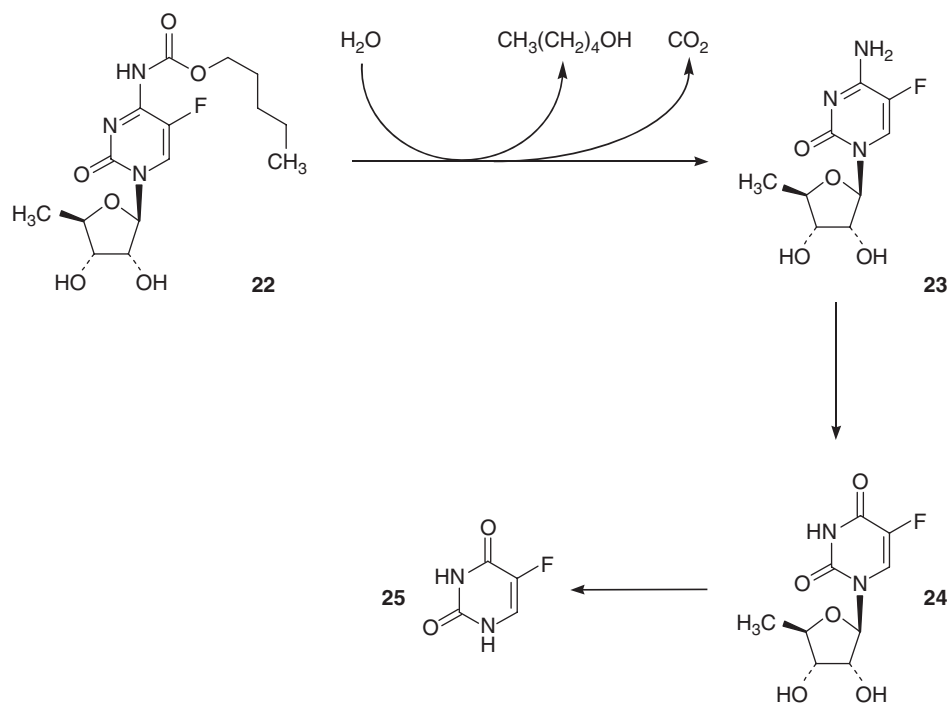


Figure 10.9 Capecitabine (22), a multistep, tumor-targeted prodrug of 5-fluorouracil (25).

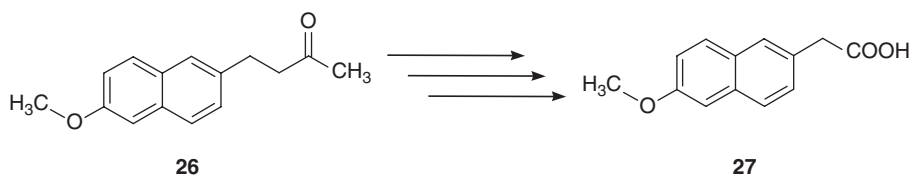


Figure 10.10 The bioprecursor nabumetone (26) and its metabolite 6-methoxynaphthylacetic acid (27), a long-acting nonsteroidal anti-inflammatory agent.

10.5.1 Nabumetone, a Long-Acting NSAID Prodrug with Low Gastric Toxicity

A rather complex and intriguing example of bioprecursors is afforded by *nabumetone* (26 in Fig. 10.10). In humans and experimental animals, this prodrug undergoes three major initial metabolic pathways, namely, oxidative O-demethylation, carbonyl reduction, and a complex route likely involving oxidation of the terminal $-\text{CH}_3$ to $-\text{COOH}$, followed by coenzyme A conjugation and β -oxidation to 6-methoxynaphthylacetic acid (27; 6MNA) [95,96]. The latter route is the major one in humans, where it accounts for at least one-third to one-half of a dose. 6MNA is the active metabolite of nabumetone. It is characterized by a very long serum half-life in humans (about 24 h, range 17–74 h), because of a slow hepatic clearance and absence of renal clearance [97,98]. The anti-inflammatory and analgesic activities of 6MNA are explained by its inhibition of cyclooxygenase, with a modest selectivity toward cyclooxygenase-2 (COX-2) [99,100].

A valuable feature of this metabolite is its affinity for synovial fluids where it reaches substantial concentrations, making it useful in chronic inflammatory arthropathies.

One of the reasons why nabumetone is discussed here is its low gastric toxicity that sets this prodrug apart from other NSAIDs [101–103]. In numerous toxicological and clinical studies, nabumetone has indeed confirmed a high therapeutic index (ratio of toxic dose overtherapeutic dose). It now appears that this low gastrointestinal toxicity is due to two factors. First, the nonacidic nabumetone has limited affinity for the gastric mucosa, where its concentrations remain low; and not being able to inhibit cyclooxygenase, it cannot cause COX-related significant damages in the gastrointestinal (GI) tract. As for its active metabolite 6MNA, its presence was undetectable in rat gastric mucosa given nabumetone orally [104]. In addition, nabumetone itself has shown an intrinsic gastroprotective effect by inhibiting neutrophil functions. Specifically, neutrophils are agents in NSAID-induced lesions in the GI tract. Indeed, NSAIDs such as indometacin facilitate their infiltration in rat gastric mucosa and induce their respiratory burst. These effects were inhibited by nabumetone.

10.5.2 Clopidogrel and Prasugrel, the Masking of a Highly Reactive Metabolite

The masking of a reactive agent to improve its therapeutic index is aptly exemplified by the successful anti-aggregating agent *clopidogrel* (**28** in Fig. 10.11) [105–107]. This compound, whose molecular mechanism of action was poorly understood for years, is now known to be a prodrug. But while its major metabolic route in humans (about 80% of a dose) is indeed one of ester hydrolysis, this reaction leads to the inactive acid. Most of the remainder of a dose of clopidogrel is activated in a three-step sequence. First, cytochromes P450s (3A and 2C19) oxidize clopidogrel to *2-oxo-clopidogrel* (**29**). This is followed by a CYP-catalyzed sulfoxidation to the *cyclic sulfoxide intermediate* **30** [106]. The latter hydrolyzes spontaneously to a highly reactive *sulfenic acid metabolite* (**31**), which forms a covalent S–S bridge with a thiol group in platelet ADP receptors, thereby causing their long-lasting inactivation. Interestingly, the same activation mechanism appears to account for the potent and irreversible inhibition of human CYP2B6 by clopidogrel [108].

The sulfenic intermediate can also be reduced by glutathione, leading to the corresponding thiol (Fig. 10.11), which until 2009 was believed to be the active metabolite [109]. This thiol has three stereogenic centers, namely, the original C(7) of (*S*)-configuration, the C(3)–C(16) double bond of (*Z*)-configuration, and C(4), whose absolute configuration appears to be (*R*).

The recent discovery of a sulfenic acid as the active metabolite of clopidogrel [106] raises the question of the enzymes catalyzing its formation. Indeed, sulfoxidation is a metabolic reaction catalyzed by CYPs as well as flavin-containing monooxygenases (FMOs), of which at least three enzymes exist in humans (FMO1, FMO2, and FMO3) [3,5]. This broadens the prospect of future investigations on the genetic and drug interaction factors that influence clopidogrel activation.

The metabolic pathway of activation of clopidogrel indeed offers opportunities for *genetic factors* and drug-drug interactions (DDIs) to influence its clinical outcome [110]. And indeed, patients taking clopidogrel and who were carrying loss-of-function *CYP2C19* alleles (*2, *3, *4, or *5) were recently found to have a higher rate of cardiovascular event [111]. In healthy subjects administered clopidogrel, carriers of at least one *CYP2C19* loss-of-function allele (30% of the studied population) had a relative

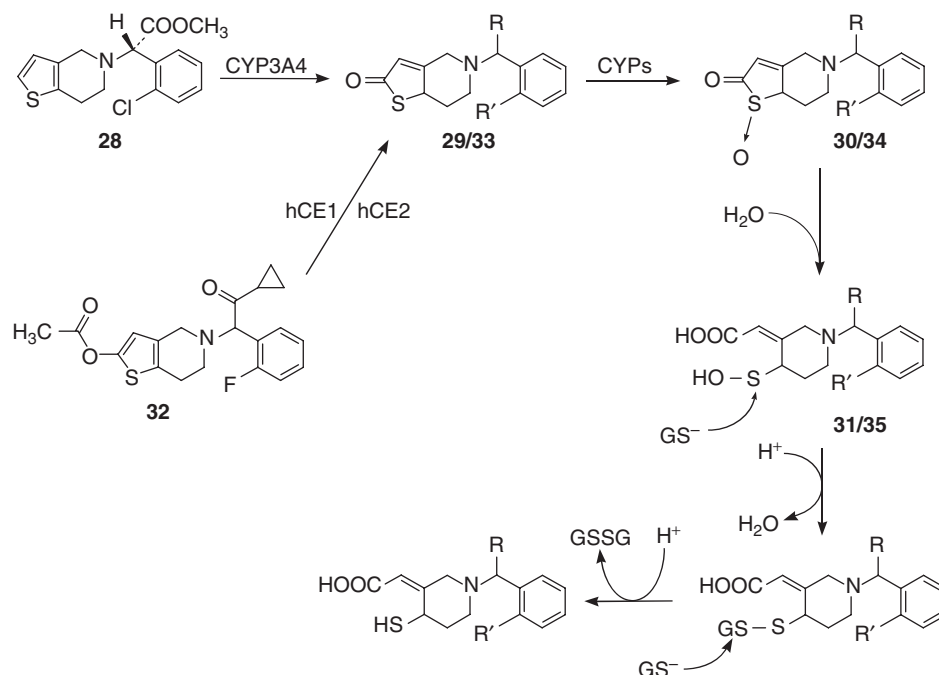


Figure 10.11 A schematic comparison of the mechanism of activation of the anti-aggregating bioprecursors clopidogrel (**28**) and prasugrel (**32**) to their (re)active sulfenic acid metabolite **31** and **35**, respectively. The lower part of the figure shows a mechanism of glutathione-mediated reduction of the sulfenic acid to the corresponding thiol, which was believed for years to be the active metabolite.

reduction of one-third in plasma exposure to the thiol metabolite, a decrease that corresponded to a reduction in maximal platelet aggregation [112]. Other studies showed that reduced-function or loss-of-function alleles of *CYP3A* genes also contributed to the phenomenon of clopidogrel resistance [113].

There are also reports of DDIs in increasing or decreasing clopidogrel activation and activity. Thus, treatment with the antituberculosis drug rifampin (a well-known *CYP3A4* inducer) enhanced clopidogrel activity such that some non- and low responders became responders [113]. In contrast, proton-pump inhibitors (e.g., lansoprazole and omeprazole), which are substrates and inhibitors of *CYP2C19* and *3A4*, negatively affected clopidogrel response and increased platelet aggregation [114].

A recently marketed analogue of clopidogrel (**28**) is *prasugrel* (**32**) [115,116]. In contrast to the former, activation of the latter begins with a CES-catalyzed hydrolysis [117–121]. Indeed, C(2) in prasugrel carries a phenolic oxygen, whose deacetylation allows tautomerization to the thiolactone **33**, a close analogue of 2-oxoclopidogrel (**29**). Formation of the intermediate phenol is demonstrated by the isolation of its *O*-glucuronide in human plasma and urine, as well as in laboratory animals. As for the thiolactone **33**, its activation by sulfoxidation to **34** can be postulated in analogy with that of clopidogrel, followed by hydrolytic ring opening to the active *sulfenic acid metabolite* **35**, which shares the same molecular mechanism of action as the active metabolite of clopidogrel.

Prasugrel is about 10 times more potent than clopidogrel, a fact ascribable to its extensive hydrolysis to the thiolactone **33**. Cytochrome P450 has been known to be involved in prasugrel activation, a finding which at the time was difficult to reconcile with its activation by hydrolysis. The discovery of a reaction of sulfoxidation as a second and key step in the activation of thienopyridines [106] seems to resolve this difficulty, while suggesting also a complementary role for FMOs (see above). The fact that prasugrel activity appears less sensitive than clopidogrel to drug interactions and genetic factors [115] is compatible with this view.

There is one noteworthy stereochemical difference between the two prodrugs, since prasugrel is used as the racemate. As for its thiol metabolite, it was found to occur as four stereoisomers, the two most abundant ones having an (*R*)-configured C(4) atom in addition to the original (*R*)- and (*S*)-configurations at the original center of chirality.

10.5.3 Tirapazamine, a Bioreductive Prodrug

Bioreductive prodrugs are designed to be activated by a metabolic reaction of reduction in target tissues or organisms with a low oxygen concentration. Such a state of hypoxia may favor reduction reactions because of a relative lack of oxygen, but the major factor accounting for the shift toward reduction is the hypoxia-increased expression of genes coding for reductive enzymes. A variety of chemotherapeutic agents (antibacterial, antiparasitic, or anticancer agents) are in fact prodrugs activated by a variety of reductases [122,123]. Such prodrugs include nitroarenes, quinones, amidoximes, platinum(IV) complexes, and *N*-oxides exemplified here with *tirapazamine* (**36** in Fig. 10.12) [124–128].

This much-studied agent is inactivated by two-electron reduction steps catalyzed by quinone reductase, losing its two oxygen atoms. Its activation, in contrast, is to a radical (**37**) by a one-electron reduction catalyzed by NADPH-cytochrome P450 reductase and multiple reductases in the nucleus. Loss of water yields the *benzotriazinyl radical* **38**, whose strong reduction potential allows it to cause DNA single-strand and double-strand breaks, thereby being reduced to **39**. Back oxidation of the radical **37** by molecular oxygen is a reaction of inactivation, which can occur in aerobic cells. In other words, cellular toxicity will depend first and foremost on oxygen levels in the cells. Of interest is also the fact that simple monosubstitution of tirapazamine can alter its lipophilicity enough to markedly improve extravascular transport and activity against target cell [128].

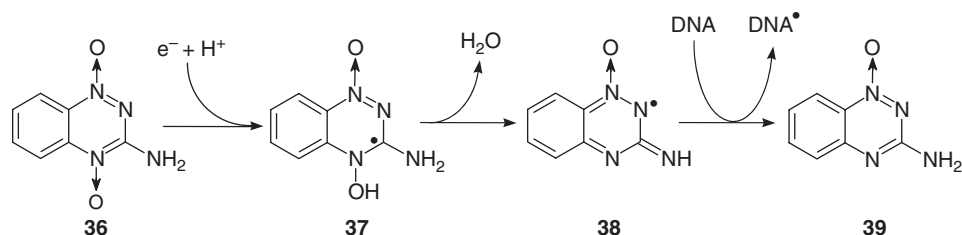


Figure 10.12 Tirapazamine (**36**), a bioreductive antitumor prodrug, and its molecular mechanism of activation to the benzotriazinyl radical **38**, which reacts with DNA.

10.6 METABOLIC DRUG-DRUG INTERACTIONS: THE CASE OF ANTI-HIV PROTEASE INHIBITORS (PIs)

DDIs at the level of metabolism result from drug A inducing or inhibiting one or more enzyme(s) acting on drug B [13]. Other types of DDIs involve interactions at the level of transporters or receptors. As such, DDIs result from a biological system being altered by the action of a xenobiotic, a process we have classified as a PD response. Significantly, this biological response is of indirect interest in our context and offers a perfect illustration of the PK/PD interplay outlined in Section 10.2.1. To illustrate the phenomenon, this section gives a short overview of DDIs produced by or seen among PIs used in antiretroviral therapy. The focus will be on the PI *ritonavir* (RTV), a classical and well-known inhibitor of the metabolism of other drugs [129–133].

RTV was first used at high doses as the sole PI in anti-HIV combinations. Its high toxicity at high doses and a less favorable profile have led to its current use being restricted to that of a pharmaco-enhancer of other PIs such as *atazanavir*, *darunavir* (DRV), *fosamprenavir*, *indinavir*, *lopinavir*, *saquinavir*, and *tipranavir*. Indeed, RTV is a substrate of CYP3A, the efflux transporter P-glycoprotein (P-gp), and to a lesser extent CYP2D6. More importantly, RTV is a potent inhibitor of CYP3A4, an inhibitor of CYP2D6 and P-gp, and a moderate inhibitor of CYP2C9.

The antiretroviral drugs with which RTV is being associated have their own inhibitory effects, resulting in complex effects. For example, the AUC and $C_{\max}$ of the C-C chemokine receptor type 5 (CCR5) antagonist *maraviroc* were increased four- and twofold, respectively, by a DRV-RTV combination [132]. In contrast, the DRV-RTV combination modestly increased or decreased the AUC and $C_{\max}$ of some reverse transcriptase inhibitors. As for agents in other therapeutic classes (e.g., antimicrobials, proton-pump inhibitors, and antidepressants), the effects of the DRV-RTV combination varied from drug to drug. For example, the AUC and $C_{\max}$ of ketoconazole increased two- to threefold, while those of sertraline decreased twofold; smaller effects were seen in the PK behavior of other drugs.

To complicate matters further, RTV is now known to be an enzyme inducer in addition to being an inhibitor [130]. Thus, it is an inducer of CYP3A, although its inhibitory interactions with this enzyme are far more prevalent and may be related to “inhibition-associated induction.” At high doses, RTV induces CYP1A2, thereby increasing the metabolic clearance of theophylline, caffeine, and olanzapine, and CYP2C9 (acenocoumarol, phenytoin, phenobarbital, etc.). At high and low doses, it also induces CYP2B6 (bupropion, meperidine, efavirenz, sertraline, etc.), CYP2C19 (methadone, proton-pump inhibitors, etc.), and UDP-glucuronosyltransferases (zidovudine, raltegravir, valproate, oral contraceptives, oxazepam, morphine, nonsteroidal anti-inflammatory drugs, etc.).

10.7 CONCLUSION

This chapter follows a double thread. Along the main thread, as obvious for the layout, we see the intrinsic activity of the drug decreasing, while that of the metabolite(s) increases. Thus, the reader moves from (i) drugs with no active metabolite (Section 10.2.4) to (ii) drugs having active metabolite(s) (Section 10.3) to (iii) intrinsically inactive prodrugs. For the vast majority of (active) drugs having active

metabolites, bioactivation is found to occur by CYP-catalyzed oxidation, as illustrated here with benzodiazepines, codeine, tramadol, and tamoxifen. Among prodrugs, a discrimination is made between carrier-linked prodrugs, whose activation is by cleavage (generally hydrolytic) of a promoiety (Section 10.4), and bioprecursors (Section 10.5), where a redox reaction creates a pharmacophoric or reactive group.

The second thread in the chapter is that of biological levels of complexity and it may seem to be followed erratically. Indeed, metabolic bioactivation may be discussed at various biological levels, from the molecular and enzymatic to the clinical. Here, there is no logical sequence, but we have tried to achieve a reasonable balance that would appeal to most readers. Depending on the breadth and value of the available information, some examples were indeed focused mainly on clinical outcomes, while others deal in greater details with biochemical aspects. Sometimes a convincing causal link can be deduced between the biomolecular and clinical levels. In other cases, the causal link may fail to be fully convincing, pointing to a need for further studies.

While these were not the proper topic of the chapter, pharmacogenetic differences and DDIs are also briefly discussed and exemplified in later sections. These serve to strengthen the link we tried to draw between the biochemical and the clinical levels of drug metabolism.

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11 Role of Metabolism in Toxicity of Drugs in Humans

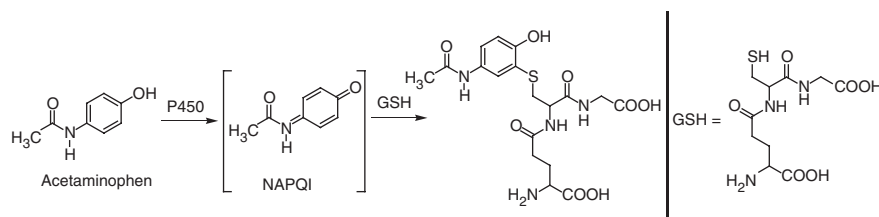
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11.1	Introduction	1
11.2	Screening for reactive metabolites	3
11.3	Structure–toxicity relationships	4
11.4	Examples of medicinal chemistry tactics to eliminate/minimize reactive metabolite formation	10
11.5	Should incorporation of toxicophores be prohibited in drug design?	14
11.6	Value of reactive metabolite trapping and covalent binding studies in the prediction of drug toxicity	15
11.7	Daily dose/low systemic reactive metabolite exposure as a mitigating factor for IADRs	17
11.8	Concluding remarks	20
	References	20

11.1 INTRODUCTION

Metabolite-dependent toxicity is an increasingly important issue for the pharmaceutical industry. In particular, the formation of electrophilic reactive metabolites with drug candidates has received much attention, considering that the liability has been associated with genotoxicity, target organ toxicity, drug–drug interactions, and possibly, idiosyncratic adverse drug reactions (IADRs). IADRs constitute a rare, toxic drug reaction found only in the drug-treated patients clinically and is not reproducible in animal experiments [1]. In an increasing number of cases, evidence suggests that this toxicity can be initiated by the production of chemically reactive, electrophilic metabolites, which covalently bind to macromolecules such as proteins and nucleic acids, unless otherwise detoxified by glutathione *S*-transferases or other scavenging phase II drug-metabolizing enzymes [2–6]. The concept of drug metabolism to chemically reactive metabolites that covalently modify proteins leading to toxicity was first demonstrated in the 1970s with the anti-inflammatory agent acetaminophen by Brodie *et al.* at the National Institutes of Health [7–10]. Mechanistic studies that established



Scheme 11.1 P450-catalyzed bioactivation of the anti-inflammatory agent acetaminophen.

the cytochrome P450-mediated bioactivation of acetaminophen to a reactive quinone-imine intermediate [NAPQI (*N*-acetyl-*p*-benzoquinone imine)], capable of depleting the levels of the endogenous antioxidant glutathione (GSH) and/or binding covalently to liver macromolecules (Scheme 11.1) has served as a paradigm for drug toxicity assessment over the decades [11]. Acetaminophen is a major cause of drug-related morbidity and mortality in humans, capable of producing hepatic necrosis after a single toxic overdose [12].

Given the sophistication of twenty-first century bioanalytical methodology, the detection of a bioactivation process can be relatively straightforward; however, the downstream consequences of this metabolic event as it relates to IADRs remain unclear [13,14]. The hapten hypothesis proposes that covalent modification of protein(s) by a reactive metabolite is followed by cleavage to peptide fragments by a proteasome. These peptides are transported to the cell surface after binding to the major histocompatibility complex class I (MHC-I) and presented to the immune system. It is believed that abnormal peptides, when recognized by cytotoxic T-lymphocytes as non-self-peptides, induce the toxic immune reaction finally leading to cell death. If so, then idiosyncrasy would reside on (i) formation of the chemically reactive metabolite, (ii) degradation of the reactive metabolites, (iii) recognition of drug-modified proteins as non-self-proteins by the immune system, and (iv) immune-mediated IADR in susceptible patients [13–16].

Possibly, the most important susceptibility factor for IADRs is genetic variability [17]. Genetic polymorphisms have a strong influence on drug metabolism and may increase risk of IADRs. For example, polymorphism of the *N*-acetyltransferase 2 (NAT2) gene differentiates fast from slow acetylators; the latter have increased susceptibility to toxicity of certain aniline-containing drugs such as isoniazid, sulfamethoxazole, dapsone, and procainamide [18,19]. The major route of elimination of these drugs in humans involves *N*-acetylation of the aniline moiety by NAT2, resulting in the neutral amide metabolites. In an NAT2-deficient population, the aniline motif in isoniazid is hydrolyzed by amidases, liberating hydrazine, which is toxic in its own right; likewise, sulfamethoxazole, dapsone, and procainamide are biotransformed by P450 enzymes to yield cytotoxic and protein-reactive metabolites that include *N*-hydroxyaniline derivatives and the subsequent two-electron oxidation products, that is, the nitroso intermediates. The reactivity of the nitroso metabolites of these drugs with GSH and/or proteins in target organs has also been demonstrated [20–22]. There is also a strong possibility that components of ingested foods, including herbal supplements, can modulate drug metabolism and therefore increase IADR risk [22].

Considering that a detailed understanding of the mechanisms of IADRs remain poorly understood, it is currently impossible to *accurately predict* which new drugs will

be associated with a significant incidence of IADRs, and this poses a significant challenge in drug discovery/development. Despite the disadvantage, there are a few *default* assays in place that could likely reduce the probability of occurrence of IADRs with new drugs. Because it is now widely appreciated that reactive metabolites, as opposed to the parent molecules from which they are derived, are responsible for the pathogenesis of some IADRs, most pharmaceutical companies have implemented procedures to evaluate a drug candidate's potential to undergo metabolic activation (bioactivation), with the goal of eliminating or minimizing reactive metabolite formation by rational structural modification of the lead chemical class.

11.2 SCREENING FOR REACTIVE METABOLITES

Screens for assessing reactive metabolite formation with drug candidates include protein covalent binding studies and/or trapping of reactive metabolites with nucleophiles. Covalent binding studies can be used to quantitatively estimate irreversible incorporation of radioactivity into proteins, following incubation of radiolabeled material with appropriate biological matrix (e.g., NADPH-supplemented liver microsomes) or through direct *in vivo* administration to preclinical species [23]. Either tissue or blood/plasma can be examined for the degree of *in vivo* covalent binding. However, covalent binding may require multiple dosing to establish the true impact since reactive metabolites formed after the first dose may be efficiently trapped by the endogenous antioxidant, GSH, and eliminated from the body. Once GSH is depleted, the extent of covalent binding with cellular macromolecules may increase rapidly, resulting in toxicity. Despite its utility, the technique has limited value in the early lead optimization phase in drug discovery because of the prerequisite need for synthesis of radiolabeled material for every compound of interest.

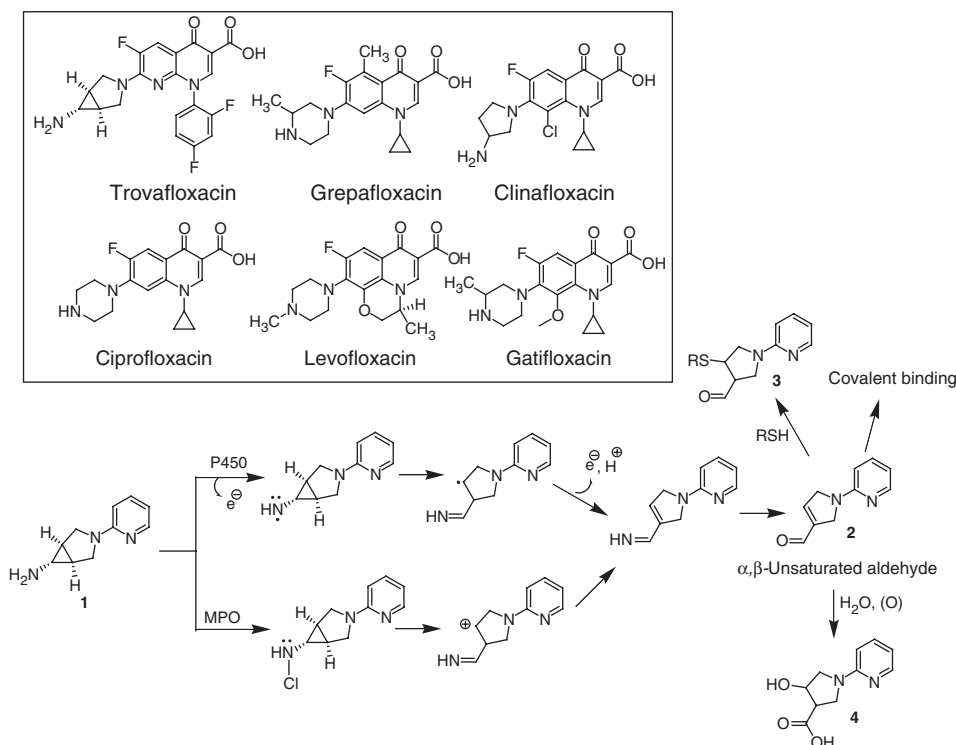
With the exception of acyl glucuronides and certain cyclic iminium ions, reactive metabolites are unstable and are not detectable in circulation. Their formation *in vitro* and *in vivo* can be inferred from the characterization of stable conjugates derived from reaction with chemical trapping agents. The trapping of reactive metabolites with exogenous nucleophiles to yield stable conjugates amenable to structural characterization by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and/or NMR spectroscopy has been exploited extensively to assess bioactivation potential of drug candidates in early discovery. Qualitative *in vitro* screening of reactive metabolite formation involves "trapping" studies conducted with NADPH-supplemented human liver microsomes and exogenously added GSH. Alternate metabolism vectors, such as liver cytosol, liver S9 fractions, hepatocytes, neutrophils, can be used instead of liver microsomes to probe the contribution of non-P450 enzymes to reactive metabolite formation. GSH adducts are relatively easy to detect by LC-MS/MS since the conjugates display a characteristic loss of 129 Da on collisional activation in MS/MS experiments of the molecular ions [24]. Since 129 Da corresponds to the loss of the pyroglutamate moiety of GSH, monitoring the loss of 129 Da in a constant neutral loss mode provides a generic and selective means to identify GSH adducts. The constant neutral loss scan will provide the molecular weight of the GSH adduct, and subsequent product ion scans can provide structural information about the nature of the adduct. Several modifications to the GSH-trapping assay have been made to accommodate screening of large numbers of compounds that are typically synthesized in discovery [25–28]. The

presence of the soft nucleophilic thiol group in GSH and its derivatives ensures efficient conjugation with soft electrophilic centers on most reactive metabolites (e.g., Michael acceptors including quinonoids, α,β -unsaturated carbonyl compounds, epoxides including arene oxides, alkyl halides) to yield stable thiol conjugates. Reactive metabolites containing hard electrophilic centers (e.g., DNA-reactive aldehydes) will preferentially react with hard nucleophiles such as amines (e.g., semicarbazide and methoxylamine), amino acids (e.g., lysine), and DNA bases (e.g., guanine and cytosine) to afford the corresponding Schiff base [29,30]. Electrophilic iminium species that are generated via metabolism of acyclic and cyclic tertiary amines can be trapped using cyanide anion, which is a hard nucleophile [31,32]. A clear-cut advantage of the reactive metabolite trapping methodology is that it does not require radiolabeled material and is amenable to high throughput screening. As illustrated later, elucidation of the structure of GSH conjugates can provide indirect information on the structure of the reactive metabolite, thereby providing an insight into the bioactivation mechanism and hence a rationale on which to base subsequent chemical intervention strategies.

11.3 STRUCTURE–TOXICITY RELATIONSHIPS

An understanding of the biochemical basis of IADRs has aided to replace the vague perception of a chemical class effect with a sharper picture of individual molecular peculiarity. There are several instances of prototype drugs associated with toxicity, which also form reactive metabolites; elimination of the bioactivation liability in the successor agent also results in elimination of the toxicity associated with the predecessor [33–36]. Although evidence from structure–toxicity analyses is usually anecdotal, a compelling case for chemotype-based liability can be inferred as illustrated with the following examples.

The broad-spectrum antibiotic trovafloxacin has been associated with several cases of clinically symptomatic liver toxicity, including cases of acute liver failure and/or liver-injury-related fatalities. This rare but serious hepatotoxicity led to the withdrawal of trovafloxacin from the market in many countries and a black box warning with intensive monitoring requirements in the United States. With the exception of trovafloxacin, none of the other drugs in the fluoroquinolone class of antibiotics (Scheme 11.2) have been associated with idiosyncratic hepatotoxicity. Grepafloxacin was withdrawn from the market because of concerns over its cardiovascular safety, while the clinical development of clinafloxacin was terminated because of phototoxicity. Ciprofloxacin, levofloxacin, and gatifloxacin remain in the marketplace. From a structure–toxicity standpoint, trovafloxacin contains the cyclopropylamine functionality at the C-7 position of the fluoroquinolone scaffold, a feature absent in other fluoroquinolone-based antibiotics. Studies with a model cyclopropylamine derivative **1** have revealed cyclopropylamine ring bioactivation by P450 and myeloperoxidase (MPO) to a reactive α,β -unsaturated aldehyde **2** trapped as a sulfhydryl conjugate **3** (Scheme 11.2) [37]. The characterization of a hydroxycarboxylic acid metabolite of trovafloxacin (**4**) in preclinical species lends further credence for the metabolism of the cyclopropylamine ring in trovafloxacin to a reactive intermediate [38]. The formation of **4** can occur from addition of water to the α,β -unsaturated aldehyde via Michael addition followed by oxidation, as depicted for the model compound (Scheme 11.2). However, the proposal for reactive metabolite formation with trovafloxacin remains a speculation since



Scheme 11.2 Insights into trovafloxacin bioactivation via the use of a model compound containing the cyclopropylamine functionality.

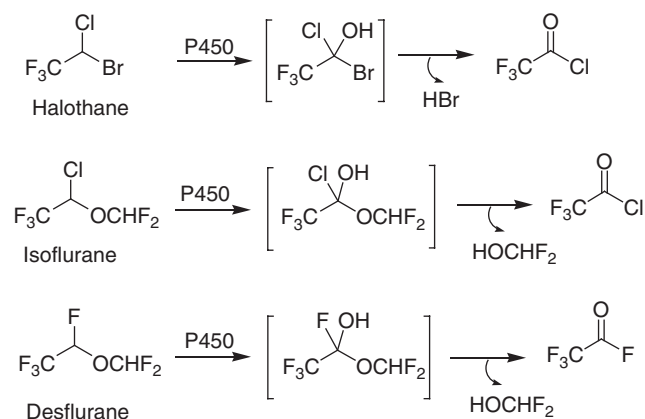
the bioactivation studies did not involve the parent fluoroquinolone; furthermore, no α,β -unsaturated aldehyde or the corresponding GSH conjugate has been detected in trovafloxacin incubations in human liver microsomes [37]. Also, the primary pathways of trovafloxacin clearance in humans include phase II metabolism (N-acetylation and N-sulfation on the primary amine group and acyl glucuronidation on the carboxylic acid moiety), with very minor contributions from phase I oxidative pathways [39].

In addition to the reactive metabolite theory, microarray analysis revealed substantial gene expression changes following treatment of human hepatocytes with trovafloxacin as compared to other marketed fluoroquinolones [40]. The expression profile induced by trovafloxacin was markedly distinct from other fluoroquinolones, in that genes involved in oxidative stress were regulated consistently by trovafloxacin. In HepG2 cells, trovafloxacin also induced oxidative stress and depleted intracellular GSH levels to a greater extent than other fluoroquinolones [41]. Finally, a potential role of inflammatory mediators in trovafloxacin hepatotoxicity has been recently disclosed [42]. In lipopolysaccharide-pretreated rats that were administered trovafloxacin, accumulation of liver neutrophil (PMN) has been observed. Prior depletion of PMN in the animals attenuated liver injury, confirming the role of PMN in the hepatotoxicity induced by lipopolysaccharide/trovafloxacin.

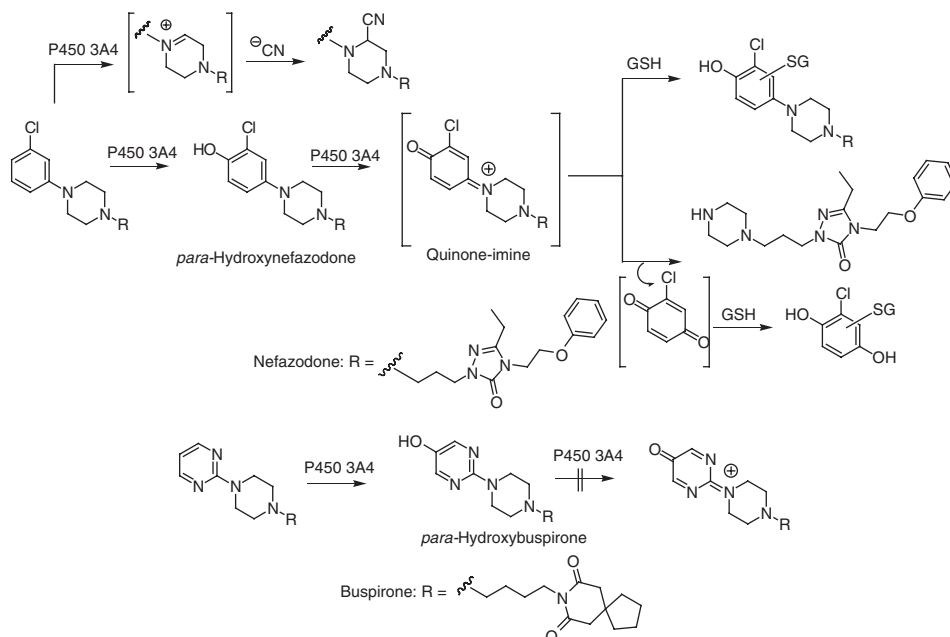
A second illustration involves a comparison of the inhaled anesthetics, namely, halothane, isoflurane, and desflurane. In susceptible patients, halothane, isoflurane, and desflurane can produce severe hepatic injury by an immune response directed against

reactive acyl halides covalently bound to hepatic proteins [43]. Approximately 20–50% halothane absorbed in the body undergoes metabolism via oxidative and reductive processes, which are catalyzed by P450 enzymes [44,45]. P450 2E1-mediated oxidation of halothane affords a highly unstable halohydrin intermediate, which spontaneously degenerates to yield trifluoroacetyl chloride, capable of reacting with protein and thiol nucleophiles [46,47]. The use of halothane derivatives, isoflurane and desflurane, both of which are only metabolized between 1% and 3%, is associated with a significantly lower incidence of hepatotoxicity. From a structure–toxicity standpoint, the relative incidence of hepatotoxicity appears to directly correlate with the extent of acyl halide formation by P450, which in turn is governed by the leaving group ability of the respective substituents within the drugs. As is seen in Scheme 11.3, halothane, which exhibits the greatest incidence of hepatotoxicity in the clinic, undergoes the most conversion to reactive acyl chloride, a feature that can be attributed to the presence of bromide substituent, which is a good leaving group. In contrast, isoflurane and desflurane also undergo oxidative metabolism resulting in the formation of reactive acyl halides, but the degree to which these anesthetics are bioactivated is significantly lower than halothane [48]. Thus, the lower yield of acylhalide formation with isoflurane may be traced back to changes in the electronic environment that reduce overall affinity toward metabolism or to the relatively poor leaving group ability of the difluoromethoxy group compared to that of bromide.

Antidepressants/antianxiolytics nefazodone and buspirone provide the third example of chemotype-specific toxicity. In the case of nefazodone, idiosyncratic hepatotoxicity resulting in more than 20 deaths has been reported since its introduction in the 1990s [49–51]. Circumstantial evidence linking nefazodone hepatotoxicity with reactive metabolite formation has been established; *para*-hydroxynefazodone, which is a circulating metabolite of nefazodone in humans, is metabolized by P450 3A4 to electrophilic quinonoid species, which are trapped as GSH adducts (Scheme 11.4) [52,53]. In addition, reactive iminium ion intermediates arising from α -carbon oxidation of the piperazine ring and its downstream metabolites have been characterized as stable cyanide conjugates (Scheme 11.4) [32,53]. Incidentally, the nefazodone bioactivation pathway catalyzed by P450 3A4 is also accompanied by mechanism-based



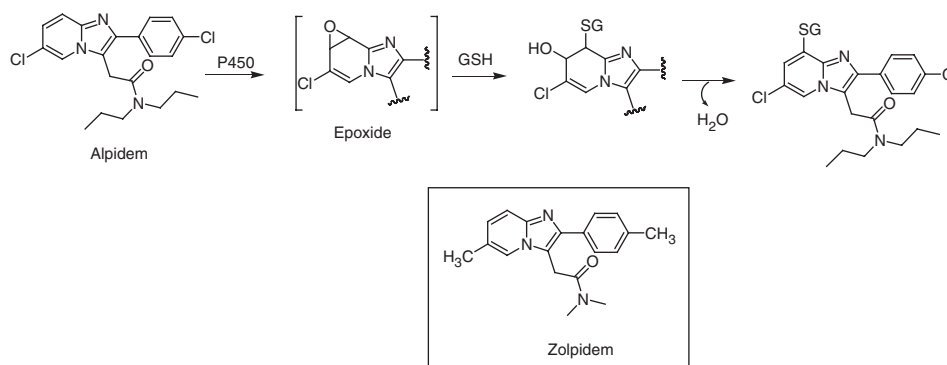
Scheme 11.3 Metabolism of inhaled anesthetics to reactive metabolites.



Scheme 11.4 Reactive metabolite formation pathways with nefazodone (hepatotoxin): comparison with the nonhepatotoxin buspirone.

inactivation of the isozyme [52]. This *in vitro* observation is consistent with nefazodone's nonlinear pharmacokinetics (due to autoinactivation) and clinical drug–drug interactions with P450 3A4 substrates [54–56]. As in the case of nefazodone, *para*-hydroxybuspirone also represents a major circulating metabolite of the anxiolytic agent buspirone (Scheme 11.4) [52]. However, failure to detect GSH conjugates in microsomal incubations of buspirone suggests that *para*-hydroxybuspirone does not succumb to reactive metabolite formation in a manner similar to that observed with *para*-hydroxynefazodone [52]. Absence of reactive metabolite formation with buspirone in a similar manner as nefazodone is consistent with *ab initio* calculations, which suggest that a weaker resonance stabilization of the oxidation products and the greater acidity make *para*-hydroxybuspirone less favorable for the two-electron oxidation (Scheme 11.4). These observations appear to correlate with the lack of idiosyncratic hepatotoxicity with buspirone, despite decades of clinical use. Furthermore, apart from the reactive metabolite liability, nefazodone (in parent form) also exhibits mitochondrial toxicity and is a potent inhibitor of the bile salt export pump (BSEP) transporter, features that are not shared by buspirone [57,58]. The reported cases of cholestasis with nefazodone can be potentially explained in part due to inhibition of the BSEP transporter [59].

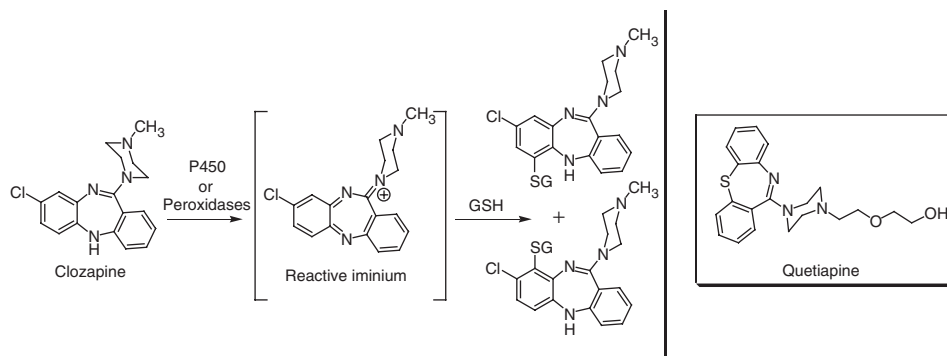
Another retrospective analysis involves the anxiolytic agents alpidem and zolpidem. Alpidem was withdrawn from commercial use within the first year of its introduction because of several cases of severe hepatotoxicity, requiring immediate liver transplant and/or leading to fatalities. In contrast, the structural analog and commercial blockbuster drug zolpidem does not possess the hepatotoxic liability. A key structural



Scheme 11.5 Differential metabolism of the anxiolytic agents alpidem (hepatotoxin) and zolpidem (nonhepatotoxin).

difference in the two drugs is the replacement of the two chlorine atoms in alpidem with two methyl groups in zolpidem. In alpidem, the chloro-imidazopyridine ring is subject to P450 bioactivation leading to the formation of a reactive epoxide that reacts with GSH to yield sulfhydryl conjugates (Scheme 11.5), which have been detected in humans [60]. In contrast, zolpidem is metabolized via oxidation of both methyl groups to the corresponding alcohol and carboxylic acid metabolites (Scheme 11.5); GSH conjugates of zolpidem have not been detected in humans [61]. Furthermore, in addition to the bioactivation liability, alpidem also exhibits potent inhibition of mitochondrial respiration and also depletes GSH in primary hepatocyte cultures, phenomena that are not observed with zolpidem even at high concentrations [61].

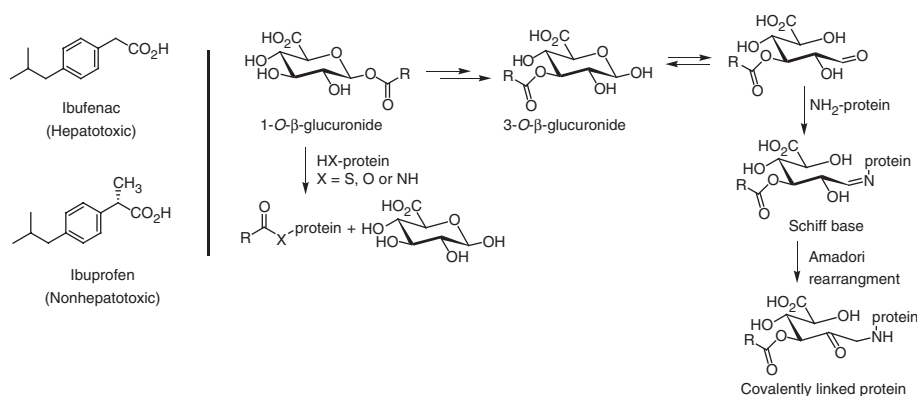
An equally intriguing example of how a subtle structural difference can influence a toxicological outcome is seen with the dibenzodiazepine derivatives and antipsychotic agents clozapine and quetiapine. While clozapine use is limited by a high incidence of agranulocytosis, hepatotoxicity, and myocarditis, quetiapine does not cause these toxic events. Clozapine exhibits covalent binding to human neutrophil proteins *in vitro*, via myeloperoxidase-mediated oxidation of the dibenzodiazepine ring to a reactive iminium ion, which covalently binds to the target tissues and also reacts with GSH (Scheme 11.6) [62,63]. Proteins covalently modified with clozapine have been observed in neutrophils



Scheme 11.6 Structure-toxicity relationships with antipsychotic drugs clozapine and quetiapine.

of patients being treated with clozapine, which reaffirms the relevance of the *in vitro* studies [64]. In the case of quetiapine, the bridging nitrogen is replaced with a sulfur atom; consequently, this drug is not bioactivated to the reactive iminium species [65]. Despite administration at doses comparable to clozapine, cases of agranulocytosis with quetiapine are extremely rare.

A final and perhaps the most interesting example is highlighted with the nonsteroidal anti-inflammatory drugs (NSAIDs) ibuprofen and ibufenac. While the NSAID ibuprofen is considered to be one of the safest over-the-counter anti-inflammatory agent in the market, its close-in analog ibufenac was withdrawn due to severe hepatotoxicity. The daily doses of both the NSAIDs were comparable, and the only structural difference between the two drugs is the presence of the α -methyl substituent in ibuprofen. Glucuronidation of the carboxylic acid moiety in most NSAIDs constitutes the principal elimination mechanism *in vivo* in humans [66–68]. Carboxylic acid glucuronides (acyl glucuronides) are reactive esters that covalently adduct to macromolecules via two different pathways (Scheme 11.7). The first is a transacylation mechanism, where a nucleophilic amino acid on a macromolecule attacks the carbonyl group of the primary acyl glucuronide leading to the formation of an acylated protein and free glucuronic acid. The second mechanism involves condensation between the aldehyde group of a rearranged acyl glucuronide and a lysine residue or an amine group of the N-terminus, leading to the formation of a glycated protein. The formation of the iminium species is reversible but may be followed by an Amadori rearrangement of the imino sugar to the more stable 1-amino-2-keto product [67,69,70]. A structural relationship between acyl glucuronide degradation to the Schiff base and covalent binding has been established [69,70]. Acyl glucuronides of ibufenac and other acetic-acid-based NSAIDs such as tolmetin and zomepirac, all of which have been withdrawn due to toxicity, exhibit the highest level of rearrangement and covalent binding, whereas mono- α -substituted acetic acids (2-substituted propionic acids) such as ibuprofen and naproxen exhibit intermediate level of acyl glucuronide rearrangement and covalent binding. Overall, these observations imply that inherent electronic and steric properties must modulate the rate of acyl glucuronide rearrangement [71,72]. Thus, in the case of ibuprofen, it is likely that presence of the α -methyl substituent slows the rearrangement of the acyl glucuronide to the electrophilic carbonyl intermediate.



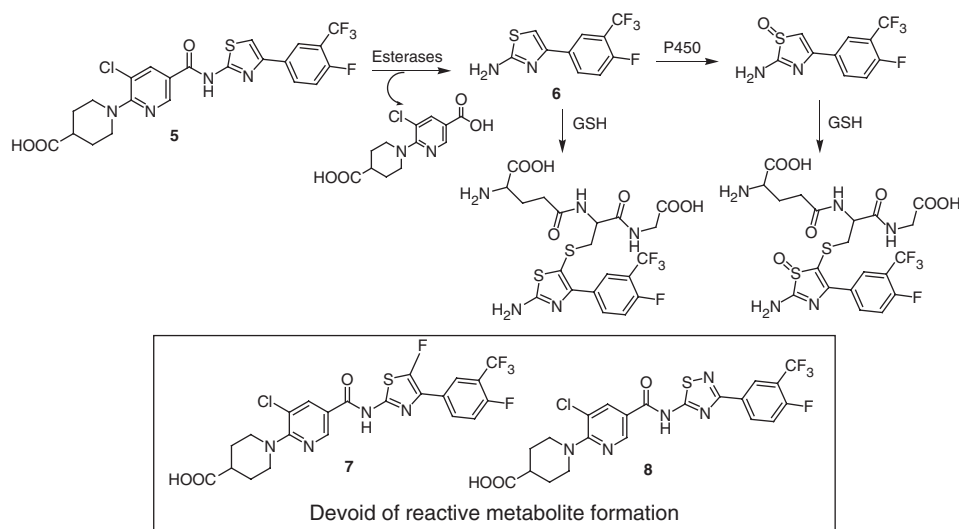
Scheme 11.7 Reaction of acylglucuronides with proteins.

It is noteworthy to point out that most of the successor drugs in the examples discussed above have not evolved from specific tactics to eliminate reactive metabolite liability in predecessor agents. However, these examples imply that by avoiding structural alerts/toxicophores in drug design, one would lessen the odds that a drug candidate will lead to idiosyncratic toxicity due to reactive metabolite formation. Given the inability to predict whether reactive-metabolite-positive compounds will ultimately cause toxicity, a general strategy adopted by many within the pharmaceutical community involves the assessment of reactive metabolite formation as early as possible in the selection of drug candidates; this in turn can influence the rational design of compounds devoid of reactive metabolite formation. In practice, however, this exercise is by no means trivial; medicinal chemistry tactics to eliminate reactive metabolite formation could confer a detrimental effect on primary pharmacology (e.g., changes in agonist/antagonist behavior and/or subtype selectivity for target receptor or enzyme) and/or pharmacokinetic attributes. Thus, chemical intervention strategies to abolish reactive metabolite formation are often an iterative process, the success of which is heavily dependent on a close working relationship between medicinal chemists, pharmacologists, and metabolism scientists.

11.4 EXAMPLES OF MEDICINAL CHEMISTRY TACTICS TO ELIMINATE/MINIMIZE REACTIVE METABOLITE FORMATION

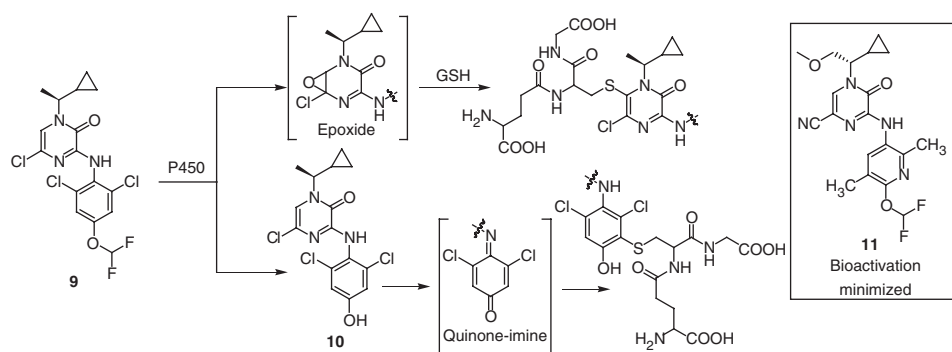
Examples of medicinal chemistry strategies to eliminate reactive metabolite formation in drug discovery are abundant in the primary literature. For instance, the presence of the 2-carboxamidothiazole toxicophore in the nonpeptidyl thrombopoietin receptor agonist **5** led to studies [73] to evaluate reactive metabolite potential of **5** via pathways involving thiazole ring scission in a manner similar to that discerned for the hepatotoxic NSAID sudoxicam [74]. Although the thiazole moiety in **5** did not undergo enzyme-catalyzed ring opening to afford reactive acylthiourea and/or glyoxal metabolites, the corresponding 4-(4-fluoro-3-(trifluoromethyl)phenyl)thiazol-2-amine (**6**), the product of carboxylesterase-mediated hydrolysis of **5** in human hepatic tissue and plasma, underwent oxidative bioactivation in human liver microsomes since trapping studies with GSH led to the formation of two conjugates derived from the addition of the thiol nucleophile to **6** and a thiazole-*S*-oxide metabolite of **6** (Scheme 11.8). Mass spectral fragmentation and NMR analysis indicated that the site of attachment of the glutathionyl moiety in both conjugates was the C-5 position in the thiazole ring. On the basis of the structures of the GSH conjugates, two bioactivation pathways were proposed, one involving β -elimination of an initially formed hydroxylamine metabolite and the other involving direct two-electron oxidation of the electron-rich 2-aminothiazole system to electrophilic intermediates. The mechanistic insights gained from the metabolism studies influenced subsequent medicinal chemistry strategies, which involved blocking the C-5 position with a fluorine atom (**7**) or replacing the thiazole ring with a 1,2,4-thiadiazole group (**8**) (Scheme 11.8). These structural changes not only abrogated the bioactivation liability associated with **5** but also resulted in compounds that retained the attractive pharmacological and pharmacokinetic attributes of the prototype agent.

A second example, which focuses on eliminating reactive metabolite liability within a chemical series, is evident in the recent work of Hartz *et al.* [75] on pyrazinone-based corticotrophin releasing factor 1 receptor antagonists and potential antidepressant/



Scheme 11.8 Medicinal chemistry strategies to eliminate reactive metabolite formation with a nonpeptidyl thrombopoietin receptor agonist **5** containing the 2-aminothiazole toxicophore.

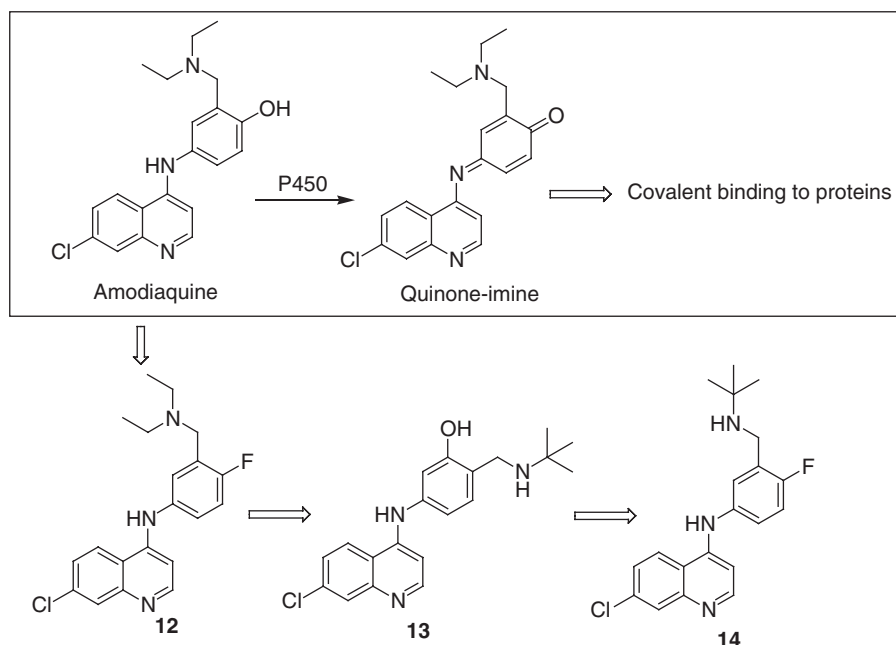
antianxiolytic drugs. The attractive *in vivo* pharmacology and disposition properties of the pyrazinone derivative **9** (Scheme 11.9) were offset by the finding that **9** was bioactivated by P450 to form reactive metabolites. In fact, a major component of the elimination mechanism of **9** in rats (~40% of drug-related material recovered in rat bile) is composed of conjugation with GSH, which is consistent with reactive metabolite formation *in vivo* [76]. Two distinct bioactivation pathways were deciphered (Scheme 11.9): (i) oxidative metabolism on the chloropyrazinone ring to yield an electrophilic epoxide and (ii) O-dealkylation of the difluoromethoxy moiety to yield a phenol metabolite **10**, two electrons in which generated the reactive quinone-imine species in a manner similar to that discerned with the 2,6-dichloro-4-hydroxyaniline analogs and NSAIDs diclofenac and lumiracoxib [77,78]. On the basis of this information, medicinal chemistry strategies were put in place to eliminate reactive metabolite liability in **9**. To eliminate bioactivation of the 2,6-dichloroaniline motif,



Scheme 11.9 Minimization of reactive metabolite formation with a pyrazinone-based corticotropin releasing factor 1 receptor antagonist **9**.

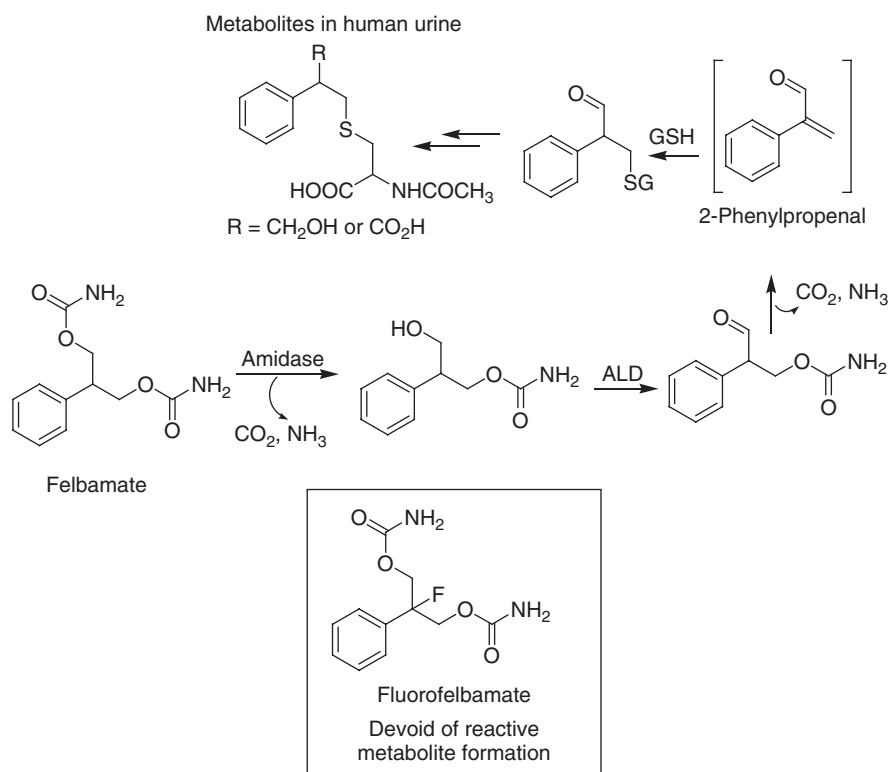
efforts were directed toward incorporation of a pyridyl group as an bioisosteric replacement. To reduce the level of 5-chloropyrazinone ring bioactivation, attempts were made to replace chlorine with the more strongly electron-withdrawing cyano group. Out of this iterative medicinal chemistry exercise emerged **11** (Scheme 11.9) with sufficiently diminished reactive metabolite formation in both rat and human liver microsomes. Consistent with the *in vitro* finding, <2–4% of GSH conjugates was recovered from rat bile following *in vivo* administration of **11**. Compound **11** also retained all the primary pharmacology and pharmacokinetic properties of the lead compound **9**.

Strategies toward eliminating metabolic activation of the electron-rich 4-hydroxyaniline motif have also been described by Park *et al.* on the antimalarial agent amodiaquine [79–83]. The clinical use of amodiaquine is somewhat restricted by several cases of hepatotoxicity and agranulocytosis; the detection of IgG antibodies in patients exposed to the drug is consistent with an immune-mediated hypersensitivity reaction [84,85]. Immune-mediated toxicity is believed to arise from the metabolism of amodiaquine to a reactive quinone-imine species that can covalently bind to cellular proteins or GSH (Scheme 11.10). Exchanging the C'-4-phenolic OH group with a fluorine results in **12** that does not undergo the obligatory two-electron oxidation process to form a electrophilic quinone-imine species (Scheme 11.10) [81]. An alternate approach to prevent reactive metabolite formation involved the isomerization of the 3' and 4' substituents in amodiaquine; from this exercise emerged analogs **13** and **14**, which were devoid of reactive metabolite formation (judged from the lack of formation of thiol conjugates) and possessed pharmacodynamic, pharmacokinetic, and safety profiles comparable to amodiaquine (Scheme 11.10) [82,83].

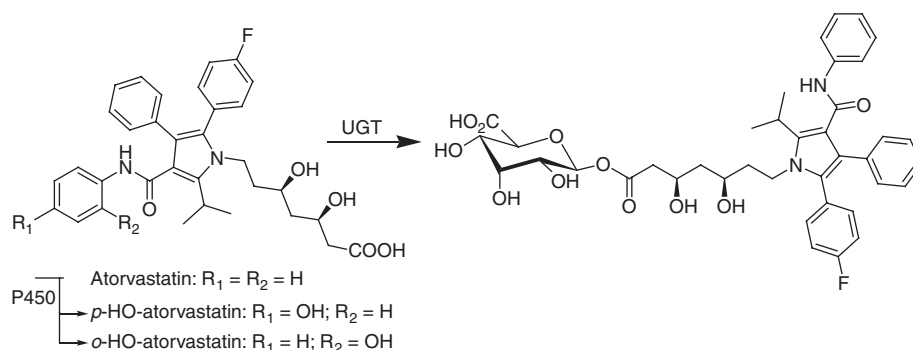


Scheme 11.10 Rational design of analogs of the antimalarial agent amodiaquine based on metabolism data.

Finally, against this backdrop, it is noteworthy to comment on the metabolism-guided design of fluorofelbamate as a potentially safe alternative to the anticonvulsant felbamate, which is associated with many cases of life-threatening aplastic anemia and hepatotoxicity, resulting in a black box label warning and extensive monitoring requirements in the United States [86]. Evidence linking reactive metabolite formation with felbamate toxicity has been presented by means of the *in vivo* characterization of mercapturic acid (downstream metabolites of GSH) conjugates of a highly reactive α,β -unsaturated carbonyl derivative 2-phenylpropenal, following felbamate administration to rats and humans [87,88]. A mechanism that is consistent with the conversion of felbamate to 2-phenylpropenal is depicted in Scheme 11.11 and involves a carboxylesterase-mediated hydrolysis of one of its carbamate groups to afford the primary alcohol metabolite, oxidation of which by alcohol dehydrogenase (ALD) to the intermediate aldehyde derivative followed by spontaneous β -elimination of the remaining carbamoyl group generates 2-phenylpropenal [87]. On the basis of this information, fluorofelbamate (Scheme 11.11) was specifically designed to eliminate the reactive metabolite liability of felbamate. Fluorofelbamate does not succumb to bioactivation because the fluorine atom prevents the β -elimination process that leads to the reactive 2-phenylpropenal [89]. Whether absence of bioactivation translates into a reduced risk of toxicity remains to be seen. Fluorofelbamate is currently undergoing clinical trials as an antiepileptic agent [90].



Scheme 11.11 Mechanism of reactive metabolite formation with the anticonvulsant agent felbamate: a rational chemical approach to circumvent bioactivation.

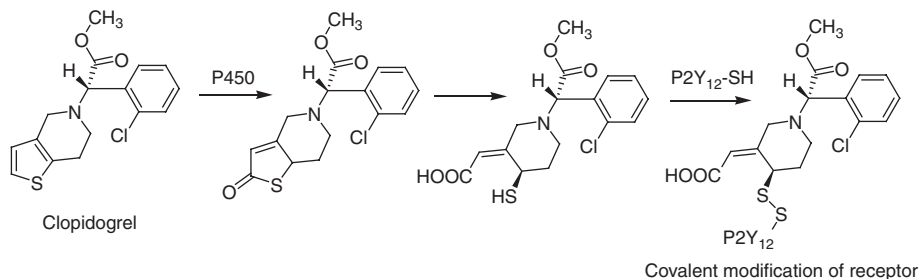


Scheme 11.12 Metabolism of atorvastatin into potentially reactive metabolites (and precursors thereof).

11.5 SHOULD INCORPORATION OF TOXICOPHORES BE PROHIBITED IN DRUG DESIGN?

The vast majority of drugs possess a phenyl ring that can be considered as a toxicophore since P450-catalyzed metabolism of the phenyl ring to a phenol proceeds through an electrophilic epoxide species. GSH conjugates of aromatic ring epoxides have been characterized with several toxic drugs (e.g., alpidem), and as such, the bioactivation sequence is the underlying cause of carcinogenic and/or hepatotoxic effects of organic solvents such as benzene and bromobenzene [91,92]. So from a practical standpoint, it is impossible to avoid a toxicophore in drug design. As such, avoiding toxicophores (other than a phenyl group) altogether in early drug discovery can lead to a missed opportunity to develop potentially important medicines. Certainly, this is the case with atorvastatin (Lipitor[®]), which not only contains the acetanilide toxicophore but also metabolism by P450 results in the formation of acetaminophen-like metabolites (Scheme 11.12) [93]. In addition, glucuronidation of its carboxylic acid moiety results in the formation of the potentially electrophilic acyl glucuronide, the likes of which have been implicated in the toxicity associated with the carboxylic acid-containing NSAIDs (Scheme 11.12) [94].

It is further interesting to note that some blockbuster drugs require reactive metabolite formation for pharmacological activity; this is achieved through the introduction of

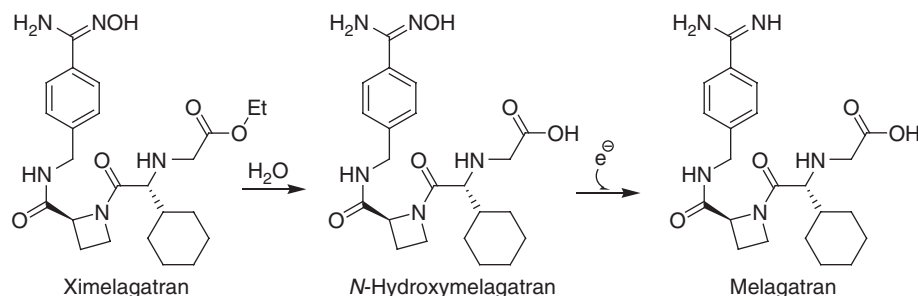


Scheme 11.13 Bioactivation of clopidogrel (Plavix[®]) to a reactive metabolite, a prerequisite for pharmacological activity.

a toxicophore in the chemical architecture. For instance, the blockbuster cardiovascular drug and P2Y₁₂ antagonist clopidogrel (Plavix[®]) is by itself inactive and requires P450-catalyzed bioactivation of its thiophene ring to form a reactive thiol metabolite, which forms a covalent disulfide bond with a cysteinyl residue on the P2Y₁₂ receptor in platelets (Scheme 11.13), a phenomenon that gives rise to its beneficial cardiovascular effects [95,96].

11.6 VALUE OF REACTIVE METABOLITE TRAPPING AND COVALENT BINDING STUDIES IN THE PREDICTION OF DRUG TOXICITY

While detection of conjugates (GSH, amino, and/or cyano) and/or covalent binding to target tissue (e.g., NADPH-supplemented liver microsomes) provides evidence for reactive metabolite formation, the data needs to be placed in proper context before making a decision on discarding a drug candidate associated with this liability. First and foremost, *a complete lack of reactive metabolite formation and/or covalent binding to liver microsomal protein is not a guarantee of a drug candidate's safety*. Reactive metabolite formation via non-P450-mediated pathways will not display a positive response in liver microsomes fortified with NADPH to test for P450 activity. For example, the felbamate bioactivation process in humans, which leads to the formation of an electrophilic α,β -unsaturated aldehyde, has never been replicated in human hepatic tissue. At therapeutically relevant concentrations of radiolabeled felbamate in *in vitro* incubations with human liver microsomes and human hepatocytes, no GSH adducts (Kalgutkar and Obach, unpublished findings) and/or covalent binding of felbamate has been discerned [97,98]. Although the reason(s) for this discrepancy remains unclear at present, in a drug discovery paradigm relying solely on reactive metabolite trapping and liver microsomal covalent binding as a means of predicting IADR potential of drug candidates, felbamate would have passed the hurdle with flying colors. Likewise, there is no *in vitro* and/or *in vivo* evidence suggestive of reactive metabolite formation with ximelagatran, the first orally active thrombin inhibitor that has been withdrawn from commercial use because of several cases of hepatotoxicity [99,100]. As such, ximelagatran does not contain any structural alerts, and its *in vivo* biotransformation in animals and humans consists of innocuous pathways involving ester hydrolysis and reduction of the amidoxime motif to afford the active ingredient melagatran (Scheme 11.14) [101]. Finally, the possibility that toxicity can arise from mechanisms other than reactive



Scheme 11.14 Metabolism of the thrombin inhibitor and hepatotoxin ximelagatran.

metabolite formation (e.g., direct mitochondrial toxicity) also needs to be taken into consideration. For instance, the finding that alpidem is a potent mitochondrial toxin and zolpidem is not can be used to rationalize the lack of hepatotoxicity associated with alpidem [61]. Similarly, the hepatotoxin nefazodone is associated with mitochondrial toxicity and inhibitory effects on BSEP transporter in the parent form, whereas the nonhepatotoxin buspirone does not lead to these liabilities; these are phenomena that can explain the differences in safety profile of the two antidepressants.

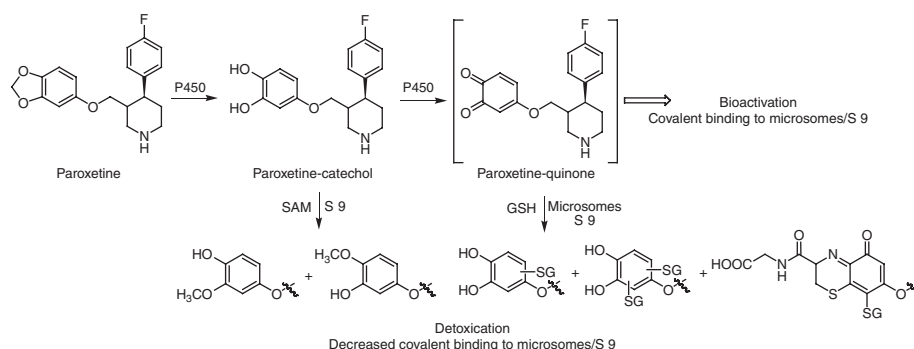
Although covalent binding studies have an advantage over the reactive metabolite trapping studies, in that they provide a quantitative estimate of covalently bound drug to proteins and therefore an indirect measure of reactive metabolite formation, *there are no studies to date that have shown a correlation between the extent of covalent binding and/or reactive metabolite formed and the probability that a drug will be associated with a high incidence of IADRs*. There are numerous examples of blockbuster drugs that are false positives in these assays, that is, they form GSH conjugates and display a degree of protein covalent modification, yet they are not associated with a significant incidence of toxicity. A striking example of this phenomenon is evident with the acetaminophen regioisomer, 3'-hydroxyacetanilide, that undergoes bioactivation yielding reactive metabolites that covalently adduct to GSH and proteins [102]. However, despite dose normalization to provide comparable levels of covalent binding *in vivo* in mice, 3'-hydroxyacetanilide does not exhibit the hepatotoxicity observed with acetaminophen. Furthermore, from a predictive standpoint, as a property itself, covalent binding *in vitro* has not been rigorously tested for its ability to distinguish between toxic and nontoxic drugs, primarily because nontoxic drugs have not been tested in covalent binding studies [23,103]. We have tested the ability of covalent binding measurements in predicting idiosyncratic hepatotoxicity by examining the binding of 18 drugs (9 hepatotoxins and 9 nonhepatotoxins) to human hepatic tissue, taking into consideration key factors such as reactive metabolite detoxication, relative importance of bioactivation leading to covalent binding versus overall metabolism, and daily dose for each drug [97,98]. While most of the hepatotoxic drugs (e.g., acetaminophen, nefazodone, tienilic acid) demonstrated covalent binding to some degree or the other, of great surprise were the findings that several nonhepatotoxic, commercially successful drugs such as buspirone, diphenhydramine, meloxicam, paroxetine, propranolol, raloxifene, and simvastatin demonstrated covalent binding. A quantitative comparison of covalent binding *in vitro* intrinsic clearance did not separate the two groups of compounds, and in fact, paroxetine and diphenhydramine, both nonhepatotoxins, showed the greatest amount of covalent binding in microsomes. Including factors such as the fraction of total metabolism represented by covalent binding and the total daily dose of each drug improved the discrimination between hepatotoxic and nonhepatotoxic drugs in liver microsomes, S9 fraction, and hepatocytes; *however, the approach still would falsely identify some agents as potentially hepatotoxic*. In the case of paroxetine, mechanistic studies further confirmed the importance of parallel metabolic and detoxication pathways in attenuating covalent binding to proteins [104]. As shown in Scheme 11.15, the catechol metabolite obtained via ring scission of the 1,3-benzodioxole group in paroxetine can partition between O-methylation by catechol-O-methyltransferase or undergo oxidation to the reactive quinone intermediate, which is efficiently detoxicated by GSH; both pathways lead to a significant reduction in covalent binding. In humans, the O-methylated catechol derivatives constitute the principal metabolic fate of the drug. When coupled with the

fact that the daily dose of paroxetine is low (20 mg), one gets some insight into the excellent safety record of this drug, despite the bioactivation liability.

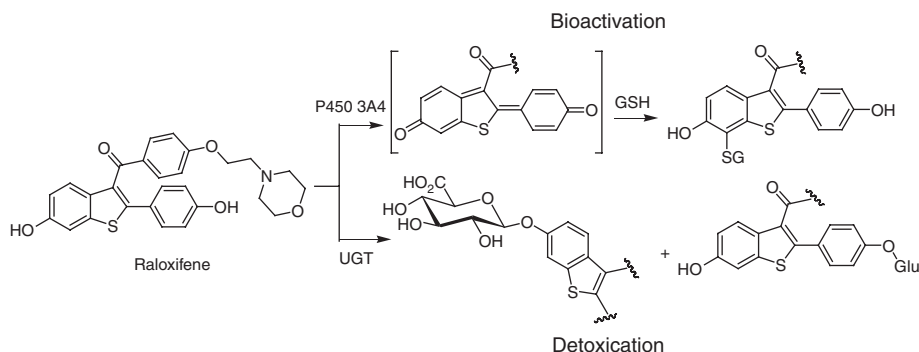
The examples discussed above pose a significant challenge toward the reliability of structural alerts, reactive metabolite trapping, and covalent binding measurements as predictors of idiosyncratic toxicity with new drug candidates. With regard to reactive metabolite formation potential, it is very important to consider factors that will influence the metabolism of structural alerts such as the presence of alternate metabolic soft spot(s) that competes with reactive metabolite formation and the existence of detoxication pathways that scavenge the reactive metabolite and/or its precursor. The importance of the first factor was illustrated earlier with the anxiolytic agents alpidem and zolpidem (Scheme 11.5). While bioactivation via epoxidation is also likely in zolpidem, the molecule does not undergo this metabolic fate; instead, the two methyl groups function as metabolic soft spots and are oxidized to the corresponding alcohol and carboxylic acid metabolites. With regard to the importance of competing, detoxication pathways, reactive metabolite formation may be discernible in standard *in vitro* systems, but the principal clearance mechanism of the drug *in vivo* may involve a distinctly different and perhaps more facile metabolic fate that does not yield reactive intermediates. This is illustrated with the selective estrogen receptor modulator raloxifene, which is known to undergo *in vitro* P450 3A4-catalyzed bioactivation on its phenolic groups to yield reactive quinonoid species; however, *in vivo*, glucuronidation of the same phenolic groups in the gut and liver constitutes the principal elimination mechanism of raloxifene in humans (Scheme 11.16) [105–107]. Thus, the likelihood of raloxifene bioactivation *in vivo* is in question when compared with the phase II glucuronidation process, a phenomenon that may provide an explanation for the extremely rare occurrence of IADRs.

11.7 DAILY DOSE/LOW SYSTEMIC REACTIVE METABOLITE EXPOSURE AS A MITIGATING FACTOR FOR IADRs

Anecdotal evidence pinpoints daily dose as a key factor in migrating IADR risks. As such, there do not appear to be examples of drugs that are dosed at <20 mg/day that



Scheme 11.15 Competing metabolic pathways of the antidepressant paroxetine. Plausible explanation for lack of IADRs despite reactive metabolite formation.



Scheme 11.16 Bioactivation and competing detoxication pathways of the selective estrogen receptor modulator raloxifene.

cause IADRs (whether or not these agents are prone to bioactivation). It is likely that the improved safety of low dose drugs arises from a marked reduction in the total body burden to reactive metabolite exposure via efficient scavenging by GSH and therefore unlikely to exceed the threshold needed for toxicity. For example, olanzapine (Zyprexa®) (Fig. 11.1) forms a reactive iminium metabolite very similar to the one observed with clozapine, yet olanzapine is not associated with a significant incidence of agranulocytosis [64,108]. One difference between the two drugs is the daily dose; clozapine is given at a dose of >300 mg/day, while the maximum recommended daily dose of olanzapine is 10 mg/day. A second illustration of this concept is evident on comparison of the thiazolidinedione derivatives troglitazone, rosiglitazone, and pioglitazone. While rosiglitazone has been associated with several cases of cardiotoxicity, the idiosyncratic hepatotoxicity associated with troglitazone, which led to its withdrawal from the market, has not been observed with rosiglitazone as well as pioglitazone. As noted earlier, both the chromane and the thiazolidinedione ring systems in troglitazone succumb to P450 3A4-catalyzed bioactivation to form reactive metabolites [109,110]. While rosiglitazone and pioglitazone do not contain the chromane ring system found in troglitazone, they do contain the thiazolidinedione scaffold. Consistent with the findings with troglitazone, both rosiglitazone and pioglitazone have been shown to undergo thiazolidinedione ring scission mediated by P450 enzyme(s) in human microsomes, resulting in reactive metabolites that are trapped by GSH [111,112]. While bioactivation of the thiazolidinedione ring is a common theme in these drugs, a key difference lies in their daily doses—troglitazone at 200–400 mg/day and rosiglitazone and pioglitazone both at <10 mg/day. Additional examples of this phenomenon are illustrated with tadalafil (Cialis®) and the antihypertensive prazosin (Minipress®) (Fig. 11.1). The methylenedioxyphenyl group in tadalafil undergoes P450 3A4-catalyzed bioactivation to an electrophilic catechol, a process that also leads to the suicide inactivation of P450 3A4 activity *in vitro* [113]. However, to date, there are no reports of IADRs or P450 3A4 drug–drug interactions associated with tadalafil use at the recommended dose of 10–20 mg/day. Likewise, there are no reports of IADRs with prazosin at the recommended daily dose of 1 mg/day, despite the bioactivation of the pendant furan ring to electrophilic intermediates, which can be trapped with semicarbazide and GSH [114].

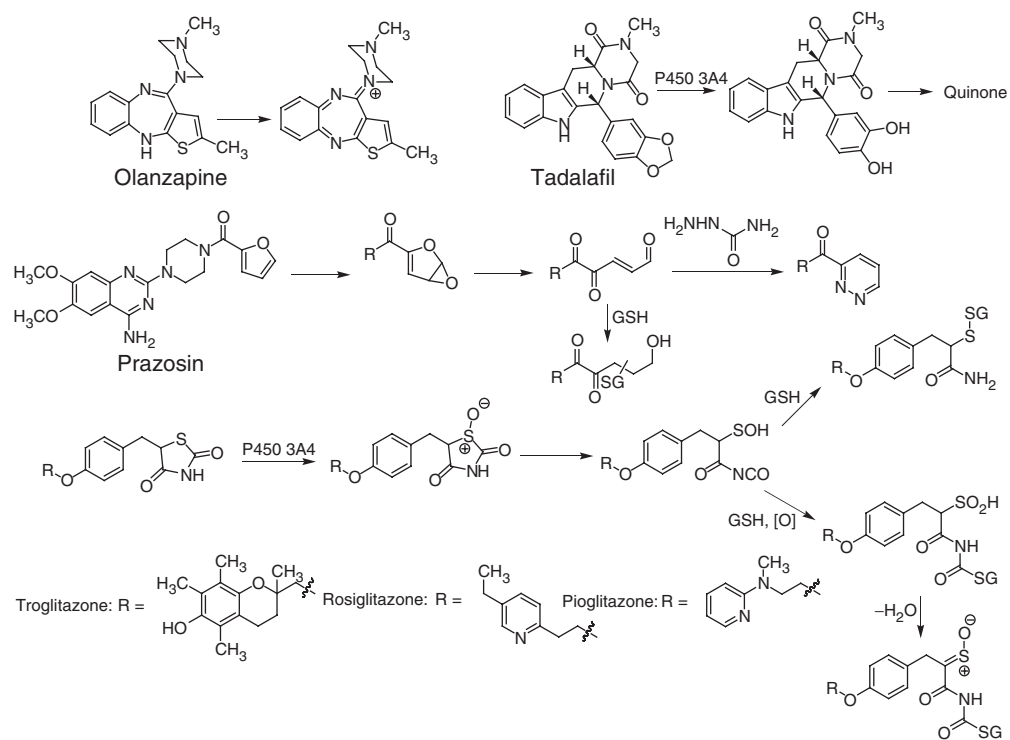


Figure 11.1 Examples of low daily dose drugs devoid of IADRs, despite bioactivation liability.

11.8 CONCLUDING REMARKS

The issue of reactive metabolites continues to receive widespread interest in the pharmaceutical industry. GSH-trapping assays in human liver microsomes are being incorporated into the discovery screening funnel early on to assess the risk of reactive metabolite formation. Early knowledge concerning bioactivation liability with a chemical series allows an insight into the structure–reactive metabolite relationships and the promise to deliver superior early development candidates. However, as discussed in this chapter, interpretation of the output from these assays is confounded by the fact that many successfully marketed drugs are positive in GSH-trapping assays. Therefore, caution must be exercised in deprioritizing a compound based on a positive result, so that the development of a useful and potentially profitable compound will not be unnecessarily halted. Appropriate consideration also must be given for drug candidates intended to treat previously unmet medical needs. Available evidence suggests that detection of reactive metabolites within a chemical series does not warrant an instant demise of the compounds per se, but it does trigger some additional due diligence including the evaluation of competing detoxication pathways of the reactive metabolite and/or its precursor by phase I/phase II enzymes and an estimation of the human dose based on pharmacokinetic/pharmacodynamic studies in preclinical species. GSH conjugation to reactive metabolites is not necessarily a bad attribute; instead, it reaffirms the ability of the endogenous sulfhydryl antioxidant to efficiently scavenge the electrophilic reactive intermediate. It is only in cases where the concentration of the reactive metabolite formed is so high that it depletes the endogenous antioxidant pool leading to toxicity, as has been demonstrated with acetaminophen. The ability to predict the potential of a drug candidate to cause IADRs is dependent on a better understanding of the pathophysiological mechanisms of such reactions. IADRs are too complex to duplicate in a test tube, and their idiosyncratic nature precludes prospective clinical studies. Genetic factors also appear to have a crucial role in the induction of IADRs. Until we develop a better understanding of the risk of idiosyncratic toxicity arising from the formation of reactive metabolites, the advancement of potent (low dose) drug candidates with only a limited propensity to form reactive intermediates would appear to be the most favored strategy in an ideal world.

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12 Idiosyncratic Drug Reactions and the Potential Role of Metabolism

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12.1	Summary	1
12.2	Background	2
12.3	Association between the formation of reactive metabolites and the risk of IDRs	2
12.4	Clinical characteristics of idiosyncratic drug reactions	3
12.5	Major hypotheses of idiosyncratic drug reactions	21
12.6	Implications of reactive metabolites for the mechanisms of idiosyncratic drug reactions	30
12.7	Classification of drugs based on their IDR pattern	32
12.8	Summary and conclusions	39
	References	39

12.1 SUMMARY

There is a large amount of circumstantial evidence that supports the hypothesis that most idiosyncratic drug reactions (IDRs) are caused by reactive metabolites rather than the parent drug. When the total daily dose is included in the calculation, there is a rough correlation between the amount of covalent binding and the risk that a drug will be associated with a significant incidence of IDRs, especially those affecting the liver. However, there are drugs that form a large amount of reactive metabolites and rarely cause IDRs, and there are drugs such as ximelagatran that do not appear to form reactive metabolites and yet had to be withdrawn from the market because of an unacceptable risk of IDRs.

There are a wide variety of proteins that are targets of covalent binding, and clearly, not all covalent binding is associated with the same risk of causing an IDR. It is likely that most IDRs are immune mediated, although this is not without controversy, especially with respect to idiosyncratic liver toxicity. There are several hypotheses that address the issue of how drugs initiate an immune response, but in the absence of valid animal models, they are very difficult to rigorously test. These hypotheses include the hapten hypothesis, the danger hypothesis, the p-I (pharmacological interaction) hypothesis, epigenetic effects leading to activation of the immune system, direct activation of antigen-presenting cells, and some of the newer biological agents simply appear

to alter the balance in the immune system and do not involve reactive metabolites. It is likely that the mechanism by which different drugs induce an immune response is different, at least in detail. There are also hypotheses that do not involve the immune system such as mitochondrial toxicity and the inflammagen hypothesis.

In the absence of valid animal models, it is important to use the clinical characteristics of IDRs to evaluate the hypotheses. A very important characteristic is the usual delay between starting a drug and the onset of the IDR. Another important characteristic is that different drugs can cause different types of IDRs in different people and even the same drug can cause different types of IDRs in different people. This is most easily explained by an immune mechanism in which this variability is due to variability in T-cell-receptor specificity, which is different even in identical twins. However, until we have a much better understanding of the detailed mechanisms of IDRs, it will be difficult to predict which drug candidates are likely to cause a relatively high incidence of IDRs and which patients are at high risk of such adverse reactions.

12.2 BACKGROUND

Adverse drug reactions are a major source of patient morbidity and mortality [1]. The most common type of adverse drug reaction involves an extension of the therapeutic effect (i.e., pharmacodynamics) of the drug, for example, warfarin-induced bleeding. In principle, such adverse reactions are predictable and preventable, but in practice, that can be difficult. Less common but making up about 10–20% of adverse reactions are idiosyncratic. Although less common, IDRs are often serious, even life threatening, and their unpredictable nature makes them very hard to deal with. Attempts to avoid IDRs also add significant cost and uncertainty to drug development [2].

There is no universal definition for IDRs [3]. Idiosyncratic means particular to an individual, and therefore, IDRs do not occur in most patients with normal use of a drug. It is the mechanistic implications of the term that are inconsistent. The term *hypersensitivity reaction* is often used for these adverse reactions, but it implies that the reactions are immune mediated, and in the absence of definitive evidence, the term *idiosyncratic* should be used. However, it is confusing because when allergists and clinical immunologists use the term *idiosyncratic drug reaction*, it usually excludes any adverse reaction that is immune mediated, and most IDRs appear to be immune mediated. In this chapter, adverse reactions that involve the therapeutic effects of the drug, such as warfarin-induced skin necrosis, are not discussed, even though such reactions can certainly be particular to an individual. Until we have a better mechanistic understanding of IDRs the term will be vague, and when the mechanisms are clear, the term may become obsolete.

12.3 ASSOCIATION BETWEEN THE FORMATION OF REACTIVE METABOLITES AND THE RISK OF IDRs

The work by the Miller and others [4] demonstrated that the ability of many chemicals to induce cancer was based on their ability to form chemically reactive metabolites that bind to DNA. This concept was translated to adverse drug reactions when it was discovered that acetaminophen-induced liver toxicity is caused by a reactive metabolite [5]. It is clear that drug-induced anaphylaxis is mediated by IgE antibodies

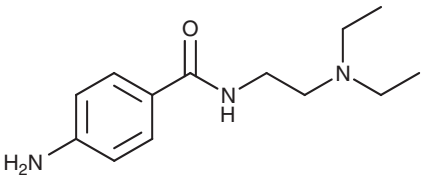
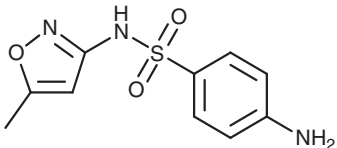
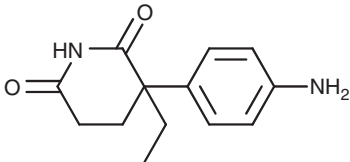
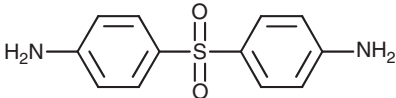
against drug-modified proteins. In the case of penicillin, the parent drug is reactive and no reactive metabolite is required, but in most cases, it is a reactive metabolite of the drug that results in the drug-modified protein [6]. In general, contact hypersensitivity is also caused by chemically reactive species [7]. Other types of drug-induced skin rashes also appear to be due to reactive metabolites [8]; however, it is difficult to prove that most IDRs are caused by reactive metabolites. Most of the evidence is circumstantial, such as in a series of gaseous anesthetics (halothane, isoflurane, and desflurane), there is a correlation between the amount of reactive metabolite formed and the incidence of idiosyncratic liver toxicity [9]. By their very nature, most reactive metabolites bind to targets close to where they are formed. The highest concentration of drug-metabolizing enzymes is in the liver, and this is presumably why hepatotoxicity is the most common type of IDR leading to drug candidate failure or withdrawal from the market. Therefore, in an attempt to “derisk” drug development, drug candidates have been screened for reactive metabolites that covalently bind to protein [10]. If the total daily dose is included in the calculation, there is a correlation between the amount of covalent binding of reactive metabolite and the risk that a drug will cause idiosyncratic liver toxicity; however, the correlation is far from perfect [11,12]. Therefore, it appears that not all covalent binding is associated with the same IDR risk, as discussed in Section 12.6.2. In addition, some drugs such as ximelagatran, pyrazinamide, and allopurinol are associated with a significant incidence of IDRs, and yet, they do not appear to form reactive metabolites (Utrecht JP, unpublished observations) [13].

12.4 CLINICAL CHARACTERISTICS OF IDIOSYNCRATIC DRUG REACTIONS

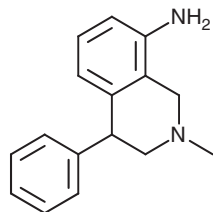
Given that we have few valid animal models and it is impossible to do controlled experiments in humans to study IDRs, it is important to gain mechanistic clues from the clinical characteristics of IDRs. In fact, it is essential for any mechanistic hypothesis to be consistent with the clinical characteristics/manifestations of these adverse reactions. One very important characteristic of IDRs is that, with few exceptions, there is a delay between starting the offending drug and the onset of the IDR unless there has been previous exposure to the drug [3]. In fact, because the exposure to halothane is brief, even though exposure to halothane-modified proteins is presumably much longer than exposure to the parent drug, halothane-induced hepatotoxicity almost always occurs after more than one exposure [14]. An often-stated characteristic is that IDRs are dose independent—this is not true. What is true is that most patients will not have an IDR at any dose, and because, by definition, the mechanism does not involve the therapeutic effect of the drug, there is no reason that the dose–response relationship in susceptible patients for the IDR will fall in the same range as that for the therapeutic effects. In fact, there is always a dose below which no patient will have an IDR [3].

It is also important to note that different people can have IDRs involving the same drug and having different characteristics, which presumably reflects differences in the mechanism of the IDR in different people [15]. Another important observation is that most drugs that can cause a relatively high incidence of a specific type of IDR can cause several different types of IDRs, although the spectrum can be different for different drugs (Table 12.1). This suggests that some types of structures are prone to causing

TABLE 12.1 Drugs Associated with Multiple Types of IDR

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Procainamide		Aromatic amine oxidized to hydroxylamine, nitroso metabolites, and <i>N</i> -chloramine [16,17]	Agranulocytosis [18] Lupus [19] Thrombocytopenia [20] Red cell aplasia [21]
Sulfamethoxazole		Aromatic amine oxidized to hydroxylamine, nitroso metabolites, and <i>N</i> -chloramine	DRESS [22] SJS/TEN Lupus [23] Agranulocytosis [22] Thrombocytopenia DILI
Aminogluthethimide		Aromatic amine oxidized to hydroxylamine, nitroso metabolites, and <i>N</i> -chloramine	Agranulocytosis [24] DILI Lupus [25] Pancytopenia [26]
Dapsone		Aromatic amine oxidized to hydroxylamine, nitroso metabolites, and <i>N</i> -chloramine	Agranulocytosis [27] Aplastic anemia [28] DRESS [29]

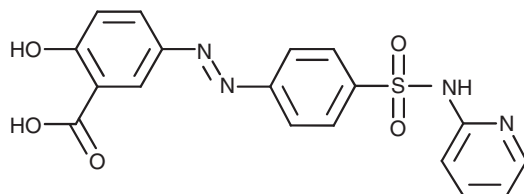
Nomifensine



Aromatic amine oxidized to hydroxylamine, nitroso metabolites, and *N*-chloramine

Hemolytic anemia [30] Lupus [31]

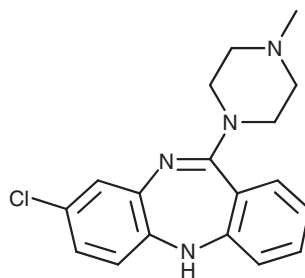
Sulfasalazine



Diazo molecule that is reduced to aromatic amines

DILI [32] Agranulocytosis [33] Aplastic anemia Hemolytic anemia SJS/TEN [34] Alveolitis [35] Lupus [36]

Clozapine

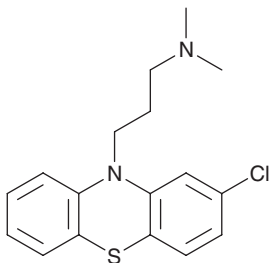
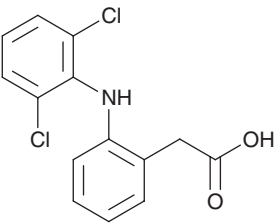
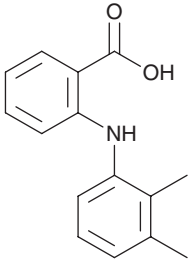


Nitrenium ion

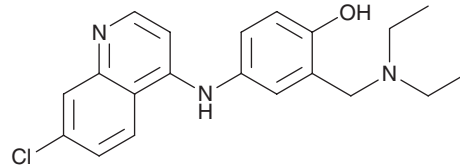
Agranulocytosis [37] DILI [38] Myocarditis [39]

(continued overleaf)

TABLE 12.1 (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Phenothiazines (e.g., chlorpromazine)		Free radical? Iminoquinone?	Cholestatic DILI [14] Agranulocytosis[40] Lupus [41]
Diclofenac		Iminoquinone [42]	DILI [43] Agranulocytosis [44] Aplastic anemia [45] Thrombocytopenia [46] Hemolytic anemia [47]
Mefenamic acid		Iminoquinone	Hemolytic anemia [48] DILI [49] SJS/TEN [50]

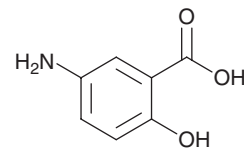
Amodiaquine



Iminoquinone

Agranulocytosis [51] DILI [52]

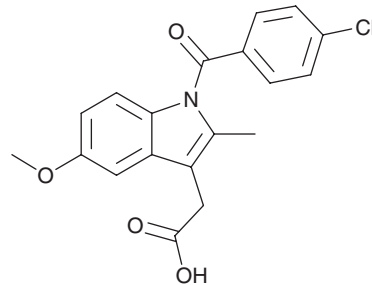
5-Aminosalicylic acid



Iminoquinone

Agranulocytosis [32] DILI
Maculopapular rash Urticaria
($<1\%$)

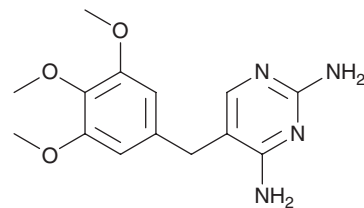
Indomethacin



Iminoquinone

DILI [53] Agranulocytosis [54]
SJS/TEN ($<1\%$)

Trimethoprim

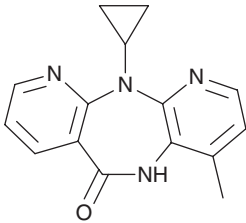
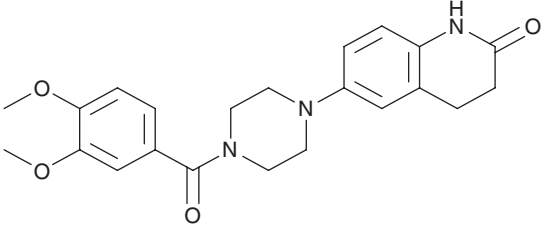
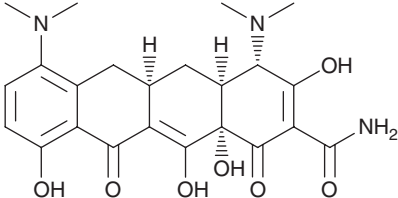


Iminoquinone methide [55]

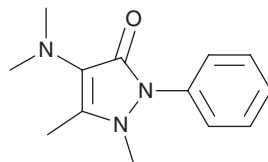
SJS/TEN [56] Anaphylaxis
Fixed drug eruption [57]
Thrombocytopenia [58] DILI [59]

(continued overleaf)

TABLE 12.1 (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Nevirapine		Quinone methide [60] Benzylic sulfate	SJS/TEN [61] DILI [62]
Vesnarinone		Quinone iminium ion [63]	Agranulocytosis [64]
Minocycline		Quinone iminium ion [65]	Autoimmune hepatitis [66] DILI [67] Lupus [68] DRESS [69]

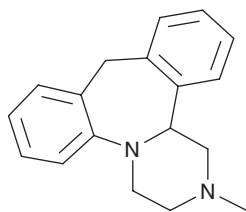
Aminopyrine



Dication [70]

Agranulocytosis [71]

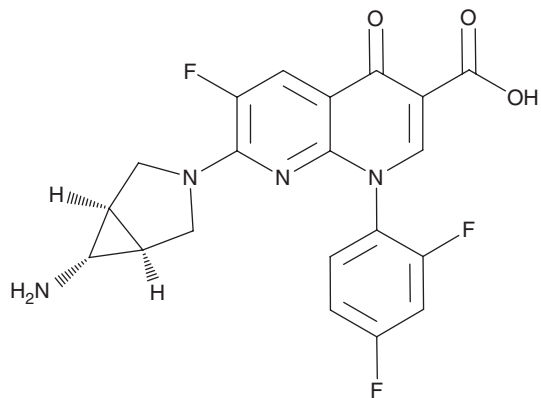
Mianserin



Iminium ion [72]

Agranulocytosis [73] Aplastic
anemia [74] DILI [75]

Trovafloxacin

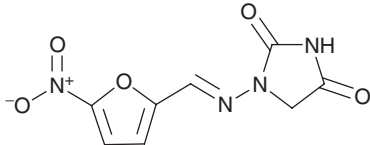
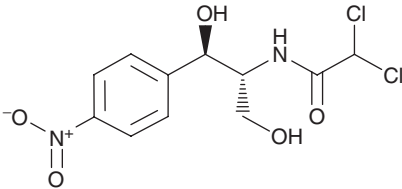
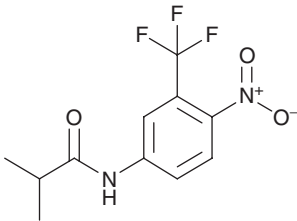
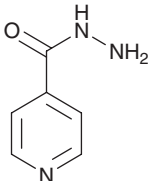


Cyclopropyl amine oxidized to a free
radical and Michael acceptor [76]

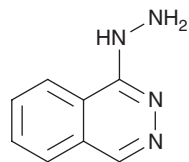
DILI [77] TEN (one case
report) [78]

(continued overleaf)

TABLE 12.1 (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Nitrofurantoin		Nitro reduced to nitroso and free radicals	Pulmonary fibrosis [79] Autoimmune hepatitis [80]
Chloramphenicol		Nitro reduced to nitroso and free radicals Acid chloride	Aplastic anemia [81] Agranulocytosis [82] Thrombocytopenia
Flutamide		Iminoquinone Diiminoquinone Free radical [83]	DILI [84] Agranulocytosis [85]
Isoniazid		Hydrazine oxidized to a diazonium ion and free radicals [86–88]	DILI [89] Lupus [90] Red cell aplasia [91] Hypersensitivity reaction

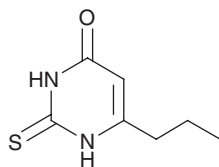
Hydralazine



Hydrazine oxidized to a diazonium ion and free radicals [92]

Lupus [93] DILI [94]

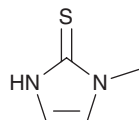
Propylthiouracil



Thionosulfur oxidized to sulfenic acid and chloride [95]

Agranulocytosis [96] **DILI** [97] Aplastic anemia [98] Lupus [99]

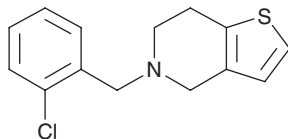
Methimazole



Thionosulfur oxidized to sulfenic acid and chloride [95]

Agranulocytosis [96] **DILI** Aplastic anemia [100] Lupus

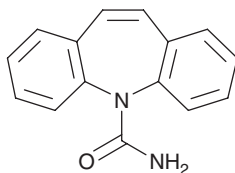
Ticlopidine



Epoxide Thiophene *S*-oxide
Thiophene *S*-chloride [101]

Agranulocytosis [102] **Thrombocytopenia** [103] Aplastic anemia [104] DILI [105] Lupus [106]

Carbamazepine

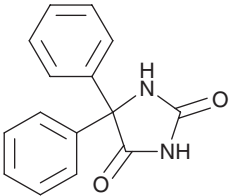
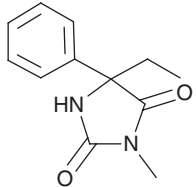
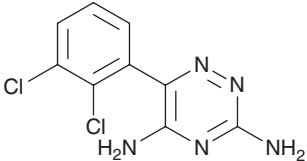
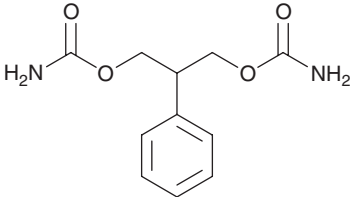


Epoxide, iminoquinone [107] Free radical [108] *o*-Quinone Acridine Carboxaldehyde [109]

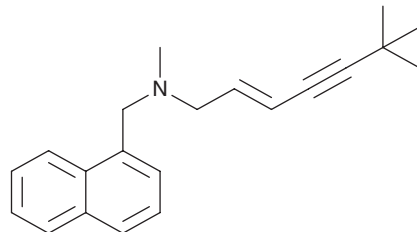
Hypersensitivity [110] **SJS/TEN** [111] Lupus [112] DILI Agranulocytosis[44] Aplastic anemia [113]

(continued overleaf)

12 **TABLE 12.1** (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Phenytoin		Epoxide Free radical <i>o</i> -Quinone	DRESS [114] SJS/TEN [115] Lupus [116] DILI [117] Agranulocytosis [44] Red cell aplasia [118] Interstitial pneumonia [119]
Mephenytoin		Epoxide Free radical <i>o</i> -Quinone	Agranulocytosis [120] Aplastic anemia Thrombocytopenia
Lamotrigine		Epoxide [121]	DRESS [122] SJS/TEN [123] DILI [124] Agranulocytosis [125]
Felbamate		Michael acceptor [126]	Aplastic anemia [127] DILI [128] TEN (one case report [129]

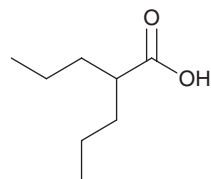
Terbinafine



Michael acceptor [130]

DILI [131] Lupus [132] Rash
[133] Agranulocytosis [134]

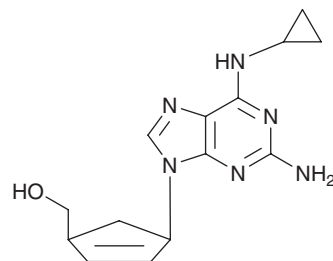
Valproic acid



Michael acceptor [135]

DILI with steatosis [14]
Pancreatitis [136]
Thrombocytopenia [137]

Abacavir

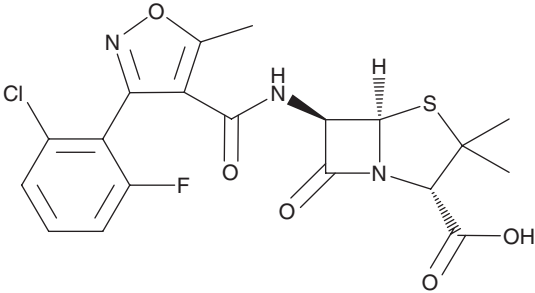
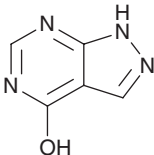


Michael acceptor [138]

Hypersensitivity [139]

(continued overleaf)

TABLE 12.1 (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Flucloxacillin		β -Lactam	DILI [140] Skin rash Urticaria
Allopurinol		None known	DRESS [141] TEN DILI [142]

Abbreviation: SJS, Stevens–Johnson syndrome.

“?” denotes that the reactive metabolite is not clear.

IDRs, and this is consistent with the ability of some drugs to be metabolized to reactive metabolites, which can lead to different types of IDRs. The following sections are a description of the major types of IDRs.

12.4.1 Autoimmunity

By definition, autoimmunity is an immune response against “self.” Many, if not most, chronic diseases have a strong autoimmune component. These diseases include type I diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, autoimmune thyroiditis, systemic lupus erythematosus, and myasthenia gravis. Even diseases such as arteriosclerosis and Alzheimer’s disease may have an autoimmune component. Drugs can also precipitate an autoimmune response.

12.4.1.1 Generalized Autoimmunity (Lupus-Like Syndrome). Several drugs cause a generalized autoimmune syndrome that resembles idiopathic systemic lupus erythematosus [143]. Although drug-induced lupus is usually milder than idiopathic lupus and usually resolves when the drug is discontinued, there are no absolute criteria that separate idiopathic and drug-induced lupus [144]. The drug associated with the highest incidence of a lupus-like syndrome is procainamide. Almost all patients treated with procainamide for an extended period develop antinuclear antibodies, and about 20% of patients develop clinically evident autoimmunity, although the manifestations are usually less severe than those of idiopathic lupus [144]. Patients are often treated with procainamide for more than a year before the onset of symptoms, but once the drug is stopped, the symptoms usually resolve in a matter of weeks, even though, being an autoimmune syndrome, the antigens are still present.

Procainamide is oxidized by macrophages to reactive hydroxylamine and nitroso metabolites, and this has the potential to activate macrophages [145]. This could be involved in the mechanism of autoimmunity, and most of the drugs that cause a lupus-like syndrome are also oxidized to reactive metabolites by macrophages [146]. In addition, procainamide and hydralazine also inhibit DNA methylation, which can lead to lymphocyte activation, and this could also play an essential role in the induction of autoimmunity, at least for these two drugs [147]. We have found that penicillamine, isoniazid, and hydralazine, which also induce lupus-like syndrome, react with carbonyl groups on macrophages and lead to their activation [148,149]. This is likely to play an essential role in the ability of these drugs to induce autoimmunity. There are many new biological drugs that inhibit various arms of the immune system, which have been developed to treat autoimmune diseases such as multiple sclerosis and inflammatory bowel disease. Paradoxically, most of these drugs can also induce autoimmunity, presumably by altering the delicate balance of the immune system [150].

12.4.1.2 Organ-Specific Autoimmunity. There are several drugs that cause an autoimmune reaction that is limited to one organ. Examples include α -methyl dopa-induced hemolytic anemia and nitrofurantoin- and minocycline-induced autoimmune hepatitis. These syndromes are described in greater detail below.

12.4.2 Skin Rash

The most common type of IDR is the skin rash. It is likely that this is because the skin acts as a barrier to the outside world and is immunologically very reactive. Furthermore,

on the basis of their clinical characteristics and histology, it is likely that virtually all skin rashes are immune mediated [151]. Rashes can vary from a mild maculopapular rash to very severe rashes such as toxic epidermal necrolysis (TEN) in which a large portion of the skin basically dies, and TEN is associated with a high (~30%) mortality rate.

If skin rashes are commonly caused by reactive metabolites, which seems likely, an important question is whether the reactive metabolite must be formed in the skin. In the case of penicillin, it is the parent drug that is chemically reactive, but its reactivity is low so that it can freely circulate and local reactive metabolite formation is not required. Most reactive metabolites are formed by cytochrome P450s, and although low levels of these enzymes are present in the skin [8], it is not clear that sufficient P450-mediated metabolism occurs in the skin to induce an immune response. Probably, the best evidence is for fragrances that can cause contact hypersensitivity reactions. Some undergo spontaneous oxidation to reactive species, but others require metabolic activation [152]. Macrophages and other antigen-presenting cells, which are present in the skin, contain myeloperoxidase that can oxidize some drugs to reactive metabolites, and given their importance to an immune response, reactive metabolite formation of some drugs by these cells could result in an immune response [153,154]. The activity of some metabolic enzymes in the skin, such as sulfotransferases, is high, and there are some cases in which sulfation can lead to a reactive metabolite [155,156]. It is also possible that an immune response is initiated elsewhere in the body, and the skin becomes a bystander because of its high immunosurveillance activity. This is consistent with the observation that a reactive metabolite may be responsible for initiating an immune response, but then the immune response spreads so that lymphocytes recognize the parent drug [157]. Although drugs can cause other types of rashes, such as fixed drug eruptions, the discussion is limited to urticaria, maculopapular rashes, and Stevens–Johnson syndrome/TEN.

12.4.2.1 Urticaria. Otherwise known as *hives*, urticaria is classically an allergic reaction mediated by IgE antibodies. When it is caused by drugs such as penicillin it is mediated by IgE antibodies against drug-modified proteins [158]. This is a classic case of a drug or reactive metabolite acting as a hapten to modify protein, and in some patients, this leads to an immune response. This is a clear case where chemical reactivity of the drug or metabolite is an essential aspect of the mechanism. In the case of penicillin, this can be demonstrated by skin tests in which a small amount of penicillin-modified polylysine is injected into the skin and results in an immediate wheal and flare response in sensitive patients, which is mediated by IgE antibodies. Penicillin binds to proteins in all patients, and it probably induces an antibody response in most patients; however, in most cases, this immune response is predominantly an IgG response, which does not result in an adverse reaction. It is only when the immune response is predominantly an IgE response, leading to a sufficient concentration of IgE antibodies, that urticaria results.

Some patients have what is called *chronic urticaria* in which they can have daily episodes of urticaria not precipitated by any known drug or chemical exposure. This condition is not well understood, but it appears to represent an autoimmune reaction [159]. In some cases, chronic urticaria appeared to follow a typical allergic reaction to a drug, and it may be that in these cases, this allergic reaction triggered an autoimmune reaction resulting in chronic urticaria. In some patients, urticaria can be induced by

cold or exercise; obviously, there are no antibodies against cold. Therefore, urticaria is not always a true allergic reaction to a chemical allergen.

12.4.2.2 Maculopapular Rash. The most common type of rash induced by drugs is a maculopapular or morbilliform rash. This can be an isolated finding, or it can be part of a more general response to a drug such as the generalized hypersensitivity reactions described below. In many cases, this rash resolves despite continued treatment with the drug. In general, drugs that cause more serious rashes such as TEN in some patients will cause maculopapular rashes at a higher frequency than the more severe rashes, and early in the course of the rash, it can be difficult to differentiate the two. However, some drugs commonly cause maculopapular rashes and rarely, if ever, cause TEN.

It appears that most drugs that cause maculopapular rashes form reactive metabolites, but it is hard to demonstrate that this is an essential part of the mechanism because this type of rash is not mediated by antibodies with an easily determined specificity. It appears that these rashes are mediated by CD4⁺ T cells [151]. This could explain why they are usually mild because CD4 interacts with major histocompatibility complex (MHC)-II, which is not present on most cells, and therefore, most cells would escape significant damage. Specifically, MHC-II is not normally expressed on keratinocytes, the major component of the epidermis; however, inflammation can result in the induction of MHC-II expression on keratinocytes and this could be part of the mechanism of these rashes [160].

12.4.2.3 Stevens–Johnson Syndrome/Toxic Epidermal Necrolysis (TEN). It appears that TEN and Stevens–Johnson syndrome share a very similar mechanism, but TEN is more severe and, by definition, involves more than 30% of the total skin area [161]. A classic feature of TEN is Nikolsky sign, where the application of lateral pressure to the skin results in its detachment. As indicated above, drugs that cause TEN, such as sulfonamides, allopurinol, carbamazepine, and phenytoin, more frequently cause mild maculopapular rashes. A major difference in the mechanism appears to be that TEN is mediated by CD8⁺ cytotoxic T cells [111,151]. This results in a more severe rash because CD8 binds to MHC-I, which is present on all nucleated cells, including keratinocytes, and therefore, the immune response can cause extensive damage to the skin. As with other IDRs, the drugs involved, with the possible exception of allopurinol, form reactive metabolites, but the mechanistic role of reactive metabolites remains to be determined.

12.4.2.4 Generalized Hypersensitivity Reaction/Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS). As the name implies, DRESS (drug reaction with eosinophilia and systemic symptoms) is a systemic immune-mediated reaction that is virtually always associated with a rash, but it also includes various other organs in the body and is usually associated with fever and eosinophilia [162,163]. This syndrome can be caused by many of the same drugs that cause TEN, such as carbamazepine, sulfonamide antibiotics, and allopurinol. However, the mechanism of this immune reaction appears to be fundamentally different from that of TEN because in Han Chinese, carbamazepine-induced TEN is strongly associated with the MHC-I gene HLA B5701, but this is not a risk factor for DRESS [164]. There is relatively new evidence that this syndrome involves reactivation of viruses by the drug, and this is discussed further in Section 12.5.1.4.

12.4.3 Idiosyncratic Drug-Induced Liver Injury (DILI)

Drug-induced liver injury (DILI) is the most common reason for removal of a drug from the market, and evidence of DILI during clinical trials can prevent a drug candidate from being approved [165]. Mild liver injury may be even more common than drug-induced skin rashes, but the liver is not visible, and so if the injury is asymptomatic, it is unlikely to be detected. It is likely that the reason that the liver is so commonly the target of IDRs is because it is the site of most drug metabolism and reactive metabolite formation. In fact, an immune response in the liver usually results in immune tolerance, and unlike skin transplants [166], immune rejection of liver transplants is relatively easy to prevent.

The involvement of reactive metabolites in the pathogenesis of most DILI is widely accepted [10,167]. However, this is difficult to definitively demonstrate, and there are probably cases that do not involve a reactive metabolite. The best example is probably ximelagatran, which does not appear to form a reactive metabolite. This drug was a promising anticoagulant, but it had to be withdrawn from the market because of an unacceptable risk of liver failure. It is a peptidomimetic drug and appears to bind reversibly to specific MHC-II molecules. Furthermore, the presence of these MHC-II molecules (DRB1*07 and DQA1*02) is a strong risk factor for ximelagatran-induced liver failure [168]. Pyrazinamide is also associated with a relatively high incidence of liver failure [169], and yet, it is a simple molecule with no evidence of a reactive metabolite.

The involvement of the immune system in the mechanism of DILI is more controversial. In some cases, such as DILI caused by halothane, tienilic acid, and dihydralazine, the DILI is associated with the presence of antidrug and/or autoantibodies against the P450 that formed the reactive metabolite of the drug [170]. This is taken by most people as evidence that DILI is immune mediated. However, it is unlikely that these antibodies actually mediate the liver damage. Most reactive metabolites of drugs that are associated with DILI are quite reactive and bind mostly to intracellular antigens. The resulting modified protein will be presented on the surface of hepatocytes in the context of MHC-I. Therefore, liver damage is more likely to be cell mediated, in particular, CD8⁺ cell mediated because CD8 binds to MHC-I. However, immune responses rarely produce only a cellular or an antibody response, and the antibody response may represent more an indication that the immune system has been activated rather than an indication of what arm of the immune system is involved in causing liver damage.

However, antibodies have not been detected in most cases of DILI, although to be fair, in most cases, it is likely that no one has looked for antidrug antibodies because the required ELISA reagents would have to be synthesized. Hepatotoxicity that is not associated with antibodies and other evidence of an allergic reaction such as fever and rash has been classified by some as metabolic idiosyncrasy [14]. One may not consider the absence of allergic symptoms to be significant evidence against an immune mechanism, and there are no cases in which a polymorphism in drug metabolism is sufficient to explain the idiosyncratic nature of idiosyncratic DILI [3]. Some DILIs are not associated with immune memory, that is, the onset of clinical symptoms of liver injury does not occur more rapidly on rechallenge of a patient who has previously experienced DILI to a specific drug. This represents stronger evidence against an immune mechanism; however, there are many immune-mediated reactions that are not associated with immune memory.

As mentioned above, the response to the same drug can vary from patient to patient. In one early report of isoniazid-induced DILI, one patient who was rechallenged developed fever within 4 h and shortly after the alanine aminotransferase (ALT) had increased more than 10-fold. In contrast, another patient with a history of isoniazid-induced DILI was treated with isoniazid for a month before there was an increase in ALT [89]. In many patients, isoniazid is stopped when there is a significant increase in ALT, but when they are later rechallenged, there is no increase in ALT, especially if the starting dose is lowered. It is also the case with ximelagatran that rechallenge of patients who had developed DILI on the drug usually did not lead to recurrence, even though ximelagatran appears to be immune mediated based on its association with specific HLA (human leukocyte antigen) genotypes (DRB1*07 and DQA*02) [168]. It is also provocative that there were cases of ximelagatran-induced DILI that did not occur until about a month after the drug was stopped [171]. It is hard to understand how this could be an antidrug immune response; it is more consistent with a drug-induced autoimmune reaction. There are other examples of IDRs starting after the drug has been stopped, as discussed below.

Some drugs such as minocycline, nitrofurantoin, and α -methyldopa can cause DILI with clear markers of an autoimmune reaction [15]. The cases of DILI that are associated with autoantibodies usually have a longer delay—often more than a year between starting the drug and the onset of liver injury. Drugs that cause autoimmune liver damage can also cause the more typical type of idiosyncratic DILI with a time to onset of about a month. Although one of the characteristics that has been used as evidence that DILI is not immune mediated is a long delay between starting the drug and the onset of injury, for example, troglitazone-induced DILI, it is possible that this is actually because the reaction has an autoimmune component even if it is not associated with the characteristic autoantibodies typical of autoimmune hepatitis: antinuclear and anti-smooth-muscle antibodies for type I autoimmune hepatitis and antiliver/antikidney microsome-I antibodies for type II autoimmune hepatitis [15].

12.4.4 Hematologic Idiosyncratic Drug Reactions

Drugs can affect any of the formed elements of the blood, either by damage to precursors in the bone marrow or by peripheral destruction of cells. The most common disorders are anemia in which the red blood cell count is decreased, thrombocytopenia in which the platelet count is decreased, agranulocytosis in which the neutrophil count is decreased, and aplastic anemia in which all three of these cells are decreased.

12.4.4.1 Anemia. The classic drug associated with idiosyncratic anemia is α -methyldopa, which causes an antibody-mediated autoimmune hemolytic anemia [172]. It is clearly immune mediated because it is associated with anti-red-cell antibodies that cause hemolysis, but the antibodies are not drug dependent, that is, they cause hemolysis in the absence of the drug [173]. It is not clear how α -methyldopa induces this autoimmune response, but being a catechol, it can form an *o*-quinone reactive metabolite, and it can also cause a lupus-like generalized autoimmune syndrome [174] and idiosyncratic liver toxicity that can have an autoimmune component [175]. At present, the most common cause of idiosyncratic drug-induced hemolytic anemia is the third-generation cephalosporins, and most of the drugs associated with hemolytic anemia are either chemically reactive or form reactive

metabolites. Some drugs, such as α -methyldopa, are mediated by autoantibodies, and others are mediated by drug-dependent antibodies [176]. The most common hypothesis of how these antibodies are induced is the hapten hypothesis, although a more recent hypothesis is that the drug (or reactive metabolite) alters the red cell membrane resulting in binding of other proteins to the altered membrane [176].

Another type of anemia is pure red cell aplasia, which reflects absent or dysfunctional red cell precursors. One such example is caused by erythropoietin treatment, which can induce antibodies against erythropoietin, a factor required for red cell development. This appears to be due to denaturation of the protein because the incidence of pure red cell aplasia is very dependent on the formulation. Specifically, the incidence increased when human serum albumin was removed from the erythropoietin formulation [177]. Pure red cell aplasia can also be caused by drugs such as procainamide and isoniazid, which can also induce other autoimmune syndromes [21,91].

12.4.4.2 Thrombocytopenia. The most common cause of idiosyncratic immune-mediated thrombocytopenia is heparin, which can induce the formation of antibodies against the complex of heparin and platelet factor 4, leading to platelet aggregation and consumption of the platelets [178]. Even though this IDR is mediated by antibodies, there is no immune memory, and if a patient with a history of heparin-induced thrombocytopenia is rechallenged after the antibodies have cleared from the blood, there is usually no recurrence, and if thrombocytopenia recurs, it has the same time course as in a naive patient [179].

There appears to be many mechanisms by which other drugs can cause thrombocytopenia [180]. Drugs such as penicillin can act as haptens and induce drug-dependent antibodies that lead to thrombocytopenia. Quinine causes thrombocytopenia that appears to be mediated by antibodies that bind to the drug and form immune complexes that then bind to platelets leading to their destruction as an “innocent bystander.” This appears to involve antibodies that are naturally weakly autoreactive with epitopes on the platelet membrane and bind more strongly after binding to the drug. Drugs such as procainamide, penicillamine, and gold salts can also induce the formation of autoantibodies that result in thrombocytopenia [181]. Another interesting mechanism involves tirofiban and eptifibatide, which cause an immediate destruction of platelets because of naturally occurring antibodies that appear to recognize a drug-induced conformational change in the membrane protein GPIIb-IIIa [182]. Abciximab, an antibody against GPIIb-IIIa, can also cause an immediate onset of thrombocytopenia because of naturally occurring antibodies that recognize the modified protein [183]. It can also cause thrombocytopenia that begins six days after abciximab treatment has been stopped, which is mediated by antibodies against drug-coated platelets that remain in the circulation [184].

12.4.4.3 Agranulocytosis. A lack of granulocytes is referred to as *agranulocytosis*, and it is usually defined as a peripheral neutrophil count of <500 neutrophils/ μL [185]. Neutrophils are a common target for cytotoxic drugs used to treat cancer because the very rapid turnover of these cells makes them especially susceptible to agents that damage DNA; agranulocytosis caused by cytotoxic drugs is not idiosyncratic. Many drugs can cause idiosyncratic agranulocytosis [186].

If idiosyncratic drug-induced agranulocytosis is caused by reactive metabolites, it is likely that the enzyme involved is myeloperoxidase, which is the major oxidative

enzyme in neutrophils. We and others have shown that most of the drugs that are associated with a relatively high incidence of idiosyncratic agranulocytosis are oxidized to reactive metabolites by myeloperoxidase. Such drugs include clozapine, aminopyrine, procainamide, propylthiouracil, amodiaquine, dapsone, and vesnarinone [63,70,101,154,187–189].

12.4.4.4 Aplastic Anemia. The diagnosis of aplastic anemia cannot be confidently made without a bone marrow biopsy that shows a marked decrease in the cellularity of the bone marrow with replacement of normal hematopoietic cells by fat cells [190]. This leads to a lack of neutrophils, platelets, and red blood cells, which is eventually fatal if not reversed. The cause of most aplastic anemia is unknown (idiopathic). It sometimes follows a viral infection such as viral hepatitis, but about 10% is caused by drugs. There is strong evidence that aplastic anemia, both idiopathic and drug induced, is immune mediated. In particular, it appears to be mediated by cytotoxic T cells that are characterized by the production of interferon- γ [191]. As a result, both idiopathic and idiosyncratic aplastic anemia usually respond to immunosuppression. It has been noted that chloramphenicol-induced aplastic anemia often occurs well after the drug is stopped, sometimes more than two months after, and the longer the delay, the more severe the aplastic anemia is likely to be [82]. This is consistent with an autoimmune mechanism, but there is no direct evidence for this hypothesis. There is some overlap in the drugs that cause agranulocytosis and aplastic anemia, but there are also significant differences [154]. A major difference is that the target of aplastic anemia is a very early cell close to the stem cell and therefore recovery is much slower than for agranulocytosis, which involves much more differentiated cells, and in some cases, it appears that only mature neutrophils are destroyed.

12.5 MAJOR HYPOTHESES OF IDIOSYNCRATIC DRUG REACTIONS

The mechanisms of IDRs are open to debate because, with a few exceptions such as penicillin-induced anaphylaxis, the evidence is circumstantial rather than definitive. Prospective mechanistic studies in humans are virtually impossible, and there is a paucity of animal models. In addition, most of the animal models involve acute toxicity and probably do not involve the same mechanism as the analogous IDR that occurs in humans, given the low incidence and especially the delay in onset in man. There are several working hypotheses (discussed in detail later), but without a battery of valid animal models, testing these hypotheses represents a major challenge. An essential test of any model or hypothesis is whether it fits the characteristics of clinical IDRs.

12.5.1 Immune-Mediated Mechanisms

The idiosyncratic nature and delay between starting a drug and the onset of the adverse reaction described earlier suggest that most IDRs are immune mediated. By definition, drug-induced autoimmunity must be immune mediated. There is ample evidence that most drug-induced skin rashes are immune mediated. Some hematological IDRs are clearly mediated by antibodies. It appears that most drug-induced aplastic anemia is immune mediated because it usually responds to immunosuppression as discussed earlier. Aminopyrine-induced agranulocytosis appears to be mediated by drug-dependent

antineutrophil antibodies [192]. In contrast, some drug-induced agranulocytosis has characteristics that suggest that it is not immune mediated. Specifically, phenothiazine-induced agranulocytosis is said to result from toxic suppression of bone marrow rather than being immune mediated [193]. Clozapine-induced agranulocytosis also does not usually recur rapidly on rechallenge, and this lack of immune “memory” also suggests that it might not be immune mediated [194]. However, these characteristics are consistent with an immune mechanism because there are other IDRs with similar characteristics that are clearly immune mediated, as described earlier. The most controversial issue is whether cases of idiosyncratic liver toxicity that are not associated with other evidence of an immune response, for example, fever, rash, and antibodies, are immune mediated. In fact, even the evidence that the liver injury associated with drugs such as halothane, in which there does appear to be immune memory and there are antidrug antibodies, is not conclusive, and not everyone believes that halothane-induced liver failure is immune mediated.

It is likely that the vast majority of IDRs are immune mediated. Certainly not all, or even most, immune-mediated reactions are associated with features such as fever and rash. In addition, there are several examples of clearly immune-mediated reactions that do not exhibit immune memory [3]. One possible reason for the lack of immune memory is that the immune response has an autoimmune component and this results in deletion or silencing of the memory T cells. However, until better evidence for an immune mechanism for some IDRs is obtained, this still must be considered a hypothesis open to debate.

The following are the major mechanistic hypotheses for immune-mediated IDRs.

12.5.1.1 Hapten Hypothesis. The hapten hypothesis is based on very old experiments that found that small molecules did not induce an immune response unless they were able to covalently bind to macromolecules [195]. A small molecule that is not immunogenic unless it is bound to a macromolecule is referred to as a *hapten*. This became part of the self–nonself view of what induced an immune response, in which the immune system differentiates between self and nonself or foreign at an early age and only responds to nonself molecules [196]. In this case, the covalent binding of small molecules to proteins results in them becoming “foreign,” and this leads to an immune response (Fig. 12.1). Presumably, the reason why covalent binding of the hapten is required is for antigens (in this case, drug-modified protein) to be presented to T cells, they must first be processed. Processing essentially involves hydrolysis of the proteins to peptides, and if the interaction of the drug with the protein was readily reversible, it would not survive processing. There is compelling evidence that the hapten hypothesis is correct for some IDRs; specifically, for IDRs such as penicillin-induced allergic reactions that are mediated by antibodies against drug-modified proteins, as discussed in Section 12.4.2.1. However, that does not mean that all IDRs are mediated by an immune response to drug-modified proteins.

There are many examples in which drugs induce autoantibodies rather than antidrug antibodies; this does not fit the simple hapten hypothesis. There are also many examples, such as halothane-induced hepatitis, that are associated with both antibodies against drug-modified proteins and native proteins [197]. Two possible mechanisms related to the hapten hypothesis by which covalent binding of a reactive metabolite to proteins could induce autoantibodies are epitope spreading and presentation of cryptic peptides. When an immune response is induced against drug-modified proteins, the

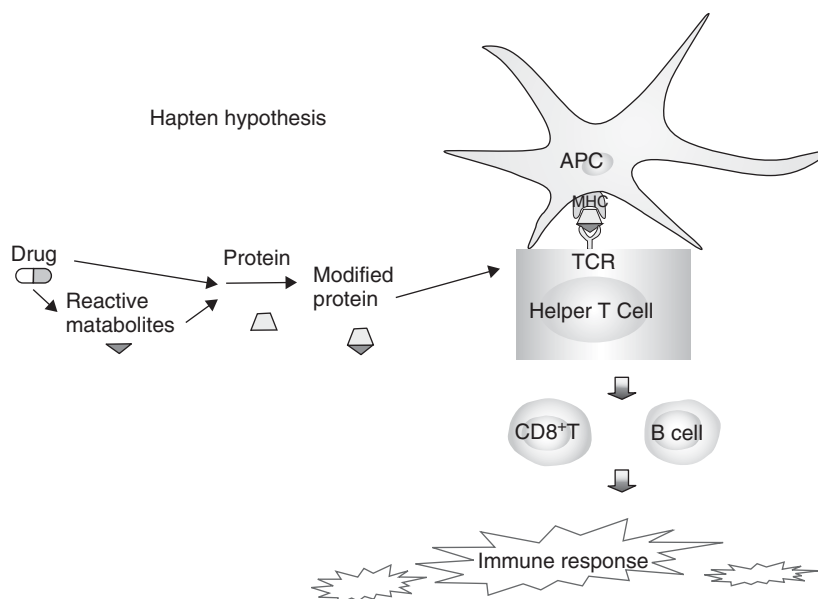


Figure 12.1 Hapten hypothesis. A drug, or more commonly a reactive metabolite of the drug, binds to protein and this makes the protein foreign. The modified protein is taken up by anti-gen-presenting cells (APCs), processed to smaller peptides, and these drug-modified peptides are presented in the context of MHC-II to T cells. TCR, T-cell receptor. (See color insert.)

epitope recognized will be different for different T cells, with some recognizing mostly the drug and others, mostly the peptide portion of the modified protein. As the immune response progresses, it may spread to recognize only the native peptide [198,199]. In contrast, the concept of cryptic peptides involves a change in the way the drug-modified protein is processed because of the change in its structure. If this leads to a change in the major site of hydrolysis of the peptide, the immune system may be exposed to peptides it has not seen before, at least not in high concentrations, and therefore, they may be recognized as foreign [200].

12.5.1.2 Danger Hypothesis. In recent years, it has become apparent that the self–nonself picture of the immune response is incomplete because not all autoreactive T cells are deleted, and there is no difference in the manner in which self and nonself antigens are presented to T cells. It was also found that at least two signals were required in order to activate T cells [201]. Signal 1 refers to the recognition of the processed antigen by the T-cell receptor. A second signal, referred to as *signal 2*, is also required for activation. This second signal consists of costimulation of T cells by interaction of various molecules on the antigen-presenting cell, such as B7 (otherwise known as *CD80/86*), with receptors on the T cell, such as CD28. The level of molecules such as CD80/86 on antigen-presenting cells is usually low unless the cell is activated, and therefore, activation of antigen-presenting cells is required to induce an immune response. There are various receptors on antigen-presenting cells called *toll-like receptors* that recognize molecules on pathogens, and binding of these molecules to toll-like receptors leads to activation of the cells [202]. However,

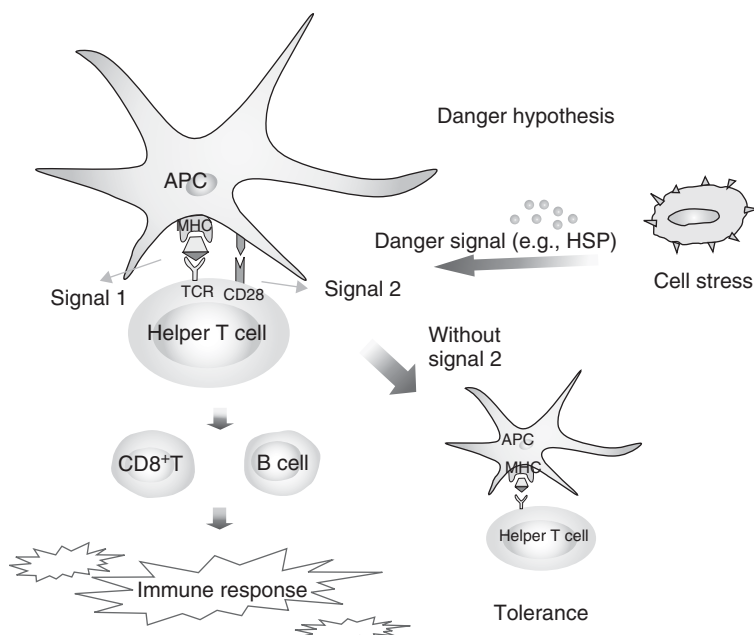


Figure 12.2 Danger hypothesis. A drug or reactive metabolite not only modifies protein but also causes cell damage. This damage causes the damaged cells to release danger signals such as heat shock proteins (HSPs), which in turn activate antigen-presenting cells leading to the upregulation of costimulatory molecules on antigen-presenting cells. This costimulation of T cells represents signal 2 where the recognition of the modified peptides by the T-cell receptor represents signal 1. The presence of both signal 1 and signal 2 leads to helper-T-cell activation, while signal 1 in the absence of signal 2 leads to immune tolerance. (See color insert.)

Matzinger suggested that differentiation between self and nonself was difficult and unnecessary. She suggested that unless something represented a danger to an organism, it could ignore it [203]. Furthermore, she suggested that it is the damaged tissue that determines not only if there will be an immune response but also the nature of that response [204]. It was also discovered that some of the “danger signals” that were produced by damaged tissue bind to the toll-like receptors that are activated by pathogens [205]. Reactive metabolites could cause cell damage that leads to the release of danger signals and upregulation of costimulatory molecules such as B7, thus producing signal 2 (Fig. 12.2). Reactive metabolites might also induce an autoimmune reaction by inducing cell damage that leads to a generalized activation of the immune system.

If this hypothesis is correct, it could explain why some drugs that covalently bind to proteins are not associated with a significant incidence of IDRs; if the reactive metabolite does not cause cell damage and the generation of a consequent danger signal, it would not induce an immune response. Alternatively, some other factors such as an infection could produce a danger signal and this could be a risk factor for an IDR. There are some cases where an infection or surgery appears to be a risk factor for an IDR [206]. However, this is not always the case, and it is important to remember that not all infections have the same effect and the effect of acute infections

on the immune system are quite different than chronic infections. The danger and hapten hypotheses are not mutually exclusive, and both protein modification (hapten hypothesis) and cell damage (danger hypothesis) may be required to induce the immune responses that lead to many IDRs as illustrated in Fig. 12.2. The danger hypothesis is attractive and it is likely to be part of the picture; however, it is difficult to rigorously test.

12.5.1.3 *p-I Hypothesis.* Pichler found that clones of T cells from patients with a history of sulfamethoxazole-induced IDRs proliferated in the absence of metabolism of the drug [207]. He also found similar effects with some other drugs. From this, he reasoned that drugs must be able to generate an immune response by binding reversibly to the antigen-presenting cell MHC/T-cell-receptor complex much like the p-I of a drug binding to its receptor. This led to the name p-I hypothesis [208]. In the case of sulfamethoxazole, this seems strange because sulfamethoxazole is a primary aromatic amine and virtually all primary aromatic amines given at a significant dose are associated with a significant incidence of IDRs, presumably because they are readily metabolized to reactive metabolites [209]. The unstated assumption in the p-I hypothesis is that the T cells are responding to the molecule that initially induced the immune response. We have found that this basic assumption is incorrect. Specifically, in a model of immune-mediated nevirapine-induced skin rash, even though the drug requires hydroxylation in order to induce a skin rash, T cells from animals with nevirapine-induced skin rash produced much more cytokines when stimulated with the parent drug than the hydroxylated metabolite. In fact, even if the skin rash was induced by treatment with the hydroxylated metabolite rather than the parent drug and the animals had never been exposed to the parent drug, the T cells responded much more to the parent drug [157]. Even though this is an animal model, it provides definitive evidence that there can be differences between what stimulates an immune response and what T cells respond to later in an immune response. In addition, the use of clones in Pichler's experiments, which were selected based on the growth in the presence of drug rather than primary lymphocytes, may select for cells that respond to the parent drug. In fact, a more recent study found that lymphocytes from patients with IDRs to sulfamethoxazole responded much better to the nitroso metabolite than to the parent drug [210]. There may be some examples such as ximelagatran, which does not appear to form a reactive metabolite, where the drug induces an immune response through the p-I mechanism. In fact, ximelagatran, which looks like a peptide, was found to bind directly to DRB1*07 and DQA1*02, and these genotypes are a significant risk factor for ximelagatran-induced liver toxicity [168]. However, it is unlikely that this is a common mechanism for the induction of an IDR.

12.5.1.4 *Viral Reactivation.* It was observed many years ago that patients with mononucleosis who were treated with ampicillin had a very high incidence of skin rash [211]. More recently, it was found that many cases of DRESS are associated with reactivation of a virus, most commonly, a herpes virus [212,213]. It is not totally clear exactly what role the virus and the drug play in these IDRs, although it seems likely that the drug somehow decreases the immune response, thereby allowing reactivation of the virus, and the virus could act as a danger signal to help produce an immune response against the drug.

12.5.1.5 Unfolded Protein Response. A large number of protein molecules are synthesized every second, and they have to be folded and moved to where they are needed. When there is an accumulation of abnormally folded proteins, there is a specific response referred to as the *unfolded protein response* [214]. This response can result in inflammation [215]. There are many things that can lead to the accumulation of unfolded proteins, but if the reactive metabolite of a drug modifies a protein, it could interfere with the normal folding of a protein and this could lead to an immune response. However, there are no clear examples in which this has led to an IDR. To some degree, this is a variant of the danger hypothesis.

12.5.1.6 Epigenetic Effects Such as Inhibition of DNA Methylation. A major mechanism by which gene expression is controlled is epigenetic [216]. Methylation of the 5-position of cytosine in DNA inhibits gene expression, and most DNA cytosine is methylated. A decrease in DNA methylation in B cells leads to their activation, and autoimmune diseases such as lupus are associated with decreased DNA methylation [217]. Some drugs such as procainamide and hydralazine can inhibit DNA methylation, and these drugs are associated with the induction of a lupus-like autoimmune syndrome, as discussed in Section 12.4.1.1 [147]. This mechanism could also contribute to other types of IDRs; however, there are no good examples where epigenetic effects have been shown to result in an IDR other than autoimmunity.

12.5.1.7 Direct Activation of Antigen-Presenting Cells. As discussed in Section 12.5.1.2, a second signal resulting from the activation of antigen-presenting cells is required for the induction of an immune response. This activation could be accomplished by danger signals produced by damaged tissue, but it is possible that a drug or reactive metabolite could directly activate antigen-presenting cells. We found that drugs that react with aldehydes such as penicillamine, hydralazine, and isoniazid directly activate antigen-presenting cells, apparently by reacting with signaling molecules on these cells that have reactive carbonyl groups [148,149]. These drugs can cause autoimmune reactions. Antigen-presenting cells also possess myeloperoxidase that can generate reactive metabolites that have the potential to activate these cells [218]. One likely example is the reactive nitroso metabolite of sulfamethoxazole, which is formed by myeloperoxidase and can activate dendritic cells [153].

12.5.1.8 Disturbing Immune System Balance. Many new biological agents work by inhibition of various arms of the immune system. Paradoxically, these agents can result in an immune-mediated IDR, presumably by altering the balance of the immune system. The exact mechanism is unknown, but because virtually all biological agents that target the immune system can cause various forms of autoimmune reactions, even though their specific targets are different, simply altering the balance of various arms of the immune system appears to have the potential to cause autoimmunity. Other agents may act by a similar mechanism, but this is more difficult to demonstrate.

12.5.2 Non-Immune-Mediated Mechanisms

Although the characteristics of IDRs suggest that they are immune mediated, the evidence remains circumstantial, and at least some IDRs may not be immune mediated. The involvement of the immune system in idiosyncratic DILI is the most controversial.

12.5.2.1 Metabolic Idiosyncrasy. Zimmerman [14] classified DILI that was not associated with features of an immune response such as antidrug antibodies, fever, and rash as metabolic idiosyncrasy. In general, this classification has been accepted. Drugs that fit this category include isoniazid, ketoconazole, and troglitazone. On average, cases that fit this description occur later and are more severe. The explanation for toxicity associated with these drugs is that it is caused by polymorphisms in metabolism that lead to a difference in response. However, polymorphisms in the metabolism of isoniazid, specifically *N*-acetyltransferase 2 and Cyp 2E1, confer a slight increase in risk of liver toxicity [219], the increase in risk is much too small to explain the idiosyncratic nature of severe cases of isoniazid-induced liver toxicity. Such a mechanism also does not explain the delay between starting the drug and the onset of toxicity or the difficulty producing an animal model with characteristics similar to the idiosyncratic reaction that occurs in humans. In some cases, there is an amnestic response to drugs classified as metabolic idiosyncrasy, such as isoniazid, as mentioned in Section 12.4.3, and ketoconazole. In one case of ketoconazole-induced DILI, a patient developed hepatitis 60 days after starting ketoconazole, and 30 months later, she was inadvertently treated with ketoconazole and within 48 hours she again developed hepatitis. However, in many cases, there is no prompt recurrence on rechallenge [220]. When there is a very rapid response on rechallenge, it strongly supports an immune mechanism; when there is a lack of an amnestic response, it is consistent with an autoimmune reaction [15]. Although polymorphisms in drug metabolism undoubtedly represent a risk factor for some IDRs, it is likely a minor mechanism of IDRs per se.

12.5.2.2 Mitochondrial Toxicity. The hypothesis that many cases of idiosyncratic liver toxicity involve mitochondrial damage is currently receiving much attention. Mitochondria supply the cell with energy through the Krebs's cycle, oxidative phosphorylation, and fatty acid β -oxidation, which could all be potential targets of toxicity for drugs and their reactive metabolites. Mitochondria also control cell death via apoptosis/necrosis through the opening of the mitochondrial permeability transition pore and the release of cytochrome *c*. Mitochondria have their own genome, which is vulnerable to oxidative damage because of the lack of protective histones and its close proximity to the inner membrane, which is the major source of endogenous reactive oxygen species in a cell. In addition, lipophilic cationic drugs can accumulate in the mitochondrial matrix because of the negative membrane potential [221]. There are several copies of mitochondrial DNA (mtDNA) in each mitochondrion and there are hundreds of mitochondria in a hepatocyte; therefore, if a drug or its reactive metabolite caused damage to mtDNA, the cell could continue to function normally (also known as *functional reserve*) until the cumulative damage reached a certain threshold and this could explain the delayed onset of idiosyncratic liver toxicity [222].

Much of the evidence for mitochondrial injury being responsible for DILI comes from *in vitro* studies using high concentrations of drug; this is not credible on its own to support the hypothesis. For instance, Xu *et al.* studied over 300 compounds in human hepatocytes using fluorescent probes and found that many drugs that cause idiosyncratic liver toxicity caused a perturbation in a marker associated with mitochondrial function, while drugs that are not associated with liver toxicity usually did not [223]. However, the study used concentrations of drugs that were 100-fold the therapeutic $C_{\max}$ achieved in humans. This was justified by saying that the kinetics could be different in some people and that the concentration in the liver during absorption would be higher than

the blood concentration. However, if this were the mechanism of DILI, it should be possible to induce DILI in animals simply by giving higher doses of the drug. In addition, several drugs, such as isoniazid and flutamide, that have been proposed to cause DILI by injuring mitochondria were considered negative and nimesulide, which is also believed to cause DILI via mitochondrial damage, was considered positive because of a marker for reactive oxygen species, but it was negative with respect to the mitochondrial marker. Furthermore, some of the agents that appeared the most dangerous in the assay, such as hydrochlorothiazide, were considered “hits” because there have been reported cases of liver toxicity, but these drugs very rarely cause idiosyncratic liver toxicity and, thus, could be viewed as false positives.

Probably, the best *in vivo* model for mitochondrial involvement in idiosyncratic liver toxicity comes from studies using the Sod2 heterozygous (Sod2+/-) mice. Sod2 is the mitochondrial form of superoxide dismutase that detoxifies superoxide anions, which are generated by the electron transport chain as an inevitable side product. Therefore, mice deficient in this enzyme would be expected to have increased oxidative damage to their mtDNA and proteins. This might make them more susceptible to drug-induced mitochondrial toxicity; however, the results have been conflicting. In one of the first published studies, troglitazone caused an increase in markers of mitochondrial oxidative stress and an approximately twofold increase in ALT and hepatic necrosis in Sod2+/- mice, but not wild-type mice after four weeks of treatment [224]. But another study by Fujimoto *et al.* was not able to reproduce these results [225]. Specifically, plasma ALT increased in both the mutant and wild-type mice, and no hepatic necrosis was observed. This discrepancy in findings could be attributable to differences in the route of administration, solvent, or formulation. Ong used a crystalline form of troglitazone in Solutol-HS 15 (composed of polyglycol esters of 12-hydroxystearic acid and polyethylene glycol) and administered it intraperitoneally. In contrast, Fujimoto treated the mice orally with an amorphous coprecipitate of troglitazone in carboxymethylcellulose solution. Solutol-HS 15 has been found to be a weak inhibitor of CYP3A4 and could affect the metabolism of troglitazone, which is a CYP3A4 substrate [226]. In addition, Solutol-HS is a fatty acid analog, which has the potential to cause or potentiate mitochondrial damage analogous to valproic acid. Another study using Sod2+/- mice involved flutamide, where treatment for four weeks caused hepatic necrosis, a mild increase in ALT, and an increase in serum lactate in Sod2+/- mice but not in wild-type mice. Microarray analysis of gene expression showed that there was decreased expression of 12 of the 13 mitochondrial genes coding for subunits in the electron transport chain complexes and 7 of the 22 mitochondrial genes coding for mitochondrial tRNA (mtRNA), which suggests a selective damage to mtDNA. However, these changes in mtRNA levels occurred in the wild-type mice as well [227].

It is essential that any hypothesis be consistent with the clinical picture of idiosyncratic DILI. The histological finding of microvesicular steatosis that occurs in many cases of valproate-induced liver toxicity is quite strong evidence for mitochondrial involvement [228], and genetic defects affecting mitochondrial function are a risk factor for valproate-induced IDRs [229]. Other clinical evidence for mitochondrial involvement in DILI comes from electron microscopy findings of mitochondrial abnormalities (swelling and loss of cristae) in patients who developed liver toxicity with nucleoside reverse transcriptase inhibitors or fialuridine, which are believed to inhibit mtDNA replication because they are nucleoside analogs [230,231]. Nucleoside reverse transcriptase inhibitors are associated with increases in ALT; however, it is often difficult

to differentiate effects of the drug from those of the HIV or associated infections [232]. There are inherited mtDNA depletion syndromes that are also associated with steatosis and lactic acidosis that manifest in infants [233]. This type of toxicity is cumulative and delayed in onset as suggested by Boelsterli. However, steatosis and lactic acidosis are not typical of idiosyncratic liver toxicity. It is hard to understand how damage to mtDNA that is sufficient to cause severe DILI leading to liver failure would not also cause steatosis and/or lactic acidosis. It is also hard to understand how damage other than damage to mtDNA would be delayed and cumulative because damage to proteins with a short half-life should occur more rapidly than is typical for idiosyncratic DILI. The most serious cases of DILI are usually those that progress after the drug is stopped, and that is consistent with a mechanism involving DNA damage, which is usually cumulative. However, the same drugs that are responsible for such severe reactions much more commonly cause mild DILI that resolves despite continued treatment with the drug [234]. Such a picture is not consistent with a mechanism that involves mtDNA damage. It is intriguing to note that two studies have found an association between mitochondrial Sod polymorphisms and the risk of idiosyncratic DILI [235,236]. In one case, the association was with hepatocellular damage in patients being treated with antituberculosis drugs, and in the other study, the association was with a cholestatic/mixed injury type of DILI caused by a variety of other drugs. In both cases, the increased risk was associated with the genotype that had the higher mitochondrial Sod concentration. This is difficult to reconcile with the mitochondrial Sod heterozygote mouse model.

In summary, there is strong evidence that the primary mechanism of DILI for a few drugs involves mitochondrial damage. This DILI is characterized by steatosis and/or lactic acidosis. It seems unlikely that this is the mechanism for most idiosyncratic DILI. It is possible that mitochondrial toxicity requires a specific genetic defect to become manifest, but if that is true, then *in vitro* assays using random cells should not work, and if a genetic defect is not required, it should be possible to reproduce idiosyncratic DILI in animals using high doses. All drugs developed in the last several decades have been tested in animals at high doses that result in blood levels higher than the therapeutic concentrations in humans. In general, these conditions did not result in DILI even for those drugs that were later found to cause an unacceptable risk of idiosyncratic liver failure. Therefore, it seems unlikely that simple mitochondrial injury is sufficient to explain most idiosyncratic DILI. However, mitochondria are very important for normal cell function, and it is quite plausible that milder mitochondrial damage that is not sufficient to cause steatosis or lactic acidosis could produce cell stress and a danger signal that stimulates an immune response leading to DILI. Such a hypothesis is consistent with the characteristics of idiosyncratic DILI, and therefore, mitochondrial damage may be an important part of the overall mechanism of DILI. If this hypothesis is correct, it could still form the basis to screen drug candidates for their potential to cause DILI, although to date, such screening methods have not proven to be very accurate.

12.5.2.3 Inflammagen Hypothesis. Roth has proposed that most idiosyncratic DILI are not mediated by the immune system, at least not the adaptive immune system [237]. He found that the combination of lipopolysaccharide (LPS) and drugs such as ranitidine caused acute liver injury [238]. There are many issues with this hypothesis. First, the observed toxicity is acute and does not fit the delayed-onset characteristic of

idiosyncratic liver injury. Although it was reasoned that the usual delay in idiosyncratic liver injury could be due to the chance occurrence of an inflammatory stimulus, this would predict a random time to onset. It would not predict the observation that with the exception of a very limited number of drugs, such as telithromycin [239], idiosyncratic response virtually never occurs immediately on first exposure, or with the exception of a few other drugs, especially drugs that cause autoimmune hepatotoxicity, rarely occurs after six months of treatment [3]. The histology in this model, which is dominated by neutrophils [238], also does not fit the typical histology observed with idiosyncratic liver toxicity, which is usually dominated by lymphocytes and is similar to that of viral hepatitis [14]. Although LPS can cause acute hepatic damage, the response to LPS is rapidly downregulated [240]. Specifically, previous exposure to other toll-like receptor agonists prevents LPS hepatotoxicity [241]. The most serious cases of idiosyncratic liver toxicity are those in which the damage progresses even after the drug is stopped, but this model has never been extended in time to determine the natural course of the injury. On the basis of the danger hypothesis, we have tried to use LPS and other toll-like receptor agonists along with drugs such as isoniazid and nevirapine to produce a model of idiosyncratic liver injury, but without success (Utrecht JP, unpublished observations). One of the problems is that such inflammatory stimuli rapidly downregulate cytochrome P450 synthesis [242], which markedly decreases reactive metabolite formation. The inflammagen model also would not be of use in predicting the risk of idiosyncratic liver toxicity because it is positive with ranitidine, which is a safe over-the-counter drug, but negative for isoniazid, which is associated with the highest incidence of idiosyncratic liver toxicity of any commonly used drug.

12.6 IMPLICATIONS OF REACTIVE METABOLITES FOR THE MECHANISMS OF IDIOSYNCRATIC DRUG REACTIONS

As stated in Section 12.3, there is a large amount of circumstantial evidence that chemically reactive metabolites of drugs are involved in mechanism of most idiosyncratic drug reactions. The question becomes exactly what role do they play and what types of reactive metabolites are most likely to cause IDRs.

12.6.1 Implications of Reactive Metabolites for Specific Mechanistic Hypotheses

The requirement for reactive metabolites is consistent with the hapten hypothesis, which requires covalent modification of macromolecules in order to induce an immune response. This also fits the observation that many IDRs are associated with antibodies against drug-modified proteins or even autoantibodies, which could be induced because of epitope spreading or the formation of cryptic peptides as discussed in Section 12.5.1.1.

The requirement for the formation of reactive metabolites is also consistent with the danger hypothesis as discussed in Section 12.5.1.2. There are many ways by which reactive metabolites could cause cell stress resulting in the release of danger signals including mitochondrial injury. We have used microarray studies to search for evidence of danger signals generated by drugs that cause a relatively high incidence of IDRs. Although we and others have found changes in gene expression that could represent a danger signal, we have not observed any consistent patterns that could be used to screen

drug candidates for the risk that they would cause IDRs [243–246]. Furthermore, it is very difficult to determine if the changes that were observed actually represent danger signals.

The p-I hypothesis specifically does not require a reactive metabolite, although a reversible interaction between the drug and the MHC/T-cell-receptor complex, the basis for the p-I hypothesis, could produce signal 1 and a reactive metabolite could produce signal 2 consistent with the danger hypothesis. Therefore, the p-I hypothesis does not preclude the involvement of a reactive metabolite.

It is not clear what the implications of reactive metabolites are for the viral reactivation hypothesis, although it is possible that a reactive metabolite could be responsible for inhibition of the immune system and allow viral reactivation. A requirement for reactive metabolites is consistent with the unfolded protein response because it is presumably covalent binding of the reactive metabolite that interferes with normal protein folding. A reactive metabolite is not required for epigenetic effects because it appears that it is the parent drug in the case of procainamide and hydralazine that inhibits DNA methylation. Direct activation of antigen-presenting cells does not absolutely require a reactive metabolite, but certainly a reactive metabolite could be responsible for activation, and in the case of penicillamine, hydralazine, and isoniazid, it appears to be direct binding of the parent drug to specific active carbonyl groups on the antigen-presenting cells that is responsible for the activation, as discussed in Section 12.5.1.7. It is very unlikely that biological agents such as antibodies form reactive metabolites, so the IDRs associated with these agents do not involve reactive metabolites. With the nonimmune hypotheses there is no specific requirement for a reactive metabolite, but any of the three proposed mechanisms could involve a reactive metabolite.

12.6.2 Different Types of Reactive Metabolites are Associated with Different IDR Risks

There appears to be a correlation between the amount of covalent binding and the risk that a drug will cause idiosyncratic hepatotoxicity as discussed in Section 12.3; however, it is not possible to determine exactly how good this correlation is because it always involves extrapolation from *in vitro* to *in vivo* or from animals to humans. It is simply not feasible to give a person a large dose of radiolabeled drug and then do a liver biopsy to determine the extent of covalent binding. Furthermore, it is clear that different drugs are associated with a different spectrum of IDRs; therefore, assuming that reactive metabolites are responsible, different reactive metabolites must have different effects.

One important characteristic of a reactive metabolite is the location of formation; generally, metabolite reactivity is inversely proportional to the distance the agent is capable of traveling through cells and tissues. However, there are a few reactive species such as β -lactams and acyl glucuronides that circulate freely. This means that they have the potential to cause IDRs anywhere in the body. In addition, they can also react with extracellular proteins. This is in contrast to metabolites whose reactivity compels them to react with the closest target molecule, which is often the enzyme that formed them. Indeed, such reactive metabolites only bind to intracellular proteins. The significance of this difference is that intracellular proteins are presented in the context of MHC-I, which usually results in a cell-mediated immune response. In contrast, extracellular antigens can be presented in the context of MHC-II and lead to an antibody-mediated

response. A good example of this difference was demonstrated by experiments by Hopkins *et al.* in which agents that predominately reacted with serum proteins induced a type 2 cytokine response, while those that reacted with intracellular proteins produced a type 1 cytokine response [247]. It is interesting to note that in most cases the highest degree of covalent binding is in the liver even if the IDR involves a different organ. For example, the covalent binding of clozapine and nevirapine is higher in the liver than in the bone marrow and skin, respectively; however, the most common IDR associated with clozapine is agranulocytosis and that for nevirapine is skin rash (Utrecht JP, unpublished observations). Reactive metabolites formed by the myeloperoxidase system in antigen-presenting cells have obvious implications for immune-mediated reactions. It is unclear whether the skin has sufficient P450 activity to bioactivate drugs that cause skin rashes.

Another obvious important characteristic of a reactive metabolite is its chemical reactivity. As mentioned above, if a reactive metabolite is very reactive, it is likely to bind to the enzyme that formed it. In addition, some reactive metabolites are “hard” and selectively react with “hard” nucleophiles such as lysine amino groups, while others are “soft” and selectively react with “soft” nucleophiles, especially the thiol group of cysteine. The terms *soft* and *hard* refer to the degree to which the electrophilic or nucleophilic site is concentrated (hard) or diffuse (soft). However, there appears to be many additional parameters that determine what proteins a reactive metabolite will react with. For example, we compared the pattern of protein binding for three drugs that are oxidized to reactive metabolites by the myeloperoxidase system of neutrophils and cause agranulocytosis. In particular, clozapine and vesnarinone are oxidized to soft electrophiles that selectively bind to thiols. Yet when incubated with activated neutrophils, the pattern of proteins to which they bind was very different [248]. The binding of procainamide was also different, but, although procainamide also forms a soft electrophile, its chemical reactivity is a bit different. Hanzlik has compiled a database of the proteins that are covalently modified by reactive metabolites of drugs and other xenobiotics, and the diversity and difference between different reactive metabolites is surprising [249]. This suggests that physical characteristics such as how different chemical species interact noncovalently with proteins play an important role in determining which proteins are the major targets of binding. It is not surprising that binding to some proteins would be more likely to cause IDRs than others, but it is not clear what the most important protein targets might be with respect to causing IDRs. It is plausible that quinone-type reactive metabolites are the most common type of reactive metabolite that is associated with IDRs, and some types such as acyl glucuronides are much less likely to cause IDRs.

12.7 CLASSIFICATION OF DRUGS BASED ON THEIR IDR PATTERN

There appears to be patterns of the type of IDRs that different drugs cause. Part of the pattern is likely based on the duration of drug therapy. Specifically, halothane is used acutely for anesthesia and therefore is unlikely to cause autoimmunity, which usually requires a prolonged period of treatment. In some cases, the pattern may not be completely known because the drug was withdrawn before large numbers of patients were treated.

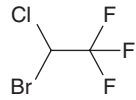
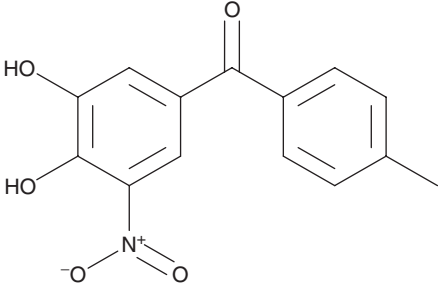
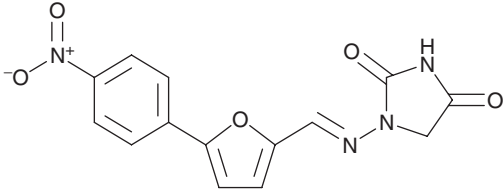
12.7.1 Drugs Causing Multiple Types of IDRs, Often Including Autoimmunity

There are many drugs that can cause multiple types of IDRs (Table 12.1). These tend to be older drugs that usually are known to form reactive metabolites. These drugs include aromatic amines such as procainamide, sulfamethoxazole, aminogluthethimide, and nomifensine; nitro compounds such as nitrofurantoin and chloramphenicol; hydrazines such as isoniazid and hydralazine; thiono sulfur compounds such as methimazole and propylthiouracil; and drugs that form quinone-type reactive metabolites such as indomethacin, minocycline, trimethoprim, and amodiaquine. Many drugs such as carbamazepine form several different types of reactive metabolites [108]. Yet even with drugs that are structurally similar, such as sulfamethoxazole and dapsone, the spectrum of IDRs varies from one drug to another and from one patient to another. It is almost certain that these are immune-mediated IDRs in which the structures to which the immune response is directed varies from one drug to another and from one patient to another. This variability is consistent with a mechanism in which the range of proteins modified by a reactive metabolite and the variability of the specificity of T-cell receptors (which are even different in identical twins) determines the variability of the IDR [15]. In one individual, the highest affinity T-cell receptor may recognize a drug-modified hepatic protein and lead to DILI, whereas in another individual, the highest affinity T-cell receptor may recognize a drug-modified skin protein or even a cryptic self peptide leading to a skin rash or an autoimmune reaction, respectively. If the mechanism were mediated by toxicity to a specific target such as mitochondria, it would be almost impossible to explain how a single drug could cause such a variety of IDRs.

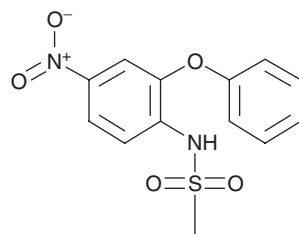
12.7.2 Drugs in Which the Dominant IDR is DILI

There are some drugs that cause IDRs that are dominated by DILI (Table 12.2). Therefore, it is not possible to argue that the IDR caused by these drugs must be immune mediated based on this variability. In many cases, such as with halothane, it is likely that the reason that the toxicity is limited to the liver is because most of the reactive metabolite is formed in the liver and the reactive metabolite is too reactive to escape in significant concentrations. In some cases, such as with tolcapone, the drug may have been on the market for too short a time to detect other types of DILI. DILI caused by drugs such as halothane and tienilic acid appears to be immune mediated because it is associated with antidrug or autoantibodies and is associated with immune memory, that is, it occurs more rapidly on reexposure to the drug. In the case of some drugs such as ketoconazole, no antibodies have been described but there are cases demonstrating a rapid response on rechallenge, which strongly supports an immune mechanism as described above [220]. In the case of many drugs, we simply do not have rechallenge data. With drugs such as bromfenac and lumiracoxib, the drugs were withdrawn quickly, and given their structures, bromfenac is a primary aromatic amine and lumiracoxib is a close analog to diclofenac, it is likely that the mechanisms are similar to other drugs in Table 12.1 with similar structures. It is possible that the mechanism of DILI for some of the drugs in Table 12.2 involves a different mechanism that does not involve the immune system such as mitochondrial damage. However, the evidence for specific nonimmune mechanisms is weak, and such mechanisms do not fit the clinical characteristics of IDRs. It is likely that some IDRs involve a combination

TABLE 12.2 Drugs Primarily Associated with DILI

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Halothane		Acid chloride [250]	DILI [251]
Tolcapone		Nitro reduced to nitroso, hydroxylamine, and free radicals [252]	DILI [253]
Dantrolene		Nitro reduced to nitroso, hydroxylamine, and free radicals [252]	DILI [254] Pleurisy and pericarditis [255]

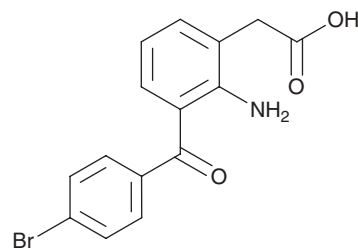
Nimesulide



Diiminoquinone [256]

DILI [257] **Hemolytic anemia** [258]
Generalized
pustulosis[259] Fixed
drug eruption [260]

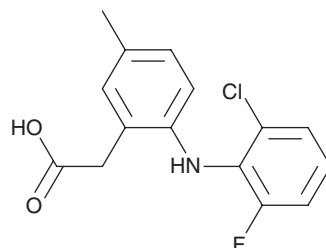
Bromfenac



Aromatic amine oxidized
to hydroxylamine,
nitroso metabolites,
and *N*-chloramine (see
procainamide)

DILI [261] (quickly
withdrawn, given its
structure, it likely
would have caused
other IDRs)

Lumiracoxib

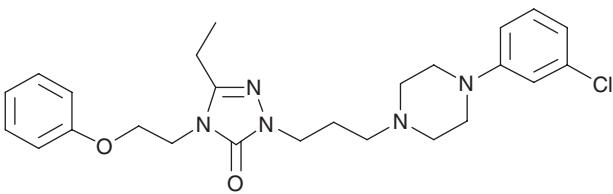
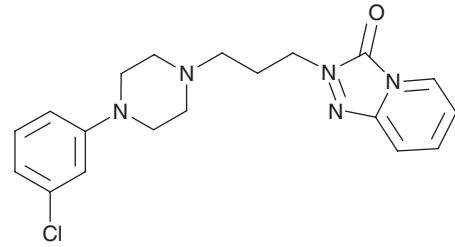
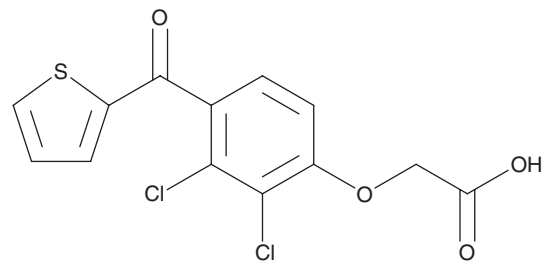


Iminoquinone (see
diclofenac)

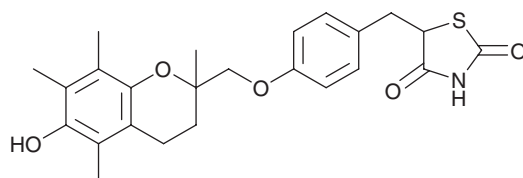
DILI [262] (given the
structure, the pattern is
probably similar to
diclofenac but it was
withdrawn early)

(continued overleaf)

TABLE 12.2 (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Nefazodone		Quinone iminium ion [263]	DILI [264]
Trazodone		Quinone iminium ion [263]	DILI [265]
Tienilic acid		Thiophene epoxide [266]	DILI [267]

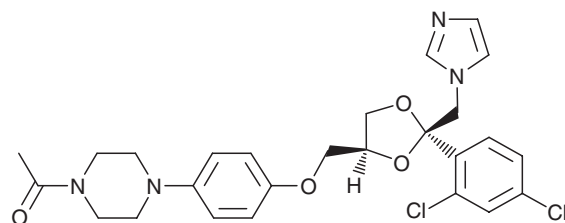
Troglitazone



Sulfenic acid Isocyanate
Quinone methide
[268]

DILI [269]

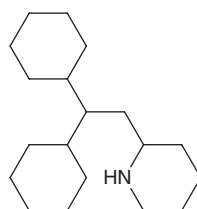
Ketoconazole



?

DILI [270] Anaphylaxis
[271]

Perhexiline

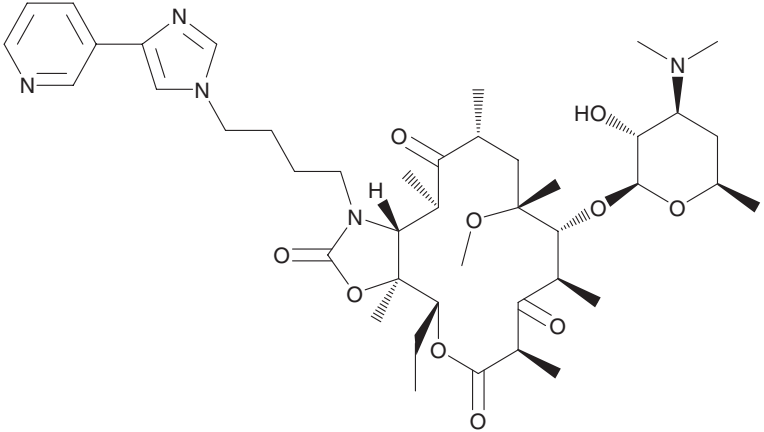
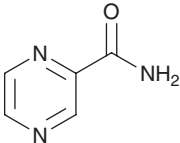


?

DILI with steatosis
[272]

(continued overleaf)

TABLE 12.2 (Continued)

Drug	Structure	Reactive Metabolite	Idiosyncratic Reaction
Telithromycin		?	DILI [239]
Pyrazinamide		None known	DILI [273]

Drugs that are associated with predominately idiosyncratic liver injury along with the reactive metabolite(s) that have been proposed to be responsible for causing the liver injury. In some cases, the identity of the reactive metabolite is inferred from analogy with other drugs that have similar structures.

“?” indicates that no reactive metabolite has been described.

of mechanisms such as mitochondrial injury or epigenetic effects leading to an immune response, but this remains to be clearly demonstrated.

12.8 SUMMARY AND CONCLUSIONS

It is apparent that there is a large amount of circumstantial evidence to suggest that reactive metabolites are responsible for most IDRs. If the total daily dose is taken into account, there is a correlation between the amount of covalent binding of a drug and the risk that the drug will cause idiosyncratic liver toxicity. However, the correlation is far from perfect; there are drugs that form reactive metabolites and are not associated with a significant risk of IDRs, and there are drugs such as ximelagatran that cause serious IDRs but probably do not form a reactive metabolite. The formation of substantial amounts of reactive metabolite in a drug candidate that will be given at medium to high dose is a significant liability, and screening for reactive metabolites is likely to make drugs safer. However, there are significant false negatives and false positives in such screens no matter how carefully they are done. At present, it is not clear exactly how reactive metabolites cause IDRs, and some might even argue whether reactive metabolites cause IDRs. In most cases, the clinical characteristics of IDRs are most consistent with an immune mechanism. Progress in preventing IDRs will depend on finding answers to these questions. Valid animal models are important tools to answer these questions (see the chapter titled *Regulation of Drug Metabolizing Enzymes and Transporters in Infection, Inflammation, and Cancer*), but it is essential that any model or hypothesis has characteristics consistent with the IDRs observed in humans.

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13 Metabolism of Psychotropic Drugs

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13.1	Summary	1
13.2	Psychotropic drugs with metabolism by CYP2D6	2
13.3	Impact of CYP2C19	8
13.4	Impact of CYP2C9	8
13.5	Other enzymes or metabolic pathways	9
13.6	Conclusions	12
	References	12

13.1 SUMMARY

The major depressive disorder schizophrenia and related disorders are among the most important causes of death and disability worldwide [1]. These disorders are highly prevalent, typically chronic or recurrent conditions with a substantial impact on public health. Therapy with antidepressant drugs is the standard of care for clinical depression; likewise, therapy with antipsychotics is required for schizophrenia.

The most important enzymes in psychotropic drug metabolism are the cytochrome P450 enzymes CYP2D6, CYP2C19, or CYP2C9. The functional impact of other drug-metabolizing enzymes including CYP1A2, CYP2A6, CYP2B6, CYP3A4, -5, and -7 or phase II enzymes in psychopharmacology is less, but some agents are cleared predominantly by these latter pathways.

The metabolism of psychotropic drugs may be assessed by *in vitro* studies and/or with *in vivo* pharmacokinetic studies. The clinical importance of drug metabolism is mainly determined by alteration of the individual activity of a relevant drug-metabolizing enzyme. This is frequently the case in drug–drug interactions, leading to enzyme inhibition or enzyme induction. Alternatively, individual patient

factors, such as pharmacogenetic polymorphisms, may contribute to variability in CYP isozyme activity.

Owing to the discrepancy between *in vitro* predictions of CYP-mediated metabolism and *in vivo* results on drug exposure, drug metabolism from *in vivo* studies will be preferentially presented in this chapter. A multitude of clinical metabolism data for psychotropic drugs exists because of the recognized importance of individual differences in drug exposure in clinical practice. As many psychotropic drugs are metabolized to equally active metabolites [2], more than one CYP isoform may be important in metabolism and exposure data of both metabolite and parent drug have to be taken into account. For many antidepressants or antipsychotic drugs, therapeutic drug monitoring is practiced regularly.

In the following pages, specific metabolism of antidepressant and antipsychotic drugs will be presented for the main CYP enzymes such as CYP2D6, CYP2C19, CYP2C9, and CYP1A2 (Table 13.1). Further, those metabolic pathways that are not typically used will be described for specific psychotropics. Special focus will be paid on the impact of genetically caused variations in enzyme activity and on drug–drug interactions, leading to changes in enzyme activity and drug metabolism (Table 13.2).

13.2 PSYCHOTROPIC DRUGS WITH METABOLISM BY CYP2D6

13.2.1 Antidepressants

13.2.1.1 Tricyclic Antidepressants. Depending on the amine side chain, tricyclic antidepressants (TCAs) can be classified into tertiary TCAs and secondary TCAs. Tertiary TCAs include amitriptyline, clomipramine, doxepin, trimipramine, and imipramine; and secondary TCAs include desipramine, nortriptyline, and protriptyline. Tertiary TCAs are first demethylated into secondary TCAs, which are further hydroxylated and conjugated. In the liver, CYP2D6 mediates the hydroxylation reactions [3–7] and CYP2C19 is responsible for demethylation of the parent drug. In certain cases, CYP3A4 may also act as a minor pathway for oxidative metabolism. Both hydroxylated and demethylated metabolites are pharmacologically active, and the demethylated metabolites are tricyclic drugs in themselves, for example, nortriptyline and desipramine (desmethyl metabolites of amitriptyline and imipramine, respectively). Nortriptyline and desipramine are mainly hydroxylated to less active or inactive metabolites [5,8]. For dose adaptation, the sum of pharmacologically active moieties (parent drug + demethylated metabolite) must be taken into account if data are available from therapeutic drug monitoring.

Stereoselective metabolism by CYP2D6 has been reported for trimipramine metabolism toward the less active L-trimipramine [6]. For doxepin, CYP2D6 polymorphisms affects only the clearance of the less active E-isomer [9]. For these two tricyclics, dose adjustments are usually based on the active drug compound (active enantiomers or isomer plus demethylated metabolite).

For several TCAs, no data on the specific enzymes involved in hydroxylation or demethylation reactions are available. However, structural similarity to other tricyclics such as imipramine implicates that CYP2D6 and CYP2C19 might be involved in metabolism of these tricyclics also.

An extremely high clearance was described for a few CYP2D6 ultrarapid metabolizers (UM) from studies with nortriptyline and desipramine, which are heavily

TABLE 13.1 List of Psychotropic Drugs and their Metabolic Pathways (Modified and Extended from [59])

	Not Any Data	<i>In Vitro</i> Data Only	Renal Excretion, Mainly	Phase II Enzymes, Mainly	CYP1A2, CYP2B6, or CYP3A4, Mainly	<i>In Vivo</i> Studies on Polymorphic Enzymes CYP2D6, CYP2C19, and CYP2C9
Antidepressant drugs	Iprindole Isocarboxzid Setiptiline Viloxazine	Amineptine Amoxapine Dibenzipine Doslepine Dothiepin Lofepramine Protriptyline	Milnacipran	Phenelzine Tranlycypromine	Bupropion Tianeptine Reboxetine	Amitriptyline Citalopram Desipramine Doxepin Duloxetine Fluoxetine Fluvoxamine Imipramine Maprotiline mianserin Mirtazapine Moclobemide Nefazodone ^a Nortriptyline Paroxetine Sertraline Trazodone Trimipramine Venlafaxine
Antipsychotic drugs	Benperidol Chlorprotixen	Chlorpromazine Remoxipride	Amisulpride Sulpiride	Raclopride	Bromperidol Iloperidone	Aripiprazole Clopenthixol

(continued overleaf)

TABLE 13.1 (Continued)

	Not Any Data	<i>In Vitro</i> Data Only	Renal Excretion, Mainly	Phase II Enzymes, Mainly	CYP1A2, CYP2B6, or CYP3A4, Mainly	<i>In Vivo</i> Studies on Polymorphic Enzymes CYP2D6, CYP2C19, and CYP2C9
	Fluphenazine Fluspirilen Mazapertine Nemonapride Pipamperon Promethazine Prothipendyl Trifluoperidol Triflupromazine	Sertindole Melperone			Perospirone Quetiapine Ziprasidone Clozapine	Clozapine ^a Flupenthixol Haloperidol Levomepromazine ^a Olanzapine Perazine Perphenazine Pimozide ^a Risperidone Thioridazine Zotepine Zuclopenthixol
Anxiolytic drugs	—	—	—	Lorazepam Oxazepam Temazepam	Alprazolam Buspirone Clonazepam Flunitrazepam Midazolam Triazolam	Diazepam
Psychostimulants	—	—	—	—	Armodafinil Modafinil	Amphetamines Atomoxetine Methylphenidate

^aDrugs that are minor substrates of CYP2D6 or CYP2C19 according to *in vivo* studies.

TABLE 13.2 Psychotropics Causing Clinically Relevant Drug–Drug Interactions Via CYP Inhibition

Inhibitory Potency	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Strong ^a	Fluvoxamine	—	—	Bupropion Fluoxetine Paroxetine	—
Moderate ^b	—	—	—	Duloxetine Moclobemide Sertraline	Nefazodone
Weak ^c	Moclobemide	Fluvoxamine	Fluvoxamine Fluoxetine Moclobemide Modafinil	Chlorpromazine Citalopram Clomipramine Clozapine Doxepin Escitalopram Fluphenazine Haloperidol Levomopromazine Perphenazine Pimozide Risperidone Venlafaxine	Fluvoxamine Fluoxetine Paroxetine

^aGreater than fivefold increase in AUC or >80% decrease in clearance.

^bTwo- to fivefold increase in AUC or 50–80% decrease in clearance.

^c1.25- to 2-fold increase in the plasma AUC, 20–50% decrease in clearance or *in vitro* data only.

dependant on CYP2D6 for their metabolism. For nortriptyline, one UM carrying 13 active CYP2D6 genes was included, which was responsible for the very high mean of clearance in this group [10,11]. The frequency of CYP2D6 gene duplications varies between 10% and 50% in certain ethnic populations, including Eastern African, Arabian, and Pacific populations. In Europe, the UM phenotype is relatively rare at ~1–5% [12].

TCAs are known inhibitors of CYP1A2, CYP2C19, and CYP2D6, although the rank order varies between individual drugs. Nortriptyline and desipramine are usually considered as the least problematic of TCAs in terms of drug interactions since they are weak inhibitors of CYP2D6. The tertiary amine TCAs potently inhibit both CYP1A2 and 2C19 and are therefore more complicated to use with other drugs.

13.2.1.2 Selective Serotonin Reuptake Inhibitors. Some SSRIs (selective serotonin reuptake inhibitors) such as fluoxetine and paroxetine are potent inhibitors of CYP2D6 activity. These drugs also rely on CYP2D6 for their own oxidative metabolism. Therefore, multiple dosing causes decreased CYP2D6-mediated metabolism of the drugs themselves. Multiple dosing of these agents can also convert extensive metabolizers (EMs) of CYP2D6 into slow metabolizers [13–16]. Besides CYP2D6, paroxetine inhibits CYP2B6 quite strongly and causes a fourfold increase in plasma concentration of desipramine, a drug metabolized both via CYP2B6 and 2D6, when coadministered

[17]. Fluoxetine and its long-lived metabolite norfluoxetine are formed by CYP2D6 but also via other CYPs (2C9, 2C19, and 3A4). Owing to these alternative metabolic pathways, the plasma levels of fluoxetine are not usually affected by selective CYP inhibitors. Both fluoxetine and norfluoxetine are potent inhibitors of CYP2D6. However, fluoxetine and norfluoxetine inhibit also other CYPs besides CYP2D6. They have been shown to increase the concentrations of risperidone (a substrate of CYP2D and 3A4) when concomitantly used.

Sertraline and its active metabolite desmethylsertraline are partially metabolized by CYP2D6. Sertraline is considered to be a potent inhibitor of CYP2D6 when its daily doses exceed 150 mg. For sertraline and citalopram, no influence of CYP2D6 polymorphisms on pharmacokinetic parameters have been detected [13,18].

13.2.1.3 Other Antidepressants. Bupropion is primarily cleared by CYP2B6 and its metabolites are known to be potent inhibitors of CYP2D6. After treatment, phenotypically EMs of CYP2D6 appear pharmacokinetically similar to poor metabolizers (PMs) [19,20]. Thus, care should be exercised when bupropion is used concomitantly with drugs metabolized by CYP2D6, especially those with a narrow therapeutic index. In about 100 subjects, no major effects of CYP2B6 genotypes on bupropion pharmacokinetics were identified [21].

There are contradictory data regarding the effects of CYP2D6 poor metabolism status on the tetracyclic antidepressant maprotiline. Patients receiving monotherapy with 150 mg maprotiline showed no differences in steady state concentrations because of the debrisoquine metabolizer status [22]. A study with healthy volunteers receiving 100 mg maprotiline over seven days revealed differences similar to those detected in tricyclics [23].

For mianserin, CYP2D6 mediates enantioselective hydroxylation of the more active *S*-(+)-mianserin. For the racemic drug mirtazapine, *S*-(+)-mirtazapine clearance significantly depends on CYP2D6 activity but no such effect on *R*-(-)-mirtazapine clearance was found [24]. For moclobemide, reboxetine, and trazodone, CYP2D6 polymorphisms do not seem to have a major influence on metabolism in humans [25–30].

Venlafaxine is a chiral drug with both enantiomers transformed by CYP2D6 to the equipotent *O*-desmethylvenlafaxine [31–33]. A higher risk for cardiotoxic events and other adverse drug effects may exist in individuals lacking CYP2D6 activity [34]. Cases of severe arrhythmias have been reported in four patients treated with venlafaxine who all were CYP2D6 PMs [35]. On the other hand, efficacy was improved in individuals with high CYP2D6 activity in a metaanalysis of four studies [36].

13.2.2 CYP2D6 in the Metabolism of Antipsychotics

The influence of CYP2D6 polymorphisms on antipsychotic drug metabolism has been studied in humans for aripiprazole, clozapine, flupentixol, haloperidol, levomepromazine, olanzapine, perazine, perphenazine, pimozide, risperidone, thioridazine, and zuclopenthixol. The antipsychotic drug aripiprazole was studied for polymorphic CYP2D6 metabolism before marketing, and a 60% higher exposure of the total active moieties (parent drug and dehydroaripiprazole) was detected in PMs compared to EMs (aripiprazole product insert). In an observational study with schizophrenic patients receiving different doses, similar dependence on the genetically defined CYP2D6 phenotype was detected for flupentixol as well as for perazine [37].

For thioridazine, a recent study has revealed a 30% higher drug exposure in individuals who are CYP2D6 PMs [38]. This result corresponds to data from a study in healthy volunteers administering single doses of the drug [39], but it is in contrast to another study where smaller differences were found [40].

For haloperidol, CYP2D6 deficiency leads to a 60–70% lower clearance indicating that CYP2D6 is a principal pathway [9,41] but other studies have not reported significant CYP2D6 differences [42,43]. A significantly higher risk for extrapyramidal side effects in PMs was observed probably because of higher levels of the metabolite, the reduced form of haloperidol. Patients with UM genotype had the worst clinical outcome measured by the positive and negative symptoms scale which might be due to subtherapeutic haloperidol concentrations [9].

For perphenazine, thioridazine, and zuclopenthixol, steady state plasma drug concentrations decreased and resulting pharmacokinetic parameters were affected relative to those following single dose administration because of autoinhibition of CYP2D6. For olanzapine, although CYP1A2 is mainly involved in metabolism, in a comparative clinical study, steady state concentrations were correlated with CYP1A2 activity (influenced by smoking) but not by the CYP2D6 genotype [44]. Because zuclopenthixol is only metabolized partly via CYP2D6 and is also sulfoxidated and glucuronidated, CYP2D6 genotype seems to be less important [45].

Hydroxylation of risperidone at the 9-position is the most important metabolic pathway mediated mainly by CYP2D6 [46–49]. The total sum of plasma risperidone and 9-OH risperidone (paliperidone) is usually used as a determinant for risperidone pharmacological activity in therapeutic drug monitoring [50]. Since the sum of risperidone and 9-OH risperidone does not differ between CYP2D6 PMs and EMs, CYP2D6 polymorphism was hypothesized not to be important [50–53]. However, several studies have found an impact on 9-OH-risperidone plasma levels and CYP2D6 activity on risperidone effects. For example, in a recent report with children with pervasive developmental disorder treated with risperidone, serum prolactin level was positively correlated with the number of functional *CYP2D6* genes and serum 9-OH risperidone [54]. This and other studies have led to the proposal that the plasma profile for CYP2D6 PMs (characterized by higher risperidone than 9-OH risperidone concentrations) may be more “toxic” than for other phenotypes [55]. Accordingly, in one large study of adverse drug effects in 360 patients, PMs had more than a threefold increased risk (odds ratio, OR = 3.4) for significant risperidone adverse drug reaction (ADRs) and a sixfold increased risk of discontinuing risperidone (OR = 6.0) because of ADRs than EMs [56]. Thus, in the case of risperidone, clinicians should consider treating CYP2D6 PMs with an alternative antipsychotic drug or careful dosing under plasma concentration monitoring.

13.2.3 CYP2D6 in the Metabolism of Psychostimulants and Atomoxetine

Methylphenidate and amphetamines have a complex metabolism in which CYP2D6 has been postulated to play only a minimal role. In the case of atomoxetine, CYP2D6 is the principle enzyme responsible for the elimination of this drug, and atomoxetine itself is not known to inhibit any CYP. CYP2D6 metabolizer status affects the efficacy and tolerability of atomoxetine treatment, and coadministration of potent inhibitors of CYP2D6 results in altered pharmacokinetics similar to CYP2D6 PMs [57,58]. Thus, potent inhibitors of CYP2D6 (e.g., paroxetine and fluoxetine) should be coadministered with caution and careful monitoring of the treatment should be exercised.

13.3 IMPACT OF CYP2C19

There are currently over 50 available medications that are primarily metabolized by the CYP2C19 enzyme. The 2C19 substrate medications include widely used pharmaceuticals, such as most of the proton pump inhibitors (omeprazole, esomeprazole, lansoprazole, and pantoprazole), proguanil, and phenytoin, and some commonly prescribed psychotropic medications. A number of antidepressants are metabolized primarily or partially by CYP2C19, including amitriptyline, clomipramine, citalopram, and escitalopram [59]. In contrast, doxepin, imipramine, nortriptyline, sertraline, and moclobemide have substantial, but not exclusive, 2C19 substrate metabolic clearance.

Sertraline is metabolized by five cytochrome P450 enzymes (i.e., CYP2D6, CYP2C9, CYP2B6, CYP2C19, and CYP3A4). Consequently, the inhibition of any single CYP does not result in a major change in the pharmacokinetics of sertraline. However, CYP2C19 PMs have been shown to have higher serum levels of sertraline at a standard dose than do normal metabolizers. CYP2C19 phenotype may be of relevance for the clinical outcome of sertraline treatment, but thus are no unambiguous recommendations for dose adjustments available. The inhibition of CYP2B6 is reported to have a similar effect. The contribution of CYP2C9 and CYP3A4 to sertraline metabolism is minimal unless there is impaired CYP2C19 and CYP2B6 metabolic activity. The major metabolite of sertraline, desmethylsertraline, is a weak serotonin transporter reuptake inhibitor and does not have an important clinical pharmacological effect. Venlafaxine is minimally metabolized by the 2C19 isozyme.

The 2C19 isozyme plays a relatively circumscribed role in the metabolism of antipsychotic medication. It is substantially involved in the metabolism of clozapine and plays a minimal role in the metabolism of thioridazine. The 2C19 isozyme plays a primary role in the metabolism of diazepam. Diazepam is metabolized predominantly by 2C19 to nordiazepam, and then by 3A4 to oxazepam, which is then conjugated and excreted in urine. Diazepam is also metabolized to temazepam and then to oxazepam via CYP3A4. Although diazepam clearance has been shown to be decreased in CYP2C19 PMs or when inhibitors of CYP2C19 are concomitantly administered, the clinical impact is generally considered to be relatively small. The role of CYP2C19 in the metabolism of other benzodiazepines has not been well demonstrated.

13.4 IMPACT OF CYP2C9

13.4.1 Antidepressants

The cytochrome P450 2C9 gene (CYP2C9) codes for an enzyme that facilitates the oxidation of about 100 medications. These include drugs with a narrow therapeutic index, such as warfarin and phenytoin, as well as many of the nonsteroidal inflammatory drugs (NSAIDs). CYP2C9 is a polymorphically expressed enzyme, and some of the SNPs (single nucleotide polymorphisms) within this gene have been identified as contributors to the wide interindividual variation in the pharmacokinetics of certain drugs. The main variant alleles *2 and *3 are present in roughly 35% of Caucasian population, but they are much less prevalent in Asian and black populations.

Amitriptyline and fluoxetine are substantially metabolized by CYP2C9, and this CYP can play an important role in clearance if other metabolic pathways are

nonfunctional. Amitriptyline is demethylated by both CYP2C9 and CYP3A4 to produce nortriptyline, an active metabolite [60]. All existing data show only a minor contribution of CYP2C9 toward the interindividual pharmacokinetic variability of TCAs. Data on amitriptyline is based on *in vitro* studies only, and a small difference in kinetics between carriers of CYP2C9*1/*1 and *3/*3 was shown for trimipramine and doxepin [61]. Amitriptyline is known to be an inhibitor of CYPs, 1A2, 2C19, and 2D6.

Fluoxetine is metabolized primarily via N-demethylation by CYP2D6. Demethylation results in the production of norfluoxetine, which is also biologically active. Fluoxetine is metabolized secondarily by CYP2C9. Given the slow elimination of fluoxetine and the subsequent required secondary clearance of norfluoxetine, fluoxetine has the longest functional half-life of any SSRI antidepressants. In addition to its own metabolism by CYP2D6, fluoxetine is a suicide CYP2D6 inhibitor, which means that a strong and long-lasting CYP2D6 inhibition takes place after several doses of fluoxetine; and as a result, it may participate in drug interactions with other drugs that are metabolized by CYP2D6.

13.5 OTHER ENZYMES OR METABOLIC PATHWAYS

13.5.1 CYP1A2

There are over 40 currently available drugs that are primarily metabolized by the 1A2 isozyme. These include some commonly prescribed psychotropic medications such as antipsychotics clozapine and olanzapine and antidepressants fluvoxamine and mirtazapine. The 1A2 enzyme also plays an important secondary role in the metabolism of other psychotropic drugs when their primary pathways are not functional. This is the case with several TCAs. Besides psychotropics, CYP1A2 has a pivotal role in the metabolism of caffeine, theophylline, naproxen, tacrine, tizanidine, and some triptans such as frovatriptan and zolmitriptan. CYP1A2 is also responsible for metabolic activation of polycyclic aromatic hydrocarbons found in cigarette smoke.

The induction of CYP1A2 by smoking results in increased metabolic clearance and necessitates higher doses to achieve effective pharmacotherapy for drugs relying CYP1A2 [62]. During smoking cessation, the CYP1A2 activity will decrease and accumulation of parent drug will take place if dosing is not adjusted accordingly. Monitoring serum levels of drugs with narrow therapeutic indices (e.g., clozapine) during these transition periods is strongly recommended. The average plasma concentration of clozapine in smokers is about 50% of nonsmokers at the same dose. Seven to 12 cigarettes per day are necessary for maximal induction [63]. Schizophrenia patients smoke up to three times more than the general population and more than most psychiatric patients. When psychiatric hospital units were mandated to become nonsmoking in the United States, newly admitted patients who were smokers and who were taking clozapine had altered clearance of clozapine as CYP1A2 induction “wore off” during an extended stay. An opposite effect occurred on discharge. Many other psychotropics have been shown to have decreased concentrations in smokers: alprazolam, chlorpromazine, duloxetine, fluvoxamine, haloperidol, mirtazapine, olanzapine, and thioridazine.

13.5.1.1 Antidepressant Medications. The only antidepressant that is metabolized primarily by the CYP1A2 enzyme is the SSRI fluvoxamine. It has no active metabolites. It is a pan-CYP inhibitor, and it potently inhibits CYP1A2. Fluvoxamine has shown to raise serum clozapine concentrations drastically (about two- to threefold) whereas ciprofloxacin, a less potent inhibitor of CYP1A2 has a much weaker effect on clozapine metabolism. A recent study suggests that initiating treatment with 50 mg/day of fluvoxamine plus 100 mg/day of clozapine can be utilized with careful monitoring of levels [63].

Additionally, several other antidepressants including duloxetine, clomipramine, and imipramine [64–66] are substantially metabolized by the CYP1A2 enzyme. Mirtazapine and amitriptyline are minimally metabolized by CYP1A2, but the CYP1A2 genotype may be important for the metabolism of these antidepressants if their primary enzymes are inactive.

Duloxetine is an antidepressant that blocks the reuptake of serotonin and norepinephrine. It has also been reported to have an analgesic effect in patients with pain related to diabetic peripheral neuropathy. In addition to CYP1A2, CYP2D6 plays a role in the metabolism of duloxetine [67]. To a lesser extent, duloxetine blocks reuptake of dopamine at the receptors.

Clomipramine is a TCA that has been widely used for the treatment of obsessive-compulsive symptoms. In addition to CYP1A2, clomipramine is metabolized by CYP2C19, CYP3A4, and CYP2D6.

Mirtazapine is an antidepressant with a tetracyclic chemical structure that enhances both noradrenergic and serotonergic neurotransmission. In addition to CYP1A2, mirtazapine is metabolized by CYP2D6 and CYP3A4 [68]. Amitriptyline is a TCA that inhibits serotonin and noradrenaline reuptake almost equally. The major metabolic pathway for amitriptyline is primarily demethylation via CYP2C19 and results in the active metabolite, nortriptyline. Alternative minor pathways for the metabolism of amitriptyline are primary hydroxylation by CYP2D6 and demethylation by CYP1A2, CYP2C9, and CYP3A4 [69].

13.5.1.2 Antipsychotic Medications. The cytochrome P450 enzyme 1A2 catalyzes biotransformation of several antipsychotics [70]. Clozapine and olanzapine are predominantly metabolized by the CYP1A2 enzyme, and chlorpromazine is substantially metabolized by CYP1A2. Thioridazine and haloperidol are minimally metabolized by CYP1A2.

Clozapine was the first atypical antipsychotic. It has one major metabolite, which is also pharmacologically active. CYP1A2 is primarily responsible for clozapine metabolism. However, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 can provide alternative metabolic pathways. Olanzapine is an atypical antipsychotic that is primarily metabolized by CYP1A2 and secondarily by CYP2D6 and is further glucuronidated by uridine diphosphate glucuronosyltransferase (UGTs). Olanzapine does not inhibit or induce the CYP system. Induction of CYP1A2 by cigarette smoking is known to lead to reduced olanzapine concentrations and inhibition of CYP1A2 by fluvoxamine is known to increase olanzapine plasma levels. This latter drug interaction has been used as the basis for a strategy of increasing the concentration of olanzapine in smokers by administering nontherapeutic low dose fluvoxamine to smokers taking olanzapine [71]. Concomitant use of olanzapine with UGT-inhibitor probenecid and a wide spectrum inducer, carbamazepine, has shown

to reduce and increase its clearance, respectively [72,73]. Chlorpromazine was the first phenothiazine used to treat psychotic patients and has minimal effect on the serotonergic pathways. It is metabolized by both CYP1A2 and CYP2D6.

Haloperidol is a typical antipsychotic drug. It is primarily metabolized by CYP3A4 and CYP2D6. However, CYP1A2 also contributes to haloperidol's metabolism, as has been demonstrated by evaluating patients who have received a CYP1A2 inhibitor and subsequently experienced increased serum levels of haloperidol [74].

Thioridazine is a phenothiazine that is a racemic compound with two enantiomers. Both enantiomers are metabolized by CYP2D6, but the CYP1A2 metabolism becomes important for patients who are taking thioridazine and who are poor 2D6 metabolizers.

Some data exist on higher drug concentrations and higher risks for tardive dyskinesia in schizophrenic patients who are smokers and carriers of CYP1A2 genotypes with reduced inducibility (C/A polymorphism at position 734 in intron 1 and G/A polymorphism at position -2964 in the 5'-flanking region of CYP1A2) but with contradictory results [75-77].

13.5.1.3 Other Drugs. Melatonin, melatonin receptor agonist, ramelteon (used as a treatment for insomnia), and melatonin analog, agomelatine (used as an antidepressant) rely mainly on CYP1A2 for their metabolism. Coadministration with fluvoxamine has shown to lead to significantly increased plasma levels of these drugs [78,79].

13.5.2 Drugs Metabolized by CYP2B6, CYP3A4, and Other Pathways

13.5.2.1 Antidepressants. CYP2B6 is the main metabolic enzyme in bupropion metabolism, and polymorphisms in CYP2B6 may be relevant for bupropion, but the differences due to genotype are small [21]. Certain non-nucleoside reverse transcriptase inhibitors (efavirenz, nelfinavir) and viral protease inhibitors (ritonavir) have been shown to inhibit bupropion metabolism, but convincing documentation concerning the clinical relevance of this interaction is currently lacking.

13.5.2.2 Antipsychotics. For iprindole, isocarboxazid, setipiline, and viloxazine, elimination occurs mainly via conjugation reactions (glucuronidation, acetylation, and sulfation). The specific phase II enzymes catalyzing these reactions are not known. Renal excretion is the principle route for phenelzine, tranlylcypromine, and milnacipran. Tianeptine as well as reboxetine seem to be mainly metabolized by CYP3A4 [80]. Buspirone is metabolized by CYP3A4 to the active metabolite, 1-(2-pyrimidinyl)-piperazine (1-PP) [81]. Inhibitors of CYP3A4, for example, diltiazem, erythromycin, grapefruit juice, itraconazole, and nefazodone have shown to greatly increase plasma buspirone concentrations and side effects. Coadministration of buspirone and CYP3A4 inhibitors should be preferably avoided, or the dose of buspirone should be greatly reduced during the concomitant treatment. Buspirone is also vulnerable to potent CYP3A4 inducers such as rifampin and others.

Elimination pathways other than those involving cytochrome P450 enzymes are important for the following antipsychotics: sulpiride, amisulpride, paliperidone (renal excretion), raclopride (glucuronidation, sulfation), and zotepine (flavin-monooxygenases involved). CYP3A4 is the main enzyme involved in metabolism of bromperidole, iloperidone, perospirone, and quetiapine [82,83]. Ziprasidone is metabolized primarily by aldehyde oxidase with a minor contribution of CYP3A4. Wakefulness-promoting drugs armodafinil and modafinil are metabolized mainly

by CYP3A4 and glucuronide conjugation. For chlorpromazine, remoxipride, and sertindole, only *in vitro* data exist on involvement of CYP2D6 [83]. Remoxipride and sertindole were withdrawn from the market because of adverse drug reactions (aplastic anemia and arrhythmia), but sertindole was recently been reintroduced to the European market. Melperone is described as potent inhibitor of CYP2D6, but studies on impact of variability in CYP2D6 activity (through genetic polymorphisms) on melperone metabolism do not yet exist [84].

13.6 CONCLUSIONS

Twenty years ago, a clinician would not have been able to anticipate possible drug interactions involving psychotropics. There has been an avalanche of data about these drugs since then. With the help of the information about the metabolism of psychotropics in this chapter, clinicians are now in a much better position to “predict” possible drug interactions. Further, the information about what drugs demonstrate P450 cytochromal genetic variations provide guidance to clinicians in deciding who would be candidates for P450 CYP genetic testing.

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14 Metabolism-Based Consequences of Polytherapy with Antiepileptic Drugs

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14.1	Summary	1
14.2	Introduction	2
14.3	Mechanisms of drug interaction	3
14.4	Metabolic interactions and hepatic isoenzymes	3
14.5	Enzyme induction	4
14.6	Enzyme inhibition	5
14.7	Antiepileptic drugs licensed for clinical use	5
14.8	Antiepileptic drugs currently undergoing development	20
14.9	Conclusions and future perspectives	22
	Acknowledgments	22
	References	22

14.1 SUMMARY

Antiepileptic drugs (AEDs) are the mainstay of epilepsy treatment, and there are now 22 AEDs licensed for clinical use and more are under development. Because of the long-term nature of epilepsy treatment and the fact that invariably patients will be prescribed nonepilepsy drugs consequent to comorbidities, the potential for pharmacokinetic interactions in general and metabolic interactions in particular is substantial. The older first-generation AEDs (carbamazepine, phenobarbital, phenytoin, and valproic acid) undergo metabolism via common cytochrome P450 (CYP) isoenzymes that are highly inducible and readily inhibited, and these AEDs are associated with many hundreds of metabolic interactions. Additionally, carbamazepine, phenobarbital, phenytoin, and valproic acid have a substantial ability to induce and/or inhibit hepatic metabolism. Although some of the new second- and third-generation AEDs are susceptible to inhibition and induction of their own metabolism (e.g., eslicarbazepine acetate,

felbamate, lamotrigine, oxcarbazepine, tiagabine, topiramate, and zonisamide), overall, they are less interacting because they are not substantial inducers or inhibitors of hepatic metabolism. Furthermore, AEDs such as gabapentin, levetiracetam, pregabalin, and vigabatrin, which are primarily renally excreted, are not amenable to interference. Similarly, AEDs such as lacosamide and rufinamide that are not metabolized by CYP isoenzymes or uridine glucuronyl transferases are also not amenable to interference. There are various new putative AEDs under development, and it would appear that these AEDs also have a low propensity for metabolic interactions. Clinically, there is always a choice, and all things being equal, particularly if neither the physician nor the patient wishes to worry about metabolic interactions and their consequence (induction can result in seizure breakthrough, while inhibition can result in concentration-related toxicity), the AEDs of choice are gabapentin, lacosamide, levetiracetam, pregabalin, and vigabatrin. However, in many clinical settings polytherapy with known interacting drugs is unavoidable, and it is best practice to manage such interactions by undertaking graded therapeutic dose adjustments that are guided by blood level measurements of the affected drug.

14.2 INTRODUCTION

Approximately 1% of the world population is affected by epilepsy, a chronic disorder that usually persists for many years and often for a lifetime [1]. The mainstay of treatment is the use of AED monotherapy, and this approach can render ~65% of newly diagnosed patients seizure-free [2]. For the remaining 35% of patients, prescribing polytherapy (two or more drugs) AED regimens so as to achieve optimal seizure control is a common practice. However, for the majority of these patients, little additional benefit is achieved from the use of polytherapy AEDs, as intolerable adverse effects commonly occur as a consequence of pharmacokinetic and/or pharmacodynamic interactions.

As a class of drugs, the AEDs are associated with more interactions than any other therapeutic drug class, and this is because (i) most AEDs have a narrow therapeutic index so that even small changes in their plasma concentration can result in seizure exacerbation or increased adverse effects; (ii) the most widely used AEDs (carbamazepine, phenobarbital, phenytoin, and valproic acid) have a substantial ability to induce and/or inhibit hepatic metabolism; (iii) additionally, these AEDs, along with some of the newer AEDs (e.g., eslicarbazepine acetate, lamotrigine, tiagabine, topiramate, oxcarbazepine, felbamate, and zonisamide), are susceptible to inhibition and induction of their own metabolism [3–6]. Additional confounding factors include the following: (i) those patients who respond to monotherapy may also experience the consequences of AED interactions as AEDs are added and withdrawn during the optimization of their monotherapy drug regimen; (ii) polytherapy AED regimens are often required to treat patients with multiple seizure types; (iii) since epilepsy is a chronic condition, many patients will inevitably develop comorbid diseases or other debilitating conditions and disorders, which will require the coadministration of non-AED drugs, and in this setting, the potential for drug interactions is considerable; (iv) the increasing use of over-the-counter medications and dietary supplements, many of which have unknown constituents and are of variable quality, that can interact with AEDs; and

(v) AEDs are increasingly used to treat other nonepilepsy conditions such as mood disorders, migraine, and pain, thereby further increasing the possibility of combined use with other drugs [7,8]. Finally, with regard to new AEDs, although with time they may be licensed for monotherapeutic use, these drugs, at least when first licensed, can only be prescribed for adjunctive therapy; therefore, polytherapy will be the only option for new AEDs, and their propensity to interact is a major consideration during their clinical evaluation.

This chapter reviews the mechanisms of interaction that can occur with AEDs, with particular emphasis on metabolic interactions. Subsequently, the AEDs are reviewed in alphabetical order in terms of their metabolism, their effects on other drugs, and the effect of other drugs on their metabolism. Finally, AEDs under development are reviewed.

14.3 MECHANISMS OF DRUG INTERACTION

Interactions can be divided into two groups: pharmacokinetic and pharmacodynamic interactions.

1. *Pharmacokinetic Interactions* These involve a change in the absorption, usually gastrointestinal; distribution, usually binding to serum albumin; metabolism, usually by isoenzymes of CYP and uridine glucuronyl transferases (UGTs); or elimination, usually renal excretion, of the affected drug and consequently altering concentrations (levels) at the site of drug action. Pharmacokinetic interactions are particularly prevalent and are associated with a change in plasma level of either the drug or its metabolite(s) or both.
2. *Pharmacodynamic Interactions* Pharmacodynamic interactions occur at the cellular level where drugs act and can occur between drugs that have either similar or opposing pharmacological mechanisms of action. Because these interactions are not associated with any change in plasma drug level, they are less well recognized and documented. Nevertheless, they are of major clinical significance.

14.4 METABOLIC INTERACTIONS AND HEPATIC ISOENZYMES

By far, the most clinically significant pharmacokinetic interactions with AEDs are those related to changes in hepatic metabolism and involve induction or inhibition of drug metabolism. With the exception of gabapentin, levetiracetam, pregabalin, and vigabatrin, all AEDs undergo hepatic metabolism and consequently are susceptible to inhibitory and/or induction interactions. Although metabolic drug interactions may involve changes in any one of the numerous enzymes involved in drug metabolism, by far, the most important are those associated with the CYP and UGT systems. The CYP system is particularly important because it is responsible for the oxidative metabolism of many drugs and exogenous compounds, as well as many endogenous compounds such as prostaglandins, fatty acids, and steroids.

14.4.1 CYP Enzymes

The CYP enzyme system consists of a superfamily of isoenzymes that are located in the smooth endoplasmic reticulum, primarily in the liver but also in many other tissues (e.g., intestine, kidney, brain, and placenta). Although in man ~60 different CYP isoenzymes have been identified, 7 isoenzymes (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) are known to be responsible for the metabolism of 95% of all drugs [9,10]. The most abundant isoenzyme is CYP3A4, which accounts for ~30% of all CYPs, has the broadest substrate specificity and is involved in more than 50% of all drug metabolism [11]. Three isoenzymes (CYP2C9, CYP2C19, and CYP3A4) are of particular importance in relation to AED interactions. Valproic acid, felbamate, and stiripentol are potent inhibitors of CYPs, while eslicarbazepine acetate, topiramate, oxcarbazepine, and rufinamide are moderate inhibitors. In contrast, carbamazepine, phenytoin, phenobarbital, and primidone act as broad-spectrum inducers of CYPs, while lamotrigine, oxcarbazepine, rufinamide, and topiramate act more specifically on CYP isoenzymes. Noteworthy is the fact that some AEDs (oxcarbazepine, rufinamide, topiramate) act as both inhibitors and inducers of CYPs.

14.4.2 Epoxide Hydrolases

Microsomal epoxide hydrolase is involved in xenobiotic metabolism and plays an important role in the metabolism of carbamazepine, phenobarbital, and phenytoin. Carbamazepine-10,11-epoxide (carbamazepine epoxide), the pharmacologically active metabolite of carbamazepine, has been implicated in various important interactions. Valproic acid and felbamate are potent inhibitors of epoxide hydrolase, while phenytoin, phenobarbital, and primidone act as inducers.

14.4.3 Uridine Glucuronyl Transferases

Various families of UGTs have been identified, of which UGT1 and UGT2 appear to be the most important in drug metabolism [12]. The UGT1A3 and UGT2B7 isoforms are involved in the O-glucuronidation of valproic acid, and UGT1A4 has been found to be the major isoform responsible for the metabolism (N-glucuronidation) of lamotrigine. Valproic acid inhibits several UGTs, while carbamazepine, phenobarbital, phenytoin, and primidone are inducers.

14.5 ENZYME INDUCTION

Enzyme induction is the consequence of an increase in enzyme protein resulting from an increase in gene transcription that is mediated by intracellular receptors. The resultant elevated enzyme activity results in an increase in the rate of metabolism of the affected drug, leading to a decrease in plasma level and possibly a reduction in the therapeutic response. If the affected drug has a pharmacologically active metabolite, induction can result in increased metabolite levels and possibly an increase in drug toxicity. The time course of induction is usually gradual and dose dependent and is dependent on the rate of enzyme synthesis and/or degradation as well as the time to reach plasma steady-state levels of the inducing drug. The latter is usually the rate-limiting step and

only occurs at a time which is approximately five elimination half-lives of the inducing drug. Similarly, the rate-limiting step in deinduction is the elimination of the inducing drug. When the inducer is removed, plasma levels of the affected drug will increase and serious adverse effects can occur if the dose of the affected drug is not reduced.

Enzyme induction represents a common problem in the management of epilepsy. Phenobarbital, carbamazepine, and phenytoin are potent inducers of CYPs, although phenytoin and carbamazepine appear to be less potent inducers at doses used clinically.

14.6 ENZYME INHIBITION

Enzyme inhibition is the consequence of competition by drugs to bind to the same enzymic site, resulting in a reduction of enzyme activity and a decrease in the rate of metabolism of the affected drug. Inevitably, plasma levels are elevated, and this can result in clinical toxicity. Inhibition is usually competitive in nature and therefore dose dependent and tends to begin as soon as sufficient levels of the drug inhibitor are achieved, which usually occurs within 24 hours of inhibitor addition. The time to maximal inhibition will depend on the elimination half-life of the affected drug and the inhibiting agent.

Among the AEDs, valproic acid, topiramate, and felbamate have been associated with inhibitory interactions. Furthermore, while topiramate and felbamate are primarily selective inhibitors of CYP2C19, valproic acid is considered to be a broad-spectrum inhibitor of hepatic metabolizing enzymes, as it inhibits CYP2C9, UGTs, and microsomal epoxide hydrolase.

An AED may be the affected drug or the cause of an interaction. In fact, with some drug combinations, the hepatic metabolism of both the AED and the other drug is altered. For example, during comedication with erythromycin and carbamazepine, carbamazepine plasma levels are elevated two- to fourfold due to inhibition of carbamazepine metabolism. Conversely, the effectiveness of standard dosages of erythromycin is reduced because carbamazepine enhances the metabolism of erythromycin. Other bidirectional interactions include those between topiramate and phenytoin and between valproic acid and lamotrigine.

14.7 ANTIEPILEPTIC DRUGS LICENSED FOR CLINICAL USE

14.7.1 Carbamazepine

Carbamazepine is metabolized in the liver, primarily by CYP3A4 with some contribution by CYP2C8, to carbamazepine epoxide, which is pharmacologically active. Carbamazepine epoxide is in turn metabolized, via epoxide hydrolase, to an inactive *trans*-carbamazepine diol. Overall, carbamazepine has a substantial propensity for metabolic interactions and acts as an inducer of metabolism.

14.7.1.1 Effect of Carbamazepine on Other Drugs. Carbamazepine induces both the metabolism of drugs metabolized by various CYPs and UGTs and its own metabolism. Autoinduction usually occurs within one week of carbamazepine initiation and completes within three to four weeks [13].

Carbamazepine induces the metabolism of many AEDs, the clinical significance of which varies from modest, where the partial loss of efficacy resulting from the decreased plasma level of the affected drug is compensated for by the antiepileptic effect of the added carbamazepine, to substantial, where the decrease in AED level is so pronounced that seizure control will be lost unless the dosage of the affected drug is increased. Modest effects occur with oxcarbazepine, phenytoin, primidone, and rufinamide, while substantial effects occur with clobazam, clonazepam, ethosuximide, lamotrigine, stiripentol, topiramate, tiagabine, valproic acid, and zonisamide (Table 14.1). Carbamazepine can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2).

Carbamazepine also induces the metabolism of many non-AED drugs (Table 14.3), the magnitude of which is particularly substantial for those drugs that undergo extensive first-pass metabolism, so that plasma levels may be decreased significantly. Such drugs include antifungals (e.g., itraconazole, ketoconazole), antihelmintics (e.g., albendazole, praziquantel), antituberculous agents (e.g., indinavir, nelfinavir), and dihydropyridine calcium antagonists (e.g., nimodipine, nifedipine) [14–17]. Additionally, carbamazepine can induce the metabolism of various antineoplastic agents (e.g., 9-aminocamptothecin, teniposide, methotrexate), so that their plasma levels decrease significantly [18–20].

14.7.1.2 Effect of Other Drugs on Carbamazepine. When considering the effect of other drugs on carbamazepine, one also needs to consider the effects on the active metabolite which is rarely measured clinically. For example, valproic acid inhibits epoxide hydrolase, the enzyme that is responsible for the metabolism of carbamazepine epoxide and has no effect on CYP3A4. Thus, carbamazepine-related toxicity can occur in the absence of any change in carbamazepine plasma levels [21,22]. Similarly, while the antipsychotic quetiapine does not affect plasma carbamazepine levels, plasma carbamazepine epoxide levels increase three- to fourfold, causing carbamazepine-related toxicity. In contrast, fluoxetine inhibits the metabolism of carbamazepine so that both carbamazepine and carbamazepine epoxide plasma levels increase and patients show significant signs of toxicity with what might appear to be only small increases in carbamazepine plasma levels [23,24]. Interestingly, felbamate increases carbamazepine epoxide plasma levels but decreases carbamazepine plasma levels. While phenobarbital and phenytoin can induce the metabolism of carbamazepine, stiripentol inhibits its metabolism increasing plasma levels several fold [25].

There are numerous non-AED drugs that are potent inhibitors of CYP3A4, and their coadministration with carbamazepine can result in carbamazepine-related toxicity. Such drugs include antidepressants (e.g., fluoxetine, fluvoxamine, nefazodone, trazodone, viloxazine), antimicrobials (clarithromycin, erythromycin, fluconazole, isoniazid, itraconazole, josamycin, ketoconazole, metronidazole, ritonavir, troleandomycin), and the calcium channel blockers diltiazem and verapamil [4,6]. Because up to threefold increases in plasma carbamazepine levels can occur, this can result in serious clinical toxicity.

14.7.2 Clobazam

Clobazam is metabolized in the liver, primarily by desmethylation, to *N*-desmethyloclobazam, which is pharmacologically active. Clobazam also undergoes

TABLE 14.1 Interactions Between Antiepileptic Drugs (AEDs): Expected Changes in Plasma Concentrations (Levels) When an AED is Added to a Preexisting AED Regimen

		Preexisting AED																				
		CBZ	CLB	ESL	ESM	FBM	GBP	LCM	LTG	LEV	OXC	PB	PHT	PGB	PRM	RFN	STP	TGB	TPM	VPA	VGB	ZNS
A d d e d A E D	CBZ	AI	CLB↓ DMCLB↑	ESL-a	ESM↓	FBM↓	↔	↔	LTG↓	↔	H-OXC↓	↔	PHT↑↓	↔	PRM↓ PB↑	RFN↓	STP↓	TGB↓	TPM↓	VPA↓	↔	ZNS↓
	CLB	?	—	?	?	?	NA	NA	?	↔	↔	?	PHT↑	NA	PRM↓	?	?	?	?	VPA↑	NA	?
	ESL-a	?	?	—	?	?	NA	NA	LTG↓	NA	?	?	PHT↑	NA	?	?	?	?	TPM↓	?	NA	?
	ESM	↔	NA	NA	—	NA	NA	NA	NA	NA	↔	↔	↔	NA	NA	?	?	NA	NA	VPA↓	NA	NA
	FBM	CBZ↓ CBZ-E↑	CLB↓ DMCLB↑	?	?	—	NA	NA	↔	NA	↔	PB↑	PHT↑	NA	?	?	?	?	?	VPA↑	↔	?
	GBP	↔	NA	NA	NA	FBM↑	—	NA	NA	↔	NA	↔	↔	?	NA	NA	NA	NA	↔	↔	NA	NA
	LCM	↔	NA	NA	NA	NA	—	↔	↔	↔	NA	↔	↔	NA	NA	NA	NA	NA	↔	↔	NA	NA
	LTG	↔	↔	?	↔	NA	NA	↔	—	↔	NA	↔	↔	↔	↔	↔	?	NA	↔	VPA↓	NA	ZNS↑
	LEV	↔	↔	NA	NA	NA	↔	↔	↔	—	NA	↔	↔	↔	↔	↔	NA	NA	NA	↔	NA	NA
	OXC	CBZ↓	?	?	?	?	NA	↔	LTG↓	NA	—	PB↑	PHT↑	NA	?	?	?	?	TPM↓	↔	NA	?
	PB	CBZ↓	CLB↓ DMCLB↑	?	ESM↓	FBM↓	↔	NA	LTG↓	↔	H-OXC↓	AI	PHT↑↓	↔	NCCP	RFN↓	STP↓	TGB↓	TPM↓	VPA↓	↔	ZNS↓
	PHT	CBZ↓	CLB↓ DMCLB↑	ESL↓	ESM↓	FBM↓	↔	↔	LTG↓	↔	H-OXC↓	PB↑	AI	↔	PRM↓ PB↑	RFN↓	STP↓	TGB↓	TPM↓	VPA↓	↔	ZNS↓
	PGB	↔	NA	?	NA	NA	?	NA	↔	↔	NA	↔	↔	—	NA	NA	NA	↔	↔	↔	NA	NA
	PRM	CBZ↓	CLB↓ DMCLB↑	?	ESM↓	FBM↓	↔	NA	LTG↓	↔	?	NCCP	PHT↑↓	NA	—	RFN↓	STP↓	TGB↓	TPM↓	VPA↓	↔	ZNS↓
	RFN	CBZ↓	?	?	?	?	NA	NA	LTG↓	NA	?	PB↑	PHT↑	NA	?	—	?	?	↔	↔	NA	?
	STP	CBZ↑	CLB↑ DMCLB↑	?	?	?	NA	NA	?	NA	?	PB↑	NA	NA	PRM↑	?	—	?	?	VPA↑	NA	?
TGB	↔	NA	?	NA	NA	NA	NA	NA	NA	↔	↔	↔	↔	?	?	?	—	NA	VPA↓	NA	NA	
TPM	↔	?	?	NA	?	NA	↔	↔	NA	?	↔	PHT↑	↔	↔	?	?	?	—	VPA↓	NA	?	
VPA	CBZ-E↑	?	?	ESM↑↓	FBM↑	↔	↔	LTG↑	↔	↔	PB↑	PHT↓ ^a	↔	PB↑	RFN↑	?	↔	TPM↓	—	↔	ZNS↓	
VGB	↔	NA	NA	NA	↔	NA	NA	NA	NA	NA	↔	PHT↓	NA	↔	RFN↓	NA	NA	NA	↔	—	NA	
ZNS	CBZ↑↓	?	?	NA	?	NA	NA	↔	NA	?	↔	PHT↑	NA	↔	?	?	NA	NA	↔	NA	—	

Drug Abbreviations: CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide (active metabolite of CBZ); CLB, clobazam; DMCLB, *N*-desmethylclobazam (active metabolite of CLB); ESL-a, eslicarbazepine acetate; ESM, ethosuximide; FBM, felbamate; GBP, gabapentin; H-OXC, 10-hydroxyoxcarbazepine (active metabolite of OXC); LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PGB, pregabalin; PHT, phenytoin; PRM, primidone; RFN, rufinamide; STP, stiripentol; TGB, tiagabine; TPM, topiramate; VPA, valproic acid; VGB, vigabatrin; ZNS, zonisamide.

Abbreviations: AI, autoinduction; NA, none anticipated; NCCP, not commonly coprescribed; ↔, no change; ↓, a usually minor (or inconsistent) decrease in plasma level; ↓↓, a usually clinically significant decrease in plasma level; ↑, a usually minor (or inconsistent) increase in plasma level; ↑↑, a usually clinically significant increase in plasma level; ?, not known, an interaction could occur.

^aFree (pharmacologically active) level may increase.

TABLE 14.2 Effects of Antiepileptic Drugs on Oral Hormone Contraceptive Metabolism

Antiepileptic Drug	Accelerates Metabolism and Therefore Compromises Contraception	Does Not Accelerate Metabolism and Therefore Does Not Compromise Contraception
Carbamazepine	Yes	—
Clobazam	—	Yes
Clonazepam	—	Yes
Eslicarbazepine acetate	Yes	—
Ethosuximide	—	Yes
Felbamate	Yes	—
Gabapentin	—	Yes
Lacosamide	—	Yes
Lamotrigine ^a	—	Yes
Levetiracetam	—	Yes
Oxcarbazepine	Yes	—
Phenobarbital	Yes	—
Phenytoin	Yes	—
Pregabalin	—	Yes
Primidone	Yes	—
Rufinamide	Yes	—
Stiripentol ^b	Unknown	—
Tiagabine	—	Yes
Topiramate (>200 mg/day)	Yes	—
Valproic acid	—	Yes
Vigabatrin	—	Yes
Zonisamide	—	Yes

^aLamotrigine has no effect on the estrogen component of a contraceptive pill and in most patients will not compromise contraception. However, lamotrigine can enhance the metabolism of the progesterone component so that progesterone blood levels decrease by ~10%. This effect may be clinically significant in some patients prescribed the progesterone-only contraceptive pill.

^bThe effect of stiripentol on hormonal contraception is unknown. However, theoretically, stiripentol may increase plasma levels of hormonal contraceptives and thus necessitate lower doses to be prescribed. Because stiripentol has a very limited indication, it is not expected to be prescribed during pregnancy or to women of childbearing potential.

metabolism by hydroxylation to form other metabolites, namely, 4-hydroxyclobazam and 4-hydroxydesmethyloclobazam. The *N*-desmethyloclobazam metabolite contributes significantly to the efficacy of clobazam. Overall, clobazam has a low propensity for metabolic interactions.

14.7.2.1 Effect of Clobazam on Other Drugs. Clobazam can inhibit the metabolism of phenytoin, primidone, and valproic acid so that their plasma levels increase (Table 14.1). Clobazam does not interfere with the metabolism of other non-AED drugs. The metabolism of oral contraceptives is unaffected by clobazam, and therefore, clobazam does not compromise contraceptive control (Table 14.2).

14.7.2.2 Effect of Other Drugs on Clobazam. The prominent interaction feature of clobazam is its vulnerability to hepatic induction so that carbamazepine, felbamate,

TABLE 14.3 Non-AED Drugs Whose Plasma Levels are Decreased Consequent to Induction by Enzyme-Inducing AEDs (Carbamazepine, Phenobarbital, Phenytoin, and Primidone)

Analgesics	<i>Carbamazepine:</i> fentanyl, meperidine, methadone, and paracetamol <i>Phenytoin:</i> fentanyl, meperidine, methadone, paracetamol, and pethidine <i>Phenobarbital:</i> fentanyl, meperidine, methadone, and paracetamol <i>Primidone:</i> fentanyl, meperidine, methadone, and paracetamol
Antimicrobials	<i>Carbamazepine:</i> albendazole, delavardine, doxycycline, efavirenz, erythromycin, indinavir, isoniazid, itraconazole, ketoconazole, mebendazole, nelfinavir, nevirapine, praziquantel, ritonavir, saquinavir, zidovudine <i>Phenobarbital:</i> albendazole, chloramphenicol, delavardine, doxycycline, efavirenz, indinavir, itraconazole, ketoconazole, metronidazole, nelfinavir, nevirapine, praziquantel, ritonavir, saquinavir, zidovudine <i>Phenytoin:</i> albendazole, delavardine, doxycycline, efavirenz, erythromycin, indinavir, itraconazole, ketoconazole, mebendazole, nelfinavir, nevirapine, praziquantel, ritonavir, saquinavir, voriconazole, zidovudine <i>Primidone:</i> delavardine, efavirenz, indinavir, nelfinavir, nevirapine, ritonavir, saquinavir, zidovudine
Antineoplastic drugs	<i>Carbamazepine:</i> 9-aminocamptothecin, docetaxel, methotrexate, paclitaxel, procarbazine, teniposide, vincristine <i>Phenobarbital:</i> 9-aminocamptothecin, docetaxel, etoposide, ifosfamide, methotrexate, paclitaxel, procarbazine, teniposide, vincristine <i>Phenytoin:</i> 9-aminocamptothecin, busulfan, cyclophosphamide, docetaxel, etoposide, ifosfamide, irinotecan, methotrexate, paclitaxel, procarbazine, teniposide, topotecan, vincristine <i>Primidone:</i> although the effect of primidone on the metabolism of antineoplastic drugs has not been determined, similar interactions would be expected as those described for phenobarbital
Cardiovascular drugs	<i>Carbamazepine:</i> amiodarone, dicoumarol, felodipine, nimodipine, nivaldipine, phenprocoumon, warfarin <i>Phenobarbital:</i> alprenalol, dicoumarol, felodipine, nifedipine, nimodipine, propranolol, quinidine, verapamil, warfarin <i>Phenytoin:</i> amiodarone, dicoumarol, digoxin, disopyramide, felodipine, losartan,^a mexiletine, nimodipine, nisoldipine, quinidine, verapamil, warfarin^b <i>Primidone:</i> dicoumarol
Immunosuppressants	<i>Carbamazepine:</i> cyclosporine A <i>Phenobarbital:</i> cyclosporine A <i>Phenytoin:</i> cyclosporine A, sirolimus, tacrolimus <i>Primidone:</i> cyclosporine A

(continued overleaf)

TABLE 14.3 (Continued)

Psychotropic drugs	<i>Carbamazepine</i>: alprozolam, amitriptyline, citalopram, clobazam, clomipramine, clonazepam, clozapine, desipramine, desmethylclomipramine, diazepam, doxepin, fluphenazine, haloperidol, imipramine, mianserin, midazolam, mirtazapine, nefazodone, nortriptyline, olanzapine, paroxetine, protriptyline, quetiapine, risperidone, sertraline, thioridazine, ziprasidone <i>Phenobarbital</i>: alprozolam, amitriptyline, citalopram, clobazam, clomipramine, clonazepam, chlorpromazine, clozapine, desipramine, desmethylclomipramine, diazepam, fluphenazine, haloperidol, imipramine, mianserin, midazolam, nefazodone, nortriptyline, olanzapine, paroxetine, protriptyline, quetiapine, risperidone, ziprasidone <i>Phenytoin</i>: alprozolam, amitriptyline, citalopram, clobazam, clomipramine, clonazepam, clozapine, desipramine, desmethylclomipramine, diazepam, fluphenazine, haloperidol, imipramine, mianserin, midazolam, mirtazapine, nefazodone, nortriptyline, olanzapine, paroxetine, protriptyline, quetiapine, risperidone, thioridazine, ziprasidone <i>Primidone</i>: alprozolam, amitriptyline, citalopram, clobazam, clomipramine, clonazepam, desipramine, desmethylclomipramine, diazepam, fluphenazine, haloperidol, imipramine, midazolam, nefazodone, nortriptyline, olanzapine, paroxetine, protriptyline, quetiapine, risperidone, ziprasidone
Steroids	<i>Carbamazepine</i>: cortisol, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, steroid oral contraceptives <i>Phenobarbital</i>: cortisol, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, steroid oral contraceptives <i>Phenytoin</i>: cortisol, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, steroid oral contraceptives <i>Primidone</i>: steroid oral contraceptives
Miscellaneous	<i>Carbamazepine</i>: bupropion, cisatracurium, doxacurium, lidocaine, omeprazole, pancuronium, rapacurium, rocuronium, theophylline, vecuronium <i>Phenobarbital</i>: bupropion, lidocaine, omeprazole, theophylline <i>Phenytoin</i>: bupropion, cisatracurium, doxacurium, lidocaine, omeprazole, pancuronium, rapacurium, rocuronium, theophylline, vecuronium <i>Primidone</i>: bupropion, lidocaine, rocuronium

^aOnly pharmacologically active carboxylic acid metabolite (E3174) is affected.

^bA biphasic interaction occurs, where initially phenytoin acts as an inhibitor and subsequently induction prevails.

phenobarbital, phenytoin, and primidone enhance its metabolism, resulting in a decrease in clobazam plasma levels but with a concurrent increase in levels of the pharmacologically active metabolite *N*-desmethylclobazam. It is not known whether the increase in metabolite levels compensates for the decrease in clobazam levels, but typically, higher doses of clobazam are warranted during comedication with these AEDs. Noteworthy is the interaction with stiripentol, which serves as a potent inhibitor, so that both clobazam

and *N*-desmethyloclobazam plasma levels increase by manyfold [26]. Cimetidine can increase clobazam levels by ~17% without affecting plasma *N*-desmethyloclobazam levels.

14.7.3 Clonazepam

Clonazepam is metabolized in the liver, primarily by CYP3A4, to 7-amino-clonazepam that is in turn metabolized by acetylation, via *N*-acetyltransferase, to form 7-acetamido-clonazepam. Clonazepam is also hydroxylated (isoenzymes not identified) to form 3-hydroxyclonazepam. Although the 7-amino-clonazepam metabolite retains some pharmacological activity, none of the other metabolites of clonazepam are pharmacologically active. Overall, clonazepam has a minimal propensity for metabolic interactions.

14.7.3.1 Effect of Clonazepam on Other Drugs. To date, there have been no reports of clonazepam affecting the clearance of other AEDs or non-AED drugs. The metabolism of oral contraceptives is unaffected by clonazepam, and therefore, it does not compromise contraceptive control (Table 14.2).

14.7.3.2 Effect of Other Drugs on Clonazepam. The AEDs carbamazepine, lamotrigine, phenobarbital, and phenytoin can increase the clearance of clonazepam and decrease clonazepam plasma levels, while amiodarone and ritonavir can decrease its clearance and increase clonazepam plasma levels.

14.7.4 Eslicarbazepine Acetate

Eslicarbazepine acetate is rapidly metabolized (hydrolysis) in the liver to its pharmacologically active metabolite, eslicarbazepine (also known as *S*-licarbazepine), by esterases (91%). Other minor metabolites, which are pharmacologically active, include *R*-licarbazepine (~5%) and oxcarbazepine (~1%). Eslicarbazepine (33%) is subsequently metabolized by conjugation with glucuronic acid. Although the metabolites of eslicarbazepine acetate are pharmacologically active, they contribute to <5% of activity. Overall, eslicarbazepine acetate has a minimal propensity for metabolic interactions.

14.7.4.1 Effect of Eslicarbazepine Acetate on Other Drugs. Eslicarbazepine can increase plasma levels of phenytoin presumably via inhibition of either CYP2C9 or CYP2C19, while lamotrigine and topiramate plasma levels are decreased. Additionally, eslicarbazepine can decrease plasma levels of warfarin [27]. Eslicarbazepine can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2).

14.7.4.2 Effect of Other Drugs on Eslicarbazepine Acetate. Carbamazepine and phenytoin can increase the clearance of eslicarbazepine and decrease eslicarbazepine plasma levels. To date, there have been no reports of other drugs affecting the clearance and plasma levels of eslicarbazepine [27].

14.7.5 Ethosuximide

Ethosuximide is metabolized in the liver by hydroxylation to form isomers of 2-(1-hydroxyethyl)-2-methylsuccinimide, of which at least 40% are glucuronide conjugates. Metabolism is primarily mediated by CYP3A and to a lesser extent by CYP2E and CYP2B/C. Overall, ethosuximide has a low propensity for metabolic interactions.

14.7.5.1 Effect of Ethosuximide on Other Drugs. At therapeutic doses, ethosuximide is not associated with induction or inhibition of hepatic enzymes, and to date, there has been no reports of ethosuximide affecting the clearance of other drugs. The metabolism of oral contraceptives is unaffected by ethosuximide, and therefore, it does not compromise contraceptive control (Table 14.2).

14.7.5.2 Effect of Other Drugs on Ethosuximide. Carbamazepine, phenobarbital, phenytoin, and primidone can induce the metabolism of ethosuximide and decrease its plasma levels, while valproic acid can inhibit ethosuximide metabolism and increase its plasma levels (Table 14.1). Furthermore, while rifampicin can induce the metabolism of ethosuximide and decrease its plasma levels, isoniazid and ritonavir can inhibit its metabolism and increase plasma levels.

14.7.6 Felbamate

Only 50% of the administered dose is metabolized in the liver, by CYP3A4 and CYP2E1, to form two hydroxylated metabolites (*para*-hydroxyfelbamate and 2-hydroxyfelbamate; 10–15%) and a variety of other unidentified polar metabolites, some of them being glucuronides or sulfate esters. Overall, felbamate has a moderate propensity for metabolic interactions and acts as an inhibitor of metabolism.

14.7.6.1 Effect of Felbamate on Other Drugs. Felbamate is both an inhibitor of drugs metabolized by CYP2C19 and β oxidation and an inducer of drugs metabolized by CYP3A4 [28]. Thus, felbamate inhibits the metabolism of phenobarbital, phenytoin, and valproic acid and increases their plasma levels but decreases the level of carbamazepine (18–31%) while increasing carbamazepine epoxide levels (33–57%) [29]. Felbamate can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2).

14.7.6.2 Effect of Other Drugs on Felbamate. Carbamazepine, phenytoin, and phenobarbital can induce the metabolism of felbamate, so that its clearance is increased by 40–82% [28]. To date, there have been no reports of other non-AED drugs affecting the metabolism of felbamate.

14.7.7 Gabapentin

Gabapentin is not metabolized, is excreted unchanged in urine, and, as such, is neither a hepatic enzyme inducer nor an inhibitor. To date, gabapentin has not been associated with any clinically significant metabolic interactions.

14.7.8 Lacosamide

Lacosamide undergoes moderate metabolism in the liver, by demethylation to *O*-desmethyl lacosamide (30%). Other unidentified metabolites constitute a further 30% of the metabolism. CYP2C19 is considered to be primarily responsible for the formation of *O*-desmethyl lacosamide, and none of the metabolites are pharmacologically active.

14.7.8.1 Effect of Lacosamide on Other Drugs. Although lacosamide undergoes moderate hepatic metabolism, it is essentially non-CYP-dependent and, as such, is not amenable to interference. To date, lacosamide has not been associated with any clinically significant metabolic interactions affecting other drugs [30].

14.7.8.2 Effect of Other Drugs on Lacosamide. To date, the metabolism of lacosamide has not been affected by other drugs, and thus is not associated with any clinically significant metabolic interactions [30].

14.7.9 Lamotrigine

Lamotrigine undergoes extensive metabolism in the liver, by conjugation with glucuronic acid, to various metabolites including, 2-*N*-glucuronide (76% of dose) and 5-*N*-glucuronide (10% of dose), a 2-*N*-methyl metabolite (0.14% of dose), and other unidentified minor metabolites (4% of dose). The isoform that is responsible for the *N*-glucuronidation of lamotrigine is UGT1A4. The metabolites of lamotrigine are not pharmacologically active. Overall, lamotrigine has a moderate propensity for metabolic interactions.

14.7.9.1 Effect of Lamotrigine on Other Drugs. Lamotrigine is a weak inhibitor of UGT, and it induces its own metabolism and causes a small decrease in plasma levels of clonazepam and valproic acid [31]. To date, there have been no reports of lamotrigine affecting the clearance and plasma levels of other non-AED drugs. The metabolism of oral contraceptives is unaffected by lamotrigine, and therefore, it does not compromise contraceptive control (Table 14.2).

14.7.9.2 Effect of Other Drugs on Lamotrigine. Carbamazepine, methsuximide, oxcarbazepine, phenobarbital, phenytoin, and primidone can increase the clearance of lamotrigine and decrease lamotrigine plasma levels. In contrast, valproic acid can decrease the clearance of lamotrigine and increase lamotrigine plasma levels. Sertraline can increase lamotrigine plasma levels twofold. Acetaminophen, olanzapine, rifampicin, and ritonavir can decrease lamotrigine plasma levels by ~20% [32,33].

14.7.10 Levetiracetam

Levetiracetam undergoes minimal metabolism with ~30% of the dose metabolized by hydrolysis to a deaminated metabolite. This metabolism is independent of the hepatic CYP system and is via a type B esterase enzyme located in whole blood. The metabolites of levetiracetam are not pharmacologically active.

14.7.10.1 Effect of Levetiracetam on Other Drugs. Levetiracetam does not undergo hepatic metabolism but undergoes minimal metabolism in whole blood [34]. Consequently, it is not amenable to interference. To date, levetiracetam has not been associated with any clinically significant metabolic interactions affecting other drugs [35,36].

14.7.10.2 Effect of Other Drugs on Levetiracetam. To date, the nonhepatic metabolism of levetiracetam has not been affected by other drugs, and thus, the drug is not associated with any clinically significant metabolic interactions [35,36].

14.7.11 Oxcarbazepine

Oxcarbazepine is rapidly metabolized in the liver to its pharmacologically active metabolite, 10-hydroxycarbazepine (also known as *licarbazepine*), by stereoselective biotransformation mediated by cytosolic arylketone reductase. 10-Hydroxycarbazepine comprises of a racemic mixture of 80% S-form (active) and 20% R-form (inactive). 10-Hydroxycarbazepine is subsequently primarily metabolized by conjugation with glucuronic acid. Minor amounts (4% of dose) of 10-hydroxycarbazepine are oxidized to an inactive 10,11-dihydroxy metabolite.

Metabolites (other than 10-hydroxycarbazepine) are not pharmacologically active [37,38]. Overall, oxcarbazepine has a low propensity for metabolic interactions.

14.7.11.1 Effect of Oxcarbazepine on Other Drugs. *In vitro* studies show that both oxcarbazepine and 10-hydroxycarbazepine have little or no capacity to inhibit the CYP1A, CYP2D, and CYP2E families. However, CYP2C19 is significantly inhibited by oxcarbazepine and consequently can increase plasma levels of phenobarbital and phenytoin by 14% and 40%, respectively [39]. Oxcarbazepine can induce the metabolism of carbamazepine and topiramate and decrease their plasma levels. Additionally, oxcarbazepine induces the metabolism of lamotrigine, via an effect on UGTs, and decreases lamotrigine plasma levels by 29% [40]. Felodipine plasma levels can decrease by 28% when oxcarbazepine is administered concurrently [41]. Oxcarbazepine can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2).

14.7.11.2 Effect of Other Drugs on Oxcarbazepine. Because oxcarbazepine is metabolized by noninducible ketoreductases, its disposition is largely unaffected by CYP inducers. However, coadministration with carbamazepine, phenobarbital, phenytoin, and primidone can increase the clearance of 10-hydroxycarbazepine and decrease its plasma levels [42]. Additionally, verapamil can decrease 10-hydroxycarbazepine plasma levels, while viloxazine can increase its plasma levels [43,44].

14.7.12 Phenobarbital

Phenobarbital undergoes significant metabolism in the liver to two major metabolites, *p*-hydroxyphenobarbital (~20–30% of administered dose), which partially (50%) undergoes sequential metabolism to a glucuronic acid conjugate, and 9-D-glucopyranosylphenobarbital (~25–30% of administered dose), a nitrogen

glucosidation conjugate. CYP2C9 plays a major role in the metabolism of phenobarbital to *p*-hydroxyphenobarbital, with minor metabolism by CYP2C19 and CYP2E1. The identity of the UGT enzyme responsible for the formation of 9- β -glucopyranosylphenobarbital is unknown. The metabolites of phenobarbital are not pharmacologically active. Overall, phenobarbital has a substantial propensity for metabolic interactions and acts as an inducer of metabolism.

14.7.12.1 Effect of Phenobarbital on Other Drugs. Phenobarbital induces the metabolism of many drugs, particularly those metabolized by CYP2C and CYP3A subfamilies and UGTs. The time course of induction (and deinduction) primarily depends on the half-life of phenobarbital, which is long (70–140 h). Consequently, induction typically begins within approximately a week, with maximum induction occurring two to three weeks after initiation of phenobarbital therapy. Phenobarbital can increase the clearance and decrease the plasma levels of many AEDs including carbamazepine, clobazam, clonazepam, diazepam, ethosuximide, felbamate, lamotrigine, midazolam, oxcarbazepine, phenytoin, rufinamide, stiripentol, tiagabine, topiramate, valproic acid, and zonisamide (Table 14.1). Phenobarbital also induces the metabolism of many non-AED drugs (Table 14.3), the magnitude of which is particularly substantial for many drugs. Phenobarbital can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2) [6].

14.7.12.2 Effect of Other Drugs on Phenobarbital. Inhibitors of CYP2C9 should have an effect on the metabolism of phenobarbital; however, the effect is small because only ~20% of metabolism occurs through this pathway. Felbamate, oxcarbazepine, phenytoin, rufinamide, stiripentol, and valproic acid can decrease the clearance of phenobarbital and increase its plasma levels. The effect of valproic acid is particularly profound because valproic acid inhibits not only CYP2C9-dependent hydroxylation but also phenobarbital glucuronidation, with phenobarbital plasma levels increasing by ~100%. Additionally, chloramphenicol, dicoumarol, and propoxyphene can increase phenobarbital plasma levels [6].

14.7.13 Phenytoin

Phenytoin undergoes extensive metabolism in the liver by hydroxylation to various metabolites, the principal metabolites being 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH; 67–88%) and a dihydrodiol derivative (7–11%). The isoenzymes responsible for the hydroxylation of phenytoin are CYP2C9 (~80%) and CYP2C19 (~20%). In excess of 60% of *p*-HPPH is subsequently glucuronidated and excreted in urine. The metabolites of phenytoin are not pharmacologically active. Overall, phenytoin has a substantial propensity for metabolic interactions and acts as an inducer of metabolism.

14.7.13.1 Effect of Phenytoin on Other Drugs. Phenytoin increases the metabolism of drugs that are metabolized by CYP2C and CYP3A subfamilies and UGT enzymes. Consequently, phenytoin can decrease plasma levels of many AEDs including carbamazepine, clobazam, clonazepam, ethosuximide, felbamate, lamotrigine, oxcarbazepine, phenobarbital, primidone, rufinamide, stiripentol, tiagabine, topiramate, valproic acid, and zonisamide (Table 14.1) [6].

Phenytoin also induces the metabolism of many non-AED drugs and decreases their plasma levels (Table 14.3). The magnitude of which is particularly substantial for many drugs. Phenytoin can increase plasma levels of chloramphenicol [6] and can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2) [6].

14.7.13.2 Effect of Other Drugs on Phenytoin. Carbamazepine and phenobarbital can increase the clearance of phenytoin and decrease its plasma levels. Carbamazepine, in addition, can inhibit phenytoin metabolism and increase plasma phenytoin levels. The AEDs clobazam, felbamate, oxcarbazepine, phenobarbital, rufinamide, stiripentol, topiramate, and zonisamide can decrease the clearance of phenytoin and increase its plasma levels (Table 14.1).

Phenytoin metabolism can be inhibited by a variety of non-AED drugs, resulting in increases in phenytoin plasma levels. Such drugs include allopurinol, amiodarone, azapropazone, bleomycin, chlorphenamine, clarithromycin, chloramphenicol, cimetidine, clinafloxacin, cotrimoxazole, disulfiram, dextropropoxyphene (propoxyphene), dicoumarol, diltiazem, doxifluridine, erythromycin, esomeprazole, fenyramidol, fluconazole, 5-fluorouracil, fluoxetine, fluvoxamine, imipramine, indinavir, isoniazid, itraconazole, methylphenidate, metronidazole, miconazole, nelfinavir, nifedipine, omeprazole, risperidone, ritonavir, saquinavir, sertraline, sulfinpyrazone, tamoxifen, tegafur, ticlopidine, trazodone, verapamil, viloxazine, and voriconazole (Table 14.3) [6].

Phenytoin metabolism can be induced by a variety of non-AED drugs, resulting in decreases in its plasma levels. These include drugs such as acyclovir, carboplatin, carmustine, cisplatin, dexamethasone, diazoxide, etoposide, loxapine, methotrexate, rifampicin, St. John's wort, sucralfate, theophylline, and vinblastine [6].

14.7.14 Pregabalin

Pregabalin is not metabolized, is excreted unchanged in urine, and, as such, is neither a hepatic enzyme inducer nor an inhibitor. To date, pregabalin has not been associated with any clinically significant metabolic interactions.

14.7.15 Primidone

Primidone is metabolized in the liver to two primary metabolites, namely, phenobarbital and phenylethylmalonamide (PEMA). Phenobarbital subsequently undergoes metabolism to two metabolites, *p*-hydroxyphenobarbital and 9-D-glucopyranosylphenobarbital. PEMA is pharmacologically active but has a much lower potency than primidone and phenobarbital. Overall, primidone has a substantial propensity for metabolic interactions and acts as an inducer of metabolism.

14.7.15.1 Effect of Primidone on Other Drugs. Because phenobarbital is invariably present during long-term primidone treatment, all the effects of phenobarbital on other drugs described previously for phenobarbital can be expected with primidone, and all the interactions affecting phenobarbital will also affect the phenobarbital derived from primidone (for further details, see Section 14.7.12).

14.7.15.2 Effect of Other Drugs on Primidone. Phenytoin and carbamazepine can accelerate the conversion of primidone to phenobarbital, which results in decreased primidone plasma levels and increased phenobarbital levels (for further details, see Section 14.7.12).

14.7.16 Rufinamide

Rufinamide is metabolized in the liver, primarily by hydrolysis which is not CYP dependent, to CGP 47292, which is subsequently excreted in urine (~70%) and feces (~9%). Approximately 7% is excreted in urine as minor acyl-glucuronide metabolites of CGP 47292. The metabolites of rufinamide are not pharmacologically active.

Overall, rufinamide has a minimal propensity for metabolic interactions.

14.7.16.1 Effect of Rufinamide on Other Drugs. Rufinamide can decrease plasma levels of carbamazepine and lamotrigine, but increases plasma levels of phenobarbital and phenytoin (Table 14.1). In contrast, rufinamide can decrease plasma levels of triazolam [45–47]. Rufinamide can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2).

14.7.16.2 Effect of Other Drugs on Rufinamide. Carbamazepine, phenobarbital, phenytoin, primidone, and vigabatrin can increase the clearance of rufinamide and decrease rufinamide plasma levels. In contrast, valproic acid can decrease the clearance of rufinamide and increase its plasma levels

To date, there have been no reports of other drugs affecting the clearance of rufinamide and affecting rufinamide plasma levels [45–47].

14.7.17 Stiripentol

Stiripentol is metabolized in the liver, primarily by desmethylation and glucuronidation, to 13 different metabolites. Precise identification of enzymes involved in its metabolism is not known, but the principal enzymes are considered to be CYP1A2, CYP2C19, and CYP3A4. The metabolites of stiripentol are not considered to be pharmacologically active. Overall, stiripentol has a substantial propensity for metabolic interactions and acts as an inhibitor of metabolism.

14.7.17.1 Effect of Stiripentol on Other Drugs. Because stiripentol has limited use in the management of epilepsy, its exact interaction profile has not been identified. However, because stiripentol is a potent inhibitor of CYP2C19, CYP3A4, and CYP2D6, caution needs to be exercised if clinical circumstances require combining stiripentol with drugs that are metabolized by these isoenzymes [e.g., citalopram, omeprazole (CYP2C19); chlorpheniramine, calcium channel blockers, statins, codeine (CYP3A4); propranolol, fluoxetine, sertraline, haloperidol, tramadol (CYP2D6)]. Stiripentol can inhibit the metabolism of carbamazepine, phenobarbital, valproic acid, and clobazam and its pharmacologically active metabolite *N*-desmethyclobazam and increase their plasma levels. The effect on clobazam and *N*-desmethyclobazam is particularly profound with levels increasing up to fourfold [25,26,48].

14.7.17.2 Effect of Other Drugs on Stiripentol. Carbamazepine, phenobarbital, phenytoin, and primidone can increase the clearance of stiripentol and decrease stiripentol plasma levels. To date, there have been no reports of other drugs affecting the clearance and plasma levels of stiripentol [25,26,48].

14.7.18 Tiagabine

Tiagabine is metabolized in the liver, primarily by CYP3A4, to two 5-oxo-tiagabine isomers (E5 and Z-5) that represent ~60% of metabolism. The remaining 40% of metabolites have yet to be identified [49]. The metabolites of tiagabine are not pharmacologically active. Tiagabine is neither an enzyme inducer nor an enzyme inhibitor, and therefore, its propensity to interact is minimal.

14.7.18.1 Effect of Tiagabine on Other Drugs. Tiagabine can decrease valproic acid plasma levels through an unknown mechanism (Table 14.1) [50]. To date, there have been no reports of tiagabine affecting the clearance and plasma levels of other non-AED drugs. The metabolism of oral contraceptives is unaffected by tiagabine, and therefore, it does not compromise contraceptive control (Table 14.2).

14.7.18.2 Effect of Other Drugs on Tiagabine. Carbamazepine, phenobarbital, phenytoin, and primidone can increase the clearance of tiagabine by 50–60%, via an action on CYP3A4, and decrease tiagabine plasma levels [51].

14.7.19 Topiramate

Topiramate is not extensively metabolized in patients on monotherapy or in patients not prescribed enzyme-inducing drugs, and typically, 40–50% of a topiramate dose is excreted unchanged via the kidneys. However, in the presence of enzyme-inducing drugs, metabolism becomes an important feature of the elimination of topiramate and this value is doubled. Metabolites thus far identified include two hydroxy and two diol metabolites, as well as several glucuronide conjugates, none of which constitutes more than 5% of an administered dose. Although the specific CYP isoenzymes for the metabolism of topiramate have not been identified, it is evident that isoenzymes induced by carbamazepine and phenytoin play a major role [52]. The metabolites of topiramate are not pharmacologically active. Overall, topiramate has a low propensity for metabolic interactions, with its metabolism both induced and inhibited by other drugs.

14.7.19.1 Effect of Topiramate on Other Drugs. Topiramate is a weak inducer of CYP enzymes and weakly inhibits CYP2C19 [53]. Topiramate can decrease the clearance of phenytoin and increase phenytoin plasma levels, while it can increase the clearance of valproic acid and decrease valproic acid plasma level. With regard to non-AED drugs, topiramate can increase the clearance and decrease the plasma levels of digoxin, glibenclamide, pioglitazone, risperidone, and sumatriptan, while it can decrease the clearance and increase the plasma levels of amitriptyline, haloperidol, hydrochlorothiazide, lithium, and metformin [54,55]. Topiramate can induce the metabolism of the estrogen component of an oral contraceptive pill and therefore may reduce the effectiveness of oral contraception (Table 14.2).

14.7.19.2 Effect of Other Drugs on Topiramate. Carbamazepine, oxcarbazepine, phenobarbital, phenytoin, primidone, and valproic acid can increase the clearance of topiramate and decrease its plasma levels (Table 14.1) [56,57]. Amitriptyline, lithium, metformin, propranolol, and sumatriptan can decrease the clearance of topiramate and increase its plasma levels [6].

14.7.20 Valproic Acid

The metabolism of valproic acid in the liver is extensive and complex, in that it involves multiple metabolic pathways including O-glucuronidation, β oxidation, ω oxidation hydroxylation, ketone formation, and desaturation. To date, in excess of 25 metabolites have been identified. Valproic acid glucuronide and 3-oxo-valproic acid are by far the most abundant metabolites (~40% and 33% of an administered dose, respectively). Hydroxylation to form 4-ene-valproic acid and other metabolites is via the action of CYP2A6, CYP2C9, CYP2C19, and CYP2B6 isoenzymes, while O-glucuronidation is mediated by UGT1A3 and UGT2B7 isoforms. Some metabolites are pharmacologically active; 2-ene-valproic acid and 4-ene-valproic acid have anticonvulsant activity that is similar in potency to that of valproic acid itself. Overall, valproic acid has a substantial propensity for metabolic interactions and acts as an inhibitor of metabolism.

14.7.20.1 Effect of Valproic Acid on Other Drugs. Valproic acid is a broad-spectrum inhibitor of hepatic metabolism, including inhibition of CYP2C9, CYP2C19, UGT1A4, and epoxide hydrolase [58]. Additionally, its metabolism can be both induced and inhibited. Valproic acid can decrease the clearance and increase plasma levels of carbamazepine epoxide, ethosuximide, felbamate, lamotrigine, lorazepam, midazolam, phenobarbital, and rufinamide (Table 14.1). Valproic acid can also increase the clearance and decrease plasma levels of topiramate and zonisamide. Of these interactions, three stand out: the inhibition of the metabolism of phenobarbital and lamotrigine and that of the active metabolite of carbamazepine, carbamazepine epoxide. During comedication with phenobarbital, phenobarbital plasma levels increase over two to three weeks by about 30–50% in most patients, but in some patients, the increase can be twofold. Inhibition of lamotrigine metabolism results in a twofold increase in lamotrigine plasma levels and the inhibitory effect is maximal at the relatively low valproate dose of 500 mg/day [59]. The particular significance of this interaction relates to the risk of lamotrigine-induced skin rashes, which is dependent on the rate of rise in plasma lamotrigine levels. Valproic acid inhibition of epoxide hydrolase results in an increase in plasma carbamazepine epoxide levels. The significance of this interaction is based on the fact that carbamazepine toxicity occurs in the absence of an effect on plasma carbamazepine levels [21].

With regard to non-AED drugs, valproic acid can decrease the clearance and increase the plasma level of amitriptyline, aripiprazole, clomipramine, chlorpromazine, lopinavir, naproxen, nimodipine, nortriptyline, paroxetine, and zidovudine [6,60]. The metabolism of oral contraceptives is unaffected by valproic acid, and therefore it does not compromise contraceptive control (Table 14.2).

14.7.20.2 Effect of Other Drugs on Valproic Acid. Carbamazepine, lamotrigine, phenobarbital, phenytoin, primidone, and topiramate can increase the clearance of valproic acid and decrease valproic acid plasma levels (Table 14.1).

In contrast, clobazam, felbamate, and stiripentol can decrease the clearance of valproic acid and increase valproic acid plasma levels. The effect of felbamate is via inhibition of the β oxidation pathway. With regard to non-AED drugs, amikacin, diflunisal, meropenem, naproxen, paripenem, rifampicin, and ritonavir can increase the clearance and decrease valproic acid plasma levels. In contrast, chlorpromazine, fluoxetine, isoniazid, and sertraline can decrease the clearance and increase valproic acid plasma levels [6,60].

14.7.21 Vigabatrin

Vigabatrin is not metabolized, is excreted unchanged in urine, and, as such, is neither a hepatic enzyme inducer nor an inhibitor. To date, vigabatrin has not been associated with any clinically significant metabolic interactions [6,60].

14.7.22 Zonisamide

Zonisamide undergoes acetylation to form *N*-acetyl zonisamide and reduction to form 2-sulfamoylacetylphenol, the latter being subsequently glucuronidated. The reduction of zonisamide to 2-sulfamoylacetylphenol is mediated by the CYP3A4 isoenzyme [61]. Metabolites are not pharmacologically active. Overall, zonisamide has a low propensity for metabolic interactions.

14.7.22.1 Effect of Zonisamide on Other Drugs. Although zonisamide is not an enzyme inducer, it appears to act as a weak enzyme inhibitor. *In vitro* studies on the inhibitory characteristics of zonisamide showed that drugs metabolized by CYP3A4, CYP1A2, or CYP2D6 should not be affected, but drugs metabolized by CYP2A6, CYP2C9, CYP2E1, and CYP2C19 may be slightly inhibited [62]. A 16% increase in phenytoin plasma levels can be expected in patients coprescribed with zonisamide [63], consequent to an inhibition of CYP2C19, while carbamazepine plasma levels have been reported to both increase and decrease (Table 14.1) [64]. The metabolism of oral contraceptives is unaffected by zonisamide, and therefore, it does not compromise contraceptive control (Table 14.2). To date, there have been no reports of zonisamide affecting the clearance and plasma levels of non-AED drugs.

14.7.22.2 Effect of Other Drugs on Zonisamide. Carbamazepine, phenobarbital, phenytoin, primidone, and valproic acid can increase the clearance of zonisamide and decrease its plasma levels (Table 14.1) [65–67]. Lamotrigine can increase zonisamide plasma levels twofold [68], while risperidone can decrease zonisamide plasma levels [69].

14.8 ANTIEPILEPTIC DRUGS CURRENTLY UNDERGOING DEVELOPMENT

14.8.1 Brivaracetam

Brivaracetam undergoes metabolism, primarily through hydrolysis and secondarily through hydroxylation mediated by CYP2C9, CYP2C19, CYP3A4, and to a negligible

extent by CYP2B6 and CYP2C8. Enzyme-inducing AEDs and rifampicin decrease plasma brivaracetam levels, while brivaracetam can increase the plasma levels of phenytoin and carbamazepine epoxide [70].

14.8.2 Ganaxolone

Ganaxolone is metabolized, primarily by CYP3A4/3A5, to yet unknown metabolites, which are eliminated from plasma at a rate three to six times faster than the terminal half-life of ganaxolone. Preliminary trials have not identified significant drug interactions with concomitant AEDs [71].

14.8.3 Seletacetam

Seletacetam is eliminated by hydrolysis and is excreted in urine. Its primary metabolite is an acidic inactive metabolite (ucb-101596-1), the concentration of which in plasma is ~10-fold lower than that of the parent compound. Two additional minor metabolites have been identified in urine. Overall, 25% of a seletacetam dose is excreted in the unchanged form [72]. On the basis of *in vitro* data, there is a low potential for interactions involving seletacetam. The drug interaction profile of seletacetam in the clinical setting is at present unknown.

14.8.4 Talampanel

Although its metabolic pathways have not been completely characterized in man, acetylation appears to occur, where talampanel is converted to *N*-acetyl-talampanel by hepatic *N*-acetyltransferase 2. This metabolic route is not major, but there appears to be significant interpatient differences in plasma concentrations of *N*-acetyl-talampanel, which is pharmacologically active and may also contribute to adverse effects in some patients. In an *in vitro* setting, talampanel has been shown to be an irreversible inhibitor of the CYP3A, and consequently, it would be anticipated that it may inhibit the metabolism of CYP3A substrates such as carbamazepine. Indeed, carbamazepine plasma levels are elevated during carbamazepine and talampanel coadministration. Also, there is evidence to suggest that talampanel inhibits valproic acid metabolism, resulting in increased plasma valproic acid levels. In patients with epilepsy, the clearance of talampanel is enhanced by enzyme-inducing AEDs such as carbamazepine and phenytoin, so that its half-life is reduced to about 3 h, while valproic acid does not appear to affect talampanel clearance [73].

14.8.5 Tonabersat

Tonabersat is highly bound to plasma proteins (98.2%) and undergoes metabolism by cytosolic carbonyl reductase enzymes to a reduced ketone, with negligible amounts excreted in urine unchanged. The primary metabolite is detectable in plasma. Acetyl hydroxylation via the CYP3A family of enzymes accounts for a minor hydroxyl metabolite [71]. The drug interaction profile of tonabersat in the clinical setting is at present unknown.

14.8.6 Valrocecimide

Approximately 10–20% of an administered dose is excreted unchanged in urine, and 40% is excreted in the form of valproyl glycine. Urinary valproic acid accounts for 4–6% of an administered valrocecimide dose. *In vitro* studies using CYP isoenzymes indicate that significant drug interactions with other AEDs would not be anticipated. Nevertheless, higher valrocecimide clearance and shorter half-life values were observed in patients receiving enzyme-inducing AEDs [74].

14.9 CONCLUSIONS AND FUTURE PERSPECTIVES

As a class of drugs, the AEDs are associated with more pharmacokinetic (metabolic) interactions than any other therapeutic drug class. The reason for this is that the older first-generation AEDs (carbamazepine, phenobarbital, phenytoin, and valproic acid) are widely prescribed and commonly used as first-line therapy. Additionally, these AEDs have a substantial ability to induce and/or inhibit hepatic metabolism, and consequently, their propensity to interact is substantial [4,5]. Furthermore, these AEDs, along with some of the newer AEDs (e.g., eslicarbazepine acetate, felbamate, lamotrigine, oxcarbazepine, tiagabine, topiramate, and zonisamide), are susceptible to inhibition and induction of their own metabolism [75]. Of the new AEDs, those that are renally excreted (e.g., gabapentin, levetiracetam, pregabalin, and vigabatrin) have minimal or no potential to interact, while those that undergo minimal/moderate metabolism with non-CYP- and non-UGT-dependent isoenzyme systems (levetiracetam, lacosamide) similarly are not susceptible to interference [75]. Finally, there are more new AEDs under development, and on the basis of available data, it would appear that the general trend of the developing AEDs that are associated with a reduced propensity for pharmacokinetic (metabolic) interactions continues [71].

From a clinical therapeutic viewpoint, AED metabolic interactions are best avoided by the use of drugs that are not potent CYP inhibitors or inducers and are not readily inhibited by other drugs. In reality, drug interactions caused by mutual inhibition are almost inevitable because CYP-mediated metabolism represents a major route of elimination of many drugs and because the same CYP enzymes can metabolize numerous drugs. Whenever polytherapy with known interacting drugs is contemplated, it is best practice to monitor blood levels of the affected drug before and after the introduction of the interacting drug [76]. Thus, a graded therapeutic adjustment can be undertaken so as to prevent unnecessary toxicity or seizure breakthrough consequent to the interaction.

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15 Oral Chemotherapeutic Agents

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15.1	Summary	1
15.2	Introduction	2
15.3	Factors influencing bioavailability of oral chemotherapeutic agents	3
15.4	Major classes of oral chemotherapeutic agents	10
15.5	Conclusions and future perspectives	28
	References	28

15.1 SUMMARY

Oral chemotherapeutic agents have been available for decades, and there has been an accelerated expansion in their development during the recent decade. Oral chemotherapeutic agents offer obvious advantages in terms of convenient and noninvasive administration, flexibility and adaptability of dosing, as well as financial and staff time savings. Compared to intravenous agents, oral chemotherapeutic agents generally exhibit substantial interindividual pharmacokinetic variability. Many factors contribute to this variability, including physiological, pathological, environmental, and genetic factors. Unique to oral agents, poor and variable bioavailability is one important source of the interindividual pharmacokinetic variability. This chapter discusses the factors influencing bioavailability of oral chemotherapeutic agents, with focus on the first-pass metabolism by cytochrome P450 3A4 (CYP3A4), intestinal efflux by ABCB1 transporter, and other factors such as concomitant administration of antacid medications, food effect, and surgery. Oral chemotherapeutic agents can be classified as traditional cytotoxic agents and novel molecularly targeted agents, hormonal agents, and immunomodulating agents. Each class of agents is unique with regard to its mechanisms of action, pharmacokinetics, mechanisms of drug resistance, and clinical use. This chapter provides an overview of the major classes of oral chemotherapeutic agents, particularly highlighting examples whereby genetic variations in the genes involved in the drug disposition or drug–target interaction influence the pharmacokinetics and/or response.

15.2 INTRODUCTION

Cancer chemotherapy has traditionally been dominated by intravenous drug administration. However, oral chemotherapeutic agents provide obvious advantages in terms of convenient and noninvasive administration, flexibility and adaptability of dosing, and financial and staff time savings [1]. The use of oral chemotherapeutic agents has been increased, especially in the recent decade. Modern cancer chemotherapy was introduced in the 1940s with the use of intravenous nitrogen mustard for the treatment of hematological malignancies [2]. In the 1950s, several oral chemotherapeutic agents (e.g., melphalan, busulfan, and chlorambucil) were investigated for their efficacy in cancer treatment [3–6], and the first oral chemotherapeutic agent, mercaptopurine, was approved by the United States Food & Drug Administration (FDA) in 1953. By the 1970s, many other oral chemotherapeutic agents were approved (e.g., procarbazine in 1969, lomustine in 1976, and tamoxifen in 1977). Yet, oral chemotherapy has not become popular as an oncologic drug delivery route until the recent decade, when a number of orally bioavailable small molecule kinase inhibitors (e.g., imatinib, gefitinib, erlotinib, sorafenib, sunitinib, etc.) have been developed and proved effective for the treatment of certain types of tumors in clinical settings. According to a 2008 National Comprehensive Cancer Network (NCCN) Task Force Report, it is estimated that one-fourth of the 400 antineoplastic agents currently in the pipeline are planned as oral drugs [7] and more than 20 oral chemotherapeutic agents have been currently approved by the FDA for the treatment of cancer [8].

Compared to intravenous agents, oral chemotherapeutic agents generally exhibit substantial interindividual pharmacokinetic variability. This variability can lead to therapeutic failure or severe toxicity. Multiple factors contribute to variations in the pharmacokinetics and response of chemotherapeutic agents. These include drug-specific factors (e.g., dosage form or formulation, dose, and schedule), patient's demographic (e.g., age, race, and sex), physiological (e.g., body size and composition) and pathological factors (e.g., disease status and organ functions), as well as environmental (e.g., interactions with food, environmental toxins, and other drugs) and genetic factors (e.g., genetic polymorphisms in the genes encoding drug-metabolizing enzymes, transporters, and drug targets). Unique to oral chemotherapeutic agents, absorption is the prerequisite for the systemic exposure and activity. Poor and variable oral bioavailability is one important source of the interindividual pharmacokinetic variability of oral agents. In this chapter, we discuss the factors influencing bioavailability of oral chemotherapeutic agents, with focus on first-pass metabolism, intestinal ABCB1 efflux, and other factors such as concomitant administration of antacid medications, food effect, and surgery.

Broadly, oral chemotherapeutic agents can be classified as traditional cytotoxic agents (e.g., alkylating agents, antimetabolites, and plant alkyls), novel molecularly targeted agents (e.g., small molecule TKIs, mTOR inhibitors, and HDAC inhibitors), hormonal agents (e.g., tamoxifen, letrozole, and anastrozole), and immune-modulating agents (e.g., thalidomide and lenalidomide). Traditional cytotoxic agents cause cancer cell death or cell cycle arrest by inducing a diverse spectrum of toxic DNA lesions (e.g., base damage, bulky adducts, replication lesions, DNA cross-links, single-strand breaks, and double-strand breaks) or interfering with cell division, a multiphase process involving the synthesis of nucleic acids, RNA, and DNA and nuclear division (i.e., mitosis). Molecularly targeted agents have emerged as a new generation of cancer treatment in the recent decade. Their mechanisms of action and toxicity profiles

differ from those of traditional cytotoxic agents. In contrast to traditional agents that act on rapidly dividing cells, including cancer cells, as well as normal tissue cells, novel molecularly targeted agents are designed to interfere with specific targets, which are usually located within tumor cells or surrounding cells. Therefore, novel molecularly targeted agents have a relative higher specificity toward tumor cells, providing a broader therapeutic window with less toxicity. In addition, some drugs that modulate tumor cell behavior or environment without directly attacking those cells are used for the treatment of cancer. Hormonal agents and immune-modulating agents fall into this category. Each class of agents is unique with regard to its mechanisms of action, pharmacokinetics, mechanisms of resistance, and clinical use. In this chapter, we review the major classes of oral chemotherapeutic agents, from a pharmacological and clinical perspective.

15.3 FACTORS INFLUENCING BIOAVAILABILITY OF ORAL CHEMOTHERAPEUTIC AGENTS

In general, the absorption of a solid drug from the gastrointestinal (GI) tract involves the following four steps: (i) gastric emptying and small intestinal transit flow to deliver the drug to its absorption site, (ii) dissolution of the solid drug into solution available for absorption, (iii) permeation of the dissolved drug through the GI membrane, and (iv) movement of the drug through the gut wall and liver before entering the general circulation [9]. Many physical, anatomic, and physiologic factors may influence the overall rate and extent of drug absorption from the GI tract. Included among them are the physicochemical properties of the drug (e.g., solubility, lipophilicity, and stability), the properties of the formulation (e.g., disintegration time, dissolution rate, and stability), and physiological features of the patient (e.g., the nature of the membrane, gastric emptying and intestinal transit, blood perfusion, and pH in the GI tract) [10]. Notably, incomplete dissolution, first-pass intestinal and hepatic metabolism, and efflux by transporters (e.g., ABCB1) in the gut lumen are the common causes of poor and variable oral bioavailability of chemotherapeutic agents.

15.3.1 First-Pass Metabolism

First-pass metabolism in the gut wall and liver before an oral drug enters systemic circulation is one major factor causing low oral bioavailability of some chemotherapeutic agents such as 5-fluorouracil (5-FU), doxorubicin, and docetaxel. The human cytochrome P450 (CYP) superfamily represents the most important system responsible for catalyzing the oxidation of a large number of drugs. Three subfamilies of CYPs, including CYP1, CYP2, and CYP3, contribute to the oxidative metabolism of more than 90% of clinically used drugs. CYP3A4 has the highest abundance in human liver and intestine, contributing to the first-pass metabolism and systemic clearance of many chemotherapeutic agents (e.g., cyclophosphamide, etoposide, ifosfamide, paclitaxel, docetaxel, tamoxifen, vinblastine, gefitinib, erlotinib, and imatinib).

There is significant interindividual variability in the expression/activity of CYP3A4. It has been reported that hepatic and intestinal CYP3A4 expressions and activities vary at least 10- and 6-folds, respectively, among individuals [11,12]. This substantial heterogeneity could attribute to large interindividual variation in the first-pass metabolism

and systemic clearance of oral chemotherapeutic agents that are CYP3A4 substrates. An *in vivo* phenotypic probe for CYP3A4 may have utility for prediction of the systemic exposure and selection of the dose for CYP3A substrate drugs. Commonly used CYP3A4 phenotypic probe drugs include midazolam, erythromycin, cortisol, and dexamethasone. The apparent clearance of oral midazolam is a useful indicator of combined hepatic and intestinal CYP3A4/5 activity because midazolam, after its oral administration, undergoes substantial metabolism by CYP3A4/5 in the intestine and liver [13]. A prospective study in patients with cancer demonstrates that midazolam oral clearance accounts for a substantial (~40%) portion of the interindividual variability of gefitinib oral clearance, implicating that oral midazolam test has the potential for prediction of systemic exposure and optimization of treatment with gefitinib and other TKIs that are predominantly metabolized by CYP3A4 [14].

While over 28 single nucleotide polymorphisms (SNPs) have been identified in human *CYP3A4* gene, genetic polymorphisms of *CYP3A4* account insignificantly for the interindividual variability of CYP3A4 activity *in vivo*. The substantial variation in CYP3A4 activity could be caused by induction or inhibition of CYP3A4 on exposure to a wide variety of drugs or chemicals. The list of representative CYP3A4 inducers and inhibitors is presented in Table 15.1. Note that CYP3A4 is particularly susceptible to enzyme inducers. For an oral chemotherapeutic agent that is a CYP3A4 substrate, concomitant administration of a CYP3A4 inducer can markedly reduce its oral bioavailability and systemic exposure, whereas a reverse pattern may be seen with concomitant administration of a CYP3A4 inhibitor. For example, a study in 224 patients with glioblastoma showed that the mean trough level of imatinib was reduced up to 2.9-fold in patients receiving daily oral imatinib 600 mg in combination with CYP3A4-inducing antiepileptic drugs (i.e., carbamazepine, phenytoin, and oxcarbazepine) compared to those not taking CYP3A4-inducing antiepileptic drugs [15]. Another example is represented by lapatinib, which is predominantly metabolized by CYP3A4. Concomitant administration of strong CYP3A4 inhibitors (e.g., ketoconazole) or inducers (e.g., carbamazepine) significantly alters lapatinib plasma concentrations. Therefore in the package insert, dose adjustment of lapatinib is recommended for patients who must receive concomitant strong inhibitors or inducers of CYP3A4.

It is worth noting that the oral administration of some chemotherapeutic agents such as paclitaxel and docetaxel is hampered by their low oral bioavailability, partly due to extensive first-pass metabolism by CYP3A4. Pharmacologic inhibition of CYP3A4 could substantially enhance oral bioavailability of such agents. For example, preclinical studies demonstrated that inhibition of CYP3A4 with ritonavir (a CYP3A4 inhibitor) increased the oral bioavailability of oral docetaxel by 50-fold in both wild-type and ABCB1 knockout mice [16]. Consistently, a clinical study in patients with solid tumors demonstrated that the oral bioavailability of docetaxel was 131% when it was administered concomitantly with ritonavir, suggesting that ritonavir inhibited both presystemic (first-pass) and systemic metabolism of docetaxel [17]. This example indicates that CYP3A4 modulation has clinical implication for improving bioavailability of oral chemotherapeutic agents that undergo extensive first-pass metabolism by CYP3A4.

TABLE 15.1 Oral Chemotherapeutic Agents that are Substrates, Representative Inhibitors, and Inducers of CYP3A4

Substrates (Oral Chemotherapeutic Agents)	CYP3A4 Inhibitors	CYP3A4 Inducers
Capecitabine	Amiodarone	Dexamethasone
Cyclophosphamide	Anastrozole	Efavirenz
Dasatinib	Aprepitant	Modafinil
Erlotinib	Azole antifungal (fluconazole, itraconazole, ketoconazole, miconazole, posaconazole, voriconazole)	Nafcillin
Etoposide	Clarithromycin, erythromycin	Nevirapine
Everolimus	Cyclosporine	Phenobarbital
Exemestane	Cyclophosphamide	Phenytoin, Fosphenytoin
Gefitinib	Dasatinib	Rifampin
Imatinib	Diltiazem	St. John's wort
Lapatinib	Everolimus	—
Letrozole	Fluoxetine	—
Nilotinib	Grapefruit juice	—
Pazopanib	HIV protease inhibitors (atazanavir, fosamprenavir, indinavir, nelfinavir, ritonavir, saquinavir)	—
Sunitinib	Isoniazid	—
Sorafenib	Imatinib	—
Tamoxifen	Lapatinib	—
—	Nilotinib	—
—	Sunitinib	—
—	Sorafenib	—
—	Verapamil	—
—	Vorinostat	—

15.3.2 Intestinal ABCB1 Efflux

In addition to drug-metabolizing enzymes, uptake and efflux transporters that facilitate the movement of drugs in or out of the cell are important determinants of drug disposition and response. Broadly, drug transporters are classified into two families, namely efflux transporters of the ATP-binding cassette (ABC) family and uptake transporters of the solute carrier (SLC) family. These transporters play crucial roles in the intestinal absorption, biliary excretion, renal excretion, and tissue/cellular penetration of a wide variety of therapeutic drugs [18]. The ABC transporters are responsible for the transport of diverse substrates out of the cell using adenosine-5' triphosphate (ATP) as an energy source. In the ABC transporter family, ABCB1 has been well characterized for its roles in limiting oral bioavailability of the substrate drugs. The *ABCB1* gene, also named as the multidrug resistance 1 (*MDR1*) gene, encodes a polypeptide (P-glycoprotein) that has two halves, each containing six hydrophobic transmembrane

domains and an ATP-binding domain. ABCB1, located on the apical or luminal surface of the epithelial cells, functions as an efflux transporter in restricting intestinal absorption, facilitating hepatobiliary excretion and renal excretion, and protecting the brain and fetus from xenobiotics. In addition, ABCB1 overexpression in cancer cells is implicated in multidrug resistance to chemotherapeutic agents [19].

There is evidence that intestinal ABCB1 limits the intestinal absorption of some chemotherapeutic agents such as paclitaxel, docetaxel, and vinblastine [20,21]. It has been demonstrated that the coadministration of oral ABCB1 inhibitors significantly increases intestinal absorption of taxanes and topotecan [22,23]. Thus, the use of potent and specific ABCB1 inhibitors has potential in improving the bioavailability of oral chemotherapeutic agents that are ABCB1 substrates. A clinical proof-of-concept study in patients with advanced solid malignancies demonstrated that oral docetaxel achieved an average bioavailability of 90% when it was coadministered with an ABCB1 inhibitor cyclosporine [22]. However, the use of cyclosporin as a booster for oral administration of taxanes is hampered by its unfavorable immunosuppressive effects, and a new booster is thus needed in place of cyclosporine. Elacridar (GF120918), a third-generation ABCB1(P-gp) inhibitor, has shown promise in this regard. For example, a small clinical study demonstrated that coadministration of elacridar increased bioavailability of oral etoposide by 40%–97% [23]. ABCB1 can also be induced by a number of drugs or chemicals. The lists of representative ABCB1 substrates, inhibitors, and inducers are given in Table 15.2. Notably, some medications commonly used in oncology practice such as morphine, dexamethasone, and rifampicin induce ABCB1 expression [24–26] and could potentially decrease the bioavailability of oral chemotherapeutic agents that are ABCB1 substrates. In addition, some oral chemotherapeutic agents such as small molecule tyrosine kinase inhibitors (TKIs) can modulate ABCB1 function (see Table 15.2). Although there is little clinical evidence showing the influence of ABCB1 inducers on the pharmacokinetics of oral chemotherapeutic agents, one should be cautious when potential ABCB1 inducers are coadministered with the oral chemotherapeutic agents that are ABCB1 substrates.

The human ABCB1 gene is polymorphic. The most common SNPs of *ABCB1* in Caucasians and Asians are C1236T in exon 12, C3435T in exon 26, and G2677T in exon 21, but these SNPs are much less prevalent in African-American populations [27–29]. Interestingly, the frequencies of *ABCB1* SNPs differ between patients with cancer and healthy control populations [30]. Given the important role of ABCB1 in drug absorption and disposition, genetic polymorphisms in the *ABCB1* gene may influence the drug exposure and outcome of chemotherapy. For example, it has been shown that the C3435T and G2677T polymorphisms of *ABCB1* were associated with decreased risk of capecitabine-induced hand-foot syndrome (HFS) [31]. The association of *ABCB1* polymorphisms and the clinical outcome of small molecule TKIs have been reported. A retrospective study in patients with renal cell carcinoma (RCC) treated with sunitinib has demonstrated that the 1236T-3435C-2677G haplotype was associated with improved progression-free survival [32]. The *ABCB1* polymorphisms were also associated with increased trough level of erlotinib [33]; surprisingly, the erlotinib-treated patients carrying *ABCB1* variants showed a shorter progression-free survival compared to those carrying the wild type [34]. Conflicting results on the clinical impact of *ABCB1* polymorphisms may reflect the complex disposition pathway of the substrate drugs. For example, the TKIs (e.g., gefitinib, erlotinib, sunitinib) were found to be the dual substrates for both ABCB1 and ABCG2 transporters and were also

TABLE 15.2 Chemotherapeutic Agents that are Substrates, Representative Inhibitors, and Inducers of ABCB1

Substrates (Chemotherapeutic Agents)	Inhibitors	Inducers
Anthracyclines (daunorubicin, doxorubicin, epirubicin)	Macrolides (Azithromycin, clarithromycin, erythromycin)	Chlorambucil
Capecitabine	Antiarrhythmic (amiodarone, carvedilol, diltiazem, lidocaine, verapamil)	Cisplatin
Dasatinib	Azole antifungal (ketoconazole, itraconazole)	Clotrimazole
Etoposide	Chlorpromazine	Colchicine
Erlotinib	Dasatinib	Daunorubicin
Imatinib	Dipyridamole	Dexamethasone
Melphalan	Doxazosin	Doxorubicin
Mitomycin C	Fenofibrate	Efavirenz
Mitoxantrone	Fentanyl	Erythromycin
Taxanes (paclitaxel, docetaxel)	Flavonoids	Etoposide
Rapamycin	Glyburide	Fluorouracil
Tamoxifen	Gefitinib	Hydroxyurea
Temozolomide	Grapefruit juice, garlic, green tea (catechins), orange juice	Insulin
Topotecan, irinotecan	Haloperidol	Lopinavir
Vinca alkaloids (vinblastine, vincristine, vindesine)	hydrocortisone	Methotrexate
—	Imatinib	Mitoxantrone
—	Imipramine	Morphine
—	Lapatinib	Nelfinavir
—	Levothyroxine	Nevirapine
—	Loperamide	Nifedipine
—	Lopinavir	Paclitaxel
—	Loratadine	Phenobarbital
—	Methadone	Phenothiazines
—	Midazolam	Phenytoin
—	Mitomycin C	Prazosin
—	Nelfinavir	Ritonavir
—	Nilotinib	St. John's Wort
—	Proton-pump inhibitors (PPI) (lansoprazole, omeprazole, pantoprazole)	Tacrolimus
—	Phenothiazines	Tamoxifen
—	Progesterone	—
—	Promethazine	—
—	Propafenone	Trazodone

(continued overleaf)

TABLE 15.2 (continued)

Substrates (Chemotherapeutic Agents)	Inhibitors	Inducers
—	Ritonavir	Verapamil
—	SSRIs (paroxetine, sertraline, fluoxetine)	Vinblastine
—	Sorafenib	Vincristine
—	Sunitinib	Yohimbine
—	Statins (atorvastatin, simvastatin, lovastatin)	—
—	Spironolactone	—
—	Tacrolimus	—
—	Tamoxifen	—

Data are obtained from <http://www.genemedrx.com/PGPtable.php>.

metabolized by CYP3A4. This means that potential ABCB1 effect could be marked by the activity of ABCG2 or CYP3A4. Hence, a systemic analysis of polymorphisms in multiple genes known or suspected to contribute to drug disposition and response would provide better insights on the genetic impact of oral chemotherapy.

15.3.3 Other Factors

15.3.3.1 Antacid Medications. Most chemotherapeutic agents are weak acids or weak bases. They exist in solution at equilibrium between unionized and ionized forms. It is believed that only unionized species are sufficiently lipophilic to penetrate membranes. The fraction unionized is determined by both the pK_a of the drug and the pH in the GI tract. Based on the prediction of the pH partition hypothesis, weak bases are absorbed faster (because of predominant fraction unionized) in less acidic environment (e.g., pH 8.0) than in acidic condition (e.g., pH 1.0), and the converse holds for weak acids. The pH in GI tract varies between 1.5 and 7.5. Certain drugs (e.g., H_2 blockers and proton pump inhibitors) could alter the pH of GI fluid and thereby potentially influencing the oral absorption and pharmacokinetics of oral chemotherapeutic agents such as dasatinib or nilotinib [35,36]. It is noted that the pH change caused by H_2 blockers or proton pump inhibitors lasts for hours, and therefore, drug interaction may still occur even with separate administration of oral chemotherapeutic agents from acid suppressants. For instance, the solubility and bioavailability of dasatinib are pH dependent, diminishing at low pH values. It has been shown that dasatinib exposure was reduced by 60% even when it was administered 10 h after the administration of famotidine, a histamine H_2 receptor antagonist that inhibits stomach acid production [37]. As drug–drug interaction studies for many of the combinations of chemotherapeutic and antacid agents may not be available, the combination of oral chemotherapeutic agents with antacid medications should be used with caution and be avoided if possible.

15.3.3.2 Food Effect. It has been demonstrated that food affects the GI physiology (e.g., gastric emptying, intestinal motility, acid secretion, bile secretion, enzyme secretion, and blood flow); these changes may influence drug absorption, particularly

TABLE 15.3 Influence of Food on the Bioavailability of Selected Oral Chemotherapeutic Agents

Drug [Ref.]	Effect From Food	Recommended Administration
Melphalan [40]	39% reduction of AUC and 27% reduction of bioavailability	Fasting
Lapatinib [39]	167% increase of AUC by low fat meal and 325% increase of AUC by high fat meal	Fasting
Capecitabine [44]	51% increase of capecitabine AUC by fasting but only 13% increase of 5-FU AUC by fasting	With food
Nilotinib	50% increase of AUC by high fat diet	Fasting
Dasatinib [46]	14% increase in AUC by high fat meal	With or without
Pazopanib	~Twofold increase in AUC and $C_{\max}$ with a high fat or low fat meal.	1 h before or 2 h after a meal
Everolimus [47]	Delayed $T_{\max}$ by 1.75 h, reduced $C_{\max}$ by 53%, and reduced AUC by 21%	With or without
Vorinostat [48]	38% increase in AUC_{0-8} but decreased absorption rate (2.5-h delay in $T_{\max}$). Overall, no significant effect.	With food

AUC, area under the concentration–time curve; $C_{\max}$, maximum serum concentration.

for poorly water-soluble drugs [38]. The delayed gastric emptying in the presence of food would result in delayed drug absorption, but the longer gastric residence time would allow more time for dispersion and dissolution of poorly water-soluble drugs. The secretion of bile salts and the larger volume of GI fluid after a meal may also increase the dissolution rate of hydrophobic drugs, thereby increasing the extent of absorption [38]. It has been reported that the bioavailability of some oral chemotherapeutic agents is influenced by concurrent food intake. Table 15.3 presents the examples of food effects on the bioavailability of selected chemotherapeutic agents. For example, the systemic exposure [i.e., area under the plasma concentration–time curve (AUC)] of lapatinib is increased by 2.67-fold and 4.25-fold with a low fat and high fat meal, respectively, compared to the fasting state [39]. As interindividual absorption variability increases significantly with food intake and is unpredictable, lapatinib is recommended in a fasting state. The AUC of melphalan was reduced by 40% when the drug was administered with food [40]. The pharmacokinetics of gefitinib, sunitinib, sorafenib, and imatinib are not significantly affected by concomitant food consumption [41–43]. Given potential food effect on the oral absorption and pharmacokinetics, patients should be instructed to take oral chemotherapeutic agents with or without meal. For example, the combination of capecitabine and lapatinib is approved for the second-line human epidermal growth factor 2 (HER2) positive advanced breast cancer. Patients on this combination should take lapatinib on an empty stomach daily and capecitabine with meals twice a day. Another example is the use of dasatinib or nilotinib as the second-line treatment for patients with chronic myeloid leukemia (CML) after imatinib failure. Dasatinib is commonly prescribed once daily and can be taken with or without food, whereas nilotinib is taken twice daily and should be taken on an empty stomach because its absorption is increased when taken with food and could cause serious toxicities (e.g., QT prolongation).

15.3.3.3 Surgery. Several surgical procedures (e.g., gastrectomy, bariatric surgery, and the Whipple procedure) could potentially affect the absorption and pharmacokinetics of oral chemotherapeutic agents. For example, it has been shown that prior gastrectomy significantly reduces the bioavailability of nilotinib, thereby causing decreased efficacy [30]. Capecitabine is an oral agent frequently used in GI malignancy, and the influence of gastrectomy on capecitabine pharmacokinetics is currently being evaluated in an ongoing clinical trial. Data reported on the effect of gastrectomy on drug absorption are in some instances contradictory. For example, an oral fluoropyrimidine TS-1 (Taiho Pharmaceutical) is an orally active combination of tegafur (a prodrug of fluorouracil), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase, thereby inhibiting degradation of fluorouracil), and oteracil (which inhibits the phosphorylation of fluorouracil in the gastrointestinal tract thereby reducing gastrointestinal toxicity of fluorouracil) in a molar ratio of 1:0.4:1 while one study showed the pharmacokinetics of an oral fluoropyrimidine TS-1 was not altered by gastrectomy, another study indicated an increased absorption of TS-1 after gastrectomy [49,50].

15.4 MAJOR CLASSES OF ORAL CHEMOTHERAPEUTIC AGENTS

Broadly, oral chemotherapeutic agents can be classified as traditional cytotoxic agents (e.g., alkylating agents, antimetabolites, and plant alkyloids), novel molecularly targeted agents (e.g., small molecule TKIs, mTOR inhibitors, and HDAC inhibitors), hormonal agents (e.g., tamoxifen, letrozole, and anastrozole), and immune-modulating agents (e.g., thalidomide and lenalidomide). Table 15.4 summarizes the mechanisms of action and clinical pharmacology of the major classes of oral chemotherapeutic agents that are currently available for the treatment of cancers.

15.4.1 Traditional Oral Cytotoxic Agents

Traditional oral cytotoxic agents can be grouped into alkylating agents, antimetabolites, and plant alkyloids, based on their classification and phase of the cell cycle that they act on. Alkylating agents are cell cycle nonspecific. They cause cell death by forming DNA or RNA cross-links via a process known as alkylation (i.e., substituting an alkyl group for a hydrogen atom). Antimetabolites act on the S phase of cell cycle. They interfere with nucleic acid synthesis by substituting themselves for purines or pyrimidines or by inhibiting critical enzymes involved in nucleic acid synthesis. The plant alkyloids target the G2 phase of cell cycle. They inhibit topoisomerase II, thus preventing cells from entering the M phase of cell cycle.

15.4.1.1 Alkylating Agents. Alkylating agents have been available for several decades. The oral drugs in this class include busulfan, melphalan, cyclophosphamide, chlorambucil, estramustine, altretamine, procarbazine, and temozolomide [51–54]. Among them, busulfan, melphalan, and cyclophosphamide are available as formulations for both intravenous and oral administration. The mechanism of action and pharmacokinetic characteristics of busulfan, chlorambucil, cyclophosphamide, melphalan, and temozolomide are summarized in Table 15.4. Of note, cyclophosphamide and temozolomide are prodrugs that require metabolic activation to exert their pharmacological activities.

TABLE 15.4 Mechanisms of Action and Clinical Pharmacology of Major Classes of Oral Chemotherapeutic Agents^a

Drug	Mechanisms of Action	F (%)	T _{max} (h)	T _{1/2} (h)	Metabolism	Major Circulating Metabolites	Elimination	Plasma Protein Binding
Alkylating agents								
Chlorambucil	A bifunctional alkylating agent of the nitrogen mustard type cross-linking tumor cell DNA	100	1	1.5	Rapidly metabolized to active metabolites	Phenylacetic acid mustard (active)	<1% of the dose excreted in urine as unchanged drug	~99%
Cyclophosphamide	A prodrug that is biotransformed to active alkylating metabolites cross-linking tumor cell DNA	>75	N/A	3–12	Metabolized by a mixed function microsomal oxidase system in the liver to form active alkylating metabolites	4-Hydroxy cyclophosphamide (active)	5–25% of the dose excreted in urine as unchanged drug	Weakly bound
Melphalan	An alkylating agent of the bischloroethylamine type cross-linking DNA and RNA and inhibiting protein synthesis	100	1	1.5	Eliminated from plasma primarily by chemical hydrolysis to monohydroxymelphalan and dihydroxymelphalan	Monohydroxymelphalan	Low	60–90%, mostly to HSA and AAG

(continued overleaf)

TABLE 15.4 (continued)

Drug	Mechanisms of Action	F (%)	T _{max} (h)	T _{1/2} (h)	Metabolism	Major Circulating Metabolites	Elimination	Plasma Protein Binding
Temozolomide	A prodrug that undergoes rapid nonenzymatic conversion to the DNA alkylating compound MTIC	~100	~1	1.8	Spontaneously hydrolyzed at physiologic pH to the reactive species MTIC and to temozolomide acid metabolite	MTIC (active)	5.6% of the dose excreted in urine as unchanged drug	Weakly bound (~15%)
Antimetabolites								
Methotrexate	An antimetabolite that inhibits dihydrofolic acid reductase, thereby interfering with DNA synthesis, repair, and cellular replication	~60	1–2	3–15	Undergoes hepatic and intracellular metabolism to active polyglutamated forms, and a small amount of metabolism to 7-hydroxy-methotrexate	Polyglutamated metabolites (active); 7-hydroxy-methotrexate (inactive)	Renal excretion by glomerular filtration and active tubular secretion	50%
Fludarabine phosphate (2F-ara-AMP)	A synthetic purine nucleotide antimetabolite agent that is dephosphorylated in plasma to form the active metabolite (2F-ara-A), functioning as a DNA chain terminator	50–65	1–2	3	Rapidly dephosphorylated in plasma to active 2F-ara-A, which then enters into the cell	2F-ara-A (active)	Renal clearance of 2F-ara-A represents ~40% of the total body clearance of fludarabine phosphate	19–29%

Capecitabine	A prodrug that is metabolized to 5-FU, which is further converted to the active FdUMP and FUTP, thus interfering with DNA, RNA, and protein synthesis	55	0.75	1.5	Capecitabine is hydrolyzed by carboxyesterase in the liver to 5'-deoxy-5-fluorocytidine (5'-DFCR) that is subsequently converted by cytidine deaminase in most tissues (including tumors) to 5'-deoxy-5-fluorouridine (5'-DFUR) which is then hydrolyzed by thymidine phosphorylase to the active drug 5-FU	5-FU	95.5 and 2.6% of administered radioactivity recovered in urine and feces, respectively.	60%, mainly to HSA
Plant alkyls Etoposide	A topoisomerase II inhibitor that acts at the premitotic stage of cell division, inhibiting DNA synthesis	50	1	4–11	Metabolized mainly by hepatic CYP3A4, and to a lesser extent by CYP1A2	<i>O</i> -desmethyl etoposide (inactive); hydroxy acid [4'-demethylepipodophyllic acid-9-(4,6- <i>O</i> -(<i>R</i>)-ethylidene- β - <i>D</i> -glucopyranoside)] (inactive)	44–60%, up to 16%, and <6% of administered radioactivity recovered in urine, feces, and bile, respectively	97%, mainly to HSA

(continued overleaf)

TABLE 15.4 (continued)

Drug	Mechanisms of Action	F (%)	T _{max} (h)	T _{1/2} (h)	Metabolism	Major Circulating Metabolites	Elimination	Plasma Protein Binding
Tyrosine kinase inhibitors								
Imatinib	A TKI of Bcr-Abl, PDGF, and SCF, and c-Kit	98	2–4	15–18	Metabolized predominantly by hepatic CYP3A4, and to a lesser extent by CYP1A2, CYP2D6, CYP2C9, and CYP2C19	<i>N</i> -desmethyl imatinib (active)	5% of the dose excreted in urine as unchanged drug	95%, mostly to HSA and AAG
Dasatinib	A dual BCR-ABL/SRC family TKI; binding to active and inactive conformations of BCR-ABL, thereby overcoming the most common mechanisms of resistance to imatinib	~100	0.5–6	3.6–5.4	Metabolized in the liver by CYP3A4, flavin-containing mono-oxygenase-3, and UGTs	<i>O</i> -oxide desatinib (active)	<1 and 19% of the dose excreted in urine and feces, respectively, as unchanged drug	96%
Gefitinib	EGFR TKI	60	3–7	48	Metabolized predominantly by hepatic CYP3A4 and CYP3A5, and to a lesser extent by CYP2D6	<i>O</i> -desmethyl gefitinib (active)	<4% of the dose excreted in urine as unchanged drug	97%, mostly to HSA and AAG

Erlotinib	EGFR TKI	60	4	36	Metabolized predominantly by CYP3A4/5, and to a lesser extent by CYP1A2 and CYP1A1	OSI-420, OSI-413	<9% of the dose excreted in urine as unchanged drug	93%, mostly to HSA and AAG
Sorafenib	A multikinase inhibitor, inhibiting C-Raf, B-Raf (wild-type and mutant), ERK, VEGFR (types 1, 2, and 3), PDGFR- β , Flt-3, c-KIT, and p38 α , a member of the MAP kinase family	38-49	2-6	25-48	Metabolized by hepatic CYP3A4 and UGT1A9	<i>N</i> -oxide sorafenib (active)	77% and 19% of administered radioactivity recovered in feces and urine, respectively	99.5%
Sunitinib	A broad-spectrum receptor TKI, specifically inhibiting VEGFR (types 1, 2, and 3), PDGFR α and PDGFR- β , c-KIT, Flt-3, colony-stimulating factor receptor type 1, and the glial cell-line-derived neurotrophic factor receptor	59	6-12	40-60	Metabolized predominantly by hepatic CYP3A4	SU012662 (active)	61 and 16% of administered radioactivity recovered in feces and urine, respectively	95%

(continued overleaf)

TABLE 15.4 (continued)

Drug	Mechanisms of Action	F (%)	T _{max} (h)	T _{1/2} (h)	Metabolism	Major Circulating Metabolites	Elimination	Plasma Protein Binding
Pazopanib	multi-tyrosine kinase inhibitor of VEGFR-1, 2, and 3, PDGFR- α and - β , FGFR -1 and 3, cytokine receptor (Kit), interleukin-2-receptor-inducible T-cell kinase (Itk), leukocyte-specific protein tyrosine kinase (Lck), and transmembrane glycoprotein receptor tyrosine kinase (c-Fms)	—	2–4	31	Metabolized mainly by CYP3A4, with minor contribution from CYP1A2 and CYP2C8	—	Primarily via feces with renal elimination accounting for <4% of the administered dose	99%
Lapatinib	Inhibiting EGFR and HER2	N/A	3–4	14–24	Metabolized predominantly by hepatic CYP3A4 and CYP3A5, and to a lesser extent by CYP2C19 and CYP2C8	O-dealkylated lapatinib (reactive) and N-dealkylated lapatinib (reactive)	27 and <2% excreted in feces and in urine, respectively, as unchanged drug	99%, mostly to HSA and AAG
mTOR inhibitors								
Everolimus	A derivative of rapamycin, inhibiting mTOR	N/A	1–2	30	Metabolized by CYP3A4	Hydroxyl- and desmethyl-metabolites (inactive)	80% of administered radioactivity recovered in feces as metabolites	74%

Histone deacetylase inhibitors									
Vorinostat	Inhibiting the enzymatic activity of histone deacetylases HDAC1, HDAC2, and HDAC3 (Class I) and HDAC6 (Class II), thus causing the accumulation of acetylated histones and inducing cell cycle arrest and/or apoptosis of some transformed cells	43	2–10	1.3–2	The major pathways of vorinostat metabolism involve glucuronidation and hydrolysis followed by β -oxidation	<i>O</i> -glucuronide of vorinostat (inactive) and 4-anilino-4-oxobutanoic acid (inactive)	<1% of the dose excreted in urine as unchanged drug	71%	
Hormonal agents									
Tamoxifen	A nonsteroidal agent with antiestrogenic effects through competitive binding to estrogen receptor	N/A	5	120-168	Extensively metabolized by CYP3A4, CYP3A5, CYP2D6, and CYP2C9	Endoxifen (active)	65% of administered radioactivity recovered in feces	Moderate bound	
Letrozole	A nonsteroidal competitive inhibitor of aromatase, thus inhibiting the conversion of androgens to estrogens	100	1–2	48	Metabolized by CYP3A4 and CYP2A6 to inactive metabolites whose glucuronide conjugate is excreted renally.	Inactive metabolites	90% of administered radioactivity recovered in urine, among which 75% and 6% as glucuronide metabolite and unchanged drug respectively.	Weakly protein bound	

(continued overleaf)

TABLE 15.4 (*continued*)

Drug	Mechanisms of Action	F (%)	T _{max} (h)	T _{1/2} (h)	Metabolism	Major Circulating Metabolites	Elimination	Plasma Protein Binding
Anastrozole	A potent and selective nonsteroidal aromatase inhibitor, significantly lowering serum estradiol concentrations and has nodetectable effect on formation of adrenal corticosteroids or aldosterone	—	—	50	Metabolized by <i>N</i> -dealkylation, hydroxylation, and glucuronidation. Three metabolites of anastrozole have been identified in human plasma and urine. The known metabolites are triazole, a glucuronide conjugate of hydroxyanastrozole, and a glucuron	Two major inactive metabolites: triazole (a glucuronide conjugate of hydroxyanastrozole) and a glucuronide conjugate of anastrozole	Eliminated primarily via hepatic metabolism (~85%) and to a lesser extent, renal excretion (~11%)	40%
Exemestine	An irreversible, steroidal aromatase inactivator that binds irreversibly to the active site of aromatase causing its inactivation	42	1.2–2.9	24	Metabolized by CYP3A4 and aldoketoreductases	17-Hydroxy-exemestane	<1% of the dose excreted in urine as unchanged drug	90%, mostly to HSA and AAG

Immune-modulating agents								
Thalidomide	Possessing immunomodulatory, antiinflammatory, and antiangiogenic properties	N/A	2.9–5.7	5–7	Undergo nonenzymatic hydrolysis in plasma to multiple products	N/A	<0.7% of the dose excreted in urine as unchanged drug	55 and 66%, respectively, for (+)-(<i>R</i>)- and (–)-(<i>S</i>)-thalidomide
Lenalidomide	Possessing immunomodulatory, antiangiogenetic, antineoplastic properties	50	0.5–4	3	Unknown	N/A	67% of the dose excreted in urine as unchanged drug	30%

AAG, alpha-1 acid glycoprotein; CYP, cytochrome P450; F, oral bioavailability; HSA, human serum albumin; TKI, tyrosine kinase inhibitor; $T_{\max}$, time to reach maximum plasma concentration; $T_{1/2}$, elimination half-life; UGT, UDP-glucuronosyltransferase; MTIC, 3-methyl-(triazene-1-yl)imidazole-4-carboxamide; 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-2-deoxyuridinemonophosphate; FUTP, 5-fluorouridine triphosphate; PDGF, platelet-derived growth factor; SCF, stem cell factor; Flt-3, Fms-like tyrosine kinase-3; FGFR, fibroblast growth factor receptor; Bcr-Abl, breakpoint cluster region-Abelson; c-KIT, stem cell growth factor receptor; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor.

N/A, data not available.

^aData are obtained from the drug package inserts.

Oral busulfan is a relatively inexpensive and well-tolerated drug at low doses. It is widely used for benign as well as malignant hematological diseases. High dose busulfan is often incorporated into hematopoietic stem cell transplant preparative regimens, but the bioavailability of high dose oral busulfan varies widely from 10% to 100% [55]. Variable bioavailability can cause substantial variability of system exposure to busulfan. Increased systemic exposure may result in a higher incidence of toxicity such as veno-occlusive disease (VOD) [56,57], whereas inadequate drug exposure may lead to graft failure [58,59]. Owing to the large interindividual pharmacokinetic variability of busulfan at high doses, many transplant centers only use intravenous busulfan for transplant conditioning. However, the intravenous formulation of busulfan is not available in many countries and is more expensive than oral formulation. Oral melphalan is currently used for patients with multiple myeloma who are not transplant candidates [60]. Use of melphalan should be avoided for potential transplant candidates as exposure to alkylating agents can make stem cell collection difficult and delay marrow recovery post transplant [61,62]. Similar to busulphan, high dose oral melphalan is associated with variable drug absorption and substantial pharmacokinetic variability. Intravenous melphalan, however, is safer and has a much more predictive outcome than oral melphalan for transplant conditioning [63,64].

Cyclophosphamide can be administered intravenously or orally. It is well absorbed after oral administration with a bioavailability of >75%. Notably, cyclophosphamide exhibits 3.5 times more alkylating potency when given orally than when given intravenously [65]. Cyclophosphamide is a prodrug that requires biotransformation to active alkylating metabolites, principally by a mixed function microsomal oxidase system in the liver. There are multiple active metabolites of cyclophosphamide, including 4-hydroxy-cyclophosphamide, alcophosphamide, 4-keto-cyclophosphamide, phosphoramidate mustard, and acrolein. Acrolein is a known causative metabolite of hemorrhagic cystitis, a condition occurring more commonly when cyclophosphamide is administered intravenously than orally [66,67]. Recently, metronomic use (frequent administration of low dose) of oral cyclophosphamide has been increasingly used for the treatment of metastatic breast cancer, especially for elderly and/ or borderline poor performance status patients, given its favorable toxicity profile and comparable efficacy compared to conventional every 3-week administration [68,69].

Temozolomide is also a prodrug that undergoes rapid nonenzymatic conversion to the DNA-alkalating metabolite, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC). It is almost completely absorbed after oral administration. Temozolomide is approved by the FDA for the treatment of central nervous system (CNS) malignancies including glioblastoma multiforme and other solid tumors [70]. Many alkylating agents can penetrate cerebrospinal fluid (CSF) with various CSF penetration rates (AUC_{CSF}/AUC_{plasma}) [71]. The CSF penetration rate of temozolomide is 20%, giving it some advantage for sensitive CNS malignancies [72].

It is interesting to note that both intravenous and oral formulations of ifosfamide were investigated, but only intravenous administration is approved by the US FDA. Like cyclophosphamide, ifosfamide is also a prodrug that requires bioactivation in the liver. When given orally, ifosfamide caused increased neurological toxicity, probably because of high plasma levels of its toxic metabolite chloroacetaldehyde that is formed from first-pass metabolism [73]. A randomized phase II study comparing intravenous and oral administration of ifosfamide in patients with non-small-cell lung cancer (NSCLC) was prematurely terminated because of excessive neurotoxicity in the

oral administration cohort [74]. This example of enhanced toxicity with oral ifosfamide alerted the oncology community that first-pass metabolism may play a critical role in the toxicity profile of oral chemotherapeutic agents.

15.4.1.2 Antimetabolites. Antimetabolites exert antitumor activity by interfering with nucleic acid synthesis or nucleotide synthesis. The oral drugs in this class include fludarabine, methotrexate, and capecitabine. Fludarabine is a purine antagonist. Intravenous fludarabine is used to treat select hematological malignancies. It is an important component of chemotherapeutic regimens for the treatment of chronic lymphoid leukemia (CLL) [75,76]. Oral fludarabine has demonstrated comparable clinical efficacy to intravenous fludarabine and has been recently approved by the FDA for treatment of CLL [77,78].

Capecitabine is an oral fluoropyrimidine and a prodrug of 5-fluorouracil (5-FU). It is widely used for the treatment of a variety of solid tumors including colorectal, breast, and gastric cancers. Capecitabine is hydrolyzed by carboxylesterase in the liver to form 5'-deoxy-5-fluorocytidine (5'-DFCR) that is subsequently converted by cytidine deaminase in tissues (including tumor cells) to 5'-deoxy-5-fluorouridine (5'-DFUR), which is then hydrolyzed by thymidine phosphorylase to 5-FU. 5-FU is further converted to the active 5-fluoro-2-deoxyuridine monophosphate (FdUMP) and 5-fluorouridine triphosphate (FUTP), thus interfering with DNA, RNA, and protein synthesis. Three enzymes, carboxylesterase, cytidine deaminase, and thymidine phosphorylase, play important roles in the bioactivation of capecitabine. As tumor cells express higher thymidine phosphorylase, capecitabine works more specifically on tumor cells than normal tissues [79–81]. Multiple clinical studies and a large meta-analysis support that capecitabine as a single agent or in combination with other chemotherapies demonstrates equivalent clinical benefit as intravenous 5-FU for the treatment of metastatic colorectal and gastric cancers [82–87]. The toxicity profile of capecitabine is, overall, comparable to continuous intravenous infusion of 5-FU [87].

Uracil-ftorafur (UFT) is an oral fluoropyrimidine-based formulation consisting of two components: tegafur, a prodrug of 5-FU, and uracil, a dihydropyrimidine dehydrogenase (DPD) inhibitor. DPD is an important enzyme in metabolism/detoxification of 5-FU. The vast majority (~80–85%) of administered 5-FU is metabolized by DPD in the liver into the inactive form, dihydrofluorouracil (FDHU) and excreted as a fluoro- β -alanine. DPD expression has been related to tolerance and response to 5-FU-based chemotherapy. Specifically, low expression or absence of DPD has been associated with greater accumulation of 5-FU and thereby, increased risk of severe toxicities, while high expression of DPD has been associated with poor response to 5-FU [88,89]. Patients lacking the DPD enzyme may experience severe to lethal toxicities when receiving 5-FU-based chemotherapy. Genetic aberration in the dihydropyrimidine dehydrogenase (*DPYD*) gene, such as exon skipping, deletion, and missense mutation, contributes to a DPD deficiency phenotype. The prevalence of DPD deficiency varies among different races, highest in African-Americans (8%), modest in Caucasians (2.8%), and lowest in Asians (<1%) [90–92]. The most known *DPYD* SNPs associated with grade 3 and 4 toxicities are IVS14 + 1G > A, 2846A > T, 1679T > G and 85T > C [93]. In particular, the exon-14-skipping mutation IVS14 + 1G > A, a G-to-A point mutation within the 5'-splicing site of intron 14, leads to a mutant DPD that lacks amino acids 581–635 and consequently lacks catalytic activity. The allele frequency of this mutation was 0.91% in a Dutch Caucasian population [94]. In

patients heterozygous for the IVS14 + 1G > A allele, half of the mean normal activity of DPD is found, which is sufficient to lead to severe 5-FU toxicities. In patients homozygous for the IVS14 + 1G > A allele, DPD activity is completely lacking and 5-FU toxicities become life-threatening and sometimes fatal [95,96]. The idea of combination of a DPD inhibitor with 5-FU is to make everyone DPD deficient regardless of the DPD genotype, and thus no genotyping-guided dose adjustment is needed.

Similar to UFT, TS-1 is an oral fluoropyrimidine-based formulation consisting of three components: tegafur (a 5-FU prodrug), 5-chloro-2,4-dihydroxypyridine (a DPD inhibitor), and potassium oxonate (an inhibitor of orotate phosphoribosyltransferase). Potassium oxonate inhibits 5-FU phosphorylation, which is believed to be the cause of the GI toxicities associated with 5-FU [97]. There is a paucity of data directly comparing the efficacy and toxicity among these three oral fluoropyrimidines. One phase II trial conducted in South Korea showed similar efficacy and side effects between capecitabine and S-1 in elderly Korean patients [98]. At present, only capecitabine is available in the United States.

15.4.1.3 Plant Alkylolds. *pt*Etoposide (VP-16) is a semisynthetic derivative of podophyllotoxin whose first topical use for antineoplastic purposes was reported in the nineteenth century [99]. Etoposide can cause single-strand DNA breaks and cell death in G2 phase via topoisomerase II inhibition. Both intravenous and oral etoposide are available and are used widely for a variety of solid and hematological malignancies. Oral absorption of etoposide exhibits dose-dependency, with lower bioavailability at higher doses [100,101]. To overcome this problem, the prodrug of etoposide, etoposide phosphate, was investigated. However, the pharmacokinetics of etoposide phosphate was found to be similar to that of etoposide [102]. In clinical practice, the oral etoposide is rarely used as it is only approved for small cell lung cancer and it is largely used for palliative purpose with occasional use in curative intent [103,104]. The recommended oral dose is twice of the intravenous dose.

15.4.2 Novel Molecularly Targeted Oral Agents

15.4.2.1 Small Molecule Tyrosine Kinase Inhibitors (TKIs). Small molecule TKIs directly inhibit the enzymatic activity of the target protein kinase by interfering with the ATP-binding site of the tyrosine kinase (TK), thereby preventing autophosphorylation of the kinase and subsequently blocking the downstream cellular signaling pathways that play an important role in tumor growth and progression. Table 15.5 lists the FDA-approved small molecule TKIs and their main targets. The use of small molecule TKIs has significantly changed the outcomes for some cancers. For example, imatinib has had a dramatic effect on CML, and sunitinib has revolutionized the treatment of renal cell carcinoma (RCC). Meanwhile, these agents have expanded the concept of individually tailored cancer treatment because they are likely to be effective in subsets of patients whose tumors have specific molecular traits. For instance, specific mutations in the epidermal growth factor receptor (EGFR) TK domain in non-small cell lung cancer (NSCLC) are important determinants of dramatic response to the EGFR inhibitors gefitinib and erlotinib. Kinase mutations also play a role in the intrinsic or acquired resistance to TKI therapy. One example is from imatinib whereby point mutations in breakpoint cluster region-abelson (BCR-ABL) render the ABL kinase

TABLE 15.5 Small Molecule Tyrosine Kinase Inhibitors Approved by the US FDA for Cancer Treatment

Drug	Main Target	FDA-Approved Indications
Imatinib (Gleevec)	BCR-ABL, c-KIT, PDGFR	Acute lymphocytic leukemia, chronic myeloid leukemia, gastrointestinal stromal tumor
Gefitinib (Iressa)	EGFR	Non-small-cell lung cancer
Erlotinib (Tarceva)	EGFR	Non-small-cell lung cancer, pancreatic cancer
Lapatinib (Tykerb)	HER2/neu, EGFR	Breast cancer with HER2/neu overexpression
Dasatinib (Sprycel)	BCR-ABL, SRC family, c-KIT, PDGFR	Chronic myeloid leukemia, acute lymphocytic leukemia
Sorafenib (Nexavar)	B-RAF, VEGFR, EGFR, PDGFR	Renal cell carcinoma, hepatocellular carcinoma
Sunitinib (Sutent)	VEGFR, PDGFR, c-KIT, FLT-3	Renal cell carcinoma, gastrointestinal stromal tumor
Pazopanib (Votrient)	VEGFR, PDGFR, c-KIT	Renal cell carcinoma

BCR-ABL, breakpoint cluster region-Abelson; c-KIT, stem cell growth factor receptor; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor; FLT-3, Fms-like TK.

resistant to imatinib, which is associated with relapse in patients with advanced CML receiving imatinib.

Imatinib (STI 571, Gleevec®) is the first-approved small molecule TKI. It targets the Philadelphia-chromosome-associated BCR/ABL fusion TK, a genetic causal mechanism of CML. Imatinib also inhibits platelet-derived growth factor receptor (PDGFR) and c-kit, a proto-oncogene linked to gastrointestinal stromal tumors (GISTs). Imatinib is FDA approved for the treatment of CML and GISTs [105,106]. In a phase III trial, 75 patients with CML receiving imatinib achieved a major cytogenetic response, which was durable with an 85% overall survival at eight years [107–109]. Primary resistance to imatinib occurs in 10–15% of patients. Primary resistance could be due to inadequate intracellular drug concentration. Several mechanisms are implicated in the decrease of effective intracellular concentration of imatinib, among which the most important includes the increased protein binding to alpha-1 acid glycoprotein [110,111], increased drug efflux due to overexpression of ABC transporters such as ABCB1 and ABCG2 [112,113], or reduced cellular uptake of imatinib due to dysregulation of uptake transporters such as the organic cation transporter (OCT1) [114,115]. Acquired (secondary) resistance after initial response to imatinib is observed in 8–10% of patients. The most common molecular mechanism of acquired resistance to imatinib is point mutations at the kinase domain that change the conformation of the c-ABL kinase, thus reducing imatinib-binding affinity to the kinase. More than 50 different point mutations in *ABL* have been identified to be associated with imatinib resistance [116]. For example, the T315I mutation was identified in 15% of patients with CML developing resistance to imatinib [117]. The drug-resistant BCR-ABL point mutation can be found in imatinib-naïve or imatinib-treated patients with CML. To target imatinib-resistant mutant forms of BCR-ABL, second generation of ABL kinase inhibitors such as nilotinib and dasatinib have been developed. Nilotinib was specifically designed

to target mutant forms of BCL-ABL, showing ~30-fold more potency than imatinib for inhibition of mutant BCL-ABL [116]. Dasatinib is a potent inhibitor of BCL-ABL, Src-family kinases, KIT, and PDGFR. On the basis of promising data from phase I and II trials, dasatinib has been recently approved for the treatment of patients with imatinib-resistant CML or Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL). About 50% of patients with acquired resistance to imatinib achieve major cytogenetic response to dasatinib and nilotinib [118,119].

Gefitinib and erlotinib are orally bioavailable, small molecule EGFR-TK inhibitors that target the ATP cleft within the EGFR-TK to prevent autophosphorylation of the receptor, and thus inhibit tumor growth and metastasis. Treatment with gefitinib or erlotinib results in dramatic clinical response in ~10–30% patients with NSCLC depending on ethnic origin, sex, and smoking history [120–122]. It is clear that specific types of somatic mutations of the EGFR gene confer sensitivity or resistance to EGFR TKI [123,124]. The somatic activating EGFR mutations present in the first four exons (18 through 21) of the TK domain [125–127]. The most common activating mutations include in-frame deletions in exon 19 (e.g., deletion of amino acid residues leucine 747 to glutamic acid 749), single nucleotide mutation (e.g., L858R in exon 21 and G719 in exon 18), and in-frame duplications and/or insertions in exon 20. Overall, the frequency of EGFR-TK-activating mutations is 5–20%, occurring more common in East Asians, women, never smokers, and patients with adenocarcinoma [128]. This mirrors the clinically defined subsets of patients who were most likely to respond to EGFR-TK inhibitors. Like other TKIs, acquired resistance occurs in virtually all NSCLC tumors that initially respond to EGFR-TK inhibitors. The emergence of acquired resistance is conferred by a second point mutation in the TK domain. The most noteworthy clinically relevant mutations associated with resistance to EGFR-TK inhibitors include T790M in exon 20, and D761Y, a T790M-like secondary mutation, in exon 19 [129,130].

Sorafenib, sunitinib, and pazopanib are orally active, small molecule multitargeted TKIs with effects on tumor cell proliferation and tumor angiogenesis. Sorafenib was initially developed as a Raf kinase inhibitor. It was subsequently found to inhibit a variety of kinase receptors, including vascular endothelial growth factor receptors (VEGFR)-1, -2, and -3; PDGFR- β ; FMS-like tyrosine kinase 3 (FLT-3); stem cell factor receptor (KIT); and glial-cell-line-derived neurotrophic (RET) receptor tyrosine kinases. Similarly, sunitinib is a potent inhibitor for VEGFR-1, -2, and -3; c-KIT; PDGFR- α and - β ; FLT-3; colony-stimulating factor receptor type 1 receptor; and RET receptor tyrosine kinase (RET). Pazopanib inhibits vascular epidermal growth factor receptor-1, -2 and -3, PDGFR- α and - β , and c-kit. These agents directly and indirectly regulate tumor growth and angiogenesis. They have been approved for the treatment of advanced RCC, with a response rate of 4% (sorafenib), 25–36% (sunitinib), and 30% (pazopanib). Sorafenib and sunitinib have also been approved for the treatment of hepatocellular carcinoma and GI stromal tumor, respectively. Notably, before their approval, there was no accepted standard treatment for advanced RCC, as previously approved agents [interferon (IFN)- α and interleukin (IL)-2] showed minimal benefit and significant side effects. The multitargeted TKIs have demonstrated clinical benefit and are becoming components of standard of care in patients with metastatic RCC. However, almost all patients develop resistance to treatment eventually, and cure is rarely seen. The majority of patients have transient benefit from therapy before tumors regrow and metastasize (i.e., acquired resistance), and a

small fraction of patients fail to show even initial clinical benefit (i.e., intrinsic resistance). Yet, the exact mechanisms of tumor resistance to these agents are not fully understood.

Lapatinib is a dual TKI that target two members of the human EGFR family, ErbB-1 (EGFR) and ErbB-2 (HER-2) [131]. Although it has EGFR inhibition, its antitumor effect is exclusively observed in HER-2-overexpressing breast cancer [132]. Lapatinib, as a single agent, has modest clinical antitumor activity and can be combined with trastuzumab, cytotoxic agents, and/or endocrine therapy [133–135]. Lapatinib is currently approved only for trastuzumab refractory patients. It is unknown if lapatinib has the same efficacy as trastuzumab in both the metastatic and adjuvant settings. However, ongoing trials will answer this question.

15.4.2.2 mTOR Inhibitors. The mTOR is a serine–threonine kinase and a key protein in the PI3K-AKT pathway. Sirolimus, initially known as rapamycin, forms a complex with its receptor FKBP12 and inhibits the mTOR complex 1 (Mtorc1) [136]. Sirolimus was originally developed as an antifungal drug. It was found to have multiple functional characteristics including antiproliferative, immunosuppressive, and antineoplastic effects. Sirolimus is currently approved by the FDA for use as an immunosuppressant after organ and bone marrow transplant but not as an anticancer agent. Everolimus (oral formulation) and temsirolimus (intravenous formulation) are the analogs of sirolimus. Both demonstrate anticancer activity. Temsirolimus was approved for advanced RCC in poor risk categories in the first-line setting, and everolimus was approved for the second-line treatment for metastatic RCC after failing VEGF inhibitors [137,138].

15.4.2.3 Histone Deacetylase Inhibitors. Vorinostat or suberoylanilide hydroxamic acid (SAHA) is a HDAC inhibitor. Epigenetic modulation of gene expression by histone acetylation was found to play a role in oncogenesis in many cancer types [139]. HDAC inhibitors induce histone acetylation, apoptosis, and cell cycle arrest. In a phase II study in patients with refractory cutaneous T-cell lymphoma, vorinostat demonstrated an overall response rate of 30%, with a median duration of response more than six months. This result led to FDA approval of vorinostat for the treatment of patients with refractory cutaneous T-cell lymphoma [140]. The efficacy of vorinostat has been investigated in multiple other cancers including solid tumors. One randomized placebo-controlled phase II study showed that vorinostat in combination with carboplatin and paclitaxel showed superior response rate when compared with carboplatin, paclitaxel, and placebo [141].

15.4.3 Hormonal Agents

Tamoxifen, a selective estrogen receptor (ER) modulator, is a standard endocrine therapy for the treatment and prevention of ER-positive breast cancer. ER-positive breast cancers are often dependent on estrogen for growth. Selective ER modulators bind to the ligand-binding domain of an ER and block the binding of estrogen. This prevents conformational changes of the ER that is required for its association with coactivators, thus blocking transcriptional activation functions of the ER and subsequently reducing or eliminating estrogen-driven proliferation of ER-positive tumors. Tamoxifen is a prodrug that requires metabolic activation to exert its pharmacological activity. The

metabolism of tamoxifen is involved in hepatic phase I enzymes (including CYP3A4, CYP3A5, CYP2C9, CYP2C19, CYP1A2, CYP2B6, and CYP2D6, as well as flavin-containing mono-oxygenase-1 and -3) and phase II enzymes (such as sulfotransferase 1A1 (SULT1A1)) [142–144]. Specifically, tamoxifen is metabolized by hepatic CYP enzymes (mainly by CYP2D6 and CYP3A4/5) to form two main primary metabolites, 4-hydroxytamoxifen and *N*-desmethyltamoxifen. Both these metabolites are further converted to abundant and pharmacologically active 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). Endoxifen formation from *N*-desmethyltamoxifen is almost exclusively catalyzed by CYP2D6, and formation from 4-hydroxytamoxifen by CYP3A4/5 [143]. In addition, tamoxifen and its metabolites undergo phase II metabolism including sulphation and glucuronidation. Endoxifen and 4-hydroxytamoxifen show much greater affinity for the ER than tamoxifen [143,145]. While 4-hydroxytamoxifen and endoxifen have a similar antiestrogen activity, endoxifen plasma concentrations are 6- to 12-fold higher than those of 4-hydroxytamoxifen [146], suggesting that endoxifen is the predominant and crucial active metabolite responsible for the *in vivo* pharmacological activity of tamoxifen.

Given the key role of CYP2D6 in catalyzing the conversion of tamoxifen to its abundant active metabolite endoxifen, altered CYP2D6 activity due to either genetic or environmental (drug-induced or drug-inhibited) factor could directly affect endoxifen concentrations and likely the clinical outcome of patients treated with tamoxifen. There is evidence that women with nonfunctional and reduced-function *CYP2D6* alleles appear to have significantly lower circulating endoxifen concentrations than those with wild-type *CYP2D6* [147,148]. Similarly, concomitant CYP2D6 inhibitors, such as certain selective serotonin reuptake inhibitors (SSRIs) (fluoxetine and paroxetine) or selective noradrenaline reuptake inhibitors (SNRIs), have been shown to reduce the plasma concentrations of endoxifen [147,149]. It has also been shown that the use of CYP2D6 inhibitors such as SSRIs and SNRIs has a negative impact on the efficacy of tamoxifen [147]. Collectively, these data support the notion that low CYP2D6 activity, caused by genetic polymorphisms or drug interactions, leads to low levels of the active tamoxifen metabolite. The effect of CYP2D6 activity on tamoxifen pharmacokinetics also translates into an effect on clinical outcome. Despite conflicting data in some instances, the majority of retrospective studies suggest that the presence of nonfunctional or reduced-function alleles of CYP2D6 is associated with worse outcome of patients receiving tamoxifen [150,151]. Notably, a recent large retrospective analysis of 1325 patients with early-stage breast cancer treated with adjuvant tamoxifen suggests that compared to extensive metabolizer, those with decreased CYP2D6 activity (heterozygous extensive/intermediate and poor metabolizers) have significantly increased risk of recurrence as well as worse event-free survival and disease-free survival [152]. In addition, *CYP2D6* genotype has also been shown to influence the efficacy of tamoxifen as a chemopreventive agent, whereby tamoxifen-treated women with poor metabolizer phenotype was associated with a significantly higher incidence of breast cancer compared to controls [153]. Findings from these studies support a role for the *CYP2D6* genotype in the activation of tamoxifen and the likelihood of therapeutic benefit from testing for *CYP2D6* genotype.

Aromatase inhibitors (AIs) are also used for the treatment of hormone-sensitive breast cancer in postmenopausal women. AIs inhibit peripheral conversion of testosterone and androstenedione to estradiol. In premenopausal women whose primary

source of estrogen is ovarian production, the AIs have no clinical efficacy. In the United States, two nonsteroidal AIs, letrozole and anastrozole, and one steroidal AI, exemestane, are FDA approved as oral formulation of AIs. Studies indicate that letrozole has more potency to suppress estrogen activity than anastrozole, but it is not known if this difference is clinically relevant [154]. Although AIs can suppress estradiol levels within one day of drug initiation, clinical and/or radiographic evidence of disease response may sometimes lag behind by several weeks [155]. Multiple large randomized studies and meta-analyses clearly showed superior efficacy of AIs over tamoxifen for the treatment of receptor-positive breast cancer in postmenopausal women in metastatic, adjuvant, and neoadjuvant settings [156–163]. Although the effect of hormonal agents is thought to be primarily cytostatic, it is interesting to observe tumor regression in a substantial number of patients with receptor-positive tumors, including clinical and radiographic complete responses (CR) [164].

Abiraterone is a new agent that irreversibly inhibits cytochrome P450 17 (CYP17) α -hydroxylase, a key enzyme converting steroid to testosterone [165]. For patients with advanced prostate cancer, the initial treatment of choice is chemical or surgical castration by luteinizing-hormone-releasing hormone (LHRH) agonist with or without an androgen receptor antagonist. Ketoconazole, which is a less potent α -hydroxylase inhibitor, confers response in castration-resistant prostate cancer (CRPC), but the therapy causes some toxicity as high doses are required for adequate enzymatic suppression. Additionally, it did not improve the overall survival [166]. Abiraterone is specific to α -hydroxylase and is generally well tolerated. In a phase III trial, abiraterone improved the survival of patients with CRPC who had failed docetaxel [167].

15.4.4 Immune-Modulating Agents

Thalidomide was initially sold as a sedative drug in the late 1950s outside of the United States. Owing to its teratogenic effects, thalidomide was withdrawn from the worldwide market in 1962. The exact antineoplastic mechanism of thalidomide is unknown. It demonstrates multiple mechanisms of action including immunomodulatory and antiangiogenic effects. Thalidomide has been investigated for the treatment of many different solid and hematological malignancies. It is currently approved for the treatment of multiple myeloma and is used widely in many countries. Owing to its extreme water insolubility, only oral thalidomide is currently available. Patients with multiple myeloma often have renal impairment at the time of diagnosis. The pharmacokinetics of thalidomide was studied in the population with renal impairment. No dose adjustment is necessary for renal impairment, and no supplementation is required after hemodialysis [168]. Thalidomide is detectable in semen, and therefore, sexually active male patients and their partners should use reliable contraceptive methods to avoid pregnancy [169].

Lenalidomide is an analogue of thalidomide and has more potent immunomodulatory activity. Lenalidomide is also approved for myelodysplastic syndrome with chromosome 5q deletion [170]. Unlike thalidomide, lenalidomide has no sedative effect and causes less peripheral neurotoxicity at therapeutic doses. Renal impairment prolongs the plasma half-life of lenalidomide and significantly increases its AUC [171], and therefore care should be taken when lenalidomide is administered in patients with renal impairment.

15.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The use of oral chemotherapeutic agents has been increased, especially in the recent decade. Besides traditional oral cytotoxic agents, a number of novel oral chemotherapeutic agents with targeted mechanisms of action are available, and many more are under clinical development. An ideal oral chemotherapeutic agent is characterized by a proven efficacy, a good bioavailability and predictable pharmacokinetic profile, and good tolerability. Providing efficacy and tolerability are not compromised; oral chemotherapy offers obvious advantages in terms of patient convenience, improved quality of life, and cost savings. In particular, chronic oral administration is critical for novel molecular targeted agents that generally require a continuous drug exposure and protracted treatment course to achieve antitumor activity. It would be anticipated that oral formulations will become the primary route of chemotherapy administration in the settings of palliative care oncology and adjuvant chemotherapy.

Knowledge and understanding of the pharmacokinetics (i.e., absorption, distribution, metabolism, and elimination) and molecular mechanisms of action and resistance will allow rational use of oral chemotherapeutic agents to achieve optimal efficacy and to avoid undesirable drug interactions and toxicities. Unique to oral agents, absorption is the prerequisite for systemic exposure and activity. Low or variable bioavailability could be due to the first-pass metabolism by CYP3A4 and intestinal efflux by transporters such as ABCB1. Altered activity of CYP3A4 or ABCB1, either caused by genetic or environmental factors (e.g., drug-induced or –inhibited factors), may modulate the bioavailability and pharmacokinetics of oral chemotherapeutic agents that are CYP3A4 or ABCB1 substrates. In addition, concomitant antacid medicines or food can affect the bioavailability of certain oral chemotherapeutic agents (e.g., imatinib and dasatinib). In the era of genomics and personalized medicine, it has been increasingly recognized that the overall response to a drug is determined by the interplay of multiple genes that are involved in the drug disposition (e.g., drug-metabolizing enzymes and transporters) and drug–target interactions (e.g., target receptors and enzymes). The significance of pharmacogenomics in oral chemotherapy is exemplified by 5-FU, tamoxifen, and small molecule TKIs (e.g., gefitinib, erlotinib, and imatinib). Notably, the use of orally bioavailable, small molecule TKIs has revolutionized the treatment of certain cancers and has also expanded the concept of individually tailored cancer treatment. These agents are generally effective in subsets of patients whose tumors have specific molecular traits. Identification of genetic markers predictive of response or resistance will aid in defining the patient subpopulation that most likely derive therapeutic benefit from these novel molecularly targeted agents.

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16 Drug Metabolism and Interactions in Pediatric Patients

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16.1	Introduction	1
16.2	Drug metabolism and interaction in relation to phase I drug-metabolizing enzymes in pediatric populations	2
16.3	Drug metabolism and interaction in relation to phase II drug-metabolizing enzymes in pediatric populations	15
16.4	Critical physiological parameters that play role in drug metabolism, exposure, and interaction in pediatric populations	18
16.5	Drug clearance and exposure in relation to drug metabolism in pediatric populations	26
16.6	Clinically relevant drug interaction in relation to drug metabolism in pediatric patients	27
16.7	Limitation in investigating drug metabolism and drug interaction in pediatric patients: approaches and experimental designs to overcome such problems	30
16.8	Off-label use of drug therapy in pediatric patients	34
16.9	Conclusions	35
	References	35
	Further reading	44

16.1 INTRODUCTION

The development of an individual from a fertilized ovum to maturity radically alters an individual's anatomy and physiology. These changes of size and function, from birth onward, cause significant alterations in the PK of drugs. During the last two to three decades, hundreds of mechanistic and clinical pharmacology studies have been conducted to investigate the impact of ontogeny on the PK and response of drugs in pediatric populations. These research efforts revealed that during child development, several physiological functions change. For example, maturation of the human body

causes changes in (i) liver and kidney size relative to BW (peak value at age one and two years, respectively, and decline toward adulthood as described by Chen *et al.* [1]) (ii) liver blood flow [2]; (iii) plasma protein composition and function [3]; (iv) biosynthesis and levels of sex/growth hormones [4]; (v) expression of drug-metabolizing enzymes (DME) (e.g., temporal switch between CYP3A7 and CYP3A4, FMO1, and FMO3 at birth [5]); and (vi) transporters [6,7]. The age-associated changes in many of these factors have often been implicated in altered drug absorption, distribution, metabolism, and excretion (ADME) and, hence, in drug clearance, pharmacological response, and safety in pediatric population compared with adults [2,8,9]. Furthermore, in the critical ill pediatric patients, that is, pediatric patients, multiple drug therapies for acute and chronic conditions are often administered simultaneously, thus promoting the risk of adverse drug reactions (ADRs) or drug–drug interactions (DDIs). To avoid these from occurrence, understanding the mechanism of drug metabolism and factors that are involved in the hepatically cleared drugs can be critical to ensure the safety and efficacy of drug therapy in human populations, with particular reference to pediatric patients.

16.2 DRUG METABOLISM AND INTERACTION IN RELATION TO PHASE I DRUG-METABOLIZING ENZYMES IN PEDIATRIC POPULATIONS

DMEs play a critical role in the extent of drug biotransformation and thus the hepatic clearance of drugs. Therefore, it is not surprising that ontogeny of hepatic DMEs can significantly alter the clearance of drugs in pediatric populations in relation to that in adults. Drug metabolism mechanisms can be classified into phase I and II reactions, the former involving structural alteration of the drug molecule and the latter consisting of conjugation with another often more water-soluble moiety. Both phase I and II metabolic pathways may be immature at birth and are subject to maturational changes; however, it appears that perinatally, humans are not as functionally adapt with phase II reactions as those of phase I. Several *in vitro* and clinical studies of probe substrates and drugs used in children and adults have revealed that the ontogeny in DMEs and drug transporters is associated with observed significant differences in PK and safety of drugs between children and adults [2,6–15]. Drugs such as omeprazole, theophylline, caffeine, digoxin, cyclosporine, sirolimus, phenytoin, and voriconazole whose clearance is mediated by DMEs and/or drug transporters have different plasma clearance in children than that in adults (Fig. 16.1). The clearance of these drugs in pediatric populations can be the same as in adults, or one population can be higher/or lower than other population can be the same or differ (higher or lower) from that in adults. By elucidating the major clearance mechanism(s) of a drug and the differences and/or similarities between children and adults in DMEs and other physiological factors that affect drug disposition using *in vitro* model systems, one can predict clearance, and thus efficacious and safe dose, of the drug in children based on the known clearance and dose in adults [14,15].

Phase I reactions can be oxidation, reduction, and hydrolysis. Oxidative reactions are frequently carried out by P450 enzymes and have extensively evaluated compared to other oxidative enzymes such as flavin monooxygenases (FMO), which is different from the CYP system. Pediatric populations found to have different metabolic capacity to

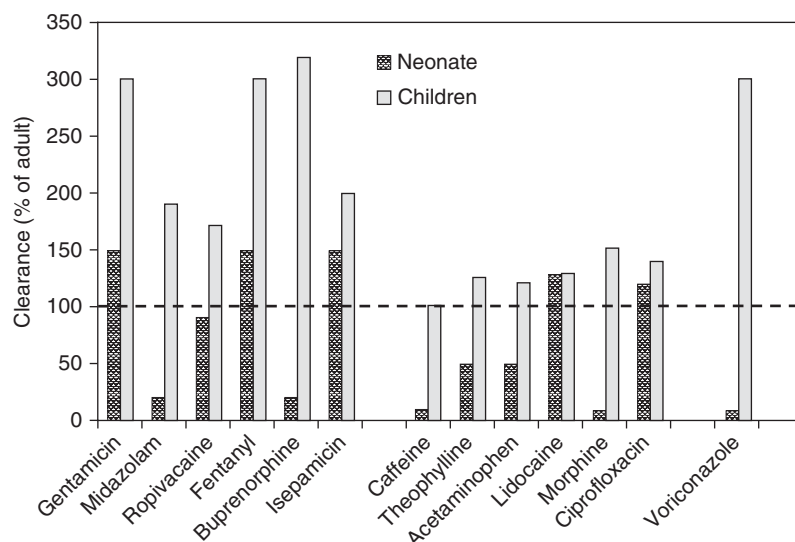


Figure 16.1 Plasma clearance of drugs eliminated by metabolism in pediatric populations compared with adults. Clearance values were presented as % of adult values (100%) shown as dashed line. *Source:* Data are published by Edginton *et al.* [8] and Yanni *et al.* [15].

drugs than in adults [4,15,16] as indicated by the difference in clearance or the presence of metabolites in plasma. In addition, there are examples of therapeutic agents that produce metabolites in children that are not present in adults. These metabolites may be responsible for some of the efficacy and/or toxicity observed with drug administration in children: an example is caffeine production in the neonate receiving theophylline, and the metabolite profile generated in children treated with sirolimus [3,17–23]. Although these recent research efforts have focused on the metabolism in relation to ontogeny of P450, other phase I oxidative enzymes such as FMO, monoamine oxidase (MAO), and alcohol dehydrogenase (ADH) [3,5,10] have been evaluated less extensively but are briefly discussed here in this chapter. In addition, the ontogeny of other phase I enzymes that responsible for the reduction and hydrolysis reactions (e.g., aldo and keto reductase, estrases, and peptidase) are mentioned in this section.

16.2.1 Phase I Oxidative Drug-Metabolizing Enzymes in Pediatric Populations

Drug metabolism occurs in almost all body organs, mainly in the liver, small intestine, kidney, and lungs; however, liver is the one that has the greatest metabolic capacity and consequently drug metabolism has been extensively studied in liver. As mentioned earlier, phase I enzymes are responsible for the biotransformation of pharmacologically active drug molecules to more hydrophilic metabolites that are subsequently eliminated by the liver, kidney, or other organs. The age-mediated changes in phase I oxidative DMEs in human liver have been extensively investigated by several groups [5,16,24–26]. Table 16.1 listed changes in the phase I enzymes across the life span as indicated by Hines and McCarver [5]. Much of the existing data that are on the developmental maturation of human hepatic biotransformation pathways are derived

TABLE 16.1 Age-Dependent Changes in Phase I Drug-Metabolizing Enzymes Activity or Protein Expression as Reported by Hines and Mccarver [5]

Gene	Prenatal Trimester			Neonates	One Month– One Year	1–10 Years	Adult
	1	2	3				
CYP1A	+	+	?	–	–	–	–
CYP1B1	?	±?	?	?	–	–	–
CYP1A2	–	–	–	–	+	+	+
CYP2A	–	–	–	+?	+	+	+
CYP2B6	–	–	?	?	?	+	+
CYP2C	–	–	–	+	+	+	+
CYP2D6	–	±	±	+	+	+	+
CYP2E1	?	+?	+?	+	+	+	+
CYP3A7	+	+	+	+	–	–	–
CYP3A4/3A5	–	–	–	+	+	+	+
FMO1	+	+	+	–	–	–	–
FMO3	±	–	–	±	+	+	+
ADH1	+	+	+	+	–	–	–
ADH2	–	+	+	+	+	+	+
ADH3	–	–	+	+	+	+	+

+, activity or protein detectable; –, no detectable activity or protein; ?, not determined; ±, activity or protein detectable, but in only a fraction of the samples examined; +?, presence or absence is controversial.

from *in vitro* hepatic microsomal studies of P450-mediated activity and to a lesser extent from clinical PK studies of probe substrates [2,10,14,25].

16.2.2 Drug Metabolism by Cytochrome P450 Enzymes (P450)

The human P450 superfamily of enzymes is encoded by 59 functional genes that have been divided into 18 families and 42 subfamilies based on divergent evolution. The vast majority of drug metabolism is carried out by 23 enzymes belonging to families 1, 2, and 3. The remaining families are more specialized enzymes involved in the synthesis and degradation of important endogenous molecules. Total P450 content in the fetal liver is 30–60% of adult values. After birth, increases are observed such that total hepatic P450 content approaches adult levels during the first 10 years of life [27,28]. The expression and *in vitro* activity of hepatic P450 enzyme isozymes follow three different patterns in development: (i) enzymes that are expressed in the fetal liver and are active toward endogenous substrates (e.g., CYP3A7) [24]; (ii) enzymes that are expressed in the early neonatal period within hours after birth but minimally expressed in fetal liver (CYP2D6 [29] and CYP2E1, [30]); and (iii) enzymes that are expressed later in neonatal development (CYP1A2 [31], CYP2C [32], and CYP3A4 [24]).

16.2.2.1 Cytochrome P450 1 Family (CYP1). The human *CYP1A1* is the main enzyme responsible for the metabolic activation of polycyclic aromatic hydrocarbons to toxic metabolites in the lung [33]. Using caffeine as a metabolic probe [34], the metabolism of drugs with CYP1A2 in pediatric populations was investigated *in vitro*, and these results were largely consistent with earlier *in vivo* results that measured urinary caffeine metabolites. CYP1A2 catalytic activity and protein levels were observed in postnatal samples with neonates at 4–5% of adult levels, 1–3 months old infants at

10–15% of adult levels, 3–12 months old children at 20–25% of adult levels, and children 1–9 years of age at 50–55% of adult levels. It was found that dietary differences can alter the rate of CYP1A2 maturation, and formula-fed infants CYP1A2 activity is faster than breast-fed infants [35]. It has been known that CYP1A2 is involved in the metabolism of aromatic amines, acetaminophen, imipramine, phenacetin, warfarin, caffeine, and theophylline [36–38]. With regard to the metabolism of caffeine and theophylline, CYP1A2 is involved in all demethylations as well as in ring hydroxylation. These metabolites were not detectable in early neonatal microsomes and became readily identifiable in infants aged one to three months [36–39], although they were present at low levels (30% of the adult level) in infants <one year and attained 50% of the adult value at one year [36]. Levels of CYP1A2 comparable to adults have been found in children three years and older [40,41]. The metabolic profile of caffeine formed by CYP1A2 has been extensively studied by Carrier *et al.* [42] and Pons *et al.* [43] and indicated differential age-mediated changes in the metabolites profile.

The human CYP2A subfamily genes family consists of three members; CYP2A6 is the most important in terms of the metabolism of drugs. Coumarin 7-hydroxylation is a probe metabolic activity for hepatic CYP2A6 [44], but the enzyme also is active toward the oxidative metabolism of nicotine [45] as well as several other protoxics [46]. It has been shown that the urinary excretion of 7-hydroxycoumarin is similar to the adult level in children of 6–13 years of age. Thus, it would appear that hepatic CYP2A6 expression is a postnatal event in liver, while an evidence of prenatal expression in extrahepatic tissues (e.g., from fetal olfactory mucosa) was reported [47].

The human CYP2C subfamily consists of four genes that encode for CYP2C8, CYP2C9, CYP2C18, and CYP2C19 enzymes and the encoded enzymes account for about 18% of total P450 in adult liver [27,28] and are responsible for the oxidative metabolism of ~29% of clinically relevant drugs [9,10]. CYP2C9 is the major hepatic CYP2C enzyme in terms of its content in liver as well as the number of drugs which it metabolizes, followed by CYP2C19 and CYP2C8. CYP2C9 substrates include the anti-coagulant, warfarin; the antidepressant, phenytoin; the nonsteroidal anti-inflammatory agents, diclofenac and ibuprofen; the antidiabetic drug tolbutamide; and angiotensin II receptor blocker, losartan [9,48], while CYP2C19 substrates include the anticonvulsant, mephenytoin [49]; the antidepressant, S-citalopram [50]; the antifungal, voriconazole [51]; and the proton pump inhibitors (PPIs), omeprazole [52–54] and lansoprazole [55].

In the case of the CYP2C family, activity can be significantly faster in children with respect to adults. *In vivo* PK data have indicated that an age-dependent decrease in phenytoin half-life was observed over the first two weeks of life [56]. PK studies in pediatric patients have indicated that the clearance of warfarin in children younger than 12 years was 44% greater than that observed in older children and adults [57]. Clearance of phenytoin that was primarily metabolized by CYP2C9 and to a lesser extent by CYP2C19 was approximately twofold higher in children younger than six years compared with that in adults [58]. The diclofenac 4-hydroxylase was also measured *in vitro* in liver microsomes from different age groups and that indicates the higher activity in children 2–10 years compared with adults [59]. Similarly, clearance of celecoxib in children aged 7–16 years was approximately double that reported for adults [60]. Therefore, as suggested by Anderson and Lynn [9], doses of drugs predominantly metabolized by CYP2C9 may need to be 50–100% higher in children than those given to adults to achieve equivalent therapeutic concentrations.

Contrasting with CYP2C9, there was no apparent change in CYP2C19 levels at birth; however, there was a trend for a near linear increase in the enzyme protein measured in liver microsomes attaining 50–75% of those observed in adults over the first five months after birth. Enzyme levels indistinguishable from adult levels were observed in postpuberty samples. These data were consistent with the (S)-mephenytoin 4'-hydroxylase activities observed in these samples. The *in vitro* activity was also determined as a function of age by Yanni *et al.* [58] and shown in Fig. 16.2a. A comparison of relative CYP2C9 and CYP2C19 levels in individual samples revealed that CYP2C19 is the dominant prenatal CYP2C enzyme, which is gradually relinquished in favor of CYP2C9 at or around birth. These *in vitro* results that described the developmental profile of both CYP2C enzymes were confirmed by the changes in maximal CYP2C9-mediated hydroxylation of diclofenac and CYP2C19-mediated hydroxylation of S-mephenytoin as a function of age of the individual from which the liver microsomes were prepared ($n = 10$ /age group ranging from 20 weeks of gestation to adult).

The PPIs omeprazole, lansoprazole, and pantoprazole that are primarily metabolized by CYP2C19 were studied by Litalien *et al.* [52,54] in children in comparison to adults. The apparent clearance (Cl/F) of PPI appeared to be faster in children than in adults. Higher metabolic capacity in children and the lower bioavailability (F) of PPI observed in children compared with adults were most likely the cause for this finding. There was some evidence of reduced PPI metabolism in newborns. This may partly account for the need in children for variable and sometimes considerably greater doses of PPIs, on a per kilogram basis, than for adults to achieve similar plasma concentrations. Kennedy [4] and Filler *et al.* [17] reported that phenytoin and sirolimus are metabolized by CYP2C9/CYP2C19 and show a higher clearance in children between 2 and 10 years of age than in adults. On the basis of our *in vitro* metabolic study and clinical PK study [15], it has recently been shown that the antifungal drug voriconazole was cleared faster in children <12 years of age than in adults (Fig. 16.1). In addition, Filler *et al.* [17] summarized the PK in children of commonly used immunosuppressive drug

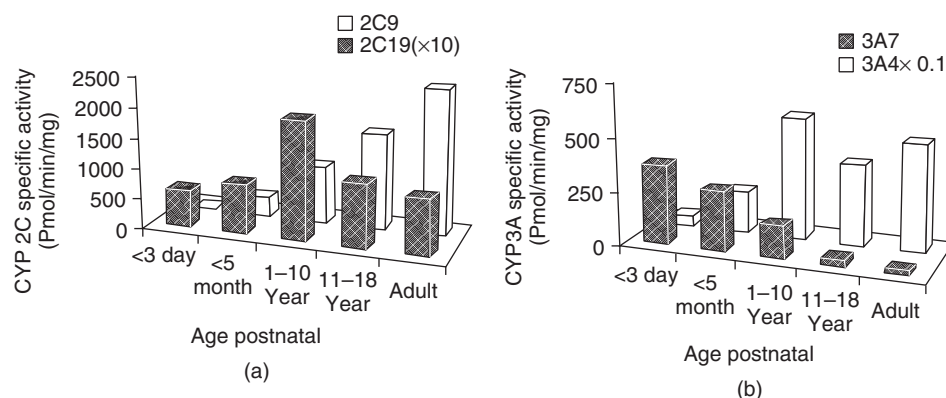


Figure 16.2 *In vitro* age-dependent changes in specific activities of DME. (a) CYP2C9 and CYP2C19 (for presentation, the activities are 10 times the actual activity); (b) CYP 3A4 (for presentation, the activity is 10 times less than actual activity) and CYP3A7 in liver microsomes. Studies were conducted as described by Yanni *et al.* [58] and Yanni [28].

sirolimus (Sir). Sir half-life and mean residence time (MRT) are both shorter than in adults. Similar to situation in adults, there is a profound DDI between cyclosporine and Sir. Of the total metabolite AUC, 77.5% was due to hydroxylated metabolites, while 39-*O*-desmethyl Sir (the main metabolite in adults) comprised only 8.4% of the metabolites. Thus, the authors then concluded that Sir drug disposition vary in children and adolescents with respect to adult patients, most likely because of developmental changes of metabolic enzymes.

The human CYP2D6 subfamily accounting for <2% of total adult hepatic P450 [27], and CYP2D6 is important for the oxidative metabolism of ~12% of clinically relevant drugs [61]. CYP2D6 is highly polymorphic with over 80 different alleles identified to date, including several complete loss-of-function, reduced function, and multiple copy number alleles that are relatively common across most ethnic groups. The well-known polymorphism of CYP2D6 is known to be present in children [62]. The isozyme CYP2D6 contributes to the metabolism of several classes of drugs, such as tricyclic and nontricyclic antidepressants, β -blockers, antiarrhythmic drugs, as well as codeine, cinnarizine, deprenyl, captopril, and ondansetron [62–66], although it is not detectable in fetal liver [63,64]. The *O*-demethylation of codeine to the active moiety, morphine [67], and dextromethorphan to dextrorphan [66], and *O*-demethyl tramadol formation clearance [68] have been used as both *in vitro* and *in vivo* CYP2D6 phenotypic probes. Dextromethorphan *O*-demethylation has been particularly useful in pediatric populations by measuring the urinary ratio of dextromethorphan/dextrorphan as noninvasive method to phenotype CYP 2D6.

CYP2D6 phenotype consistent with its genotype was achieved on average by 15 days postnatal age (PNA) and remained relatively constant over the first six months of life in a cohort of term neonates. CYP2D6 protein was undetectable in 47% of the fetal samples by 30 weeks in gestation, whereas 30% of the samples exhibited values that were at least 5% of those observed in the adult samples. In the 1- to 7-day old newborn age bracket, all samples had detectable CYP2D6 protein, but at a mean level that still was only 5% of that observed in adults. CYP2D6 protein content in this newborn group also was independent of gestational age, suggesting that the development of CYP2D6 is related to the events occur at birth. Thereafter, there was a steady, age-dependent increase in CYP2D6 protein levels such that newborns 7–28 days of age exhibited mean levels that were 30% of adults and infants four weeks to five years, 70% of adults. Dextromethorphan *O*-demethylase activity closely followed this same developmental pattern.

Dextromethorphan *O*-demethylase activity (measured by the ratio of dextromethorphan/dextrorphan) in overnight urine samples [35] was in agreement between the dextromethorphan/dextrorphan ratio and an assigned activity score based on genotype by two weeks of PNA in a cohort of term newborns [68]. There was considerable interindividual variation in dextromethorphan/dextrorphan ratio and Johnson [69] proposed that the discrepancy between the *in vitro* and *in vivo* data on CYP2D6 ontogeny may be largely explained by the added complexity of renal functional maturation that was not considered in the *in vivo* study by Blake *et al.* [35].

The complex interaction of ontogeny, CYP2D6-related pharmacogenetics, and renal elimination clearance were illustrated based on observations tramadol and *O*-demethyl tramadol disposition in infancy [68]. Tramadol (M) hydrochloride is a 4-phenyl-piperidine analog of codeine. *O*-demethyl tramadol (M1) is produced by *O*-demethylation of M by CYP2D6, and it has pharmacodynamic relevance.

N-demethyl tramadol (M2) is produced after *N*-demethylation of M, mainly by CYP3A4. In adults, the urinary log M/M2 ratio reflects CYP3A4/CYP2B6 activity, while the urinary log M/M1 ratio reflects CYP2D6 activity. Tramadol disposition can, therefore, be used as a probe to simultaneously determine the ontogeny of CYP3A4/CYP2B6 and CYP2D6. In early life, the contribution of M, M1, and M2 to overall elimination clearance of tramadol and ratios of M/M1 and M/M2 were assessed in 24-h urine collections during continuous intravenous tramadol administration. Correlations with perinatal characteristics were explored [67]. In neonates and young infants, Allegaert *et al.* [70] reported that the mean log M/M2 was 1.44 compared to an adult phenotypic ratio of 0.47. An inverse correlation between log M/M2 ratio and postmenstrual age (PMA, weeks) ($r = -0.66$, $p < 0.001$, and PNA, days) ($r = -0.5$, $p < 0.01$) was observed with a maturational half-life of the log M/M2 ratio of 16–20 weeks. In a multiple regression model, PMA remained the only significant variable to explain the interindividual variability in log M/M2 ratio. The mean urine log M/M1 was 0.94 compared with an adult phenotypic ratio of -0.1 . Significant correlations of the urine log M/M1 ratio with PMA ($r = -0.73$, $p < 0.0001$) and PNA ($r = 0.56$, $p < 0.001$) were observed. The authors reported that the weak correlations reflect that PMA only in part explains the interindividual variability in log M/M2 or log M/M1.

On the basis of observations in children and adults, it was anticipated that CYP2D6 polymorphisms also contribute to the interindividual variability in *O*-demethylation. The impact of both PMA (either <40 [×] or ≥ 40 weeks [+]) and CYP2D6 activity score on the plasma log M/M1 values is illustrated in patients with an CYP2D6 activity score of 1, 1.5, or 2 (Fig. 16.3a). The lower the log M/M1 value, the higher phenotypic CYP2D6 isoenzyme activity. There is a PMA- and polymorphism-dependent effect.

However, one has to anticipate that besides ontogeny of M1 formation (CYP2D6 related), the final phenotypic M1 concentration–time profile will also depend on renal M1 elimination as clearance mechanisms mature. This results in similar time–concentration profiles from about full-term age onward throughout infancy (Fig. 16.3b), since maturation of glomerular filtration is relatively delayed compared with CYP2D6 ontogeny [68]. On the basis of these *in vivo* observations, we confirm the hypothesis of Johnson [69] that indeed phenotypic CYP2D6 activity depends on ontogeny, CYP2D6 polymorphisms, and renal maturation.

The human CYP3A subfamily consists of four genes that encode for CYP3A4, CYP3A5, CYP3A7, and CYP3A43. It has been shown that CYP3A43 has minimal, if any xenobiotic-metabolizing activity; it is expressed at low levels and no developmental profile of this enzyme has been reported [10]. In contrast, CYP3A4, CYP3A5, and CYP3A7 combined are the most abundant hepatic members of the P450 family and account for nearly 46% of the oxidative metabolism of clinically relevant drugs [61]. These three enzymes share at least 85% sequence identity, a property that has proven to be challenging when these three enzymes protein need to be measured independently by Western blot; however, they exhibit considerable differences in substrate specificity and expression as function of age. In adult liver, CYP3A4 comprises 10–50% of the total P450 enzymes [27]; while CYP3A7 is the dominant enzyme in fetal liver, it is expressed at substantial levels (10–40% of total CYP3A levels) in the adult liver and intestine, depending on the presence of the CYP3A7*1 C allele [72]. A temporal switch was observed between CYP3A7 and CYP3A4 in terms of their expression and activity in liver as function of age. However, CYP3A7 mRNA was detected in fetal liver and

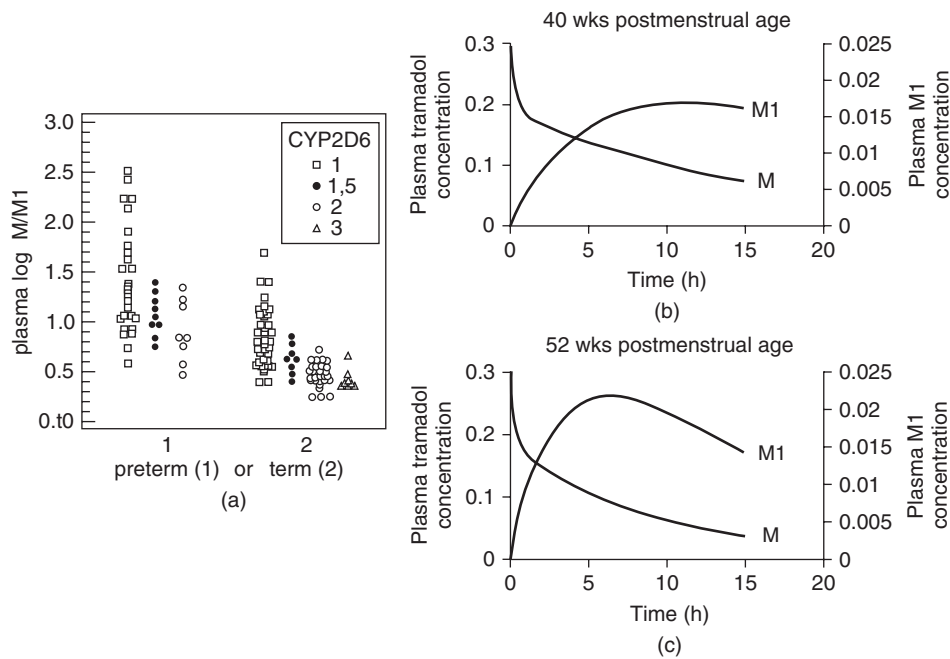


Figure 16.3 Polymorphisms and gestation age effect on interindividual variability of drug metabolism *in vivo*. (a) The impact of maturation on CYP2D6 activity as indicated by plasma log M/M1 values in patients with a CYP2D6 activity score of 1, 1.5, 2, or 3, the lower the log M/M1 value, the higher phenotypic CYP2D6 isoenzyme activity. (b) M and M1 concentration time profile from full-term age onward throughout infancy changes as maturation of glomerular filtration is relatively delayed compared with CYP2D6 ontogeny. *Source*: Figure adapted based on Allegaert *et al.* [67,70,71].

was not detected in adult livers. For CYP3A4 expression, the opposite was observed: it is not expressed in fetal liver but expressed in adult. At the protein level, CYP3A5 was shown to be expressed in a limited number of fetal (1/5; ages not specified) and postnatal (8/20) liver samples with no apparent correlation with age [73] (Fig. 16.4). Consistent with earlier studies, Stevens *et al.* [74] reported that CYP3A5 protein was observed in half of tested samples, but there was no apparent change in enzyme levels as a function of age. As shown in Fig. 16.4, CYP3A7 protein expression in liver microsomes was high but variable in all fetal samples examined; CYP3A4 was detectable in some samples, but at levels that were nearly 100-fold less than CYP3A7. After birth, there was an approximate 50% decline in neonatal CYP3A7 enzyme levels, while little or no change was observed in CYP3A4 level. The CYP3A7 continues to decrease by age, while CYP3A4 continues to increase to reach adult level at age of two years. Hines [10] indicates that CYP3A7 remains the dominant CYP3A enzyme in children up to one year after birth and that CYP3A4 expression increases steadily from birth onward, but only reaches adult levels after one year of age. CYP3A7 activity measured by dehydroepiandrosterone (DHEA) 16 α -hydroxylase in liver microsomes ranged from 0.48 to 1.31 nmol/min/mg in samples from fetal livers and in livers from neonates one to seven-days after birth; the activity then declined steadily to 0.56 nmol/min/mg in neonates between 8 and 28 days to 0.34 nmol/min/mg in infants one to three months

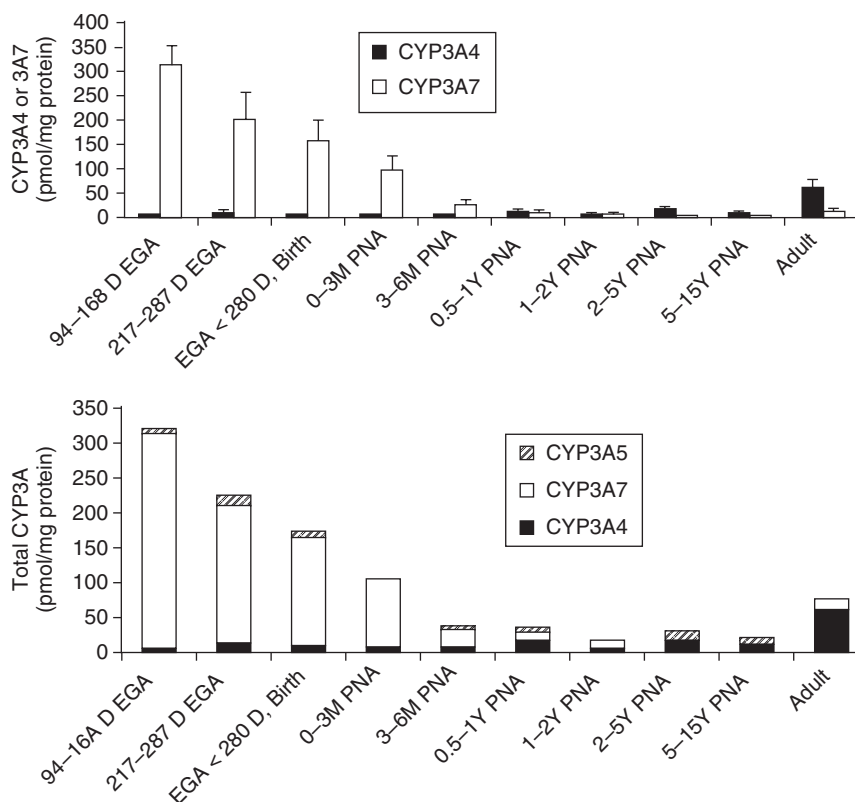


Figure 16.4 Protein expression of CYP3A isozymes, CYP3A4, CYP3A5, and CYP3A7 in liver samples as a function of age. Age ranged from 94 days of prenatal to adult. *Source:* Adapted with permission from Stevens *et al.* [74]. EGA is prenatal age, while PNA is postnatal age.

of age, to 0.17 nmol/min/mg in children from 3 to 12 months of age, and reached the lowest activity (0.07 nmol/min/mg) in adults. In contrast, CYP3A4 activity measured in liver microsomes by testosterone 6 β -hydroxylation was 0.035–0.052 nmol/min/mg, at one year of age and achieved adult levels of 0.120–0.130 nmol/min/mg microsomal protein in children older than one year of age (Fig. 16.4). In this study, the contribution of CYP3A5 activities had not been characterized or considered. However, in a slightly similar approach by Stevens (2003) [74] who considered using differential metabolism of DHEA, 7 β - and 16 α -hydroxylase activity, $V_{\max}$ and K_m parameter values for CYP3A4, CYP3A5, and CYP3A7 were 0.423, 0.0134, and 0.0125 nmol/min/mg protein and 0.433, 0.002, and 1.32 μ M, respectively. DHEA 7 β -hydroxylation was considered a preferential activity of CYP3A4, DHEA 16 α -hydroxylation of CYP3A7, and CYP3A5 levels were determined immunologically. CYP3A4 postnatal ontogeny is consistent with metabolic study of amprenavir in fetal and postnatal liver samples [75] that concluded the fetal-specific expression of CYP3A7, postnatal expression of CYP3A4, while CYP3A5 expression in a limited number of samples during both life stages. These results have been confirmed by the change of *in vitro* enzyme activity as a function of age for both CYP3A7 and CYP3A4 in liver microsomes preparations [59] (Fig. 16.2b).

The clinical implications of the temporal switch between CYP3A7 and CYP3A4 is described in PK of cisapride, a gastroprokinetic agent that was commonly used in pediatric patients with feeding intolerance, apnea, and bradycardia due to gastroesophageal reflux. Owing to a higher than acceptable incidence of cisapride-related QT prolongation in adult patients associated with excessive dose or high plasma levels of parent drug, cisapride was removed from the market and used only through a limited access program for specific diseases, including feeding intolerance in neonates. However, a study by Kearns *et al.* [2] in the same patient population demonstrated a significant increase in QT prolongation not associated with adult risk factors [76]. *In vitro* studies indicated that cisapride is metabolized primarily by CYP3A4, but not by CYP3A5 or CYP3A7 [77]; consequently, it was hypothesized that neonates would show deficient metabolic activity toward cisapride [78]. Consistent with this hypothesis, cisapride *in vitro* metabolism was measured in 50% of the livers from fetus or neonates less than seven days old and the extent of metabolism was low. The hypothesis was further confirmed in an *in vivo* study reported by Kearns *et al.* [2], in which the cisapride terminal elimination rate constant was shown to increase from $\sim 0.02 \text{ h}^{-1}$ in 30-week postconceptional prematured neonates at birth to 0.20 h^{-1} in full term—12-week-old infants. Other kinetic parameters also were consistent with compromised cisapride metabolic clearance in neonates. Thus, the differential substrate specificity of CYP3A4 and CYP3A7 combined with the developmental transition between these two enzymes are implicated with the deficient metabolic clearance of cisapride causing its accumulation that mediates the QT in neonatal patients, particularly those born prematurely, thus the risk for ADRs. Oral clearance of midazolam [79] is also markedly lower in preterm infants relative to that of younger children; it is higher in children aged 2–12 years than in adolescents aged 12–16 years [80]. Similarly, tacrolimus clearance is elevated 2.7- and 1.9-fold among children aged 1.5–5 and 5–12 years, respectively, compared with clearance in those older than 12 years [81]. Similar age-related increases in the clearance of amlodipine [82], clonazepam [83], lopinavir [84], midazolam [79], and saquinavir [85] have been observed. The average weight-corrected doses of drugs predominantly metabolized by CYP3A4 in children must be twofold higher than adult doses to achieve equivalent therapeutic concentrations [4].

Jones *et al.* [86] reported that in recent studies with coumadin (R/S-warfarin) therapy, a complication was observed by interindividual variability in metabolism with CYP3A isoforms that likely contribute to patient responses and clinical outcomes. Despite a significant focus on CYP3A4, little is known about CYP3A5 and CYP3A7 metabolism of warfarin. *In vitro* metabolism studies with recombinant CYP3A4, CYP3A5, and CYP3A7 indicated that these three CYP3A isozymes metabolized R- and S-warfarin with efficiencies that depended on the individual enzymes. For R-warfarin, CYP3A4, CYP3A7, and CYP3A5 demonstrated decreasing preference for 10-hydroxylation over 4'-hydroxylation. By contrast, there was no regioselectivity toward S-warfarin. While all enzymes preferentially metabolized R-warfarin, CYP3A4 was the most efficient at metabolizing all reactions. Individuals, namely African-Americans and children, with higher relative levels of CYP3A5 and/or CYP3A7, respectively, compared with CYP3A4 may metabolize warfarin less efficiently and, thus, may require lower doses and be at risk for adverse DDIs related to the contributions of the respective enzymes.

16.2.3 Drug Metabolism by Flavin-Containing Monooxygenases in Pediatric Populations

The FMOs are major mammalian non-CYP oxidative enzymes [87]. The most abundant adult human liver FMO isozyme is FMO3, which exists at similar level as CYP3A4 [88]. Human FMOs are localized in the endoplasmic reticulum (ER) in intact tissue and are isolated in the microsomal subcellular fraction.

FMOs are encoded by a six-member gene family (FMO1–6) and are involved in NADPH-dependent oxidative metabolism of a wide range of xenobiotics containing nucleophilic nitrogen-, sulfur-, selenium-, and phosphorus heteroatoms. Common therapeutic substrates include tamoxifen, itopride, benzydamine, olopatidine, and xanome-line [89,90]. FMO activity is not readily modulated by induction or inhibition; however, Borbás *et al.* [91] reported that hepatic FMO1 activity increased in a diabetic rat model. The ontogeny of human hepatic FMO exhibits a switch similar to that observed for CYP3A. Expression of FMO1 but not of FMO3 was detectable in fetal liver [92]. In contrast, the transcript of FMO3, but not of FMO1, was detected in adult liver samples. In a study with 240 liver samples ranging in age from 8 weeks of gestation to 18 years postnatal, the highest level of FMO1 expression was observed at 8–15 weeks of gestation [10,93]. FMO1 expression subsequently declined during fetal development and was completely suppressed within three postnatal days by a mechanism coupled to birth but not gestational age. FMO3 expression was observed at low levels at 8–15 weeks of gestation but not in subsequent time periods of fetal development. The FMO3 postnatal expression was found to be highly variable. Most individuals failed to express FMO3 in the neonatal period, but the expression was detectable by one to two years of age, as listed in Table 16.2.

It has been reported that FMO enzymes are subject to interindividual variation. Single nucleotide polymorphisms (SNPs) are associated with large differences in enzyme activity in some human subjects, mostly among Japanese and African-American populations [94]. Some FMO3 mutations have been associated with trimethylaminuria (TMA), a rare metabolic disorder caused by the inability to *N*-oxygenate dietary-derived TMA. The therapeutic implication of ontogeny in relation to FMO has not been as well documented as for P450 enzymes. To date, there are only few PK

TABLE 16.2 Age-dependent Changes in Phase I Drug-Metabolizing Enzymes Activity or Protein Expression as Reported by McCarver and Hines [26]

Gene	Prenatal Trimester			Neonates	One Month– One Year	1–10 Years	Adult
	1	2	3				
GST α 1/ α 2	+	+	+	+	+	+	+
GST μ	+	+	+	+	+	+	+
GST π	+	+	+	+	–	–	–
UGTA1	–	–	–	+	+	+	+
UGTA3	?	+	+	+	+	+	+
UGT1A6	–	–	–	+	+	+	+
UGT2B7	?	+	+	+	+	+	+
SUIT1A1	+	+	+	+	+	+	+
SULT1A3	?	+	+	+	+	+	+
SULT2A1	–	–	–	+	+	+	+

+, activity or protein detectable; –, no detectable activity or protein; ?, not determined.

studies that describe the role of FMO in the disposition of drugs in children and pediatric populations compared with adults. Orenstein *et al.* [95] reported that ranitidine, which is predominately metabolized by FMO, had a clearance value in children that was similar to the reported for adults. Impaired *in vivo* metabolism of benzydamine (marker substrate for FMO3) was found in pediatric patients with deficiency of FMO3 [96].

16.2.4 Drug Metabolism by Monoamine Oxidases in Pediatric Populations

MAOs are oxidative enzymes located in the mitochondria and involved in the metabolism of endogenous and exogenous compounds [97]. The ontogenetic development of MAO-A and MAO-B in the frontal cortex of human brain has been previously studied by Kornhuber *et al.* [98]. The temporal switch that has been reported with CYP3A and FMO was also found with MAO. MAO-A activity was found to be very high at birth and then decreased rapidly during the first two years of life and remained unchanged onward, while the MAO-B activity was low at birth, remained unchanged during early childhood, and then increased with age. The ontogeny-mediated changes of drug metabolism by MAO-A/B in human tissues have not been investigated, and it could be useful if the age-mediated changes in the metabolism by MAO of tyramine and tyramine, containing foods such as cheese, to be investigated in the gut.

16.2.5 Drug Metabolism by Alcohol Dehydrogenases in Pediatric Populations

ADH is the rate-limiting enzyme responsible for the biotransformation of ethanol. Early PK data for ethanol in neonates clearly support the poor metabolism of ethanol and its plasma accumulation and reduced clearance confirming developmental immaturity in ADH activity [99,100]. It would appear that by 12–30 months of PNA, ADH activity reached equal to or greater than that observed in adults [101,102] (Table 16.1). ADH3, also termed *formaldehyde dehydrogenase* or *S-nitrosoglutathione reductase*, plays a critical role in the enzymatic oxidation of formaldehyde and reduction of nitrosothiols that regulate bronchial tone. Considering reported associations between formaldehyde vapor exposure and childhood asthma risk, and thus potential involvement of ADH3, Thompson *et al.* [102] reviewed the ontogeny, distribution, and regulation of mammalian ADH3. The authors reported that multiple biological and chemical stimuli influence expression and activity of ADH3, including the feedback regulation of nitrosothiol metabolism and concluded that the levels of ADH3 correlate with, and potentially influence, bronchial tone. Hence, ADH3 ontogeny function relative to the effects of formaldehyde, as well as other chemical and biological exposures, might be critical to childhood asthma.

Brent [103] recently investigated the treatment of pediatric patients with metabolic acidosis caused by the catalysis of ADH to the ingested ethylene and diethylene glycol, butoxyethanol, and methanol by using drugs such as fomepizole. Fomepizole as well as ethanol are potent inhibitors of ADH; thus, they reduce the generation of toxic metabolites causing increased anion gap. The median age of these cases was 5.5 years old. All 10 patients had resolution of their metabolic acidosis. The half-times of ethylene glycol elimination ranged from 9 to 15 h during fomepizole therapy, which is faster than the 19.7 h reported in adults. The two patients who ingested diethylene

glycol or butoxyethanol all recovered. Plasma fomepizole concentrations were measured in three cases and were found to be therapeutic with apparent Michaelis–Menten kinetics, having a zero-order elimination rate of 0.6–1 mg/L/h at higher concentrations and a first-order elimination with an apparent elimination half-time of 3.9 h at lower concentrations. In conclusion, the author reported that fomepizole dosage in pediatric population is the same as used for adults.

16.2.6 Metabolism by Phase I Reductive Enzymes in Pediatric Populations

Phase I reductive enzymes-mediated drug metabolism is poorly investigated. One of these enzymes is the cytosolic aldo-ketoreductase that reduces carbonyl group-containing drugs. The enzyme is present in the liver, other tissues, and erythrocytes. It metabolized hypolipidemic drugs, such as fenofibrate, and antitumor drugs (e.g., the anthracyclines) [104,105]. Ketoreductase activity is also involved in the metabolism of prostaglandin E1 (PGE1), which is used for the treatment of peripheral arterial occlusive diseases [106]. Idarubicinol, the alcohol product of keto reductase biotransformation of idarubicin, was found in the cerebrospinal fluid (CSF) of pediatric patients receiving the drug in a phase I leukaemia study [107,108]. In the absence of additional information, it is difficult to conclude whether the presence of the reduced metabolite in the CSF is due to higher peripheral and/or central ketoreductase activity in the pediatric patients or to a different distribution of idarubicin and/or idarubicinol in this population. A 13,14-dihydro-15-ketoprostaglandin E1 is formed as an intermediary metabolite of PGE1; this metabolite is further reduced by a 15-ketoreductase to 13,14-dihydro-prostaglandin E1, the active compound. Interindividual variation in the enzymatic 15-ketoreduction of 13,14dihydro-15-ketoprostaglandin E1 was reported in human liver and children aged ≤ 10 years had a significantly lower activity than those aged ≥ 13 years [109]. The activity of such a ketoreductase has been thought to be related to sexual maturity [108,110].

16.2.7 Phase I Hydrolytic Enzymes in Pediatric Populations: The Role of Extrahepatic Metabolism

Hydrolytic enzymes, such as esterases, might display age-dependent changes in their functions. *In vivo*, drug such as propacetamol can serve as probe to assess esterase activity since it is a prodrug that hydrolyzed to paracetamol. A study by Anderson *et al.* [111] demonstrated that by predicting hydrolysis half-life in pooled population PK study in neonates, toddlers, and children, an assessment of potential age-dependent maturation of esterase during childhood can be made. The authors thereby concluded that there was no age-dependent effect on hydrolysis half-life, suggesting that esterase is already mature at birth. However, hydrolysis half-life depends on both clearance and distribution volume characteristics. Other reports showed that esterase activity is reduced in the newborn [111–112], but a rapid increase in its activity, for example, plasma pseudocholinesterase and arylesterase activity occurred from 28 weeks gestation to 1 year of age with no significant changes in activities from those of adults occurring after one year. However, the erythrocyte acetylcholinesterase levels increased significantly between 40 weeks gestation and 1 year of age [112]. Plasma esterases in infants hydrolyzed procaine hydrochloride and the insecticide paraoxon with lower rate than children older than one year and adults, although the hydrolysis rate was

faster in the mature infants than in the premature. Drugs administered in the form of an ester that require metabolism to the microbiologically active form *in vivo*, for example, erythromycin estolate and chloramphenicol palmitate, might be incompletely hydrolyzed in neonates [113]; thus, low plasma concentration of acid metabolite will be achieved and necessitate drug concentration monitoring to ensure therapeutic plasma concentrations are obtained [114]. Similarly, in a study on serum aspirin–esterase activity in epileptic patients [115], significant differences have been found in serum aspirin–esterase activity between children and adults, although in this case, the epileptic children were found to show significantly higher levels of hydrolytic activity than adult patients or control healthy subjects.

Different from esterases, peptidase has been taking a role in the treatment of children and adolescent psychiatric patients who suffer from attention deficit hyperactivity disorder (ADHD). Lisdexamfetamine (LDX) was approved by the Food and Drug Administration (FDA) for treating ADHD in children in early 2007. LDX is a prodrug, which when orally ingested is converted to L-lysine and active D-amphetamine, which is responsible for its therapeutic activity. The hydrolytic mechanism occurs by the catalysis of peptidase in blood and tissues. This unique formulation may lead to a possible reduction of the abuse potential by circumventing the first-pass metabolism. The solubility and conversion for LDX to D-amphetamine and absorption of LDX (mediated by uptake drug transporters PEPT) is not affected by variations in gastric pH, while the PK of D-amphetamine were found to be similar in pediatric and adolescent ADHD patients and healthy adult volunteers [116].

16.3 DRUG METABOLISM AND INTERACTION IN RELATION TO PHASE II DRUG-METABOLIZING ENZYMES IN PEDIATRIC POPULATIONS

16.3.1 Role of Glucuronosyltransferase Enzymes (UGT) in the Metabolism and Interaction of Drugs in Pediatric Populations

The biotransformation of many drugs, such as chloramphenicol and novobiocin, undergo critical changes during the perinatal period. Glucuronidation appears to be particularly impaired in early neonatal life, and this impairment does play a role in the genesis of neonatal “physiologic” jaundice [117,118]. Activity toward estrone was present in the fetal liver at 30% of adult values, while activities toward bilirubin, androsterone, testosterone, 1-naphthol, 2-aminophenol, 4-nitrophenol, and morphine were all present in fetal and term liver at values of <14% of those of adults [119–121]. Bilirubin glucuronidation that carried out by the isozyme uridine 5′-diphosphate glucuronosyltransferase (UGT)1A1 was particularly low in fetal liver, <1% of the activity of the adult liver [119] as well as the rate of morphine glucuronidation that catalyzed by UGT2B7 [122] was 6- and 10-fold lower in fetal liver microsomes samples than in the adult [121]. Activity toward all other substrates measured developed after birth [118] and glucuronidation may not approach adult values until three to six months of life [118], one year [123], three years [124,125], or even later [124–126] (Table 16.2). As glucuronidation is markedly deficient in most premature infants and some full-term babies [127], the accumulation of high concentrations of unchanged chloramphenicol may occur in a

neonate receiving recommended doses based on BW, resulting in serious toxicity (gray baby syndrome). Doses adjustment based on PK studies led to more precise dosage regimens on the basis of weight, gestational, and PNA. Filler *et al.* [17] summarized the PK in children and youths of commonly used immunosuppressive drug, mycophenolate mofetil (MMF). Regarding MMF, the authors were unable to demonstrate age dependency of MMF in combination with cyclosporine. By contrast, there was an inverse relationship between age and the dose-normalized mycophenolate (MPA) area-under-the-time-concentration curve (AUC) in children who received concomitant tacrolimus (Tac). Dose-normalized MPA AUCs were higher than commonly observed in adult patients. The authors hypothesized that the age dependency is related to developmental changes in the expression of the UDP-glucuronosyltransferases. In conclusion, MMF drug disposition varies in children and adolescents from adult patients, most likely because of developmental changes of biliary transporters and metabolic enzymes.

As CYPs, there are several isozymes of glucuronosyl transferases and the developmental changes of one isozyme can be different compared to the others. Therefore, characterization of glucuronidation reactions should be considered and caution should be taken in consideration when extrapolating information on developmental changes obtained with one isozyme to the others. Morphine is extensively metabolized by glucuronidation with the formation of both 3- and 6-glucuronides (M3G and M6G). M6G has been shown to be a potent analgesic in clinical studies in human subjects [128]. The metabolism of morphine was studied in children and premature neonates [129]. All the neonates and children had detectable concentrations of M3G and M6G. The M3G/morphine and M6G/morphine ratios were significantly higher in children than in neonates, thus suggesting that morphine glucuronidation capacity is enhanced after the neonatal period, although there was no difference in the M3G/M6G ratio in children and neonates, indicating a parallel development of both glucuronidation pathways that were carried out by the same isozyme [130]. It has been documented that the additional impact of PNA (first 10 days of postnatal life) on glucuronide formation, irrespective of the PMA. On the other hand, acetaminophen glucuronidation is carried UGT1A6, but UGT1A1 and UGT1A9 are also involved [131]; a relative deficiency in glucuronide conjugation of this drugs is still observed in children of 7–10 years compared with adults [126].

The phenotypic impact of glucuronidation on elimination clearance of a compound also depends on the availability of other elimination routes, either metabolic (hepatic, e.g., sulfation) or elimination (mainly renal). To illustrate the relevance of this concept, it is worth comparing the PK of acetaminophen with the PK of propofol in early neonatal life [132]. Anderson *et al.* [133] demonstrated that elimination clearance for intravenous acetaminophen depends on PMA, without any additional impact of PNA. Besides PMA, PNA is also a covariate of the proportion of glucuronidated acetaminophen to overall acetaminophen metabolic elimination, reflecting impaired glucuronidation (Fig. 16.5a). However, impaired glucuronidation activity is compensated by a phenotypically higher sulfation metabolism and even renal elimination of the mother compound, acetaminophen [133]. As a result, acetaminophen clearance only depends on PMA without any additional impact of PNA [134].

In contrast, propofol is highly lipophilic and undergoes metabolic elimination clearance. The disposition of propofol has been extensively studied in different populations

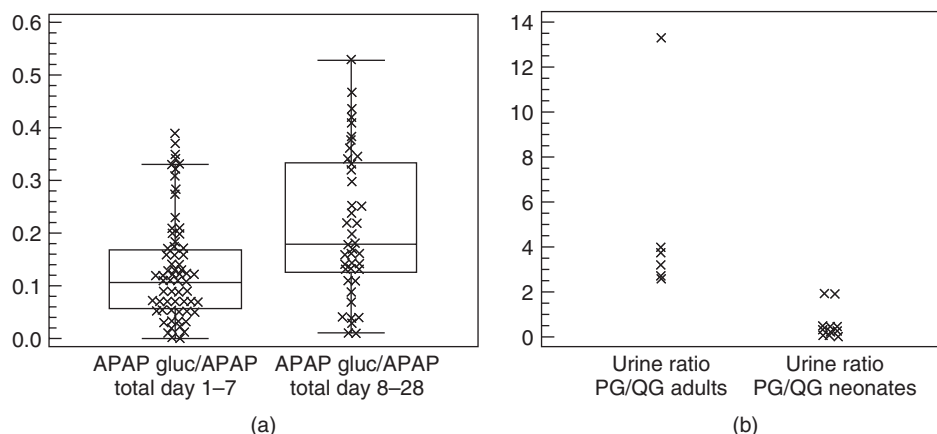


Figure 16.5 Glucuronidation of as a function and impaired glucurination at birth. (a) Demonstrated elimination clearance for intravenous acetaminophen depends on postmenstrual age, without any additional impact of postnatal age. Besides postmenstrual age, postnatal age is also a covariate of the proportion of glucuronidated acetaminophen to overall acetaminophen metabolic elimination, reflecting impaired glucuronidation. (Data summarized in Anderson *et al.* [133]) (b) Age-dependent differences in propofol clearance reflect quantitative or qualitative differences in propofol metabolism. In adults, propofol undergoes either glucuronidation [mainly through UDP-glucuronosyltransferase (UGT) 1A9, resulting in propofol glucuronide, PG] or hydroxylation [mainly cytochrome P450 (CYP) 2B6, resulting in 1- or 4-quinol, 1-Q, and 4-Q] with subsequent glucuronidation [(1-quinol glucuronide (1-QG), 4-quinol glucuronide (4-QG)] or sulfation [4-quinol sulfate (4-QS)], respectively; PG is the most contributing metabolite, whereas in neonates, the contribution of quinol metabolites is much more relevant to overall metabolism in neonates compared with glucuronidation (as described by Allegaert *et al.* [71]).

of adult and pediatric age, but observations in neonates are limited. Propofol clearance in neonates is significantly lower compared with children and displays extensive variability. Covariates of propofol clearance in neonates are PNA and PMA [133].

Since propofol clearance exclusively depends on biotransformation, it is reasonable to anticipate that age-dependent differences in propofol clearance reflect quantitative or qualitative differences in propofol metabolism. In adults, propofol undergoes either glucuronidation [mainly through UDP-glucuronosyltransferase (UGT) 1A9, resulting in propofol glucuronide, PG] or hydroxylation [mainly cytochrome P450 (CYP) 2B6, resulting in 1- or 4-quinol, 1-Q and 4-Q] with subsequent glucuronidation [(1-quinol glucuronide (1-QG), 4-quinol glucuronide (4-QG)], or sulfation [4-quinol sulfate (4-QS)], respectively. After bolus administration (3 mg/kg) in adults, PG is the most contributing metabolite while urinary excretion of unchanged propofol only marginally (<1%) contributes to propofol clearance. In neonates, the contribution of quinol metabolites is much more relevant to overall metabolism in neonates compared to glucuronidation (Fig. 16.5b) [135]. Since CYP2B6 ontogeny is also relatively delayed, this pathway cannot compensate for the PNA-dependent glucuronidation pathway and in the absence of other (primary elimination pathways), this results in overall low propofol clearance in neonates, even more pronounced in early neonatal life (< eight days postnatal).

16.3.2 Role of Sulfotransferase (SULT) Enzymes in the Metabolism and Interaction of Drugs in Pediatric Populations: Role of Extrahepatic activity

The sulfotransferase (SULT) gene family encodes at least 11 distinct enzymes that are expressed in the cytosol and they catalyze the sulfate conjugation of a variety of endogenous (e.g., hormones and neurotransmitters) and exogenous chemicals (e.g., drugs). SULTs and glucuronosyltransferases may have overlapping substrate specificities and thus complimentary biotransformation. Sulfoconjugation can overcome the impairment of glucuronidation in the pediatric population as this reaction is practically mature at birth [51,132,133]. When SULT with 2-naphthol as a substrate was investigated in human adult and fetal liver, the activity in the adults was approximately threefold higher than that in the fetuses [136] (Table 16.2). However, in fetal kidneys and gut, the activity was twice as high as in the liver, whereas it was comparable to the hepatic activity in the lungs and the adrenals [71]. The sulfate pathway is the dominant metabolic route for salicylamide and paracetamol in infancy and childhood [126]. Similarly, the neonate can use sulfate conjugation as an alternative route for morphine metabolism before glucuronidation develops [71,137]. As CYPs and UGTs, there are several forms of SULTs and the developmental pattern of these isoforms can be different, as shown for two forms of phenolsulfotransferases, SULT1A1 (substrate 4-nitrophenol) and SULT1A3 (substrate dopamine) [138–140]. SULT1A3 develops earlier than SULT1A1 and its activity is threefold higher in the human fetal compared with adult liver. At midgestation, SULT1A1 activity is lower in the human fetal than the adult liver [141]. Gilissen *et al.* [142] demonstrated the presence of SULT1A1 activity as early as 15-week gestation and failed to see any significant changes in activity during gestation or at 1 or 1.5 years PNA. Thus, SULT1A1 activity increases in the adult after quite late in childhood development or perhaps in early adult. Barker *et al.* [143] examined the age-mediated change of SULT2A1. In the liver, both activity and protein levels were low to nondetectable before 25-week gestation but then increased substantially during the latter half of gestation to approach adult levels in the neonate. A similar SULT2A1 developmental expression pattern was observed in the kidney, although activity in this tissue was 10% of that observed in the liver.

16.4 CRITICAL PHYSIOLOGICAL PARAMETERS THAT PLAY ROLE IN DRUG METABOLISM, EXPOSURE, AND INTERACTION IN PEDIATRIC POPULATIONS

In addition to the complexity of ontogeny, other covariates [144] and many physiological parameters also change with age and these could alter the drug metabolism and, hence, drug dispositions among humans.

16.4.1 Protein Binding

The use of the well-stirred model to calculate clearance requires a consideration of the age-related changes in hepatic blood flow (Q_H) and unbound fraction (f_u) as shown in Equation 16.1. Therefore, the values of Q_H and f_u , specific for the age group, must be used to obtain estimates of *in vivo* hepatic metabolic drug clearance in specific pediatric groups. It is known that plasma protein levels are lower in the newborn gradually

increase with age. At birth, human serum albumin (HSA) concentrations are close to adult levels (75–80%), while α_1 -acid glycoprotein (AAG) is initially half the adult concentration. McNamara and Alcorn [13] have reported that age-dependent changes in f_u can be estimated for a particular pediatric age group on the basis of known pediatric plasma protein-binding ontogeny data. This approach scales the unbound fraction from adult to pediatric subjects on the basis of a correlation between unbound fraction and the relative levels of the major plasma-binding proteins (albumin and AAG) assuming a constant value for binding affinity between adults and pediatric subjects according to Equation 16.2:

$$\text{Calculated } in \text{ vivo } Cl_{\text{plasma}} = \frac{Q_H \times f_u \times \text{intrinsic } Cl'_{\text{plasma}}}{Q_H + f_u \times \text{intrinsic } Cl'_{\text{plasma}}} \quad (16.1)$$

$$f_{u,\text{infant}} = \frac{1}{1 + \frac{P_{\text{infant}}(1 - f_{u,\text{adult}})}{P_{\text{adult}} f_{u,\text{adult}}}} \quad (16.2)$$

where P_{infant} is the molar plasma protein concentration in infants and P_{adult} is the molar plasma protein concentration in adult. The authors showed that the extent of drug binding to HSA in children is close to that in the adult; however, the extent of protein binding for drugs largely bound to AAG increased with age. As listed in Table 16.3, for values published by the authors, the observed f_u versus predicted f_u values in infant and compared the results to those in adults for several drugs bound to HSA or AAG. From the results in Table 16.3, for all drugs, whether bound to HSA or AAG, f_u appeared to be different in children than in adults, but the difference did not exceed more than threefold. Since AAG is also an acute phase protein, a postoperative increase has been described. It should be noted that, in addition to lower HSA concentration, bilirubin and free fatty acids may influence the extent of binding of some drugs in the newborn. This binding is competitive in nature, and a highly bound drug (e.g., rocephine) is contraindicated in neonates because of displacement of (endogenous) unconjugated bilirubin, potentially resulting in kernicterus. Nau *et al.* [136] reported that the free fatty acid elevation after birth results in increased free fractions of diazepam and its main metabolite. Hyperbilirubinemia is also found to reduce the binding of a number of acidic drugs. Elevated free fatty acids and bilirubin may explain some of the intersubject variability for a given drug within a population of infants. The effect of elevated bilirubin, free fatty acid, or lower HSA in neonatal serum may explain the difference between f_u in neonates and adults, but it is clear that caution should be exercised when using predictive models in determining f_u in pediatric populations. In a study with antifungal drug micafungin, *in vitro* serum-binding study in serum from neonates using equilibrium dialysis, f_u of micafungin was found to be eightfold higher than that in adult serum [145]. This indicated that mechanistic studies of drug serum binding as a function of age should be conducted, especially with drugs that are extremely lipophilic.

16.4.2 Hepatic Blood Flow

Developmental changes in hepatic blood flow (Q_H) are poorly understood. Age-associated changes in Q_H may influence hepatic clearance when hepatic elimination is limited by the rate of hepatic drug transport and not enzyme activity [9]. With blood flow rate-limited drugs, developmental changes in metabolic enzymes will impact

TABLE 16.3 Difference between Children and Adults in Extent of Drug Binding to Either α_1 -Acid Glycoprotein (AAG) or HAS Data Were Reported by McNamara and Alcorn [13]

Age	Major Binding Protein (% of Adult)	Drug	Child		Adult f_u
			f_u Observed	f_u Predicted	
Premature:one day	AAG (53.4)	Alfentanil	0.350	0.171	0.099
		Fentanyl	0.230	0.263	0.160
	HAS (76.4)	Salicylic acid	0.175	0.154	0.123
Term: one day	AAG (53.4)	Alfentanil	0.269	0.171	0.099
		Alprenolol	0.370	0.219	0.130
		Desipramine	0.377	0.382	0.248
		Fentanyl	0.300	0.268	0.160
		Lidocaine	0.585	0.433	0.290
		Propranolol	0.418	0.219	0.130
		Quinidine	0.400	0.219	0.130
		Sufentanil	0.201	0.137	0.078
		Verapamil	0.396	0.179	0.104
		Cisplatin	0.150	0.115	0.090
	HAS (76.4)	Diazepam	0.035	0.023	0.018
		Digoxin	0.791	0.814	0.770
		Morphine	0.690	0.727	0.670
		Paracetamol	0.632	0.591	0.525
		Penicillin G	0.520	0.466	0.400
		Phenacetin	0.610	0.514	0.474
		Phenobarbital	0.658	0.528	0.461
		Phenytoin	0.181	0.153	0.122
		Valporate	0.121	0.191	0.153
		Salicylic acid	0.056	0.045	0.053
Term: one to seven days	AAG (53.4)	Alprenolol	0.360	0.219	0.130
		Cloxacillin	0.140	0.080	0.062
Term: 7–28 days	HAS (76.4)	Ceftriaxone	0.285	0.115	0.090
3–12 months	AAG (54.9)	Lidocaine	0.320	0.426	0.090
		Quinidine	0.220	0.214	0.130
		Sufentanil	0.115	0.141	0.083
	HAS (77.2)	Ceftriaxone	0.160	0.114	0.090
		Phenytoin	0.147	0.152	0.122
		Valporate	0.143	0.107	0.085

hepatic clearance only when the enzyme activity is exceedingly under developed such as when enzyme activity becomes the rate-limiting factor for overall drug elimination. For blood flow rate-limited drugs, the use of an adult parameter or allometric scaling model for Q_H may not be appropriate for newborns and a significant portion of hepatic portal blood flow will be shunted away from the functioning hepatocytes in the first or second week of life. The Q_H values according to population age and gender are listed in Table 16.4a.

TABLE 16.4 Age-Dependent Changes in Physiological Parameters Affecting the Drug Metabolism-Mediated Drug Clearance(a) Organ Blood Flows ($1 \text{ min}^{-1} / \text{kg tissue}$), Cardiac Output, Cardiac Index, and Total Hepatic Blood Flow in the Different Age Groups

Organ	Neonate (M/F)	6 Months (M/F)	1 Year (M/F)	2 Years (M/F)	5 Years (M/F)	10 Years (M/F)	15 Years (M/F)	Adult (M/F)
BRN	0.50/0.50	0.70/0.70	0.70/0.70	0.70/0.70	0.81/0.81	0.68/0.68	0.54/0.52	0.54/0.52
HRT				0.79/1.08	(In all age groups)			
LNG ^a				0.16/0.16	(In all age groups)			
LVR ^b	0.59/0.57	0.49/0.47	0.41/0.39	0.36/0.35	0.32/0.30	0.28/0.28	0.28/0.27	0.25/0.25
KDN	1.34/1.08	2.82/2.36	2.97/2.53	2.93/2.52	3.43/2.79	3.94/2.78	4.21/3.25	4.00/3.49
SKN	0.16/0.16	0.16/0.16	0.16/0.16	0.16/0.16	0.16/0.16	0.16/0.16	0.12/0.12	0.12/0.12
STM	1.25/1.13	1.05/0.94	0.88/0.78	0.77/0.70	0.68/0.60	0.60/0.56	0.60/0.54	0.53/0.50
GUT	2.11/2.50	1.78/1.70	1.49/1.41	1.31/1.27	1.15/1.09	1.02/1.02	1.01/0.97	0.90/0.91
PSP	2.18/2.11	1.85/1.75	1.56/1.45	1.36/1.29	1.18/1.11	1.06/1.05	1.07/1.00	0.93/0.92
BNE				0/0	(In all age groups)			
BMA				0.024/0.026	(In all age groups)			
MSC				0.039/0.039	(In all age groups)			
FAT				0.024/0.026	(In all age groups)			
CRC				0.084/0.090	(In all age groups)			
CO ^c	0.58/0.55	1.35/1.22	1.68/1.52	2.06/1.90	2.88/2.63	3.90/3.74	5.77/5.15	6.79/5.69
CI ^d	2.55/2.52	3.41/3.31	3.57/3.46	3.69/3.54	3.81/3.62	3.58/3.36	3.55/3.32	3.59/3.44
Q _a ^e	0.22/0.21	0.38/0.35	0.45/0.41	0.53/0.50	0.72/0.68	1.04/1.04	1.55/1.45	1.72/1.52

Source: Tables were copied with permission [146].

^aBronchial artery.^bHepatic artery.^cCardiac output (1 min^{-1}).^dCardiac index ($1 \text{ min}^{-1} \text{ m}^{-2}$).^eTotal hepatic blood flow (1 min^{-1}).

(continued overleaf)

TABLE 16.4 (Continued)

(b) Organ/Tissue Weights (kg), Body Weights, and Total Extracellular Water in Both Sexes (M/F) in the Different Age Groups

Organ	Neonate (M/F)	6 Months (M/F)	1 Year (M/F)	2 Years (M/F)	5 Years (M/F)	10 Years (M/F)	15 Years (M/F)	Adult (M/F)
BLD ^a	0.16/0.15	0.35/0.31	0.48/0.42	0.54/0.49	0.74/0.72	1.44/1.25	2.23/1.72	3.07/2.08
BRN	0.35/0.35	0.75/0.71	0.94/0.87	1.12/1.03	1.29/1.19	1.36/1.25	1.39/1.28	1.45/1.30
HRT	0.02/0.02	0.04/0.04	0.05/0.05	0.07/0.06	0.09/0.09	0.15/0.15	0.26/0.23	0.33/0.25
LNG	0.06/0.05	0.12/0.12	0.16/0.17	0.24/0.24	0.34/0.32	0.43/0.50	0.90/0.75	1.20/0.95
LVR	0.12/0.13	0.27/0.25	0.36/0.34	0.48/0.46	0.59/0.59	0.87/0.89	1.35/1.33	1.80/1.40
KDN	0.03/0.03	0.05/0.04	0.06/0.06	0.09/0.08	0.11/0.10	0.18/0.18	0.25/0.24	0.31/0.28
SKN	0.17/0.16	0.29/0.27	0.34/0.32	0.41/0.39	0.55/0.53	0.80/0.81	2.01/1.66	3.30/2.30
STM	0.01/0.01	0.02/0.02	0.02/0.02	0.03/0.03	0.05/0.05	0.99/0.99	0.14/0.14	0.15/0.14
GUT	0.05/0.05	0.09/0.09	0.14/0.14	0.19/0.19	0.34/0.34	0.58/0.58	0.82/0.82	1.02/0.96
-cnt ^b	0.14/0.14	0.23/0.23	0.24/0.24	0.30/0.30	0.30/0.30	0.42/0.42	0.74/0.74	0.90/0.85
PSP	0.02/0.01	0.03/0.03	0.05/0.05	0.07/0.07	0.09/0.09	0.14/0.15	0.24/0.23	0.29/0.25
BNE	0.17/0.17	0.40/0.38	0.59/0.59	0.85/0.82	1.26/1.26	2.30/2.30	4.05/3.70	5.50/4.00
BMA	0.00/0.00	0.00/0.00	0.02/0.02	0.03/0.03	0.16/0.16	0.63/0.63	1.48/1.38	2.48/1.80
MSC	0.80/0.80	1.35/1.35	1.90/1.90	2.83/2.83	5.60/5.60	11.0/11.0	24.0/17.0	29.0/17.5
FAT	0.89/0.89	2.97/2.85	3.64/3.23	3.76/3.72	5.00/5.00	7.50/9.00	9.50/16.0	14.5/19.0
CRC	0.56/0.37	1.08/0.56	1.20/0.76	1.62/1.18	2.18/1.35	3.51/3.41	7.35/6.48	7.70/6.95
BW ^c	3.55/3.33	8.03/7.25	10.2/9.18	12.6/11.9	18.7/17.7	31.4/32.6	56.7/53.7	73.0/60.0
ECW ^d	38/39	27/27	29/29	29/29	27/26	23/23	22/22	21/20

^aExcluding blood in capillary spaces in the organs.^bGut contents.^cBody weight (kg).^dExtracellular water (% of BW).

16.4.3 Liver Size Relative to Body Weight

The major organs responsible for drug clearance are the liver (and kidneys). Their developmental changes in relative size affect the PK of drugs in an age-dependent manner. The most prominent observation is that the mass of liver (and kidney) relative to age is several-fold greater in preschool-age children than in young adults [10] (Fig. 16.6a). On the basis of this observation, the ratio of liver to body mass is not a constant parameter; it is considerably greater in infants and young children than in adults. This may contribute to greater hepatic clearance in children; however, the size alone can account for some, but not all the developmental differences observed in drug clearance between adults and children [147,148]. Scaling by normalization to a 70-kg individual using the three-fourth power allometric rule was used in the prediction of pediatric data based on adults data, adjusted for the differences in liver size relative to body mass in pediatric versus adult individuals [149]. Because the weight ratio of different organs may vary with age, it is recommended that age appropriate parameters ([146]; Table 16.4b) to be also used for calculating the clearance and estimation of doses in children from adult data. Yanni *et al.* [15] determined the factors responsible for the higher clearance of the metabolically cleared antifungal drug voriconazole in children age two to eight compared with adult and concluded that the ratio of liver size to body mass seems not to contribute to the difference in clearance (although the scaling factor that is the product of milligram of microsomal protein and ratio of gram liver to kilogram body mass was statistically different, greater in children compared to adults) [69].

16.4.4 Milligram Microsomal Protein per Gram Liver

Recent studies have shown that microsomal protein content also changes with age. Studies using a set of pediatric samples suggested an increase in microsomal protein content from birth (26 mg/g liver) to the average microsomal protein content of a 30 year old (40 mg/g liver), which then declined to 31 mg/g liver for the average 60 year old [150]. When correlation analysis was performed between observed and calculated clearance for 21 different drugs in adults at different milligram microsomal protein per gram of liver (MPPG) (77, 45, 21, and 34 mg/g), the best correlation was found when MPPG is 34 mg/g (Fig. 16.6b2). MPPG values determined with samples from pediatric livers indicated that MPPG increases with age, the mean MPPG value for young infants less than one year of age was as low as 10 mg/g, while the mean value for children <10 years of age was 23 mg/g, and for adults, the mean value was 31 mg/g (Fig. 16.6b). It is clear that the change in MPPG with age has an impact on altering the scaling factor value and the hepatic clearance of drugs in human population, especially between young children and adults.

16.4.5 Uptake and Efflux Drug Transporters

In addition to DMEs, transporter-mediated processes also play critical roles in the overall disposition of numerous drugs in clinical use. Besides their roles in xenobiotic transport, drug transporters often mediate important physiologic functions via transport of endogenous substrates such as amino acids, bile acids, and hormones. Transport proteins are known to affect drug bioavailability and clearance and, most importantly,

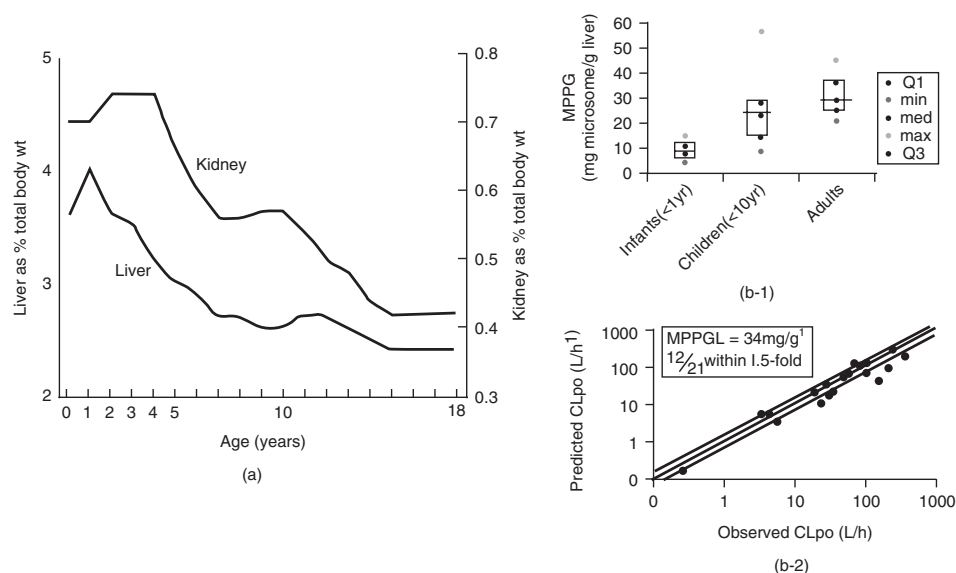


Figure 16.6 Changes in physiological parameters as a function of age. (a) Changes in relative liver and kidney mass as percentage of body weight from infancy to young adulthood (Adapted with permission from Chen *et al.* [1]). (b-1) Box plot of MPPG values measured in infants, children, and adults based on results listed in [28]; (b-2) analysis of observed oral clearance of 21 drugs and predicted values calculated by using MPPG value of 34 mg/g liver. *Source:* Adapted with permission from Barter *et al.* [150]).

efficacy in target organs [151]. Drug transporters are expressed in all body organs; however, they are most extensively investigated in those organs that are responsible for drug disposition and clearance such as the liver, kidney, and intestine [152,153]. The localization of major transporters and their role in the disposition of drugs and endogenous biomarkers are also reported by these investigators. Drug transporters can be generally separated into two major classes: uptake and efflux transporters. The role of uptake transporters is to facilitate the translocation of drugs into cells. Major members of the uptake transporters are the organic anion-transporting polypeptide family (OATP, *SLCO*), sodium–taurocholate cotransporting polypeptide (NTCP, *SLC10A1*), organic anion transporter (OAT, *SLC22A*), and organic cation transporter (OCT, *SLC22A*) families. Among the uptake transporters, certain transporters have been shown to exhibit both influx and efflux properties, while others act only as uptake transporters. For example, the OATPs are primarily considered uptake transporters that mediate transport via sodium-independent mechanisms in contrast to sodium-dependent uptake transporters such as NTCP. Efflux transporters export drugs from the intracellular to the extracellular milieu, often against high concentration gradients. The ABC efflux transporters are P-glycoprotein (P-gp, *ABCB1*), the bile salt export pump (BSEP, *ABCB11*), the multidrug resistance associated (MRP, *ABCC*) protein family, and the breast cancer resistance protein (BCRP, *ABCG2*). The efflux transporter, P-gp, has different functions in various tissues. For example, its expression is associated with a decreased absorption of substrate xenobiotics in the gut, which leads to lower bioavailability, whereas it decreased the penetration of substrate xenobiotics into the brain across the

blood–brain barrier, reducing the CNS effect or unfavorable CNS toxicity. However, in the liver and renal tubules, P-gp facilitates the excretion of substrate xenobiotics into bile and urine, respectively [152–154]. As in the other organs, drug transporters are either localized in the basolateral membrane or apical (canalicular) membrane to enhance the uptake from blood into the hepatocytes or the efflux into bile.

In humans, very little information is available on the ontogeny of drug transporter-mediated drug disposition and its effect on the extent of drug metabolism. Johnson and Thomson [6] and Yanni *et al.* [145] have recently presented results from *in vitro* investigations that described age-related changes in expression of transporters in hepatic and extrahepatic human tissues and provided an insight of the impact of these changes of the drug transporter functions on altering drug disposition.

In humans, P-gp expression in the central nervous system from neonates born at 23–42 weeks of gestational age suggested a pattern of localization similar to that in adults [2]. However, the level of expression of P-gp appeared to be lower than that in adults. Johnson and Thomson [6] in their review article reported that the mRNA and protein expression of P-gp in normal duodenal biopsies from children aged 1 month to 17 years was not statistically different and concluded that the age has no effect on the expression level. In addition, P-gp mRNA and protein in human were detected as early as 11–14 weeks of gestation in the liver and kidney but were detected only in the intestines of full-term infants. In contrast to these results, others have demonstrated an age-dependent increase of intestinal expression of MDR1, where a fourfold higher expression of MDR1 was observed in young adults than in neonates. These data indicated a postconception age-related maturation of these transporters. This conclusion was supported by a clinical study of cyclosporine by Fanta *et al.* [155] who showed that while the bioavailability of cyclosporine was independent of genetic polymorphism in MDR1 in younger children than eight years, genetic polymorphism in MDR1 codes for P-gp in children older than eight years caused 1.5-fold higher bioavailability of cyclosporine in noncompetent MDR1 subjects than in competent MDR1 subjects. These results revealed the presence of active P-gp function in children older than eight years but not in younger populations. However, when immune staining of MDR1 in children liver (less than two years of age) was compared to that in adults, localization and expression levels were similar. In humans, it has been reported that oral clearance of fexofenadine depends on the efflux transporters P-gp and organic anion-transporting peptides in the gastrointestinal tract [156]. A population PK analysis of fexofenadine in infants and children aged 6 months to 12 years revealed no significant effect of age on oral clearance of the drug [157]. This clinical data indicate that the functions of both OATP uptake and P-gp efflux transporters might mature as early as six months of age. It has been found that P-gp, MRP1, and MRP2 act synergistically with P450 to protect the organism from toxic compounds. Digoxin serves as an excellent example for ontogeny of renal elimination. The drug is not only excreted by glomerular filtration but also extensively secreted by the tubular cell by P-gp. Young children need threefold higher doses of digoxin per kilogram BW than adults. This difference cannot be explained by GFR changes alone, as GFR in young children is twofold higher than in adults per kilogram. The secretion of para-aminohippurate (PAH) demonstrates that organic anion secretion is low at birth in humans and other mammals, increases over the first few weeks of neonatal life, and then declines to adult levels [158]. This increase in organic anion secretion is disproportionate to the increase in kidney mass and is thought to reflect the specific maturation of the organic anion transport system

rather than the growth and development of the kidney itself. A wide variety of drugs are transported by OATs, including ACE inhibitors, angiotensin receptor blockers, β -lactam antibiotics, antiviral drugs, and NSAIDs, and have been prescribed to children at all ages with no reports on toxicity, implying that OATs are fully functional [1].

16.5 DRUG CLEARANCE AND EXPOSURE IN RELATION TO DRUG METABOLISM IN PEDIATRIC POPULATIONS

The changes in phase I and II DMEs as a function of age is one of the factors that affects drug clearance in pediatric populations and that has been extensively investigated *in vitro* by measuring expression and activities in tissues from different age groups [2,14–16,24,69]. Furthermore, it has been investigated in the clinical setting (*in vivo*) by comparing the hepatic metabolic clearance of DMEs probe substrates in various age groups and *in silico* using physiologically based pharmacokinetic (PBPK) models [11,12,159–161]. These studies showed that age-associated changes in phase I/II DMEs can have a significant impact on PK/pharmacodynamics (PD) of drugs whose elimination is predominately dependent on the metabolic pathway mediated by those enzymes. Accordingly, altered dosage regimens were recommended to optimize drug exposure and achieve therapeutic efficacy and safety in children [160,161]. These studies have improved understanding on the differences in PK/PD of drugs between children and adults during the last two decades, and as a result, a child is no longer considered a “miniature” adult and pediatric dosing regimens are now adjusted based on maturity of body functions, drug clearance, and exposure [160,161] and not just by simplistic (per kilogram) or more complex (body surface area, BSA, $\text{kg}^{0.75}$) scaling of an adult dose. However, more research in this field is warranted to improve our ability to estimate safe and efficacious doses in different pediatric subpopulations from our knowledge of the adult doses [69].

In the past, therapeutic or toxicological assessment in pediatric populations was done only on the basis of an approximate linear relationship between PNA and BW or surface area. Hence, dose was adjusted on the basis of age-associated differences in BW or BSA [18] according to Equations 16.3 and 16.4:

$$\text{Dose}_{\text{child}} = \text{Dose}_{\text{adult}} \times \frac{\text{BW}_{\text{child}}}{\text{BW}_{\text{adult}}} \quad (16.3)$$

$$\text{Dose}_{\text{child}} = \text{Dose}_{\text{adult}} \times \frac{\text{BSA}_{\text{child}}}{\text{BSA}_{\text{adult}}} \quad (16.4)$$

These approaches might have caused underpredicting of therapeutic dose, thus therapeutic failure, or overpredicting the dose that might have caused drug-related toxicity. It is known that the total exposure to a drug determined by the area under the plasma concentration versus time curve is not only determined by the bioavailable dose fraction (F) but also by the efficiency of the elimination mechanisms as measured by the systemic clearance (Cl_s) as shown in Equation 16.5:

$$\text{AUC}_0^\infty = \frac{F \times \text{Dose}}{\text{Cl}_s} \quad (16.5)$$

Therefore, age-dependent differences in bioavailability and clearance that altered by the extent of metabolism (if metabolism is the mechanism of elimination) usually account for differences in plasma concentrations and exposure among age groups. Scaling of a dose by simple adjustment per kilogram basis or surface area does not consider the developmental changes as a result of both growth and maturation of the physiological and biochemical processes governing bioavailability and clearance.

Clearly, because age-associated changes in body composition and organ function are not linear and can be discordant during the first decade of life, those simplified dosing approaches across the span of childhood or in various disease states may account for 90% of ADRs among hospitalized children as it was reported by Johnson [6]. Thus, therapy must be optimized by assessing the developmental differences of drug clearance that govern drug exposure. In a recent review article, Anderson and Lynn [9] published a search of the literature by using the MEDLINE database regarding age and PK (1979–July 2008) and then evaluated the literature by focusing on drugs with one primary elimination pathway (e.g., renal clearance or clearance involving a single metabolic isozyme). Their analysis revealed that children need weight-corrected doses that are substantially higher than adult doses for drugs that are metabolically eliminated solely by cytochrome P450 (P450) CYP1A2, CYP2C9, or CYP3A4. In contrast, weight-corrected doses for drugs eliminated by renal excretion or metabolism involving CYP2C19, CYP2D6, *N*-acetyltransferase 2 (NAT2), or UGT are similar in children to those in adults. The authors added that in children, bioavailability of drugs with high first-pass metabolism is decreased if CYP1A2, CYP2C9, or CYP3A4 is responsible for the metabolism. However, this report has been contradicted by other reports [157] that indicated for drugs such as voriconazole that metabolized by CYP2C19, CYP3A4, and FMO3 [15] in children required two times as higher bioequivalence dose as those of adults.

16.6 CLINICALLY RELEVANT DRUG INTERACTION IN RELATION TO DRUG METABOLISM IN PEDIATRIC PATIENTS

Metabolism-based DDIs mostly occur when one drug altered the function of DME or enzymes that are responsible for the metabolism and clearance of other drugs, resulting in significant changes in exposure and potential toxicity. A few review articles and clinical studies have recently discussed clinically relevant P450-mediated DDIs in pediatric populations. The medications that are most frequently used and are linked with major interactions in human populations, in adults as well as in pediatric patients, have been illustrated in previous sections of this chapter. Examples of the medications that are known to alter other drug's extent of P450-mediated metabolism in pediatric populations are summarized in Table 16.5. As we outlined previously, drug metabolism can be altered owing to the maturation of body functions, interaction with other medications, genetic variation, diet, etc. This can cause ADRs or DDIs in pediatric populations that are not generally seen in the adult populations. For instance,

1. altered metabolism of sodium valproate in children under three years caused higher incidence of hepatotoxicity;
2. impaired metabolism of chloramphenicol in neonates resulted in the gray baby syndrome (cyanosis and respiratory failure);

TABLE 16.5 P450 Probes in Clinical Studies in Children and Medications That Leads to Potential Drug Interactions

CYP Probe	Substrate(s)	Studies	Modulators of Metabolism	
			Inhibitor	Inducer
1A2	Caffeine	+ Caffeine breath performed in children CYP1A activity detectable in infants >33 days Pons <i>et al.</i> 1988 [36] + Decreased oral caffeine clearance in preterm infants compared with infants and adults Aranda <i>et al.</i> 1979 [18]	Theophylline	Rifampicin Carbamazepine Tobacco Phenytoin
2C9	Diclofenac	+ Systemic weight-corrected Diclofenac clearance increased in children aged four to six years by twofold compared with adults Korpela and Olkkola 1990 [162]	Fluconazole voriconazole	
CYP2C19	Omeprazole	+ Oral clearance increased in children one to six years compared with adults Andersson <i>et al.</i> 2000 [163]	Fluvoxamine	Rifampicin
	Phenytoin	+ Increased clearance in children two to eight years compared with adults Curras <i>et al.</i> 2009 [164]; Yanni <i>et al.</i> 2010 [15]	Fluoxetine	Carbamazepine
2D6	Voriconazole Dextromethorphan	+ Maturation of CYP2D6 pathway ahead of CYP3A4 Johnson <i>et al.</i> 2000 [165]. Phenotype consistent with genotype >14 days postnatal		
	Tramadol, codeine	+ CYP2D6 activity depends on ontogeny, polymorphisms, and renal maturation Allegaert <i>et al.</i> 2008 [67]		
3A	Midazolam (oral and iv)	+ IV clearance, 3–13 years sixfold higher than patients <two year and twofold higher than adults Hughes <i>et al.</i> 1996 [166]	Voriconazole	Rifampicin
	Erythromycin, warfarin	+ Oral clearance, <two years twofold higher than children >12 years Reed <i>et al.</i> 2001 [80]	Itraconazole	Phenobarbital
			Ritonavir	Efaviranez

3. altered drug metabolism caused metabolic acidosis following the use of propofol in the critically ill children; and
4. increased resistance to acetaminophen toxicity in children relative to adults, apparently because of an increased capacity for sulfate conjugation early in life.

In addition to the well-documented cases of drug interactions in pediatric patients that are described previously, there are other studies that reported significant inadequate drug therapy owing to observed metabolism-based drug interactions. For example, Leroy *et al.* [158] reported that intestinal inflammation that compromises CYP3A activities, coadministration with calcium channel blocker and tacrolimus, and genetic polymorphism of CYP3A and (P-gp) resulted in a toxic tacrolimus trough blood level in a renal transplant. In another study with tacrolimus, Moreau *et al.* [167] showed that tacrolimus trough blood concentration increased by introducing omeprazole to liver transplant pediatric patients, although these two drugs are known to be metabolized by different CYP isozymes (the former by CYP3A, while the latter by CYP2C19 and to less extent by CYP3A; Fig. 16.7). The authors concluded that, with a parallel *in vitro* metabolism study with liver microsomes, the interaction between these two drugs is based on competitive (reversible) inhibition of CYP3A-mediated tacrolimus metabolism. When CYP2C19 became saturated in extensive metabolizers (as in this patient) under administration of high dose of omeprazole (or was absent in a poor metabolizer subject), CYP3A becomes a key player in the metabolism of omeprazole and would explain the metabolically based interaction between these two drugs.

One of the therapeutic areas in pediatric patients where the metabolism-mediated drug interaction plays critical roles is the treatment of human immunodeficiency virus (HIV). It requires long-term administration of multiple antiretroviral (ARV) drugs, and usually other medications for possible infections, the occurring illnesses,

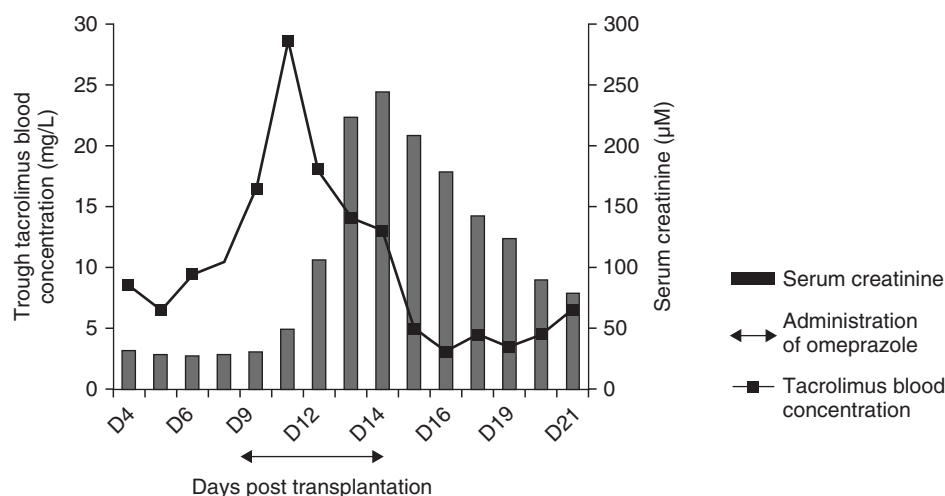


Figure 16.7 Effect of omeprazole on tacrolimus blood concentration and serum creatinine in a pediatric liver recipient. Relationship between trough tacrolimus plasma concentration and days of omeprazole administration as well as liver transplantation was presented to determine the interaction between both drugs as shown by the increase in plasma concentration of tacrolimus. The impact of omeprazole interaction on the decrease of serum creatinine was also indicated. Source: Adapted with permission from Moreau *et al.* [167].

or management of side effects. The complexity in this pharmacotherapy indicates that DDIs are inevitable.

Metabolic interactions are a major concern in HIV therapy due to the fact that most PPIs and nonnucleoside reverse transcription inhibitors (NNRTI) are substrate for CYP3A enzymes, which also metabolize 60% of therapeutic drugs [168]. For infants, children, and adolescents, the metabolic capacity of the liver can vary from <10% to >200% of adult values, depending on the age of patients and the enzyme involved [2]. Thus, interactions may be of a greater or lesser magnitude in HIV-infected children.

Ritonavir, for example, inhibits CYP3A in an irreversible, concentration-dependent manner [168]. Ritonavir increases clarithromycin AUC by 77% and almost completely inhibits the major metabolite formation [169]. Ritonavir increased the concentrations of oral and nasal fluticasone, which may result in hypothalamic–pituitary axis suppression [170]. In addition, several reports have indicated that ritonavir inhibits CYP2D6, 2C9, and 2B6, but to a lesser extent than CYP3A4, and has the ability to induce UGT, 2C9, 2C19, 1A2, and 2B6. These effects result in interactions with ethinyl estradiol, voriconazole, and theophylline. Voriconazole AUC is reduced 82% when coadministered with ritonavir [171].

On the other hand, Atazanavir inhibits UGT1A1, which leads to elevations in unconjugated bilirubin in some children, just as it does in adults. This hyperbilirubinemia may be more pronounced in children <18 months as UGT1A1 activity may not have attained adult levels yet [2]. Atazanavir inhibition of UGT1A1 increases raltegravir AUC by 41%, which is a therapeutic interaction. A phase II study observed that more patients achieved low plasma HIV RNA concentrations when received a combination of atazanavir with raltegravir [172] in adults and children.

Interestingly, the AUC of atazanavir is reduced by 74% when given with efavirenz, as this later agent was found to be an inducer of CYP3A4. Higher dose of atazanavir is recommended if combined with efavirenz, although anticonvulsant concentrations must be carefully monitored in children taking efavirenz [173].

On the basis of these information, Fletcher [174] concluded that (linear) extrapolation of the magnitude of DDIs from adults to children is problematic because of the age-associated changes in body composition and organ function. Therapeutic drug monitoring, if available, may be a useful clinical tool to ensure that safe and effective concentrations of antiretrovirals and coadministered medications are achieved.

16.7 LIMITATION IN INVESTIGATING DRUG METABOLISM AND DRUG INTERACTION IN PEDIATRIC PATIENTS: APPROACHES AND EXPERIMENTAL DESIGNS TO OVERCOME SUCH PROBLEMS

The aphorism “Children are not small adults and infants are not small children” applies not only to their anatomy and physiology but also to their drug disposition and drug response. As outlined previously, despite increasing knowledge in ontogeny and drug therapy, there is much to learn about how to determine safe and effective doses of therapeutic agents. Because of perceived ethical considerations, children are rarely included in most product submission studies. Therefore, data on dosing, efficacy, and safety that are available for adults are not usually available for children. When available, data are often not from rigorous clinical trials. Historically, 75% of the drugs have insufficient labeling information for pediatric dosing, safety, or efficacy [175].

Inadequate dosing and safety information places children at risk for adverse events and denies them potential therapeutic benefits.

Several organizations in the United States and Europe have funded research to determine the mechanisms underlying the differences in disposition of drugs between pediatric populations and adults. These efforts aim at determining the role of ontogeny in DMEs and drug transporters with the overarching goal to use this information in setting alternative approaches that predict PK parameters of drugs in children.

Several *in vitro* and clinical studies of probe substrates and drugs used in children and adults have revealed that the ontogeny in DMEs and drug transporters is associated with observed significant differences in PK and safety of drugs between children and adults. Drugs such as omeprazole, theophylline, caffeine, digoxin, cyclosporine, sirolimus, phenytoin, and voriconazole whose clearance is mediated by DMEs (and/or drug transporters) have different plasma clearance in children than in adults. However, to date, knowledge of differences in DMEs and/or transporters between adults and children does not yet allow prediction of the clearance or dose of drugs in pediatric populations based on the adult doses, prospectively. Elucidating the major clearance mechanism(s) of a drug and the differences and/or similarities between children and adults in DMEs, transporters (not addressed in this chapter), and other physiological factors that affect drug disposition using *in vitro* model systems, one can predict clearance, and thus efficacious and safe dose, of the drug in children based on the known clearance and dose in adults. Mechanistic studies should be conducted for all drugs that are currently investigating in children so that our knowledge in drug therapy in pediatric patients is improved and drug safety is ensured. However, because of ethical issues, the availability of biological samples from children to conduct retrospective studies can also be of limitation.

de Wildt *et al.* [176] discussed the challenges encountered when translating adult drug study design to children in clinical practice, using CYP3A phenotyping as an example with probes such as midazolam clearance and erythromycin breath test. Ultimately, the authors were aimed to provide tools that could support pharmacotherapy in pediatrics. As discussed in other sections, the authors have discussed and recommended the use of therapeutic probes; understand the developmental changes in the metabolism pathways and other patients specific covariates; accepting ethical challenges such as limitation of use nontherapeutic probes in phenotyping studies and sampling; considering the use of noninvasive approaches such as urinary drug to metabolite ratio (e.g., hydroxy cortisol/cortisol); and sensitive analytical assay to reduce the sample size. The use of population PK modeling has also proven to be an effective approach to determine drug clearance rate of several drug substrates [81,82,84,132–135,177], can be used to limit sampling per patient, and a means to deal with intersubject variations. Finally, microdosing has been recommended to limit risk of nontherapeutic dose and potential toxicity.

In addition to these review articles, several articles have demonstrated the usefulness of using PBPK models in predicting PK parameters of drug, drug exposure, and safety. The *in vitro* and *in vivo* data are also essential elements in building strategic PBPK; however, there has been great interest in building a pediatric-specific PBPK (P-PBPK) model (Simcyp Pediatric ADME Simulator) by the pharmaceutical industry, regulatory organizations such as the FDA, and clinical practice to improve the efficiency of drug development, especially in populations where designing and conducting clinical studies is more challenging. P-PBPK was used to simulate a number of published clinical

studies. Simulations were used to explore the likely underlying reasons for observed PK behavior of drugs in critically ill children. In addition, the use of P-PBPK models to predict complex DDIs highlighted disparities with adult populations. Prior knowledge of *in vitro* drug provides a starting point to simulate PK and multiple DDI encountered in critically ill pediatric patients [160,161].

In case of nonclinical practice, pediatric tissue banks of normal and diseased specimens should support organizations and academic institutes to conduct molecular mechanistic studies for test drugs; hence, creating a data base for *in silico* prediction of PK and dosing strategy of all drugs will be available before first time dosing in children.

Voriconazole is cleared predominantly by metabolism and *in vitro* studies have implicated CYP2C19, CYP3A4, and in addition to the P450 enzymes, FMO can metabolize voriconazole to its major circulating metabolite, the *N*-oxide [178]. Clinical studies have indicated that voriconazole clearance in children <11 years of age is higher than in adults [179,180] and children have lower oral bioavailability than adults [157]. To elucidate the source(s) of these differences, *in vitro* metabolism studies were conducted using liver tissues from adults and children, and the results were modeled to estimate *in vivo* clearance in children. *In vitro* metabolism of voriconazole by microsomes, prepared from livers of children and adults, showed that the oxidative metabolism is significantly faster in children, as indicated by shorter *in vitro* half-life ($T_{1/2}$). In contrast to similarity in the K_m value, the apparent V_{max} for oxidation of voriconazole to the *N*-oxide is approximately three times higher in children compared with adults. Thus, the microsomal system appears to mimic *in vivo* metabolic clearance of voriconazole. The ratio of Michaelis–Menten kinetic parameters, (V_{max}/K_m) of voriconazole *N*-oxidation by liver microsomes from children and adults, was used to calculate *in vivo* clearance. The calculated scaling factor, based on milligram microsomal protein per gram liver (MPPG) and liver weights that were normalized to BW, was marginally different ($p > 0.04$) between children and adults. The calculated *in vivo* clearance values were ~80% of the observed plasma clearance of voriconazole in children and adults. These results indicate that the higher clearance of voriconazole in children is the result of faster oxidative metabolism by hepatic enzymes. The *N*-oxidation by FMO3 and CYP2C19 in children is five and three times that in adults, respectively, but the estimated rate of *N*-oxidation by CYP3A4 is the same in both groups (Fig. 16.8). On the basis of this study, it has been recommended that prediction of clearance in pediatric population can be based on available PK data in adult populations and comparative data in a relevant *in vitro* assay using tissue from adults and children can be employed to provide an insight for the potential PK in clinic in pediatric patients. Several steps should be considered:

1. The design of *in vitro* studies should be guided by the known mechanism of clearance in adults, such as metabolism, biliary excretion, or renal excretion.
2. *In vitro* clearance of a drug in biological samples from adults to be experimentally determined using *in vitro* concentrations comparable to plasma drug concentrations, then intrinsic clearance to be calculated.
3. The well-stirred model can be used to calculate plasma clearance by including f_u and Q_H values corresponding to age group (Table 16.4a). For neonates, f_u has to be measured in neonatal serum. For older children, f_u were not statistically different than adults [145]; thus, the adult value of f_u can be used for older children.

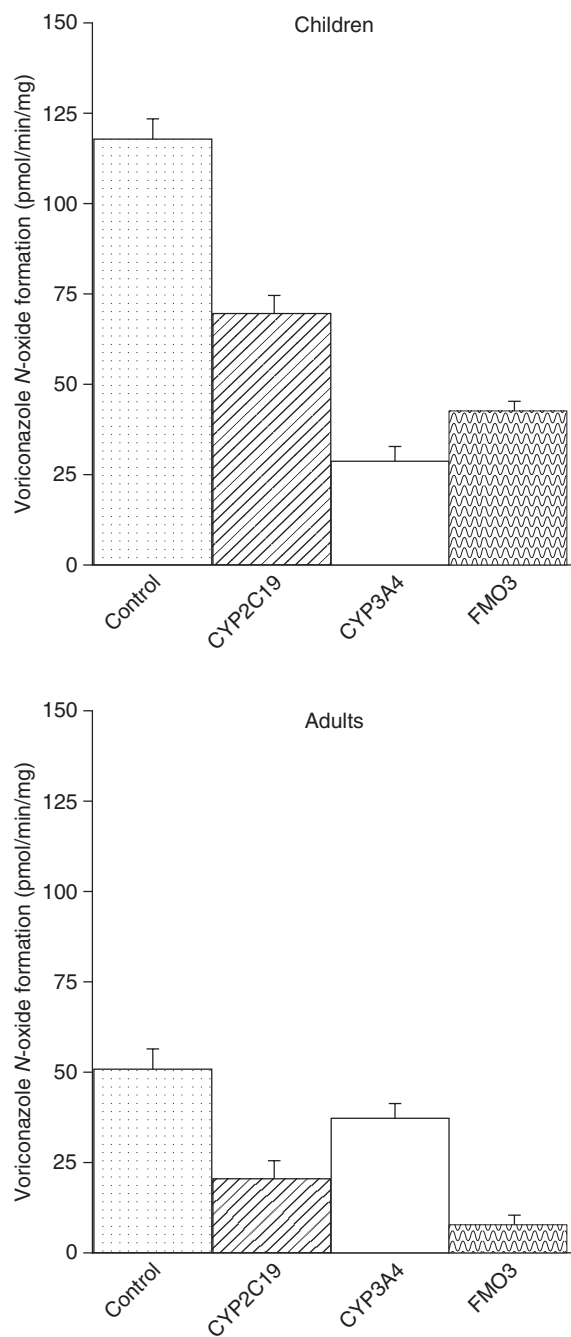


Figure 16.8 Contribution of oxidative metabolism by P450 and FMO enzymes to the higher voriconazole clearance in children compared with adults. *In vitro* metabolic studies of voriconazole using microsomal samples from children with ages two to eight–years or adults revealed the higher metabolic rate of CYP2C19 and FMO3 enzymes in children, which cause the observed higher clearance in children compared with adults. *Source*: Results adapted from Yanni *et al.* [15].

4. For a metabolically cleared drug, $V_{\max}$ for the formation of major metabolites by pediatric liver samples was determined *in vitro*. Intrinsic clearance in children was calculated by multiplying the ratio of ($V_{\max \text{ child}}/V_{\max \text{ adult}}$) by the ratio of (scaling factor for child/scaling factor for adult).
5. The good relationship of adult *in vitro* intrinsic clearance–*in vivo* clearance will ensure the prediction of the *in vivo* clearance in children based on *in vitro* studies as indicated earlier. Factors that might contribute to the observed disposition of drugs in children might also be revealed from the resulted *in vitro*–*in vivo* relationships.

16.8 OFF-LABEL USE OF DRUG THERAPY IN PEDIATRIC PATIENTS

For a drug to be marketed in the United States or Europe, the maker must submit adequate and well-controlled trials that reveal the efficacy and safety of the product when used as intended to the FDA or European Medicines Agency (EMA). These trials are the basis of the labeling or package insert instructions for approval by the FDA or EMA. Children are usually not included in most product submission studies and, therefore, the data on dosing, efficacy, and safety that are available for adults are not usually available for pediatric patients. Other data may be available but are often not from the same type of rigorous clinical trials. The use of unlicensed and off-label medicines in children is widespread and has raised an increasing concern over the last years. Historically, 50–75% [175,181] of the medicines used in children have only been studied in adults and not necessarily for the same indication [182]. The insufficient labeling information for pediatric dosing, safety, efficacy, and/or appropriate pharmaceutical formulations for use in children may expose them to unwanted overdosing, leading to adverse events or underdosing without the expected efficacy. Lacking such information often denies pediatric patient potential therapeutic benefits. Physicians who care for pediatric populations have, therefore, been forced to either withhold treatment shown to be effective in older patients or provide drugs to children in whom the dose, efficacy, and safety have not been studied. This off-label use of drugs may result in benefit, no effect, or harm, depending on how much other information about the use of the product in the pediatric population is available. The need for more studies to obtain pediatric information for medicines used in children is now a matter of consensus on a global basis [183,184]. The awareness of off-label drug usage in the daily practice by pediatricians and the need to identify specific off-label clinical priorities in pediatrics has been documented.

The Pediatric Research Equity Act of 2003, and very recently draft guidance, has been issued by the FDA with the aim to effectively manage the off-label phenomenon, enabling sponsors to distribute publications about off-label use of approved drugs to prescribers [185]. This has paved the way for the new European legislation (the “Pediatric Regulation”), which was adopted in 2007 with the objective to guide the development and authorization of medicines for use in children aged 0–17 years [186,187]. This legislation was designed to better protect the health of children in Europe. A pediatric committee was established within the EMA with the intent to provide scientific opinions on any development plan for pediatric medicines. The current global discussion of the issues related to pediatric research and regulation [188]

raises a new challenging question: is it always necessary to perform additional clinical studies in children? Studies with Tafuri *et al.* [181], by using available evidence for one of the categories of drugs most frequently used off-label in children: PPIs, showed that, in some instances, the evidence already available may be sufficient to extend the indications to children without further clinical studies. This would allow the translation of the existing evidence into clinical practice, minimizing regulatory hurdles, and avoiding the unethical replication of trials.

16.9 CONCLUSIONS

We have provided a summary of the current state of knowledge on drug metabolism in pediatric populations and interactions that could arise owing to the maturation of DME activities; other physiological parameters that have impact on drug metabolism; and/or from the effect of comedication, disease state, genetic factors, or diet. Furthermore, this chapter has provided an overview on how the differences in drug metabolism in children relative to adults and the resulting effects on the PK and pharmacodynamics of drug therapy, specifically safety and efficacy. It is anticipated that while there are several ethical issues that hinder further translational research and clinical studies in pediatric patients, the recommendations and knowledge included in this chapter can aid other scientists and clinician with their investigations, support the design of future clinical studies, and hopefully reduced off-label use of drugs in neonates and children.

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17 Population-Based Pharmacokinetic Modeling and Simulation

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17.1	Summary	1
17.2	Introduction	2
17.3	Approaches to modeling drug metabolism and interactions	2
17.4	<i>A priori</i> prediction of variability through PBPK modeling	3
17.5	<i>A posteriori</i> estimation of variability using population modeling	14
17.6	Conclusions	18
	Acknowledgments	19
	References	19

17.1 SUMMARY

Simulating and predicting interindividual variability in drug pharmacokinetics (PK) and drug–drug interactions has become an integral part of drug development and clinical therapeutics. Our understanding of the mechanisms by which anatomical, physiological, or genetic differences translate into variability is quite advanced and even, to a large extent, quantitative. PK modeling is a mature field where compartmental models and, in particular, physiologically based pharmacokinetic (PBPK) models have been used since the 1970s. PBPK models differ from empirical compartmental models, in that they describe well-identified tissues or organs, linked at least by blood flow. With the help of such models, variability can be studied either *a priori*, through purely predictive computer simulations, or *a posteriori* to discover the determinants of variability through data analysis. Predictive simulations rely on the continued development of detailed organ simulation models and QSAR/QSPR (quantitative structure–activity relationship/quantitative structure–property relationship) models, which are able to finely describe the processes of drug absorption, excretion, or metabolism at specific sites of the body. To be able to forecast the existence of sensitive subpopulations, they require the data mining of parameter values for these processes in large populations,

or at least in subpopulations, differing in sex, age, ethnicity, disease state, and so on. The development of *a posteriori* population PK analyses followed the diffusion of specialized (multilevel) statistical models able to disentangle variability from uncertainty, which is always present in clinical study data. Improved numerical techniques, such as the Bayesian Markov chain Monte Carlo (MCMC) methods, are now bridging the gap between the *a priori* and the *a posteriori* approaches, using them in a refining cycle of model calibration and model prediction. These techniques have been successfully applied to complex PBPK models. The future should see ever improving PBPK models, linked to organ physiology models and cellular effect models. We are progressing toward the development of predictive PK and pharmacodynamic analyses of drug safety and efficacy with ever finer descriptions of individual susceptibility. This drive is congruent with the current development of personalized medicine.

17.2 INTRODUCTION

Inter- or intraindividual variability in drug PK, and particularly in metabolism, translates into variability of target doses. Such variability can have direct consequences for therapeutic safety and the likelihood of toxicity, especially for compounds with narrow therapeutic windows. Therefore, understanding, simulating, and predicting interindividual variability have become an integral part of the assessment of PK in humans [1]. The mechanistic framework of mathematical PK models, and in particular PBPK models, provides the capacity to predict drug–drug interactions and interindividual variability in PK when the required information is adequately incorporated [2–5]. This chapter presents the state of the art on that question, reviewing the deterministic (mechanism-based) and stochastic (statistical) methods currently available. The corresponding pharmacodynamic aspects are not reviewed here. Those are largely beyond the scope of metabolism studies and models.

17.3 APPROACHES TO MODELING DRUG METABOLISM AND INTERACTIONS

Interindividual differences (variability), in the anatomical and physiological characteristics of humans or animals are a glaring and a common experience. Such differences affect, for example, metabolic clearance, organ volumes, and blood flow and translate quite naturally into variability in the PK of drugs and chemical substances from one individual to the next. Since PK determine, in part, effective dose and ensuing effects, PK variability has an impact on individual susceptibility. Two complementary modeling approaches have been developed to understand, evaluate, and predict that variability: *a priori* and *a posteriori* modeling.

A priori predictive variability modeling can be undertaken through deterministic descriptions of the determinants of variability or through stochastic simulations (Monte Carlo methods) [1,6–10]. Both approaches can also be combined. Deterministic modeling is made possible by the fact that some of the differences between individuals are known to be due to age-related changes, genetic makeup, sex, and so on. Such changes can be explicitly modeled in lifetime PBPK models, for example [4,11]. Monte Carlo methods, on the other hand, acknowledge the fact that part of the differences between

individuals seem to occur by chance or cannot be ascribed to obvious causal determinants. Such differences are then considered as random and the corresponding parameter values are described by statistical distributions, rather than by point estimates or simple functions of time, age, and other covariates.

A posteriori modeling of interindividual variability is data based and was originally developed in PK, where human data are routinely generated through clinical trials. The aim here is to assess, for example, whether the across-subject variability of blood concentrations for a given drug can be explained by variability in bioavailability, metabolic rate, and so on. And this is best achieved through model calibration (data fitting) in the framework of a multilevel statistical model: in our case, a “population pharmacokinetic model” [12]. The population approach was first used with classical (minimal) compartmental models [13], but the added ingredients of MCMC simulations and Bayesian inference made it amenable to the treatment of PBPK models [14]. After identifying the determinants of variability, predictive simulations can be performed to assess their impact in various therapeutic treatment scenarios.

Uncertainty is different from variability, although their effects may be both confounded and compounded. Uncertainty is primarily due to imperfect knowledge and may have various sources. For example, studying a limited number of patients introduces an element of randomness when attempting to extrapolate the results to the whole population. Also, measurements are made only with finite precision, so when data are used to fit PK or pharmacodynamic models, there is always some residual uncertainty about the estimated parameter values (including the values of parameters measuring variability). Finally, our inability to model a biological system in all its details may result from lack of understanding, simplifications, or misspecifications. This translates into parametric uncertainty for the fitted models [2,15].

While variability is irreducible and unavoidable, as it is a state of Nature, uncertainty can be reduced by new experiments or clinical trials or by a better understanding of the biology and improved models of it. Statistical population models are able to account separately for variability and uncertainty and can help disentangle the two. These models are reviewed in this chapter (Section 17.5).

17.4 A PRIORI PREDICTION OF VARIABILITY THROUGH PBPK MODELING

17.4.1 PBPK Modeling

When a chemical substance penetrates an animal body (following intentional administration or unintentional exposure), it is usually distributed to various tissues and organs by blood flow [16]. Following its distribution to tissues, the substance can bind to various proteins and receptors, undergo metabolism, or can be eliminated unchanged. The concentration versus time profiles of the xenobiotic in different tissues, or the amount of metabolites formed, are often used as surrogate markers of internal dose or biological activity [17].

Mathematical models can be used to interpolate and extrapolate (predict) such concentration–time profiles from data. Reported models range from simple compartmental [18] to very sophisticated ones [4]. PBPK models are evolved compartmental models that try to use realistic biological descriptions of the determinants of drug disposition in the body [19]. Those models describe the body as a set of compartments

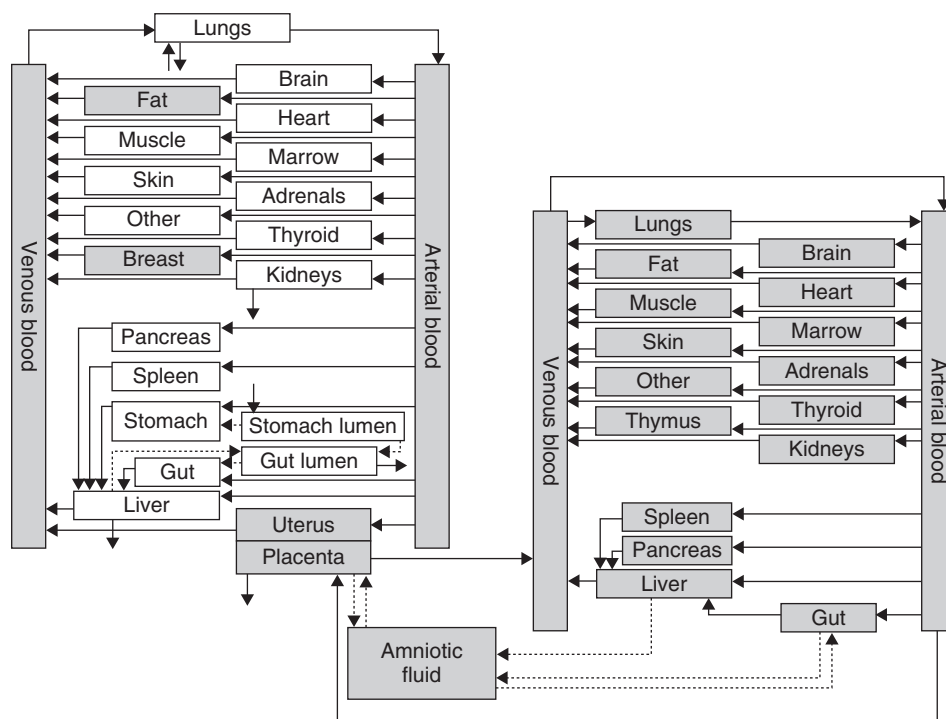


Figure 17.1 Schematic representation of a PBPK model for the model and fetus. The various organs or tissues are linked by blood flow. The parameters involved are compartment volumes, blood perfusion flow, tissue affinity constants (or partition coefficients), and specific absorption, diffusion, metabolic, and excretion rate constants.

corresponding to specific organs or tissues (e.g., adipose, bone, brain, gut, heart, kidney, liver, lung, muscle, skin, and spleen) (Fig. 17.1). Between compartments, the transport of substances is dictated by various physiological flows (blood, bile, pulmonary ventilation, etc.) or by diffusions [20,21]. Perfusion-rate-limited kinetics applies when the tissue membrane presents no barrier to distribution. Generally, this condition is likely to be met by small lipophilic substances. In contrast, permeability-rate kinetics applies when the distribution of the substance to a tissue is rate limited by the drug's permeability across the tissue membrane. This condition is more common with polar compounds and large molecular structures. Consequently, PBPK models may exhibit different degrees of complexity. In the simplest and most commonly applied form (Fig. 17.1), each tissue is considered to be a well-stirred compartment, in which the substance distribution is simply limited by blood flow. In such a model, any of the tissues can be a site of elimination. However, in Fig. 17.1, it is assumed that the gut, liver, and kidney are the only metabolizing tissues and that excretion only happens in the kidney.

Building a PBPK model requires gathering a considerable amount of data which can be categorized in three groups, namely, systemic data (physiological, anatomical, biochemical data), drug-specific data, and the model structure, which refers to the arrangement of tissues and organs included in the model [22]. In a sense, PBPK

modeling is an integrated systems approach to both understanding the PK behavior of compounds and predicting concentration–time profiles in plasma and tissues. Additional details on PBPK modeling can be found in Refs. 20 and 22–25 and in the volume titled *ADME of Drugs*, part titled *General Principles of ADME. Drug Transporters and Other Mechanisms of Transport* of this encyclopedia. Indeed, such descriptions of the body are approximate, if not rough, but a balance has to be found between precision (which implies complexity) and simplicity (for ease of use). Yet, the generic structure of a PBPK model facilitates its application to any mammalian species as long as the related system data are used. Therefore, the same structural model can approximately be used for a human, a rat, or a mouse [26].

17.4.2 Monte Carlo Methods

Predictive variability assessment with PBPK models proceeds currently with a combination of mechanism- and data-based refinements of the model structure (e.g., using age-dependent compartment volumes) [27] and stochastic modeling of the remaining random components of interindividual differences. Numerical Monte Carlo methods [28], eventually with hierarchical sampling [29], are the most widely used for the latter task. Those are best suited for complex models with many parameters. The basic assumption made by Monte Carlo simulations is that the randomness of the model state variables (e.g., blood concentration) is simply due to the randomness in the model parameter values. It is enough then to define statistical distributions for the parameters supposed to vary randomly in the population and to sample the model parameter values from those distributions. The model is then run with the needed inputs, and its outputs of interest are recorded (Fig. 17.2). That sampling-running step constitutes a basic Monte Carlo iteration. As many iterations as needed can be performed to characterize precisely the statistical distribution of the recorded outputs. Additional details of Monte Carlo methods can be found in Ref. 30.

Early attempts to use Monte Carlo methods and to simulate PK behavior in “virtual populations” date back to the mid-1980s. Jackson *et al.* [31,32] assessed the robustness of different experimental *in vivo* indices to detect and display genetic polymorphisms in human drug–metabolizing activity. Monte Carlo analyses of PBPK models were also described at that time [7,33]. These simulations were later expanded to show the effect of variability in absorption, distribution, metabolism, and excretion (ADME) parameters on the power of single time point estimates for the assessment of metabolic activity [34], the differentiation of parent drug and metabolite data in bioequivalence assessment [35], the discriminatory power of different indices of *in vivo* enzyme activity, and the optimization of sampling to assess such activity [36]. Coupled with Monte Carlo methods, PBPK modeling has been used to assess the quantitative impact of physiological and environmental factors on human variability in toxicokinetics and PK in other publications [4,6,37–39].

17.4.3 Sources of Variability in Pharmacokinetics

The overall interindividual variability in PK can be simulated by considering the variability in key system parameters in PBPK modeling[4]. Details on the prediction of interindividual variability in PK are briefly reviewed below.

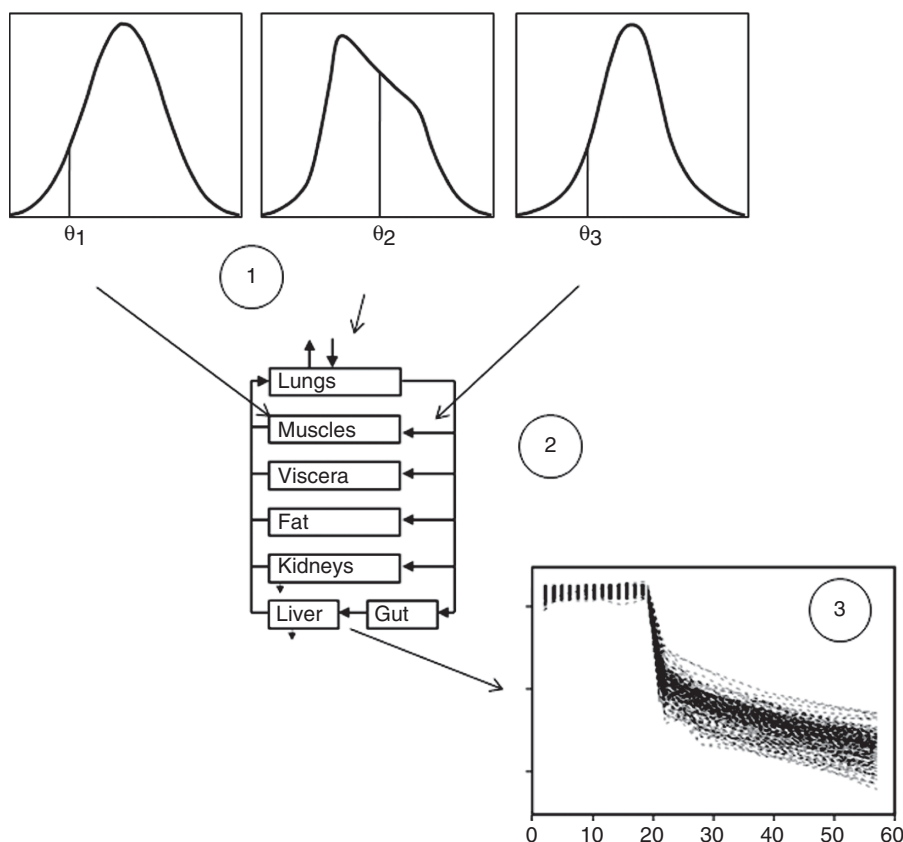


Figure 17.2 Application of Monte Carlo simulations to a PBPK model. Three steps are iterated a large number of times. Step 1: parameter values are randomly sampled out of specified statistical distribution functions. Step 2: the PBPK model is run for a particular exposure and observation scenario using the sampled parameter values. Step 3: the outputs of the PBPK model (e.g., the liver concentration at various times) are collected. Those outputs can be further analyzed for uncertainty or sensitivity analysis.

17.4.3.1 Absorption. Oral, infusion, intravenous, intramuscular, transdermal, and inhalation routes are routinely used for drug administration. Among them, the oral, transdermal, and inhalation routes are the most affected by variability.

17.4.3.1.1 Oral Absorption. Oral administration is the most common and convenient route of drug administration. Yet, it is often associated with low bioavailability and high interindividual variability. After oral administration, the drug is first released from formulation, then gets dissolved in the gastrointestinal fluid, and passes sequentially through the intestinal wall and the liver, before entering the systemic circulation. Oral bioavailability, F_{oral} , is usually defined as

$$F_{\text{oral}} = f_a \cdot F_G \cdot F_H \quad (17.1)$$

where f_a is the net fraction of dose absorbed from the intestinal tract, F_G is the fraction of dose that escapes intestinal first-pass metabolism in the enterocytes, and F_H is the fraction of dose that escapes hepatic first-pass metabolism.

The majority of drug absorption occurs in the small intestine because of its large absorptive surface area and high blood perfusion rate. The large intestine has a considerably smaller absorptive surface area than the small intestine, but it may still serve as a site of drug absorption for compounds that are not completely absorbed from the small intestine. Drug-related factors affect oral drug absorption, but physiological and biological factors, such as gastric emptying time, intestinal residence time, or regional pH in the gastrointestinal tract, mostly affect variability.

Gastric emptying strongly conditions the residence time of a drug in the stomach. It is an important factor for the initiation of oral drug absorption. Variability in gastric emptying rates results usually in variable absorption rates and sometimes even variable absorption extents. The rate of gastric emptying is influenced by both volume and composition of gastric contents. Additional factors influence the residence time of a drug in the stomach, including the nature of the dosage form, the volume of water coadministered, and presence of food [40]. Generally, the process of gastric emptying is modeled as a first-order process; however, it is in fact very complex.

Intestinal residence time can greatly affect oral drug absorption, particularly for drugs with low permeability. Yu and coworkers [41] analyzed published data for small intestinal residence time in over 400 subjects and reported a mean value of 199 min with ranges of about 1 and 6 h. Intestinal residence time appears to depend somewhat less on the dosage form (liquid vs solid) than on the gastric residence time [42]. The presence of food does not appear to influence intestinal transit [42,43].

The regional pH in the gastrointestinal tract can influence drug solubility and hence the dissolution of solid dosage forms. Gastrointestinal pH may also affect drug permeability by influencing the balance between ionized and nonionized moieties. Fallingborg and coworkers [44] measured pH profiles along the gastrointestinal tract in 39 healthy volunteers and observed pH values ranging up to two units at the same site in different subjects. In addition, the presence of food in the gastrointestinal tract can raise the pH in stomach and the proximal part of the small intestine because of the buffering capacity of proteins. Following a meal, gastric pH gradually returns to its basal value (corresponding to the fasted state) [45]. The return rate is considerably slower in elderly subjects than in young subjects [46]. Gastrointestinal pH is also affected by other factors including pathological states such as achlorhydria and hypergastrinemia [47].

In addition, various transporters are variably expressed in the apical and basolateral membranes of intestinal epithelial cells [48–52]. Much attention has been given to the efflux transporters (e.g., P-gp, MRP2, and BCRP) at the apical (brush border) membrane of the intestine, as they can limit the intestinal absorption of drugs administered orally [53–55]. Available data demonstrate that the expression levels of transporters vary along the gastrointestinal tract; for example, the relative P-gp levels increased progressively from the proximal to distal region [55]. Little is known about the pattern followed by other intestinal transporters.

Key physiochemical properties, such as solubility and permeability, can be used in empirical methods to estimate the absorption potential of a drug [56,57]. But physiological models, such as the Compartmental Absorption and Transit (CAT) model [58], have also been developed to mechanistically simulate drug absorption. The CAT

model has been further developed into the Advanced Compartmental Absorption and Transit (ACAT) model [59] and the Advanced Dissolution, Absorption, and Metabolism (ADAM) model [60], which are implemented within software GastroPlus (Simulation Plus Inc, California, USA, www.simulationsplus.com) and Simcyp Population-based ADME Simulator (Simcyp Ltd, Sheffield, UK, <http://www.simcyp.com>), respectively. In brief, these absorption models consist of physiologically based compartments corresponding to different segments of the gastrointestinal tract. A series of differential equations are used to describe drug release, dissolution, degradation, metabolism, and absorption within each segment and drug transit from one segment to the next. For each drug phase in the small intestine, for example, solid and dissolved drug, a segment is modeled as a well-stirred compartment in which different processes such as dissolution/precipitation, absorption/efflux/uptake, and transit can simultaneously happen.

17.4.3.1.2 Inhalation. The large absorptive surface area, limited metabolic enzyme activity, and active transporters in the pulmonary system make inhalation a favorable delivery strategy for systemic drugs with low bioavailability. As for other routes of administration, the absorption kinetics in the lung tissues depend on both drug- and system-related parameters. Different aspects of lung physiology and formulation composition that influence the systemic delivery of inhaled therapeutics and recent advances in inhaled drug delivery have been reviewed previously [61].

17.4.3.1.3 Dermal Absorption. The skin is the largest interface between the environment and the body and protects the body against chemical, physical, and microbial injury; loss of water; and other endogenous substances. It is involved in thermoregulation and assumes excretory functions [62]. Permeation of drug molecules across the skin occurs by passive diffusion according to the chemical activity gradient [63–65]. The outer skin layer, stratum corneum, forms a rate-controlling barrier for diffusion of most compounds. The predominant diffusional path for a molecule crossing the stratum corneum appears to be intercellular [66]. However, this path is not exclusive, and probably, most molecules pass through the stratum corneum by a combination of routes [67,68].

17.4.3.2 Distribution. Distribution here refers to the reversible transfer of drug from one location to another within the body. The processes that determine the transport of drugs in the body include their delivery to tissues by blood flow or lymphatic circulation and their ability to cross membranes, bind to proteins within blood and tissues, partition into fat, and to be subject to active uptake in tissues [69]. Those processes depend on drug-specific factors such as ionization, lipophilicity, and binding affinity to plasma proteins or influx or efflux transporter proteins. But drug distribution is also affected by physiological factors such as tissue volumes, perfusion and composition, plasma protein concentrations, hematocrit, and the expression of transporter proteins, which we briefly review here.

17.4.3.2.1 Tissue Volumes and Blood Perfusion. Tissue volumes and tissue blood flows are essential components of a PBPK model, but early systematic publications reporting physiological parameter values did not indicate the biological variability associated with those data [70,71]. Interindividual variability on those parameters has since been reviewed [72,73]. The correlation between tissue volume and perfusion

should be considered when modeling interindividual in PBPK models, because it is blood perfusion rate per unit tissue mass that is actually maintained by homeostasis [71]. Drugs can be distributed between plasma and red blood cells, and hematocrit, the percentage of total blood volume occupied by red blood cells, is influenced by factors including age, sex, seasonal influence, and habits of physical activity [74–76]. Hematocrit ranges between 40% and 54% in males and 38% and 47% in females. For drugs that get heavily distributed into the red blood cells, such as cyclosporine, hematocrit variation can affect the drug clearance [77].

17.4.3.2.2 Tissue Composition. For drugs effectively transported by blood, the tissue-to-plasma partition coefficients (K_{tp}) can be used to characterize their affinity for the various tissues of the body. These coefficients are the ratios of tissue concentration over plasma concentration in steady-state conditions. They are determined by the drug partitioning between lipids and water, as well as by their binding to common proteins present in the tissue interstitial space. Most of the studies on tissue composition (water, neutral lipids, phospholipids, etc.) were carried out between the late 1960s and the mid-1980s [78–81], and overall, data on human tissue composition and related variability are very limited.

17.4.3.2.3 Drug-Protein Binding. This is a reversible interaction of drugs with plasma proteins and is a function of drug and protein concentrations, the affinity constant for the drug-protein binding, and the number of protein binding sites [82]. The major drug-binding proteins in plasma are albumin, α_1 -acid glycoprotein (AAG), and lipoproteins. Albumin levels generally decrease with age, whereas AAG levels are not significantly affected by age. Many pathophysiological, pharmacological, and environmental factors, such as renal disease, hepatic disease, obesity, trauma, stress, surgery, pregnancy, concomitant drug therapy, or smoking, can alter the concentration of albumin and/or AAG.

17.4.3.2.4 Transporters. Numerous drug transporters are found in tissues membranes. These transporters can influence drug distribution into the tissues, particularly for drugs with low passive permeability [83]. For most drugs, transporters may influence the local kinetics in certain tissues (e.g., brain, liver), with pharmacological and toxicological consequences. Polymorphism is present in transporters, as reviewed, for example, by Ho and Kim [84].

17.4.3.2.5 Prediction of Drug Distribution. As discussed above, PBPK models provide a mechanistic and reasonably realistic description of the behavior of a substance in the body. They also give access to predictions of interindividual variability in drug distribution, through Monte Carlo simulations. Moreover, the development of mechanistic methods for predicting tissue-to-plasma partition coefficient, K_{tp} , values have greatly extended the application of PBPK modeling in drug discovery. K_{tp} values are important parameters in PBPK models. Their direct determination is costly and time consuming, as it usually involves intravenous constant infusion to animals followed by an extraction and quantification of the drug from tissue homogenates [85–87]. It is therefore of interest to estimate K_{tp} values without conducting *in vivo* animal studies. Several mechanistic equations (in essence, these are QSPRs) have been proposed to predict the tissue affinities of volatile organic compounds [88–90] or drugs [91–94].

The mechanistic basis of these parametric equations is that each tissue and plasma is a mixture of lipids, water, and plasma proteins. The assumptions are that the drug distributes homogeneously into each tissue and plasma by passive diffusion, partitions between lipids and water, and binds reversibly to common proteins present in plasma and tissue interstitial space. Values for the main compound-specific parameter of these equations can be provided by *in vitro* data on the drug lipophilicity and plasma protein binding. The species-specific tissue composition parameters can be found in the literature [95]. These equations have been extended and improved by considering drug ionization and incorporating more details on drug distribution inside tissues. Briefly, the assumptions are that all drugs will dissolve in intra- and extracellular tissue water and partition into the neutral lipids and neutral phospholipids located within cells. Compounds with at least one $pK_a \geq 7$ (ionized bases and corresponding zwitterions) are assumed to have electrostatic interactions with tissue acidic phospholipids. These compounds tend to preferentially bind to AAG. Since AAG is largely restricted to plasma, its interaction with extracellular proteins can be considered to be minimal. For other compounds, associations with extracellular proteins are an essential component, with acids and weakly basic compounds being assumed to bind primarily to albumin and neutral ones to lipoproteins [96,97]. As mentioned earlier, drug distribution into a tissue can be rate limited by either blood perfusion or membrane permeability. In the first case, the tissue can be regarded as a well-stirred system and the above calculations of partition coefficients apply. In the second case, a more realistic yet more complex model is needed and tissues need to be divided into two or more compartments to represent vascular blood, extracellular space, and intracellular space separated by permeability barriers [20,98].

17.4.3.3 Metabolism. Drug metabolism is the major mechanism for elimination of most drugs and its reactions are generally grouped into two phases. Phase I metabolism includes oxidation, reduction, hydrolysis, and hydration reactions. Phase II reactions use an endogenous compound, such as glucuronic acid, glutathione, or sulfate, for conjugation to the drug or its phase I-derived metabolite to produce more polar end products that can be more readily excreted in bile or urine. Although drug metabolism can take place in many organs, the liver has been long recognized as the major site of metabolism for most drugs. The role of gut metabolism in first-pass metabolism is also well recognized, the intestinal tissue being endowed with phase I and II enzymes, although at lower levels than those for the liver [99].

17.4.3.3.1 Hepatic Metabolism. Rane *et al.* [100] successfully predicted *in vivo* hepatic metabolic clearance in rats based on *in vitro* data obtained from rat liver microsomes, taking into consideration the hepatic blood flow rate and the unbound fraction in blood. Since then, significant progresses have been achieved on predicting human hepatic metabolic clearance from a variety of *in vitro* systems, such as human liver microsomes, recombinant enzymes, and hepatocytes [101–108].

Interindividual variability in the hepatic metabolic clearance of a drug is mainly determined by the variability of three key parameters: hepatic blood flow, the free (i.e., unbound to proteins or hepatocytes) fraction of the drug in blood, and most importantly, enzyme abundances in the liver. When drug distribution into the liver is permeability limited, variability in hepatic transporters also plays a role. Hepatic blood flow is the sum of hepatic arterial and hepatic portal vein blood flows, representing about 6.5%

and 19% of cardiac output in male adults, respectively, and 6.5% and 20.5% of cardiac output in female adults, respectively [109]. Cardiac output is dependent on body surface area as well as age [110,111]. The free fraction of the drug in blood is determined by variations in drug-binding protein concentration and hematocrit. Interindividual differences in the expressions and catalytic activities of various drug-metabolizing enzymes are often the major causes of the large interindividual variability observed *in vivo* in drug metabolism. In humans, about 15 different CYPs have been identified as being involved in drug metabolism in the liver. These P450s have been characterized to varying degrees with respect to their regulation of expression and catalytic activities [112]. Meta-analyses of data on CYP abundances in liver and their variances have been performed for Caucasians [113] as well as for Japanese and Chinese [114]. The abundances of UDP-glucuronosyltransferases (UGTs) in the human liver and their interindividual variability have also been reported [115]. Differences in metabolism that result from functional genetic polymorphisms can be inferred from the frequency of the different genotypes. PK differences between phenotypes are most relevant for drugs with low therapeutic indices. Inherited defects in several drug-metabolizing enzymes such as CYP2D6, CYP2C19, and CYP2C9 have long been recognized to be critical to the observed therapeutic efficacy or toxicity of certain drugs. It is also known that the content and activity of CYP enzymes can decline in patients with liver diseases [116].

Several models have been developed to quantify the effects of varying hepatic blood flow, fraction unbound in blood, and hepatic enzyme activities on hepatic clearance [117]. Among those, the well-stirred model has been widely used for its mathematical simplicity and practicality. The well-stirred model first assumes that drug distribution into the liver is perfusion limited with no diffusion delay and that no active transport systems are involved. It also assumes that the drug is distributed instantly and homogeneously throughout liver water and that the unbound drug concentrations in plasma and liver water are identical. When enzyme activities are measured using recombinantly expressed enzyme, human liver microsomes, or human hepatocytes, they are usually normalized using the amount of microsomal protein per gram of liver (MPPGL) or hepatocellularity (the number of hepatocytes per gram of liver (HPGL)). In that case, the variability of these normalizing factors and that of liver mass has also an impact on the model predictions. The 95% confidence interval of the geometric mean ranges from 27 to 32 mg/g for MPPGL and from 74 to 131×10^6 cells/g for HPGL [108]. A modest decrease in MPPGL with age was also reported. For liver mass, a predictive model based on body surface area and ethnic differences, based on data from more than 5000 individuals aged from birth to 28 years, is available [118,119].

17.4.3.3.2 Gut Metabolism. Several CYP enzymes (CYP1A2, CYP2D6, CYP2E1, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and CYP3A5) have been evidenced in the human small intestine, CYP3A4 being the most prominent [120,121]. Although the total content of CYP3A in the entire human small intestine is only 1% of that in the liver [121,122], the fraction of CYP3A substrates processed in the intestine is often similar to or even exceeds the fraction processed in the liver [123].

The determinants of interindividual variability in intestinal metabolism include mainly the blood flow to the small intestine, enzyme abundances in the gut wall, effective intestinal permeability, and intestinal cylindrical surface area [124]. The blood supply to the small intestine is provided by the superior mesenteric artery and constitutes about 10% of the cardiac output in an average adult [109]. In the unfed state,

mucosal blood flow represents about 80% of the total mesenteric flow, and in turn, about 60% of mucosal blood flow supplies the epithelial cells of the intestinal villi [125]. Hence, villous blood flow is about 4.8% of the cardiac output [124]. Following a meal, blood flow increase can be as high as 200% above baseline levels and persists for 2–3 h [125]. The total CYP3A content of microsomes prepared from mucosal scrapings of the duodenal and jejunal portion of 31 human donors averaged 50 pmol/mg and ranged from 18 to 151 pmol/mg [120]. Along with CYP3A4, CYP2C9 and CYP2C19 were detected in all donor intestines and their concentrations varied at least fivefold between individuals. Effective intestinal permeability (per unit surface area) is related to drug permeability but can be affected by the abundances of intestinal transporters and pH in the gut lumen. Intestinal cylindrical surface area is also variable among individuals.

A “ Q_{Gut} ” model has been developed to predict first-pass metabolism in the gut [124,126,127]. This model retains the form of the “well-stirred” model but is an empirical model rather than a fully physiological model such as the ADAM model.

17.4.3.4 Excretion. Excretion is usually defined as the irreversible loss of the chemically unchanged drug. For most drugs, excretion occurs predominantly via the kidneys. However, some drugs and their metabolites are extensively excreted in the bile. Drug excretion can also happen via saliva, sweat, breast milk, and lungs, although their contributions to overall drug elimination are often small.

17.4.3.4.1 Renal Excretion. The kidney is the major site of drug excretion. Net renal drug excretion is a combination of three processes: glomerular filtration, tubular secretion, and tubular reabsorption. The glomerular filtration of a drug is a passive process that is dependent on the unbound fraction of a drug in plasma (f_u) and renal blood flow available for filtration. Tubular secretion occurs predominantly in the proximal tubule and is mediated by several families of transporters, including the organic anion transporters (OATs) and organic cation transporters (OCTs). Tubular reabsorption can be a passive or an active transport process. Active reabsorption occurs in the proximal tubule and, similar to tubular secretion, is energy dependent, saturable, stereospecific, and likely to be associated with competitive drug interactions [128]. Active transports are mediated by several families of transporters, which play key roles in the secretion and reabsorption of many drugs. Passive reabsorption may occur throughout the nephron. It is largely due to the reabsorption of water, which concentrates the urine with respect to plasma.

The primary determinants of renal excretion are renal blood flow, plasma protein binding, urine flow, urine pH, and activity of renal transporters [129,130]. The extent of sensitivity to these parameters depends on the nature of the compound considered and the mechanisms involved in its renal elimination (e.g., glomerular filtration, active secretion, passive reabsorption). Thus, interindividual variation in these parameters determines the renal clearance. Urine flow is sensitive to individual fluid intake and administration of diuretic drugs. Urine pH can affect the ionization degrees of low molecular weight weak acids and weak bases and therefore influence their reabsorption. Nonionized drugs are usually lipophilic and are therefore likely to be reabsorbed through tubule back into the circulation [131]. Interindividual differences in urine pH are mainly related to differences in diet and physical activity [132]. Renal transporters play key roles in the secretion and reabsorption of many drugs and can significantly

contribute to the variability in renal excretion of these compounds [133]. Organic anion and organic cation transport systems are two major drug transport systems in the human kidney [134], and the effects of genetic variations in transporters on renal clearance have been investigated recently [135]. A range of pathophysiological states also influence the efficiency of individual renal elimination pathways [128,130]. It is well recognized that renal diseases alter the renal clearance and therefore the elimination of drugs. Although it is obvious that renal impairment is associated with a decrease in renal excretion of a drug and its metabolites, renal impairment has also been shown to be associated with other changes in absorption, hepatic metabolism, plasma protein binding, and drug active transport [136]. The underlying mechanisms of these effects are not yet fully understood in humans.

Current *in vitro* models to investigate the complex parallel processes for renal clearance are limited, and the prediction of renal clearance of drugs in humans is usually based on preclinical animal data.

17.4.3.4.2 Biliary Excretion. Biliary excretion is one of the primary elimination routes for xenobiotics and their conjugate metabolites [137]. Biliary excretion requires active secretory transport since drugs are transported across the biliary epithelium against a concentration gradient. Drugs excreted into the bile often undergo some degree of reabsorption along the intestine (enterohepatic circulation). If the biliary excreted drug is totally reabsorbed from the intestine, biliary secretion is a component of drug distribution. Only when there is no or incomplete reabsorption from the intestine does it become a route of elimination.

Biliary excretion is mediated by transporters in the canalicular membrane. Therefore, genetic variation in these transporters contributes to the interindividual variability in biliary excretion [138,139].

Biliary excretion of drugs can be investigated using *in vitro* systems with varying degrees of complexity spanning from the whole hepatocyte to membrane vesicles prepared from cell lines transfected with specific transport proteins [139]. Nevertheless, the predictive accuracy of these methods in humans is largely unknown owing to the lack of clinical data on the biliary excretion of drugs. *In vitro* data obtained from sandwich-cultured human hepatocytes to predict the biliary clearance for three drugs, significantly underestimated values obtained *in vivo* [140].

17.4.3.5 Drug–Drug Interactions and Coexposures. Part of the large variability in metabolism observed in humans may also be due to uncontrolled coexposures to naturally occurring food-borne substances, environmental contaminants, therapeutic drugs, or chemical substances in the workplace. Drug–drug interactions are a well-known problem falling under that umbrella [1,4,141], but its generalization to many-substance exposure remains to be better explored, even though the necessary tools are becoming available [5,142,143].

The challenge is to efficiently describe together cascades of reactions and the eventual transport and elimination of intermediate and final metabolites. The associated model can be very complex, even if limited to PK and metabolic interactions because the transport of each metabolite requires a set of differential equation of its own. Generic PBPK modeling for absorption, distribution, and excretion, coupled to a systems biology approach (able by design to deal with a large number of interacting

substances), for metabolism appears to be a promising way toward the solution of that question [144].

17.5 A *POSTERIORI* ESTIMATION OF VARIABILITY USING POPULATION MODELING

17.5.1 Motivation

Purely predictive modeling, as described above, can be cross-validated by confronting its predictions (e.g., for plasma concentration of a substance) to data obtained of a sample of individuals. In the ideal case, data and predictions agree both on average and in terms of variability and no further model refinement is attempted. But quite often, the predictions are less than “perfect” and may even be really poor (even though publication bias reduces the visibility of that case). That may be the occasion to improve the model through some calibration or data integration procedure and learn something about the true determinants of variability in a population [145]. However, some care should be taken to properly disentangle uncertainty from variability in that calibration process. In addition, when a PBPK model is examined in that context, it is important to retain the prior physiological knowledge it embodies in its parameter definition and values [14]. The “naive” approach consists in fitting individual subjects’ data separately, collecting the resulting individual parameter estimates, forming their average, and so on. That approach does not work and is actually incorrect if very precise parameter estimates cannot be obtained from the data [13,146]. For ethical, feasibility, or cost reasons, the data on individuals tend to be sparse in clinical PK or toxicokinetics. Such data usually lead to fairly uncertain parameter estimates and the so-called population approaches should then be used [147].

We review in the following section the typical PK data available for calibration, the general structure of population models, and the tools available to actually perform statistical inference using those models. As to actual applications, published population PK analyses are too numerous to be usefully surveyed here. Note that useful extensions to clinical trial optimal design [148–150], including for drug–drug interactions [151,152], have been proposed. In addition, pharmacodynamic drug interactions can also be studied with these methods [153–155].

17.5.2 Typical Population Pharmacokinetic Data

An example of population-based PK data is given in Fig. 17.3. Caffeine blood concentration was measured (for therapeutic monitoring) at one to four time points in 35 premature newborns. Measurements followed intravenous injection at various recorded times. Typical for a population data set, the data are sparse, most subjects having only one or two measurements. In such a fragile population, frequent blood sampling is counterindicated. Little could be drawn from such data if caffeine kinetics were not relatively simple (caffeine distributes uniformly in body water). In addition, body mass, a covariate measurement of body water content, was daily measured during all treatment. Taken together, blood data, covariates, and a PK model were able to predict individual and population caffeine kinetics for improved treatment and monitoring [156].

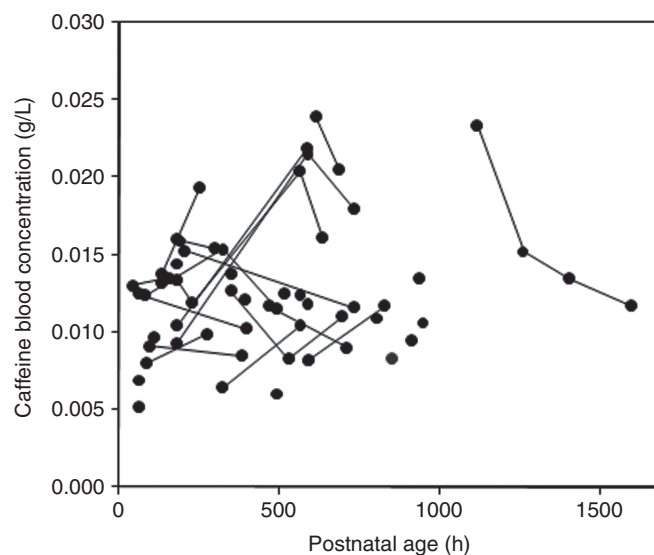


Figure 17.3 Example of population pharmacokinetic data. Caffeine concentration in venous blood was measured in 35 premature newborns at one or several times. Linked points belong to the same subject. *Source:* adapted from Ref. 156.

17.5.3 Statistical Population Models for Clinical Trials

Population models, or multilevel models, were first introduced in the context of PK studies for drug development and evaluation [157,158]. Their objective is to obtain, from data on individuals, a quantitative description of the variability of the kinetics of a compound within a large population. The same structural PK model is used to describe the data for each subject, and the model parameters are assumed to differ randomly between subjects (Fig. 17.4) [159–161].

The structural model can be of any complexity as desired, from the basic one-compartment model to a full-fledged lifetime PBPK model. The scope of population models is actually not limited to PK. Pharmacodynamic data, for example, may be analyzed with the same tools [158,162–164]. The randomness between subjects characterizes variability and can be described by a multivariate probability distribution if several parameters are supposed to vary at once, as it is often the case. In population models, information of each subject is reinforced by “borrowing strength” from the other subjects’ data and the overall estimation process is improved. Individuals’ metabolic clearance, for example, can be assumed to be lognormally distributed around a “population mean,” with a “population variance” that measures variability in the population. The population means and variances (one for each kinetic parameter supposed to vary between subjects) form the “population” parameters. They are estimated during the model calibration (data fitting) together with the parameters of each subject [2,13,165]. Other population parameters can be defined, such as parameter covariance (if some parameters are supposed to be correlated at the population level) or degrees of freedom (if a (Student) t -distribution is assumed instead of the normal distribution) [161].

For a typical population model describing interindividual variability, at the subject level, data (y) are measured at times t after exposure to dose E . A set of individual characteristics (parameters, θ) also condition the observations. Some of these characteristics

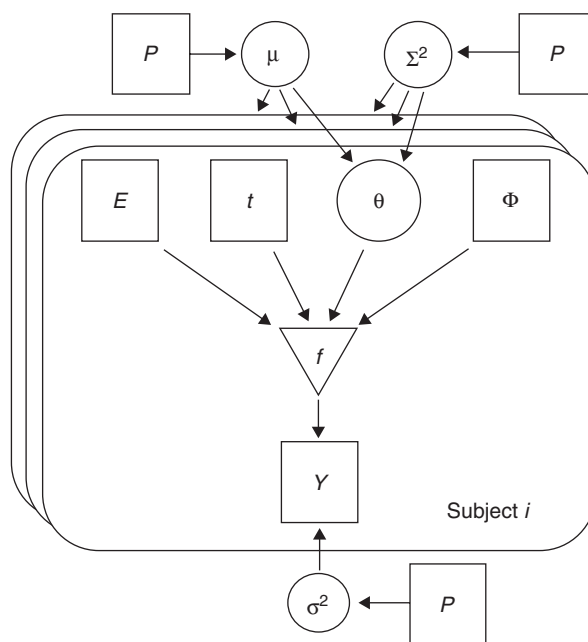


Figure 17.4 General graphical representation of a multilevel population pharmacokinetic model. The “hierarchical” or “multilevel” structure of the model is apparent, with two major components: the subject level and the population level. Symbols: P , prior distributions (only relevant in a Bayesian context); μ , mean population parameters; Σ^2 , variances of the parameters in the population; E , toxicant exposure concentrations; t , experimental sampling times; θ , unknown physiological parameters; ϕ , measured covariates; f , structural pharmacokinetic model; Y , measured drug concentrations (e.g., in plasma); and σ^2 , variance of the experimental measurements. Parameters μ and Σ^2 summarize the distribution of the individual parameters θ across the population.

may be known (e.g., sex, age, genotype), in which case they are considered as “covariates,” but usually some interesting characteristics are unknown and need to be estimated from the data. That estimation can be achieved by data fitting. In Fig. 17.4, unknown quantities are enclosed in circles and known quantities in square boxes. Besides a description of the population variability (which parameters are supposed to vary randomly between subjects), population data analysis requires the definition of two models at the individual level.

- First, a statistical model should link some “predictable” aspects of the data (e.g., their expected values or their variance) to the actual measurements (y). Deviations between measurements and their expected values may be due to limited assay precision, modeling error, or random intraindividual variability. Such deviations are typically assumed to be independent normal or lognormal random variables, with mean zero and variance σ^2 . That variance can be set to a known value (e.g., if analytical precision has been independently assessed and if modeling error is negligible). It can also be estimated along with the other parameters. The model chosen will specify the data “likelihood.” This likelihood is proportional to the probability of the observed data, given a model and its parameter values.

- Second, a PK model, f , should link the chosen “predictable” to E , t , and θ , or any other relevant “explanatory” variable.

At the population level, intersubject variability is described by considering that the individual parameters, θ , are randomly drawn from given statistical distributions, each with population mean μ and population variance $\sum^2$, as described above. Population means and variances are to be estimated together with the parameters θ and the residual variance σ^2 . The preceding mention of population means and variances does not imply that only parametric definition (e.g., Gaussian or log-Gaussian) of the population variability can be used. In fact, choosing a particular distribution shape for the population distribution of a given parameter can be problematic. What if, for example, a Gaussian shape (unimodal by definition) is used when in fact the values fall into two clearly distinct groups in the population? The estimated mean would probably fall in between the two groups but would describe neither of them correctly. An answer to that problem can be found in the use of more flexible nonparametric methods, which aim to estimate the shape itself of the population distribution [166–168].

Uncertainty enters in the above framework in two ways:

- First, through all variance sources aggregated into the residual variance σ^2 (typically, limited assay precision, modeling error, and unmodeled levels of variability). Note that it is possible to disaggregate σ^2 in order to identify separately its various components. For example, a further level in the hierarchy can be defined to deal with intraunit variability [169]. In that case, residual variance can be reduced if intraunit variability is significant. However, some measurement and modeling errors are likely to remain, even with the best available data and models. A side effect of that “noise” is to allow different model parameter values to yield adequate fits to the data (what is meant by adequate is determined by the statistical measurement model). This results in uncertainty about all the quantities estimated in the fitting process (population parameters, individual characteristics, etc., and even σ^2 if it is adjusted). This uncertainty may entangle estimates through fitting-induced correlations, which make it difficult to interpret the results when many parameters are adjusted.
- A less obvious source of uncertainty, which affects the population parameters, is the finite and often small size of the population sample studied. Even if the characteristics of each individual were perfectly known (from perfect data and models), the population parameter values inferred from a nonexhaustive sample would still be affected by uncertainty. For example; in the case of 1,3-butadiene toxicokinetics, the coefficient of variation of the population variance estimate for metabolic clearance was found to be around 50% with a sample of only 10 subjects and is in general inversely proportional to the square root of the number of subjects [148].

17.5.4 Tools for Inference on Population Models

The methods available to calibrate population models fall mostly into two groups: maximum likelihood approaches [168,170] and Bayesian approaches [161,171–173]. Both approaches are reviewed and discussed in Ref. 174.

Bayesian methods extend the maximum likelihood approach by placing prior distributions, hopefully informative, on the various unknown parameters, such as θ , μ , and Σ^2 in the above example (Fig. 17.4). In the Bayesian framework, unknown quantities are considered as random variables and their probability distributions are interpreted in terms of degrees of belief about their possible values. Before analyzing an experiment, a “prior” probability distribution, P , is constructed for each unknown to reflect current knowledge about it. Using a probability distribution to represent prior knowledge gives a unified way to account for precise to vague information available before an experiment. When little knowledge is available on a particular parameter, a noninformative distribution (i.e., flat shaped) can be used. In that case, actually, the Bayesian and the maximum likelihood methods should give similar results. If stronger prior knowledge is at hand, the prior should represent that information as appropriately as possible. Information from the literature tends to relate to average values or variances for a population and is naturally handled in a population model. With a Bayesian approach, such information can be directly used under the form of prior distributions for population parameters such as μ and Σ^2 .

Bayesian approaches have emerged as the best suited for PBPK models, given the large amount of prior information they give access to [14,173]. But as we have seen above, a large amount of that information is already encoded as prior distributions, and updating those distributions with system’s level data, is simply a matter of using Bayesian numerical methods such as MCMC simulations [175]. The availability of those methods has largely contributed to the proliferation of Bayesian analyses in applied statistics during the last 15 years.

To bridge the “bottom-up” PBPK approaches and the “top-down” population PK analysis of clinical data and to accelerate model building and covariate recognition in drug development, a “parameter estimation” module has recently been implemented in the Simcyp Simulator (version 9.30). It allows the PBPK, drug–drug interaction, and ADAM models to be fitted to observed clinical data for the purpose of estimating unknown/uncertain drug or physiological parameters.

17.6 CONCLUSIONS

A priori variability modeling and *a posteriori* population analyses use fundamentally similar models to describe the mechanisms of drug PK and metabolic interactions. Improved numerical algorithms, such as MCMC methods, and computer power are now bridging the gap between the *a priori* and *a posteriori* approaches. For example, PBPK models can now be calibrated and refined using population samples data before being used for prediction of the full population. We have not reviewed the topic of pharmacodynamic drug–drug interactions or variability. The fact is that predictive mechanistic pharmacodynamic models are less advanced than PBPK models. Arguably, they tackle more complex questions, but we are already witnessing advances on that front [176–180], with systems biology approaches potentially able to make a mechanistic continuum between kinetics, metabolism, and cascades of effects [5]. The future should see ever improving PBPK models linked to organ physiology models and cellular effect models. We are progressing toward the development of global predictive analyses of drug safety and efficacy, with ever finer descriptions of individual susceptibility. This trend is congruent with the current drive toward personalized medicine.

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18 Clinical Pharmacology in Drug Labeling. The Impact of Drug Metabolism and Clinical Pharmacology on Recommended Dose of Drugs

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18.1	Introduction	1
18.2	Recommended dose of drugs	3
18.3	Formulation and method of administration	4
18.4	Age	4
18.5	Size and Weight	5
18.6	Renal Impairment	5
18.7	Hepatic Impairment	6
18.8	Metabolic Pathways	6
18.9	Genetic polymorphism	9
18.10	Ethnic Differences	10
18.11	Individualized drug therapy	10
18.12	Conclusion	11
	References	11

18.1 INTRODUCTION

The main principle of drug labeling is to provide the prescriber with sufficient objective information to make informed prescribing decisions. The structure of US drug labels has evolved over time, and in 2006 new regulations were put in place to make the organization and chapter headings of drug labels consistent and easier to understand for both the patient and physician. Nevertheless, the labels have lengthened and the composite book of US labels (the 2011 edition of the PDR) has grown to over 3250 pages of labeling information on over 1116 of the most commonly prescribed drugs. Issues of dosing, drug metabolism, and clinical pharmacology have to be carefully reviewed for any drug prescribed to avoid unwanted consequences. Important issues

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are addressed as “black box warnings” although there is some dispute whether or not this information is used in a consistent manner [1]. The length of the label may also reflect a defensive legal side as well as the more helpful informational aspect. Despite the carefully crafted labeling information, drugs are increasingly prescribed off label, which means using approved drugs in unapproved situations [2].

Modern drugs are fully investigated before approval and marketing and tend to be very robust in dose and rarely need dose modification. This chapter discusses how we reached this state and provides some examples from the past of drugs that were inappropriately dosed or that required dose modification based on variations in drug metabolism or excretion. Recommended drug doses are currently based on a full development program providing appropriate doses to maximize efficacy and minimize safety issues. In addition, a full drug development program uses clinical pharmacology to investigate pharmacokinetics and pharmacodynamics, including potential metabolic variability and drug–drug interactions. The full description of the efficacy, safety, and clinical pharmacology information collected is then presented in the drug labeling for the use of physicians and patients. Whether or not this information leads physicians to make dosing modifications for individual subjects is discussed.

Before considering the impact of drug metabolism, it is worth considering how recommended drug doses are decided; and the factors that may influence that decision both before and after regulatory approval. For new drugs or old drugs for new indications, the process of drug development leads to a choice of dose, dosing interval, and method of administration. Appropriate formulations are then manufactured to satisfy those needs and the definitive clinical trials are usually carried out using those formulations, which are then taken through to marketing.

Selection of new drugs usually involves a choice from a range of chemicals with different physical and chemical properties, leading to potential benefits in terms of absorption, distribution, and elimination. Once selected, the chemical is tested in animals to determine the nature of any toxicity at high doses and the serum levels of drug that are associated with those toxicities. This allows the first use in man using appropriate algorithms to compare kinetics in different species and choosing a dose with an adequate safety margin [3]. From that point clinical pharmacology guides the process, but the minimum dose is considered from observation of efficacy and the maximum dose from the appearance of toxicity or maximum tolerability. This is made easier for drugs with a clear surrogate end point such as measurement of serum cholesterol or blood pressure, but it is harder for drugs with more subjective end points, such as pain, or psychiatric disorders. Thus, at the end of the development program, a recommended dose range is chosen together with appropriate formulations and a full label to guide prescribing.

The basis for the dose range is supported by clinical trials in predominantly healthy subjects, that is, those with the disease of interest but otherwise healthy. Additional studies provide information on absorption, distribution, and elimination; potential interaction with food; drug–drug interactions; and pharmacokinetics in the presence of renal and hepatic impairment. The product is then launched and subsequent information becomes available through postmarketing surveillance, phase 4 clinical trials, and other sources of information. This ultimately leads to changes in the label providing guidance to physicians and users. Thus, information from real world use leads to continuing updates to prescribing information as the product life cycle continues.

18.2 RECOMMENDED DOSE OF DRUGS

The dose of drug chosen, for marketing, at least for small molecules, is usually an arbitrary or compromise dose, dependent on number preferences. For example, a dose of 10 mg of drug a day could have been in a range of 5–15 mg and the number preference was to make a 10-mg dosage form rather than 7.5-, 8-, or 12.5-mg dose. These decisions are to some extent made by market comparisons and also by the risk–benefit profile (lower doses sacrifice efficacy for safety, whereas higher doses are more likely to be efficacious at the potential risk of higher adverse event rates). This lack of precision rarely matters because the variability of serum levels of the same drug in the same individual is usually greater than a small difference in the dose of drug. However, there are a small number of drugs that are very sensitive to variations in serum level, and they may have to be monitored carefully, either by measuring the serum drug level or by measuring the pharmacological action of the drug. Some examples are warfarin, phenytoin, and digoxin.

The US label for warfarin actually lists seven different oral presentations between 1 and 10 mg, as well as a parenteral form, and states that dosage needs to be individualized for each patient by regular measurement of clotting through the INR (international normalized ratio) [4].

Phenytoin has only one slow release form of 100 mg with an initial recommended dose of 300 mg/day and subsequent adjustments up to 600 mg/day. That allows dose adjustment with the objective to prevent seizures without inducing phenytoin toxicity. Because of the narrow therapeutic range, it is advisable to measure phenytoin serum levels until the dose is stable. It appears that the wide interindividual variability is due to CYP2C9 and CYP2C19 variants [5].

Digoxin is similar in its narrow therapeutic range and the manufacturer provides four solid oral doses staged by doubling the previous dose (62.5, 125, 250, and 500 μ g) as well as a liquid capsule and a parenteral form. In the 1970s, the Burroughs Wellcome Company changed the formulation of the branded Lanoxin (digoxin) tablet, unintentionally, making it more bioavailable. This resulted in a number of cases of digoxin toxicity and shows that dose cannot be considered independently of formulation and actual bioavailability [6].

For the vast majority of small molecule drugs, there is a wide therapeutic range so that dose selection is less critical. This has led to a number of examples of excessive recommended doses of drugs continued over many years. The recommended dose of estrogens, for example, was excessive for many years, probably because estrogens were first used in the 1940s and were not subjected to current standards of clinical pharmacology. A paper in 1948 [7] described the dose of ethinyl estradiol (which had just become available in Britain) at around 1 mg/day, whereas the current tablet size is 0.03 mg. It is not clear that the lowest effective dose of the various forms of estrogen has even now been reached, leading to confusion in the literature about the efficacy and hazards of estrogen use in women at different times of pre- and postmenopause [8].

The dose of captopril was initially 150 mg three times daily in the majority of preapproval studies and was initially approved at that level. It was subsequently used at doses closer to 25 mg two or three times daily. The adverse effect profile was reviewed soon after the launch of captopril and showed that rash and taste disturbance were clearly dose-related adverse effects when compared to doses >150 mg/day and <150 mg/day, and the efficacy was maintained at the lower dose [9].

Another area to consider in initial drug dosing is the first-time use in humans. The FDA provides extensive guidance on estimating the initial human dose [3]. This has been an area of relative safety, but a recent experience with a biological has inspired a fresh look at first in man dosing. Tegeneron 1412 is a monoclonal antibody to CD28, which was studied in animals and then caused severe life threatening reactions in all four of the initial volunteers, who were all dosed at the same time. A representative critique suggests that further nonclinical work should have been carried out before initiating human dosing and the initial dose was too high [10].

On the basis of this history, regulators have clarified and expanded the clinical pharmacology requirements for new drugs and new formulations to lead to a fuller understanding of desirable dosing. This leads to a large amount of information to include in the label for drugs that is designed to help good prescribing.

18.3 FORMULATION AND METHOD OF ADMINISTRATION

The concept of a dose is based on the formulation and method of administration. Some drugs such as fenofibrate have been reformulated to have different doses in the different formulations each providing similar activity. Fenofibrate is a relatively insoluble molecule, so altering the physical properties, such as by micronizing the active pharmaceutical ingredient changes the bioavailability. This leads to different dosages depending on the type of formulation with doses of 48 and 145 mg for the Tricor brand; 130 mg for the Antara brand; 160 and 200 mg for the Lofibra brand; and 45–135 mg for Trilipix a delayed release form. In addition, the absorption of fenofibrate is increased by 35% when given with food for all of the brands except Trilipix.

18.4 AGE

An understanding of dosing is needed at both ends of the age spectrum. It has been traditional in children to use drugs studied in adults and adjust the dose in some nonspecific way. Since 1997, the pharmaceutical industry has been encouraged to carry out studies in children because of the Food and Drug Administration Modernization Act (FDAMA), which provides additional six months of marketing exclusivity in return. This has provided more appropriate information to prescribe in children based on actual pediatric experience in the disease of interest.

As individuals get older, their renal function deteriorates leading to a need for specific dosing guidance. Groups of interest are subjects with ages from 65 to 75, 75 to 85 and older than 85 [11]. One of the first drug problems in the elderly occurred with the drug benoxaprofen, a nonsteroidal anti-inflammatory agent. Benoxaprofen was initially approved at a single dose of 600 mg/day but turned out to have a much longer half-life in the elderly population, particularly, in subjects over 80 years of age. A series of subjects, all aged over 80 years, had cholestatic jaundice and died probably related to the excessive benoxaprofen serum levels seen in this population [12]. A study suggested that the drug was being overdosed particularly in the elderly [13]. This episode led to the requirement for pharmacokinetic studies in the elderly to ensure the dosing is consistent with the younger population and adverse events are kept to a minimum [14].

18.5 SIZE AND WEIGHT

Most labeling is silent on dose adjustments for weight or BMI (body mass index). However, some attempts have been made to study different doses for different sized people. A study of heparin suggested that if doses were modified, it would lead to more rapid anticoagulation and reduced bleeding, but the concept has not been widely adopted [15]. Anticancer drugs are usually prescribed based on body surface area and are labeled as a dose per meter squared. This concept was started in the 1950s but has been questioned [16]. It has also been suggested that the interpretation of body surface area leads to underdosing of anticancer medication for the treatment of breast cancer, particularly, in subjects of low socioeconomic status [17]. Some other classes of drugs, including anesthetic agents, do have nomograms for modifying dose based on weight or BMI; but the best way of estimating the dose adjustment still has to be established [18].

18.6 RENAL IMPAIRMENT

It is clear that if a drug is excreted by the kidneys, either as the unchanged parent drug or as major metabolites, the plasma, blood, and tissue levels are likely to rise in the presence of increasing renal impairment. That has led the FDA to insist on estimating this increase in subjects with renal impairment for all chronic use drugs whether or not they are renally excreted [19]. The results of these small pharmacokinetic studies are then reported in the label with statements such as “use with caution in subjects with renal impairment.”

However, there are some drugs that become toxic as the serum levels increase, and this has led to various methods for monitoring and adjusting dose in response to renal impairment. Aminoglycoside antibiotics have traditionally been used in renal impairment even though they cause both ototoxicity and nephrotoxicity at high systemic levels. An early nomogram for gentamicin was published in 1972 that allows the dose to be modified to maintain efficacy in all degrees of renal impairment and hemodialysis, and the results were very successful in terms of outcome. Another drug that has to be used with caution in subjects with renal impairment is lithium. As lithium toxicity impairs renal function, it exacerbates the problem, and careful monitoring of lithium levels is important. It may be necessary to use hemodialysis to reduce lithium toxicity [20].

Because of the general concerns about using drugs in renal impairment, efforts have been made to offer guidelines for such prescribing that go beyond the approved label [21]. Such advice is almost certainly used in renal units where the issues are fully understood and there is very little margin of error. However, in the more general medical environment, the issues of renal impairment are probably underestimated [22]. Physicians seeking clarity on the appropriate dosing guidance in renal impairment may find confusion in the various official and secondary sources of information [23].

Renal physicians are comfortable using a limited number of drugs in subjects with severe renal failure or on dialysis, but there is some confusion in the general population who may incidentally have mild or moderate renal impairment. Although there are theoretical concerns, it does not appear that there is a major safety issue for the majority of drugs and those drugs that are well known to cause problems have appropriate dose adjustment guidelines. The use of language such as “use with caution” is probably too vague to be useful in practice.

18.7 HEPATIC IMPAIRMENT

Many drugs are metabolized by the liver either into inactive metabolites or into active moiety. It is therefore recommended that such drugs have pharmacokinetic studies in mild, moderate, and severe liver function as measured by the Child-Pugh classification. Unlike renal disease, there is no simple factor that determines the degree of liver function. The study design recommended by the FDA [24] involves studying the drug in at least eight usable subjects with the relevant amount of hepatic dysfunction and an age- and sex-matched control group. This provides pharmacokinetic information but does not really address whether or not there are consequences of chronic use in such subjects and whether or not the dose needs adjusting. There are some drugs that may be needed to modulate the hepatic disease such as prednisolone and other drugs that are needed for chronic or acute therapy in subjects who happen to have some degree of hepatic impairment, but in general, it is easier to avoid or reduce the use of therapeutic drugs in subjects with marked hepatic impairment.

A full discussion of the various factors involved in dose adjustments in hepatic dysfunction has been published [25]. They include protein binding as well as increases and decreases in efficacy as a result of potential pharmacokinetic changes in both the parent drug and its metabolites. In addition, safety considerations may be intensified in subjects with hepatic impairment. This is a consideration particularly with antineoplastic drugs, which may also contribute to the hepatic toxicity and resulting dysfunction. However, there is no clarity in dose adjustments for particular drugs even though the pharmacokinetic changes may be clear resulting in a clinical dosage trial in each subject as suggested by some authors [26].

18.8 METABOLIC PATHWAYS

The knowledge of metabolism of individual drugs is a major part of drug discovery and development starting with *in vitro* microsomal studies and following through with extensive study of metabolic pathways and the resulting potential drug–drug interactions. This is discussed extensively in this encyclopedia. Drugs with high potential for interactions are rarely chosen to develop further. However, this is an area where drug labeling is more precise and the results of pharmacokinetic drug–drug interaction studies are accurately presented. There are two potential directions of concern. Does the drug interact with other drugs because of competition, stimulation, or inhibition of a particular pathway that may be needed for the metabolism of the other drug? Alternatively, does the other drug interfere with the metabolism of the new drug because of competition, stimulation, or inhibition of the relevant metabolic substrates? This leads to presentation of the data in the label with statements about the pharmacokinetic changes.

If we use Lipitor (atorvastatin) as an example of a commonly used drug, the dosing consequences from the label can be seen.

Under the heading of drug interactions, we see the following statements:

The risk of myopathy during treatment with statins is increased with concurrent administration of fibric acid derivatives, lipid-modifying doses of niacin, cyclosporine, or strong CYP3A4 inhibitors (e.g., clarithromycin, HIV protease inhibitors, and itraconazole).

No numbers are presented to help assess the increased risk. Is it from 0.001% to 0.002%, which is a doubling, but remaining very rare; or is it from 5% to 6%, a smaller increase of the population but one that will affect many more people? Many physicians use fibrates and atorvastatin together and a combination tablet has been under development [27] with results that were encouraging and showed no increase in myopathy in a relatively small study.

The metabolism of modern drugs is well understood allowing fairly accurate statements to be made in the label.

Strong Inhibitors of CYP3A4. Lipitor is metabolized by cytochrome P450 3A4. Concomitant administration of Lipitor with strong inhibitors of CYP3A4 can lead to increase in plasma concentrations of atorvastatin. The extent of interaction and potentiation of effects depend on the variability of effect on CYP3A4.

Clarithromycin. Atorvastatin AUC was significantly increased with concomitant administration of Lipitor 80 mg with clarithromycin (500 mg twice daily) compared to that of Lipitor alone. Therefore, in patients taking clarithromycin, caution should be used when the Lipitor dose exceeds 20 mg.

Combination of Protease Inhibitors. Atorvastatin AUC was significantly increased with concomitant administration of Lipitor 40 mg with ritonavir plus saquinavir (400 mg twice daily) or Lipitor 20 mg with lopinavir plus ritonavir (400 + 100 mg twice daily) compared to that of Lipitor alone. Therefore, in patients taking HIV protease inhibitors, caution should be used when the Lipitor dose exceeds 20 mg.

Itraconazole. Atorvastatin AUC was significantly increased with concomitant administration of Lipitor 40 mg and itraconazole 200 mg. Therefore, in patients taking itraconazole, caution should be used when the Lipitor dose exceeds 20 mg.

So, it is clear that despite the known interaction, it is reasonable to use up to 20 mg Lipitor, and it may even be useful to use higher doses. How are the individual physician and patient to decide? Although no formal survey has been performed discussion with a number of practicing physicians suggests that each subject is considered in a therapeutic environment with reductions in dose to alleviate adverse events and increases in dose to maintain the desired therapeutic effect so the language in the label may be ignored unless issues arise.

Grapefruit Juice It contains one or more components that inhibit CYP3A4 and can increase plasma concentrations of atorvastatin, especially with excessive grapefruit juice consumption (>1.2 L/day).

Does this mean that subjects should not take grapefruit juice or that they should take grapefruit juice to increase the efficacy of Lipitor? The clinical answer suggests that it depends on how the subject is responding although this statement seems to cause concern to subjects on atorvastatin.

Cyclosporine Atorvastatin and its metabolites are substrates of the OATP1B1 transporters. Inhibitors of the OATP1B1 (e.g., cyclosporine) can increase the bioavailability of atorvastatin. Atorvastatin AUC was significantly increased with concomitant administration of Lipitor 10 mg and cyclosporine 5.2 mg/kg/day compared to that of Lipitor alone. In cases

where coadministration of Lipitor with cyclosporine is necessary, the dose of Lipitor should not exceed 10 mg.

The advice is clear but the maximum AUC increase is not mentioned to help understand whether to go to 20 mg Lipitor if the desired efficacy is not achieved.

Rifampin or Other Inducers of Cytochrome P450 3A4 Concomitant administration of Lipitor with inducers of cytochrome P450 3A4 (e.g., efavirenz and rifampin) can lead to variable reductions in plasma concentrations of atorvastatin. Owing to the dual interaction mechanism of rifampin, simultaneous coadministration of Lipitor with rifampin is recommended, as delayed administration of Lipitor after administration of rifampin has been associated with a significant reduction in atorvastatin plasma concentrations.

This is the most specific type of labeling language giving clear and undeniable advice, but this is the most unusual form.

Digoxin When multiple doses of Lipitor and digoxin were coadministered, steady state plasma digoxin concentrations increased by ~20%. Patients taking digoxin should be monitored appropriately.

This advice refers to digoxin rather than Lipitor and clearly implies that the dose of digoxin may need to be lowered but does not advise how best to do that. Discussions suggest that a 20% increase in digoxin serum concentrations are considered generally insignificant and digoxin dosing would not be modified unless there was an evidence of toxicity.

Oral Contraceptives Coadministration of Lipitor and an oral contraceptive increased AUC values for norethindrone and ethinyl estradiol. These increases should be considered when selecting an oral contraceptive for a woman taking Lipitor.

The implication is that lower dose oral contraceptives should be chosen, but no comments are made on whether the efficacy of those lower doses will be sustained. This would appear to be a relatively unusual situation, as the vast majority of subjects treated for excess lipids are postmenopausal. However, it becomes a serious issue in those subjects with familial hypercholesterolemia. Other statins are available that are not modulated through cytochrome P450 3A4 and that may be an alternative solution although not specifically mentioned in the label for atorvastatin.

Warfarin Lipitor had no clinically significant effect on prothrombin time when administered to patients receiving chronic warfarin treatment.

This is an important labeling statement because it discusses the outcome, that is, prothrombin time, rather than the usual presentation of pharmacokinetic drug levels.

The conclusion is that a good deal of clinical pharmacology takes place to help understand the metabolism of the drug of interest. However, whether that can be translated into practical clinical advice remains unclear, and the physician must then decide on how to deal with each drug–drug interaction issue.

18.9 GENETIC POLYMORPHISM

Variations in cytochrome P450 metabolic pathways are modified by polymorphic genetic variability. These provide opportunities to modify the drug dose and hence individualize drug therapy, to reduce adverse events and maintain efficacy. A review of the subject suggests that adverse events could be reduced, but the practical implications are not contained in current labeling [28]. A more recent paper confirms these findings and argues that science be used prospectively in drug research [29].

Some examples may be helpful. Tramadol is a centrally acting analgesic that is by itself active and has an active metabolite *O*-desmethyl-tramadol (M1). The *O*-demethylation is catalyzed by cytochrome P450 2D6 and the *N*-demethylation is catalyzed by CYP2B6 and CYP3A4. There is a wide variability in pharmacokinetics because of CYP genetic polymorphism [30]. This is reflected in the current Tramadol label, which reads as follows:

“The formation of the active metabolite, M1, is mediated by CYP2D6. Approximately 7% of the population has reduced activity of the CYP2D6 isoenzyme of cytochrome P-450. Based on a population PK analysis of Phase I studies with immediate-release tablets in healthy subjects, concentrations of tramadol were approximately 20% higher in “poor metabolizers” versus “extensive metabolizers,” while M1 concentrations were 40% lower. *In vitro* drug interaction studies in human liver microsomes indicate that inhibitors of CYP2D6 (fluoxetine, norfluoxetine, amitriptyline, and quinidine) inhibit the metabolism of tramadol to various degrees, suggesting that concomitant administration of these compounds could result in increases in tramadol concentrations and decreased concentrations of M1. The full pharmacological impact of these alterations in terms of either efficacy or safety is unknown.”

Irinotecan is an anticancer drug which can cause toxicity including leucopenia and diarrhea. Irinotecan is metabolized to form an active metabolite SN-38 which is then further metabolized by UDP-glucuronosyltransferase (UGT) 1A1 enzyme. There is genetic polymorphism of the (UGT) 1A1 enzyme [31]. The label states the following:

“The metabolic conversion of Irinotecan to the active metabolite SN-38 is mediated by carboxylesterase enzymes and primarily occurs in the liver. SN-38 is subsequently conjugated predominantly by the enzyme UDP-glucuronosyl transferase 1A1 (UGT1A1) to form a glucuronide metabolite. UGT1A1 activity is reduced in individuals with genetic polymorphisms that lead to reduced enzyme activity such as the UGT1A1*28 polymorphism. Approximately 10% of the North American population is homozygous for the UGT1A1*28 allele. In a prospective study, in which Irinotecan was administered as a single-agent on a once-every-3-week schedule, patients who were homozygous for UGT1A1*28 had a higher exposure to SN-38 than patients with the wild-type UGT1A1 allele SN-38 glucuronide had 1/50 to 1/100 the activity of SN-38 in cytotoxicity assays using two cell lines *in vitro*. The disposition of Irinotecan has not been fully elucidated in humans. The urinary excretion of Irinotecan is 11% to 20%; SN-38, <1%; and SN-38 glucuronide, 3%. The cumulative biliary and urinary excretion of Irinotecan and its metabolites (SN-38 and SN-38 glucuronide) over a period of 48 hours following administration of Irinotecan in two patients ranged from approximately 25% (100 mg/m²) to 50% (300 mg/m²).”

“When administered as a single-agent, a reduction in the starting dose by at least one level of Irinotecan hydrochloride injection should be considered for patients known to be homozygous for the UGT1A1*28 allele. However, the precise dose reduction in this patient

population is not known and subsequent dose modifications should be considered based on individual patient tolerance to treatment.”

It would appear that the science continues to improve to help understand how genetic polymorphism explains differences in toxicity and to some extent efficacy. However, based on these two examples, it is still not possible to adequately label the proposed change in dose with any precision.

18.10 ETHNIC DIFFERENCES

Ethnic factors have become an issue in drug development with a guideline generated from the International Congress on Harmonization (ICH) [32]. It is hard to tease out the effects of ethnic factors from genetic polymorphism as discussed in the last section. Ethnic factors include intrinsic factors such as genetics and metabolism and extrinsic factors such as diet, medical practice, and use of concomitant drugs, tobacco, or alcohol. There are probably no pure ethnic factors but the proportion of the population with a particular genetic polymorphism varies in different ethnic populations. It is clearly desirable to include as diverse a population as possible in clinical trials to help understand and anticipate any ethnic differences, but the practical labeling consequences are less clear. It is apparent that some drugs have been dosed lower in Japan than the United States, but there is greater concordance in dosing between Europe and the United States although there are some exceptions [33]. A full review of the literature underpinning ethnic differences has been carried out, and includes questions on the precision of the ethnic group being tested as well as differences in methodology used in different studies [34]. Modern drug development incorporates a diverse population with multicentered multinational studies becoming the norm in phase 3. However, the interpretation of this diverse data does not lead to individual drug labeling advice.

18.11 INDIVIDUALIZED DRUG THERAPY

Much has been written about personalized medicine but with a few exceptions it remains a dream rather than a reality. Looking at mean differences resulting from drug therapy in clinical trials and comparing with placebo has been the standard for 40 years. Defining subpopulations with the appropriate results from genetic testing of some description will result in several different subpopulations making recruitment for clinical trials more complex. This has been established with a few efficacy markers such as Herceptin targeting HER2, Erbitux targeting EGFR for metastatic colorectal cancer, and Gleevec targeting the cell surface tyrosine kinase receptor in gastrointestinal cells [35]. Whether it is equally possible to target subpopulations who are more likely to have adverse events remains to be seen and will present increasing complexity in clinical trial design. One of the issues is that it may be becoming easier to predict changes in serum level of active drug or active metabolites, as has been discussed, because of increasing knowledge of genetic diversity, but translating these values into meaningful dosing information remains difficult.

18.12 CONCLUSION

The drug development process leads to increasing knowledge about the metabolism of drugs, and this information is usually collected during drug development before drugs being marketed. The drug development process is intended to exclude drugs that have problematic metabolism or drug–drug interactions, and modern drugs are usually robust in their dosing regardless of the medical issues of the subjects taking the drugs. The labeling process is formalized, but it would appear not to be terribly helpful in practice in answering questions about dose modifications. Terms such as *use with caution* are prolific but all drugs should be used with caution so the term is almost meaningless. Questions on particular drug dosing have suggested that labeling be more precise and kept simple but that full information be made available so physicians can make informed decisions [36]. The prospect of individualized medicine remains an opportunity rather than a reality. In addition, it is important that medical professionals get more training on drug use at medical school and through ongoing continuing medical education, so they can make fully informed and appropriate dosing decisions [37,38].

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13 Assessing the Hepatic Disposition and Toxicity of Xenobiotics Using Primary Hepatocytes

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13.1	Introduction	1
13.2	Metabolic stability of drug candidates	4
13.3	Metabolic profiling and metabolite identification	11
13.4	Inhibition	13
13.5	Enzyme induction	17
13.6	Hepatobiliary transport	27
13.7	Hepatotoxicity	36
13.8	FDA draft guidance	41
13.9	Summary	42
	Acknowledgments	43
	Abbreviations	43
	References	44

13.1 INTRODUCTION

When developing a new drug, the goal is to produce therapeutic agents that are safe and effective at treating or preventing diseases. It is estimated that over 10% of drugs fail in clinical trials because of pharmacokinetic (PK) reasons and unexpected drug–drug interactions [1]. As such, the US Food and Drug Administration (FDA) guidelines emphasize the identification of metabolic pathways, relevant major metabolites, and the potential for drug–drug interactions of new chemical entities (NCEs), where metabolism is the principal route of elimination [2]. Consequently, preclinical drug metabolism and drug–drug interaction studies are critical for the development of orally administered drug candidates.

A number of liver-derived *in vitro* systems, such as slices, subcellular fractions (microsomes), and primary and immortalized hepatocytes, are typically utilized to assess the metabolism and toxicity of new drugs. Microsomes derived from preclinical species and human livers are the screening model of choice for assays based on high throughput drug metabolism, and these are increasingly being replaced or complemented by fresh isolated or cryopreserved primary hepatocytes [3,4]. Before utilizing primary hepatocytes for *in vitro* drug testing, it is helpful to appreciate the key architectural features and molecular factors that determine their unique phenotype *in vivo*. Hepatocytes from adult mammalian liver are highly differentiated epithelial cells and perform a wide variety of functions related to absorption, metabolism, and excretion of drugs and other xenobiotics. The cytoplasm is usually enriched in rough endoplasmic reticulum due to the secretory nature of the cells. There are also numerous areas of smooth endoplasmic reticulum which contain most of the enzymes involved in the metabolism of xenobiotics, especially the cytochrome P450 (P450) and uridinediphospho (UDP)-glucuronosyltransferase enzymes. Fat droplets may appear as inclusions in the cytoplasm, particularly in hepatocytes isolated from donors with a high body mass index (BMI). Hepatocytes are highly polarized cells and exhibit distinct domains on the plasma membrane, specifically the sinusoidal domain on the blood sides and the canalicular domain on the bile side, which is crucial for establishing the vectorial uptake, metabolism, and excretion of xenobiotics from the liver. Within the hepatocyte, there is a polarized localization of cytoplasmic organelles, such as the endoplasmic reticulum, Golgi apparatus, and the cytoskeletal elements, which aids in establishing and maintaining the overall architecture and therefore, functions of the cell [5,6].

Metabolism of drugs and other xenobiotics primarily takes place in the hepatocytes and usually occurs in two phases. Phase I metabolism involves reactions such as oxygenation, oxidation, reduction, dehalogenation and hydrolysis, that expose or introduce functional groups into the lipophilic molecules, thereby making them more hydrophilic and more readily excretable [7]. Phase II metabolism (conjugation enzymes) comprises synthetic reactions in which small endogenous molecules (e.g., glucuronic acid, glutathione, sulfate, glycine, and other amino acids) are added to the functional groups of xenobiotics or their phase I metabolites, thus making them even more polar and readily eliminated. Phase I metabolism does not necessarily precede conjugation and direct phase II metabolism, without prior oxidation/hydroxylation is common. For example, diclofenac is metabolized by direct conjugation of the parent drug as well as oxidation of the aromatic rings, usually followed by conjugation [8]. Similarly, the nonsteroidal anti-inflammatory drug (NSAID) carprofen is directly conjugated to form an ester glucuronide, and this represents the only significant pathway of metabolism [9]. Conjugated metabolites are actively transported out of the hepatocyte by efflux pumps that reside in the basolateral and canalicular (apical) membranes and excreted via bile and urine [7].

Historically, one of the major obstacles to determine the pharmacological and toxicological effects of new drugs on the liver has been the lack of adequate *in vitro* model systems. Therefore, a major goal of pharmaceutical researchers studying drug metabolism and hepatotoxicity has been to develop *in vitro* cell models that simulate *in vivo* conditions. Short-term metabolism and safety studies can be conducted with isolated hepatocytes and liver slices, as the incubation medium, and cells can be easily separated for analysis of drug metabolites [10,11]. When isolated and handled properly, primary hepatocytes from adult liver contain a broad complement of

metabolizing enzymes and transport proteins, organized in a physiologically relevant context and regulated via cellular processes that occur within the liver *in vivo* (e.g., nuclear-hormone-mediated xenosensors) [12].

It must be appreciated, however, that the behavior of the liver is, to some extent, dependent on its anatomy, and this cannot be mimicked by standard suspension culture methods. Cells in suspension inevitably lack various features of the organization of hepatocytes in the intact liver, and they cannot be used for evaluating drug-induced changes in gene expression (i.e., enzyme induction) due to their refractory nature while in suspension [13,14]. Moreover, suspended cells are spherical, entirely covered with microvilli and lack distinct membrane regions such as the sinusoidal versus canalicular domains. In addition, they lack the contact with extracellular matrix or cell–cell contacts (with either other hepatocytes or nonparenchymal cells), which appear to be important for maintaining differentiated function *in vivo* [5,12].

The development of more physiologic hepatocyte culture systems has permitted major advances in the study of hepatic gene regulation and cellular differentiation [14,15]. Many related molecular and biochemical pathways are restored and respond to experimental manipulation only after a period of many days in culture. Therefore, a major advantage of the hepatocyte culture systems compared to other *in vitro* liver preparations is their relative longevity of several days, as compared to hours for perfused liver, liver slices, or suspensions of isolated hepatocytes. Another advantage of primary hepatocyte culture, as compared to freshly isolated suspensions, may be that deficiencies of the latter, resulting from damage of the cell membrane by the collagenase treatment, are resolved during the early stages of culture. It has been suggested that recovery might include restoration of cell polarity, some hormone receptors [14,16,17], rates of protein synthesis [18], restoration of normal intracellular ions and glutathione levels, repair of DNA damaged during cell isolation [19], removal of proteolytic enzymes that may have persistent effects in fresh suspensions [20,21] and removal (as nonattached cells) of any residual damaged hepatocytes.

In addition to the repair of damage, recovery of polarity, and restoration of cell–cell interactions, a preincubation period in monolayer culture before initiating studies may also allow better definition of experimental variables by minimizing effects of prior exposure to hormones or nutrients *in vivo*, especially for primary human hepatocytes [14]. Another special advantage of the hepatocyte culture systems compared to cell suspensions or liver slices is the ease with which cells can be examined using today's high content microscopic imaging techniques, so that structural and subcellular changes induced by exposure to a drug can be readily detected [22]. Likewise, the regulation of cell–cell contacts and the mechanisms involving their re-establishment can be studied in cell culture [5]. Similar studies would be virtually impossible to perform using other *in vitro* model systems.

For applications requiring longer experimental periods, cells stably adapted to a defined *in vitro* environment, or where cell architecture and polarity are likely to be important and more sophisticated culture systems may be required for *in vitro* studies. Moreover, for investigating the hepatotoxic effects of drugs, especially on growth control, changes in gene expression, and bioaccumulation, the ability to maintain functional hepatocytes for many days or even months can be essential. For example, culturing hepatocytes in primary sandwich culture between two layers of extracellular matrix proteins (e.g., collagen type I and Matrigel) dramatically prolongs the longevity of cultures and retains many of the hepatocyte-specific functions [23]. Culturing hepatocytes

on collagen, with and without the overlay, results in morphologically distinct hepatocytes with relatively flattened nonoverlaid cells, while in sandwich culture, hepatocytes form aggregates and retain their cuboidal shape [3]. Sandwich-cultured hepatocytes can form extensive, functional bile canaliculi, while these are minimal in the absence of the overlay [24].

With the increased knowledge of the molecular and cellular factors that dictate hepatocyte structure and function *in vivo*, improved isolation, incubation, and cell culture techniques have greatly expanded the number of applications for hepatocytes during the drug discovery and development process [3,25–27]. In this chapter, several standard approaches are described for utilizing primary hepatocytes in suspension or monolayer culture to address specific issues related to hepatic metabolism, transport, and toxicity.

13.2 METABOLIC STABILITY OF DRUG CANDIDATES

13.2.1 Background

Liver-derived systems, such as microsomes, S9 fractions, and intact hepatocytes are routinely utilized to assess the metabolic stability of drug candidates. The use of intact hepatocytes is advantageous because of the presence of the major drug-metabolizing enzymes, xenobiotic transporters, and relevant cofactors [12]. Compounds can be screened and rank ordered according to *in vitro* intrinsic clearance (CL_{int}) values obtained from hepatocyte-based metabolic stability studies and *in vivo* CL_{int} extrapolated. For some therapeutic areas (e.g. neuropathic pain, Parkinson's disease and osteoporosis), long half-life drugs ($T_{1/2}$) with appropriate target potency and volume of distribution (V_d) are desirable, so that once per day or once per week dosing can be achieved [28,29]. In other instances, a short $T_{1/2}$ is desired as in the case of prodrugs or drugs for insomnia, where it is important to have a short $T_{1/2}$ so that there is no residual drowsiness during the day. Metabolic screening assays enable synthesis of more (or less) stable compounds through structure–activity relationships and prevent the progression of labile compounds prior to more costly *in vivo* studies.

13.2.2 Experimental Approaches

In vitro CL_{int} can be calculated by evaluating metabolite formation or substrate depletion using microsomes and hepatocytes [30,31]. Direct comparisons between these *in vitro* systems indicate that hepatocytes are the superior system. For metabolic stability experiments using the metabolite formation approach, a range of substrate concentrations are employed with a set of protein concentration and incubation time. The Michaelis–Menten parameters V_{max} and K_m are estimated and CL_{int} calculated according to the following equation:

$$CL_{int} = \frac{V_{max}}{K_m}$$

Information regarding metabolites is required when using this method and in some cases, authentic metabolite standards are not available to determine formation rates [30]. Furthermore, linear incubation conditions with respect to protein content and incubation time need to be employed to ensure parent depletion is not greater than 10% [31]. The

substrate depletion approach utilizes a single low concentration assumed to be much lower than the K_m value. Typically, hepatocytes or microsomes are incubated with a substrate concentration of $1\ \mu M$ and the disappearance of parent compound monitored over several time points. Monoexponential kinetics is assumed as CL_{int} is a function of the first-order rate constant, k . This approach is also referred to as the *in vitro* half-life ($t_{1/2}$) method and the equation used to calculate CL_{int} is

$$CL_{int} = \frac{0.693}{t_{1/2}} \times \frac{V}{N}$$

where $k = 0.693/t_{1/2}$, V is the incubation volume, and N is the number of cells [32,33].

The area under the concentration versus time curve is at times incorporated within similar equations derived from the substrate depletion method [34]. Assuming that the *in vitro* clearance process follows first-order kinetics, CL_{int} is calculated from the ratio of the initial amount of the test compound minus the amount remaining after 2 h of incubation and the corresponding *in vitro* AUC (area under the curve) between 0 and 2 h. In contrast to metabolite formation methodology, this approach is most effective when at least 20% of parent compound is metabolized over the incubation period [31].

The substrate depletion method is simple and amenable to use in an early discovery setting and microsomes or hepatocytes can be used. The advantage of using hepatocytes is that unlike microsomes that only extrapolate biotransformation reactions carried out by P450 enzymes, the contribution of phase II enzymes to metabolism is also assessed [35]. The use of microsomes poses the risk of underestimation of *in vivo* CL_{int} if metabolic elimination routes other than P450s predominate *in vivo*. Also, if active uptake is required for transport of drug to the enzymatic active site, this factor would be missed entirely by the use of microsomes, leading to overestimation of CL_{int} . Alternatively, liver S9 fractions could be utilized, as these contain both microsomes and cytosol. However, S9 fractions lack membranes as well as the multiple cofactors needed for the phase I and phase II reactions, thereby offering limited advantage over microsomes and the disadvantage of adding multiple relatively unstable cofactors [35]. Healthy hepatocytes with the complete cellular machinery including the additional required cofactors and membrane transporters provide the most complete system for conducting metabolic stability measurements.

13.2.3 Metabolism in Fresh and Cryopreserved Suspensions

The availability of healthy cryopreserved hepatocytes has facilitated the use of these cells in routine drug disposition studies by lessening the issues related to availability of fresh tissue. Moreover, these cells have the added advantage of consistency, as hepatocytes isolated from the same liver can be used multiple times, over a period of time [35–37]. We used the substrate depletion method to estimate *in vitro* CL_{int} in freshly isolated human hepatocytes and cryopreserved human hepatocytes processed from the same donor and calculated values in suspensions and monolayers. Our data demonstrated that metabolic activities were statistically similar between freshly isolated and cryopreserved human hepatocytes from the same preparation. We recommend that fresh or cryopreserved hepatocytes may be used for these studies, as these have comparable metabolic capacity. It is important to include the same positive controls across experiments to ensure the integrity of the individual hepatocyte preparation and validity of experimental conditions.

We prepared primary human hepatocytes from three separate tissue samples and used a portion of each preparation immediately after isolation or brief storage (<24 h) in preservation medium or after at least one month of cryopreservation. Both freshly isolated and cryopreserved hepatocytes from each preparation were subsequently characterized for metabolic activity of the major drug-metabolizing enzymes as per the methods shown in Table 13.1. The resulting metabolic activities of fresh and cryopreserved human hepatocytes from the same preparation were comparable, and statistically significant differences were not observed between activities of specific isoforms (Figure 13.1). The observed differences in metabolic activity between the individual hepatocyte preparations are due to interdonor variability and not cryopreservation. These data demonstrate that functional drug-metabolizing enzymes are maintained in

TABLE 13.1 Incubation Conditions for specific activity

Enzyme	Probe Substrate	Concentration (μM)	Incubation Time (min)
CYP1A2	Phenacetin	100	15
CYP2B6	Bupropion	500	15
CYP2C9	Diclofenac	25	15
CYP2C19	S-Mephenytoin	250	30
CYP3A	Testosterone	200	15
UGT	7-Hydroxycoumarin	100	30

Substrate incubations were performed with human hepatocytes in suspension at a density of 0.50×10^6 cells/mL in multi-well plates. Incubations were terminated by freeze on contact or with organic and samples analyzed by HPLC or LC/MS/MS analyses to determine specific activity.

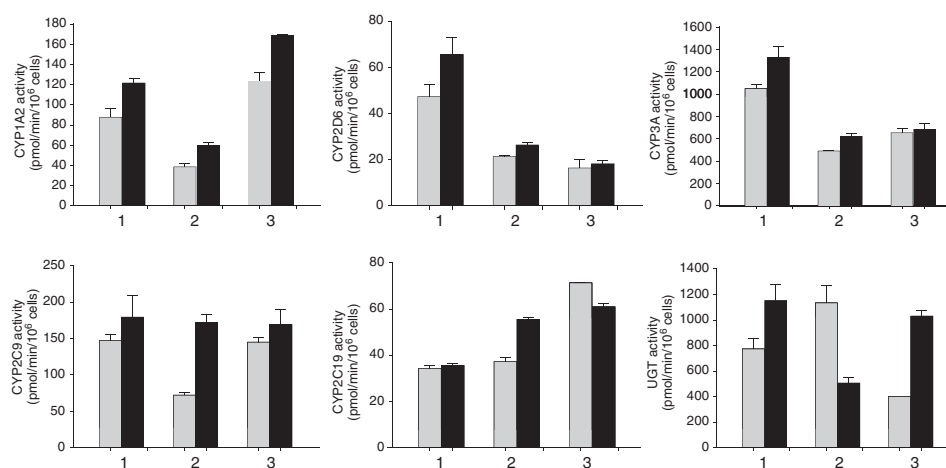


Figure 13.1 Enzyme-specific activities in freshly isolated and cryopreserved human hepatocytes from the same donor. The data represents three separate hepatocyte preparations (1, 2, and 3). The gray bars represent fresh hepatocytes and the black bars cryopreserved hepatocytes from the same preparation. Bars represent mean $\pm$ standard deviations of triplicate determinations. Missing error bars are indicative of duplicate determinations. Assay conditions are provided in Table 13.1.

cryopreserved human hepatocytes. Next, we assessed the feasibility of using cryopreserved human hepatocytes for metabolic stability evaluations. Six compounds that covered a range of known metabolic pathways were evaluated in hepatocyte preparations isolated from one donor liver. Phenacetin, midazolam and dextromethorphan were chosen as representative examples of moderate to high turnover compounds and tolbutamide (TLB) and diazepam (DZP) represented compounds belonging to a low turnover class. The substrate benzydamine was chosen as a probe for flavin-containing monooxygenase (FMO) activity to assess non-P450 activity in fresh and cryopreserved human hepatocytes. The intrinsic clearance results for moderate to high turnover compounds were comparable between fresh and cryopreserved suspensions (Table 13.2). In our studies, low turnover substrates proved challenging in short-term suspension studies, as reliable CL_{int} values were not attainable and R^2 values from linear regression analyses were below acceptable ranges (data not shown). Metabolic turnover percentages for TLB and DZP generally did not reach the 20% or greater levels that are essential when using this method [31,38].

To alleviate the possibility of severely reduced viability as a reason for decreased metabolism, cell viability was assessed immediately after thawing and removal of cryopreservation medium (fresh hepatocytes) or postthaw (cryopreserved hepatocytes) and also after a 2 h of incubation at 37°C. Viabilities at the onset of incubations were 77% and 78% for fresh and cryopreserved hepatocytes, respectively. Less than 5% loss in cell viability was observed during the incubation period for fresh hepatocytes, and a 6% decrease in viability was observed in cryopreserved hepatocytes. Our results demonstrate that cryopreserved human hepatocytes are good tools for short-term intrinsic clearance experiments and demonstrate particular suitability for moderate to high turnover compounds. As more drugs are identified as substrates of non-P450 pathways, it is critical to have confidence in the metabolic competency of cryopreserved hepatocytes as an *in vitro* tool for studying these pathways. We have conducted metabolic stability studies in hepatocyte suspensions for up to 4 h in culture. We found that incubating hepatocytes for extended periods of time in suspension cultures (i.e., >4 h) causes unacceptable drops in viability (>20%) and a differential loss of phase II metabolic rates compared to those for phase I reactions.

13.2.4 Metabolism in Cultured Fresh and Cryopreserved Primary Human Hepatocytes

In vitro metabolic stability measurements in suspension hepatocytes often underpredict *in vivo* CL_{int} , particularly for low turnover compounds. For orally administered candidate drugs, prediction of *in vivo* CL_{int} is required for dose estimations required to achieve appropriate PK for target pharmacological activity. In these cases, short-term *in vitro* suspension assays in which the substrate depletion method is used may not be applicable for CL_{int} predictions. Griffin and Houston proposed using monolayer cultures as an alternative to suspensions for estimating intrinsic clearance [39]. In their studies, seven compounds were assessed in rat suspensions and monolayers including low turnover substrates *S*-warfarin and tolbutamide and the data demonstrated that CL_{int} can be effectively measured at below 0.1 $\mu\text{L}/\text{min}/10^6$ cells.

We assessed the metabolic stability of six compounds in human hepatocyte monolayers isolated from a single donor liver. The moderate to high turnover compounds

TABLE 13.2 Comparison of Intrinsic Clearance Values Obtained in Freshly Isolated and Cryopreserved Hepatocytes Suspensions and Cultures Processed from the Same Donor Tissue

Substrate	Fresh or Cryopreserved	Hepatocyte Monolayers			Hepatocyte Suspensions			Observed <i>In Vivo</i> CL (mL/min/kg)
		CL _{int} <i>In Vitro</i> (μL/min/10 ⁶ cells)	CL _{int} Scaled (mL/min/kg)	Predicted CL _H (mL/min/kg)	CL _{int} <i>In Vitro</i> (μL/min/10 ⁶ cells)	CL _{int} Scaled (mL/min/kg)	Predicted CL _H (mL/min/kg)	
PHN	Fresh	15.3 ± 1.1	40.3 ± 2.9	13.4 ± 0.3	78.3 ± 8.0	207 ± 21	18.2 ± 0.2	19.6 ± 4.5 ^a
	Cryopreserved	9.13 ± 1.17	24.1 ± 3.1	10.9 ± 0.6	54.5 ± 4.8	144 ± 13	17.5 ± 0.2	
MDZ	Fresh	13.0 ± 2.1	34.2 ± 5.5	12.6 ± 0.8	21.5 ± 0.3	56.7 ± 0.8	14.8 ± 0.1	12.0 ± 1.6 ^b
	Cryopreserved	6.48 ± 1.29	17.1 ± 3.4	9.16 ± 1.03	13.1	34.6	12.5	7.0 ± 2.1 ^a
DXT	Fresh	2.70 ± 1.17	7.12 ± 3.09	5.11 ± 1.81	13.1 ± 1.9	34.7 ± 4.9	12.6 ± 0.7	8.6 ^c
	Cryopreserved	2.56 ± 0.66	6.77 ± 1.75	5.01 ± 1.01	8.40 ± 3.42	22.0 ± 9.0	10.2 ± 2.2	
TLB	Fresh	1.88 ± 0.32	4.95 ± 0.85	3.96 ± 0.55	N.R.	N.R.	N.R.	0.21 ± 0.04 ^a
	Cryopreserved	0.329 ± 0.146	0.869 ± 0.384	0.829 ± 0.351	N.R.	N.R.	N.R.	
DZP	Fresh	2.47 ± 0.62	6.51 ± 1.64	4.87 ± 0.94	N.R.	N.R.	N.R.	0.37 ± 0.10 ^a
	Cryopreserved	0.807 ± 0.067	2.13 ± 0.18	1.92 ± 0.15	N.R.	N.R.	N.R.	
BNZ	Fresh	1.58 ± 0.24	4.18 ± 0.65	3.45 ± 0.45	4.40	11.6	7.33	2.3 ^d
	Cryopreserved	2.14 ± 0.68	5.65 ± 1.79	4.36 ± 1.10	5.20	13.7	8.09	

Abbreviations: BNZ, benzydamine; DXT, dextromethorphan; DZP, diazepam; MDZ, midazolam; PB, phenobarbital; PHN, phenacetin; TLB, tolbutamide; N.R. not reported.

Primary human hepatocytes cultures and suspensions were incubated with a low concentration of substrate (0.5 or 1 μM) and intrinsic clearance values determined from linear regression analyses of disappearance of parent versus time profiles. *In vitro* clearances were scaled to predicted hepatic clearance as described within the text.

^aStringer *et al.* [46].

^bBlanchard *et al.* [40].

^cMcGinnity *et al.* [32].

^dObach *et al.* [33].

were incubated with the hepatocyte monolayers for up to 8 h and the low turnover compounds tolbutamide and diazepam were incubated for up to 24 h. The cell monolayers revealed no marked change in cell morphology after compound treatment, relative to vehicle control, after overnight incubations. We observed higher turnover with tolbutamide and diazepam in the hepatocyte monolayers as compared to that in suspensions. While *in vitro* CL_{int} values were statistically similar between fresh and cryopreserved monolayers, CL was slightly higher in fresh monolayers for phenobarbital, midazolam, tolbutamide, and diazepam (Table 13.2). This trend was more evident when the values were scaled to *in vivo* CL_{int} . Overall, our data are in line with other published studies comparing CL_{int} in fresh and cryopreserved human hepatocyte suspensions and monolayers [32,34,40,41].

Hepatic clearance (CL_H) can be estimated by three separate established models, each with different perceptions of liver physiology. These include the well-stirred model, parallel tube model, and dispersion model, all of which rely on inherent assumptions [30,42,43]. The fraction of drug unbound (f_u) is incorporated in the models to account for plasma protein binding. The present study utilized the well-stirred model to estimate CL_H with omission of the fraction unbound terms [32]. The following scaling factors were utilized in the equation: human liver weight = 22 g/kg body weight, hepatocellularity = 120×10^6 cells/g of liver, and hepatic blood flow = 20 mL/min/kg [32]. CL_H was compared to clearance observed in clinical studies. Predicted hepatic clearance was within the range (less than twofold) observed for *in vivo* clearance for phenobarbital, midazolam, dextromethorphan, and benzydamine in both fresh and cryopreserved hepatocyte suspensions. *in vivo* clearance for these compounds could also be predicted (less than twofold) from both fresh and cryopreserved hepatocyte monolayers. Interestingly, predictions were overestimated for low turnover compounds in the hepatocyte monolayers. Fresh monolayers overestimated tolbutamide CL by about 19-fold, while cryopreserved monolayers overestimated CL by about fourfold. A similar trend was observed with diazepam predictions where fresh and cryopreserved monolayers produced 13-fold and 5-fold overestimations in CL, respectively. These results are in agreement with a previously published study which reported overestimations of *in vivo* clearance for low clearance compounds using rat hepatocyte monolayers [39].

It is not immediately clear why fresh hepatocyte monolayers produced higher clearance than cryopreserved monolayers, as our prior studies have shown similar metabolic activity. Tolbutamide is metabolized by CYP2C9, while diazepam is metabolized by CYP2C19 and to a lesser extent, CYP3A. The CYP2C9-, CYP2C19-, and CYP3A-specific activities of the hepatocyte preparation used for this study did not differ significantly between the fresh and cryopreserved preparations (Fig. 13.1). If there was a difference in the two preparations in culture, this did not manifest in the predictions obtained for moderate and high turnover compounds. More studies are required with low turnover substrates and additional hepatocyte preparations to better understand the discrepancy between fresh and cryopreserved monolayers observed with tolbutamide and diazepam.

13.2.5 *In Vitro*–*In Vivo* Correlations

In vitro–*in vivo* correlation is the real measure of success for any given systematic approach relative to metabolic stability. Ito and Houston [44] reported a ninefold underestimation of *in vivo* CL_{int} for 52 drugs using published *in vitro* CL_{int} data derived

from human liver microsomes. A separate report examined 37 drugs in cryopreserved human hepatocytes from collated sources and reported a 4.5-fold underestimation in predicted clearance [45]. A recent study assessed compounds cleared primarily by glucuronidation in hepatocytes and microsomes. A poor *in vitro*–*in vivo* correlation was observed in microsomes, even after adding alamethicin and UDPGA in the incubations for Phase II metabolism [46]. In contrast, hepatocytes were proficient at catalyzing the phase II reactions. Overall, the general consensus is that primary hepatocytes predict hepatic clearance within two- to threefold of *in vivo* clearance values and are superior to microsomes for this purpose [35]. Currently, cryopreserved hepatocytes are the favorable model for metabolic stability studies because of the convenience of availability and because the same batch can be used multiple times, lessening concerns related to interdonor variability [35]. These results and others support the utility of cryopreserved human hepatocytes for metabolic stability and other metabolism studies (e.g., metabolic profiling and metabolite identification).

One of the main causes of discordance between *in vitro* and *in vivo* predictions and between laboratories that often goes unrecognized or underappreciated is the quality of the cell preparations—whether freshly isolated or cryopreserved. There are limited industry-wide acceptance criteria or established quality standards for primary human hepatocytes that has gained widespread adoption. As such, we propose the following criteria that can and should be considered whenever selecting a batch of cryopreserved hepatocytes for metabolic stability evaluations:

1. Consider the cellular and biochemical data that is provided with each batch of hepatocytes from the vendor. For example, most providers of hepatocytes include post-thaw viability and cell yield data, as well as the average specific activities for key P450 and Phase II enzymes, which should be within acceptable ranges. Average yields and viabilities vary somewhat between preparations and vendors but minimally they should be between 5.0 and 10.0 million cells per vial and >80%, respectively [38].
2. Information about “viability stability” over time should be provided by or obtained from the vendor to determine the suitability of preparations for suspension incubations. For instance, most metabolic stability studies require incubation times of 1–3 h. As such, it is important that the viability of the hepatocytes stays reasonably stable and does not drop more than 15–20% during that time period.
3. Average intrinsic clearance values of known substrates determined for each batch of cells should also be requested, if not provided. Typical values for low, medium, and high clearance compounds are available in literature and represent some guidance regarding the suitability of cryopreserved hepatocytes for metabolic stability assays.
4. Finally, in the event that accurate clearance values are needed for low turnover compounds, additional data about the attachment efficiency and percent confluence of the corresponding monolayers are the relevant parameters. On the basis of our experience, cryopreserved hepatocytes that exhibit >70% attachment efficiency after an initial 4–6 h attachment period generally are suitable for metabolic stability studies, with the prerequisite that suitable intrinsic clearance values are obtained for prototypical substrates during the subsequent 12- to 24-h culture period.

13.3 METABOLIC PROFILING AND METABOLITE IDENTIFICATION

13.3.1 Metabolic profiling

Metabolic profiling is the qualitative measure of the parent compound, the metabolites and their intermediates in response to actions by drug-metabolizing enzymes in biological systems. The measurement and interpretation of the metabolite profile from biological samples (typically primary hepatocytes or hepatic microsomes *in vitro*; and urine, serum, bile, or tissue extract, *in vivo*) provide a view of the exposure that a particular species has had, or will have, to the xenobiotic. The metabolism of drugs is a complex process involving one or more phase I and phase II enzyme systems. In discovery, the potential half-life of compounds is ranked based on metabolic stability, whereas in preclinical development, *in vivo* rodent and nonrodent species are used for allometric scaling to extrapolate to the human. Metabolic profiling is very useful in selecting the right preclinical nonrodent species for safety and PK assessments. For this, it is essential to choose the species with metabolites that are similar to what is expected in humans. Reasonably similar metabolites in *in vitro* systems support the relevance of a particular animal species to the assessment of a potential human risk. Moreover, knowledge of differences between a preclinical species and humans (e.g., a toxic metabolite in a particular preclinical species but not in humans) could aid in interpretation of clinical data. If there are major differences in the metabolites between the test species and humans, this would reduce the confidence in these studies as predictors of safety in humans. At the least, the species chosen should have all the major metabolites found in the human *in vitro* samples.

A decade ago, human liver microsomes were routinely used for metabolic profiling comparisons. However, this approach can lead to underprediction of metabolism, as the assumption that phase I oxidation is the major pathway of metabolism of the drug candidate is made. For example, in humans, ethinyl estradiol is predominantly metabolized by phase II metabolism into glucuronide and sulfate conjugates [47]. Human liver microsomes produce primarily the 2-hydroxyl metabolite, while human hepatocytes produce both the conjugated metabolites, similar to what is observed in human plasma samples. Microsomes underpredict ethinyl estradiol metabolism and fail to provide a true picture of metabolite exposure. Several such studies have demonstrated that while microsomes allow for the evaluation of specific phase I oxidative metabolites, intact fresh isolated or cryopreserved hepatocytes represent a more scientifically relevant experimental system, as these provide a more complete system to assess all possible metabolites. In cases where human hepatocyte samples develop a major metabolite not present in any of the animal cells, that metabolite has to be synthesized and exposed to the preclinical species, so that any potential toxicity can be assessed. These applications demonstrate the utility of hepatocytes for *in vitro* metabolic profiling and identification studies, as these have the capacity to develop phase I and phase II metabolites, similar to those formed *in vivo*.

Metabolic profiling studies are typically conducted over a short time period (2–4 h) using suspension hepatocytes that are placed in a nutrient-rich buffer or media, in a gently shaking, 37°C environment. In most cases, this is an appropriate format to conduct these experiments. In some cases, particularly for low turnover compounds, for example, *S*-warfarin and tolbutamide, this time is not sufficient for metabolism to completely occur. For these studies, we have found that similar to the intrinsic

clearance measurements, it is best to conduct overnight incubations. Since suspension hepatocytes do not thrive for more than 4 h in this environment, attached hepatocytes on a simple collagen substratum should be used, as these allows for a longer incubation and the formation of most of the possible metabolites [48].

13.3.2 Metabolite Identification

Structural identification of metabolites that have been generated by *in vitro* or *in vivo* evaluations is an integral part of preclinical and clinical drug metabolism studies. Literature indicates that the qualitative metabolite pattern of drug candidates obtained *in vitro* generally reflects that found *in vivo* [49]. *In vitro* knowledge of the structures of the major metabolites helps in predictions of the fate of drug candidates and enables the planning of timely strategies in cases where there may be toxicokinetics or hepatotoxicity issues in preclinical species or in the clinic.

The evaluation of all major human metabolites *in vitro* is emphasized in the FDA 2008 guidance “Safety Testing of Drug Metabolites.” The guidance recommends safety testing of drug metabolites that (i) account for more than 10% of parent drug AUC in human samples, (ii) are present only in human plasma, or (iii) constitute disproportionately higher levels in human samples as compared to preclinical species. Safety testing includes synthesis of the metabolite and exposure of the metabolite to a chosen preclinical species, followed by the conduct of toxicity studies. Conducting appropriate studies with hepatocytes before clinical evaluations could help in early attrition of drug candidates that have the potential to fail in the clinic due to metabolite-related toxicities.

Similar to metabolic stability and profiling measurements, a much clearer and more complete picture of the metabolites is obtained using hepatocytes versus microsomes. Improved tissue culture methodologies have enabled analysis of biliary metabolites *in vitro* [50]. By culturing primary hepatocytes in a 3D micropatterned collagen gel substrate configuration, Matsui *et al.* were able to quantify bile acids embedded in a microcavity with a diameter of 60–80 μm after directly recovering from the bile canaliculi of hepatocytes.

Previously, structural elucidation was typically performed later in preclinical development; however, over the past decade, this assessment has moved to Discovery in order to reduce later stage attrition. Owing to the larger number of compounds and the tighter time lines in Discovery, there is the need for immediately available and higher throughput methods (e.g., using cryopreserved hepatocytes and LC/MS/MS systems that utilize “smart” software to assist in the acquisition, identification, and structural characterization of metabolites). Although sensitivity can be a factor when using hepatocytes, substrate incubations at higher concentrations (i.e., 10–50 μM) and longer incubation times (i.e., 2–4 h) can circumvent this potential issue. Mass analyzers including time of flight, triple-stage quadrupole, ion traps, and Fourier transform mass spectrometry are increasingly sensitive. However, mass spectrometry (MS) alone is unable to adequately address all the caveats of structural elucidation. Additional information such as the site of biotransformation can aid structure activity relationship (SAR) to prevent undesirable PK effects when utilized in drug development. Where structural elucidation is needed, wet chemistry techniques combined with MS are useful including chemical derivatization, hydrogen/deuterium exchange, and hydrolysis.

Nuclear magnetic resonance (NMR) spectroscopy is also a valuable tool for structural characterization of metabolites. For example, LC/MS/MS was used to identify metabolites of retigabine in rat, dog, and human urine and other biological excreta [51]. While glucuronide metabolites were discovered in all three species, an additional acetylated metabolite was identified in rats and humans, which was absent in dogs. Using NMR, it was shown that the structures of retigabine metabolites in human and rat were similar across the *in vivo* urine samples and *in vitro* hepatocyte extracts. Similarly, novel metabolites of verapamil were identified in both primary human hepatocytes and human urine using LC/MS/MS and the structures elucidated using NMR [52]. All the above studies support the role of hepatocytes for metabolism studies, particularly for metabolic stability, profiling, and identification.

13.4 INHIBITION

13.4.1 Background

PK drug–drug interactions can occur when one drug alters the metabolism of a coadministered drug. Specifically, the metabolic route(s) of elimination, including most of those occurring via the P450 family of enzymes, can be inhibited by concomitant drug treatment leading to serious clinical drug–drug interactions. P450 inhibition is implicated in a majority of clinically relevant drug–drug interactions [53,54], as a P450 inhibitor can decrease metabolic clearance of a coadministered substrate drug and/or decrease formation of an active metabolite (or formation of a drug from a prodrug), resulting in decreased efficacy. This inhibition can be assessed in preclinical *in vitro* studies using hepatic microsomes and hepatocytes. The increase of plasma concentrations caused by drug interactions can be substantial, for example, the interaction between ketoconazole or itraconazole (CYP3A4 inhibitors) and triazolam (CYP3A4 substrate), which resulted in a 22- to 27-fold increase in triazolam exposure following coadministration with ketoconazole and itraconazole, respectively [55]. Unwanted effects are most obvious and expected when they involve drugs such as warfarin with a narrow therapeutic range resulting in an increase in plasma concentration.

Interactions involving enzyme inhibition have been responsible for the limited development potential, severe dosing restrictions, and even termination of development of many investigational drugs. If these interactions are not identified early in the development process, the result can be exposure to toxic drug levels in some patients, resulting in a black box warning on the product label or withdrawal of the drug from the market. A classic example of a drug interactions is with the nonsedating antihistamine Seldane® (terfenadine) and the common antibiotic erythromycin [56]. Patients treated with terfenadine along with erythromycin accumulated dangerously high blood levels of terfenadine because of the inhibition of CYP3A4 enzymes by erythromycin, resulting in potentially fatal arrhythmia. On the basis of this, the FDA issued a recall of Seldane® in 1997 and subsequently recommended that the potential of an investigative drug to be involved in drug–drug interactions be assessed relatively early in drug development.

Drugs that cause time- or metabolism-based inhibition of CYP450 enzymes may also result in potent, potentially toxic drug interactions. One such compound, mibefradil, a Ca^{2+} channel antagonist for the treatment of hypertension and

chronic stable angina pectoris caused serious drug–drug interactions, including rhabdomyolysis and renal failure with simvastatin, and symptomatic bradycardia with β -blockers [57,58]. Subsequently, mibefradil was found to be a potent inhibitor of CYP3A4, CYP2D6, and P-glycoprotein (P-gp) and a potent time-dependent inhibitor for CYP3A4/3A5 [59–61]. These serious adverse events resulted when mibefradil was coadministered with CYP3A4 substrates, likely because of time-dependent inhibition of CYP3A4 and mibefradil was withdrawn from the market a year after launch. Similarly, rofecoxib, a cyclooxygenase-2-selective NSAID, was withdrawn from clinical use in 2006 because of cardiovascular events, as rofecoxib moderately increased plasma concentrations and effects of theophylline [62] and the *R*-isomer of warfarin [63]. Therapeutic doses of rofecoxib increased plasma concentrations of tizanidine more than 10-fold, suggesting potent inhibition of CYP1A2 [64]. *In vitro* studies demonstrated that rofecoxib was a potent, mechanism-based CYP1A2 inhibitor and provided a mechanistic explanation for the interactions of rofecoxib with CYP1A2 substrates [65].

13.4.2 Experimental Approaches and Data

To understand the potential of drug candidates to cause PK-based drug–drug interactions via inhibition of drug-metabolizing enzymes, *in vitro* screens are employed to determine the extent and type of P450 inhibition. These assays typically use recombinantly expressed P450s, human liver microsomes, and human hepatocytes.

A majority of all enzyme inhibition studies are conducted using microsomes. More recently, the use of human hepatocytes to study P450 reversible and time-dependent inhibition is increasing, as primary hepatocytes provide an *in vitro* environment that closely resembles the human liver. CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 reversible inhibition has been evaluated by known isoform-specific inhibitors in primary human hepatocytes [66]. Cryopreserved hepatocytes from a mixed pool of 10 adult human donors were used. Hepatocytes (2×10^6 cells/mL) were incubated with the test inhibitor and isoform-specific substrates: 50 μ M phenacetin (CYP1A2), 50 μ M coumarin (CYP2A6), 75 μ M tolbutamide (CYP2C9), 50 μ M *S*-mephenytoin (CYP2C19), 8 μ M dextromethorphan (CYP2D6), 50 μ M chlorzoxazone (CYP2E1), and 50 μ M testosterone (CYP3A4), for up to 2 h before terminating with organic solvent. The data set demonstrated that 10 μ M furafylline (CYP1A2), 50 μ M diethyldithiocarbamate (CYP2A6), 1 μ M sulfaphenazole (CYP2C9), 10 μ M omeprazole (CYP2C19), 1 μ M quinidine (CYP2D6), 100 μ M 4-methylpyrazole (CYP2E1), and 1 μ M ketoconazole (CYP3A4) inhibited metabolite production by 91.8%, 77.6%, 78.9%, 74.7%, 89.9%, 82.7%, 94.9%, respectively. Another report compares K_i values determined in rat microsomes and freshly isolated hepatocytes using six P450 inhibitors (miconazole, fluconazole, ketoconazole, quinine, fluoxetine, and fluvoxamine) [67]. These inhibitors were included in studies utilizing four probe substrates for CYP2C, CYP2D, and CYP3A enzymes (tolbutamide and phenytoin, dextromethorphan, and midazolam, respectively). All analyses were performed under initial rate conditions with respect to incubation time, microsomal protein content, or hepatocyte density. The K_i values determined in this investigation were in good agreement between microsomes and hepatocytes.

Time-dependent P450 inhibition has also been evaluated using cryopreserved human hepatocytes for six structurally diverse compounds known to exhibit time-dependent

inhibition [68]. CYP3A4 activities were determined after incubation with amprenavir, diclofenac, diltiazem, erythromycin, raloxifene, and troleandomycin. Human hepatocytes (0.5×10^6 cells/mL) were preincubated with and without time-dependent inactivators in 48-well plates for 1 h at a final volume of 250 μ L. At the end of the preincubation, 200 μ L aliquots were transferred to 96-well tubes, centrifuged, and the resulting pellet was washed and resuspended in cell culture medium containing midazolam, the probe substrate. The kinetic parameters (k_{inact} and K_I) associated with the inactivation of CYP3A4 in human hepatocytes were derived. In addition, the kinetic parameters of inactivation (k_{inact} and K_I) were measured using human liver microsomes. In general, this side-by-side comparison indicated that the microsome-based predictions overestimate the inactivation potency observed in hepatocytes for troleandomycin, erythromycin, raloxifene, and amprenavir. The discrepancies are likely related to metabolic stability, nonspecific binding, active membrane transport, or a combination of these factors. Therefore, these factors must be considered when microsomal inactivation parameters are used to predict inhibition-based drug interactions in intact cell systems. Time-dependent P450 inhibition has also been evaluated using human hepatocytes in cultures [69]. P450 activities were determined after incubation with tienilic acid, a prototypical inhibitor of CYP2C9 and erythromycin, troleandomycin, and fluoxetine, prototypical inhibitors of CYP3A4. Human hepatocytes were cultured on six-well collagen-coated plates and treated with varying concentrations of inhibitor (0.1, 1, and 10 μ M) for up to 48 h. At several time points during the incubation period, isoform-specific substrates in cassette format were added to each well. Aliquots were removed at various time points after addition of probe substrate, and the samples were quenched by the addition of organic solvent. Phenacetin deethylation, diclofenac 4'-hydroxylation, *S*-mephenytoin 4'-hydroxylation, bufuralol 1'-hydroxylation, and midazolam 1'-hydroxylation were used as selective probe reactions for CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively. The kinetic parameters (k_{inact} and K_I) associated with the inactivation of these enzymes in human hepatocytes were derived. In addition, the kinetic parameters of inactivation (k_{inact} and K_I) were measured using recombinantly expressed enzymes and human liver microsomes. In a side-by-side comparison, it was noted that the kinetic parameters obtained using human hepatocytes were in good agreement with those values derived using recombinantly expressed enzymes and human liver microsomes [69].

We have developed hepatocyte-based methods for the identification and quantification of reversible and time-dependent inhibition for CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4, validating these assays with appropriate positive control inhibitors. Using single concentrations of the inhibitors investigated, 12–73% reversible inhibition was observed with the human hepatocytes (Table 13.3). Time-dependent inhibition in hepatocytes was assessed for a single concentration of inhibitor after a 30-min preincubation with hepatocytes and remaining activity compared to hepatocytes preincubated with vehicle only. At the inhibitor concentrations used, enzyme activities were reduced to 29–69% of control activity (Table 13.4). For CYP1A2, CYP2B6, CYP2C19, and CYP2D6, it was also observed that the 30-min preincubation with vehicle resulted in a reduction in activity of the enzymes as compared to coincubation alone (data not shown). The percentage of inhibition observed after 30-min preincubation with hepatocytes was compared to previous data generated in microsomes at identical inhibitor concentrations (Table 13.4). For all isoforms investigated, the inhibition in hepatocytes was similar to or less than the inhibition observed in human liver microsomes. This reduction in inhibition could be due to the contribution of cell

TABLE 13.3 Percentage of Reversible Inhibition in Cryopreserved Human Hepatocytes When Coincubated with Positive Control Inhibitors

Enzyme	Assay	Chemical Inhibitor	Inhibitor Concentration (μM)	Metabolic Activity in the Presence of Inhibitor (Percentage of Vehicle Control)
CYP1A2	Phenacetin <i>O</i> -deethylase	Furafylline	5	57.8 $\pm$ 1.7
CYP2B6	Bupropion hydroxylase	Thiotepa	20	53.0 $\pm$ 1.2
CYP2C8	Paclitaxel 6 α -hydroxylase	Quercetin	10	88.1 $\pm$ 3.3
CYP2C9	Diclofenac 4'-hydroxylase	Sulfaphenazole	6.25	37.3 $\pm$ 3.2
CYP2C19	(<i>S</i>)-Mephenytoin 4'-hydroxylase	Ticlopidine	1	26.8 $\pm$ 9.2
CYP2D6	Dextromethorphan <i>O</i> -demethylase	Quinidine	0.4	58.6 $\pm$ 14.3
CYP3A4/5	Midazolam 1'-hydroxylase	Ketoconazole	0.1	41.6 $\pm$ 8.3
CYP3A4/5	Testosterone 6 β -hydroxylase	Ketoconazole	0.1	60.2 $\pm$ 1.6

TABLE 13.4 Percentage of Time-Dependent Inhibition in Cryopreserved Human Hepatocytes When Preincubated with Positive Control Inhibitors

Enzyme	Substrate	Chemical Inhibitor	Inhibitor Concentration (μM)	Metabolic Activity in the Presence of Inhibitor (Percentage of Vehicle Control) Hepatocytes HLM	
CYP1A2	Phenacetin <i>O</i> -deethylase	Furafylline	1	29.3 $\pm$ 6.8	11.0–12.9
CYP2B6	Bupropion hydroxylase	Thiotepa	30	35.5 $\pm$ 2.1	10.9–12.4
CYP2C8	Paclitaxel 6 α -hydroxylase	Phenelzine	100	69.1 $\pm$ 6.0	25.4–45.2
CYP2C9	Diclofenac 4'-hydroxylase	Tienilic acid	3	58.3 $\pm$ 8.3	5.8–39.5
CYP2C19	(<i>S</i>)-Mephenytoin 4'-hydroxylase	Ticlopidine	0.5	39.4 $\pm$ 19.6	20.4–36.1
CYP2D6	Dextromethorphan <i>O</i> -demethylase	MDMA	10	55.4 $\pm$ 6.1	3.3–5.3
CYP3A4/5	Midazolam 1'-hydroxylase	Mifepristone	10	35.1 $\pm$ 5.5	5.6–22.7
CYP3A4/5	Testosterone 6 β -hydroxylase	Mifepristone	10	45.3 $\pm$ 4.5	6.2–9.5

Abbreviations: HLM, human liver microsomes; MDMA, 3,4-methylenedioxymethamphetamine.

membranes and/or uptake/efflux transporters, limiting the access of the inhibitors to the CYP enzymes. We also quantified the time-dependent inhibition of CYP3A4/5 by mifepristone, using midazolam 1'-hydroxylase activity as a marker for CYP3A4/5 activity. The inhibition parameters were determined to be $K_I = 23.1 \pm 2.1 \mu M$ and $k_{\text{inact}} = 0.101 \pm 0.003 \text{ min}^{-1}$. We demonstrated that hepatocytes can be used for the determination of reversible and time-dependent inhibition; however, it is difficult to prove that any inhibition observed is a result of metabolic activation. Further work will require a focus on conditions that would optimize a controlled environment for metabolic activation.

In another work reported by Mao *et al.*, cryopreserved human hepatocytes were used to compare the accuracy of enzyme inhibition predictions in a protein-free system combined with the fraction unbound in human plasma for inhibitor(s) with those obtained with protein-containing incubations [70]. Hepatocytes were incubated with or without CYP3A, CYP2C9, or CYP2D6 inhibitors at multiple concentrations, in human plasma or tissue culture media. The data indicated a general underprediction when using free systemic plasma concentrations. Hepatocytes suspended in the plasma proved to be a simple, accurate predictor of drug–drug interactions and superior to the protein-free approach [70].

Over the past 10–15 years, hepatocytes in primary culture have become widely recognized as the “gold standard” for predicting *in vivo* P450 induction in humans. In contrast, progress is just now being gained toward understanding the use of hepatocytes in inhibition/drug interaction studies. It is clear that the use of hepatocytes in these studies is advantageous in that the study of both phase I and phase II processes together with drug efflux and/or uptake and cellular accumulation can be assessed. Further studies should focus on further optimization of the experimental conditions and on understanding how the obtained results can be used in *in vitro*–*in vivo* predictions of drug–drug interactions mediated via P450 inhibition.

13.5 ENZYME INDUCTION

13.5.1 Background

Induction of hepatic drug-metabolizing enzymes can have significant consequences on the PK and toxicity of drugs. Chronic dosing of a compound that is an inducer can increase the expression of drug-metabolizing enzymes and transporters, which often leads to a concomitant increase in the activity of these clearance pathways. Consequently, any coadministered drug that utilizes these same clearance pathways will be more rapidly eliminated, resulting in lower drug concentrations and may become less effective. For example, rifampin can decrease the *in vivo* AUC of coadministered drugs by 70–90% (e.g., when coadministered with (*R*)-verapamil [71]), thereby significantly decreasing efficacy. Induction of P450s has been shown to result in toxicity by increasing reactive metabolite production or the CYP1A-mediated bioactivation of procarcinogens [72]. Induction effects are further complicated, as foods (e.g., grapefruit juice), cultural and lifestyle-related agents (e.g., alcohol and nicotine), and herbal supplements (e.g., St. John's Wort) can also induce enzyme activities. Chronic exposure to inducing agents has been shown to disrupt cellular homeostasis, leading to endocrine disruption, altered inflammatory response, and bone density loss [73–76]. Potent inducers such as rifampin and St John's Wort simultaneously upregulate multiple genes including CYP3A4, CYP2B6, UDP-glucuronosyltransferase (UGTs), and

glutathione-S-transferase (GSTs), as well as multiple transporters such as MDR1 (multidrug-resistant protein 1) and organic anion-transporting polypeptides (OATPs).

In most cases, the inducer directly or indirectly activates a cytosolic receptor complex causing it to translocate to the nucleus of the cell where the complex is converted to an active transcription factor, leading to increased transcription and translation of target genes that possess the corresponding DNA binding sequences in their promoter regions [77]. Although increased levels of these enzyme systems can occur by the stabilization of mRNA and/or proteins (e.g., induction of CYP2E1) [78,79], inductive events that lead to clinically significant changes in the clearance or bioactivation of drugs predominantly occur by a receptor-mediated process that causes increased production of an enzyme or other protein initiated at the level of gene transcription. These clinical effects can be predicted qualitatively *in vitro* using primary hepatocytes in sandwich culture.

In hepatocytes, the principle nuclear receptors, the aryl hydrocarbon receptor (AhR), the pregnane X receptor (PXR), and the constitutive androstane receptor (CAR) control the expression of key P450 enzymes involved in drug metabolism including CYP1A (AhR), CYP2B, CYP3A, and CYP2C subfamilies (PXR and CAR); the Phase II enzymes such as UGTs, glutathione-S-transferases, sulfotransferases; and transporters including MDR1, MRP2 (multidrug-resistance-associated protein 2), OATPs, and organic cation transporters (OCTs). Under appropriate culture conditions, primary hepatocytes retain relevant levels of the major drug-metabolizing enzymes, transporter proteins, nuclear receptors, and transcription factors, while thus far no immortalized or transformed hepatic cell line (e.g., HepG2, Fa2N4, or HepaRG) has been reported to contain the complete complement of enzymes, transporter proteins, and all corresponding receptor signaling cofactors. As such, primary hepatocytes in a sandwich configuration remain the model of choice for *in vitro* induction studies [14].

13.5.2 Experimental approaches

As stated above, under appropriate conditions, primary human hepatocytes express the biochemical and molecular machinery necessary to respond to enzyme inducers much as they would *in vivo* [80–82]. Currently, the most relevant culture model for induction is the sandwich culture system in which freshly isolated or cryopreserved hepatocytes are seeded on collagen, type I and allowed to attach and sandwich with an extracellular matrix overlay, typically hydrogels of collagen or Matrigel® [14,24,83]. This format provides a more “natural” environment for the hepatocytes, which retain liver-like cell morphology and form functional bile canaliculi [24,84,85]. In this culture configuration, the cumulative effects of metabolism, induction, time-dependent inhibition, and metabolite effects replicate the more steady-state-like effects on P450 activity and gene expression [86].

Tissue culture media also influences the morphology, integrity, and cellular function of hepatocytes. Our data indicate that Williams E Medium supports a higher basal CYP3A expression relative to the Dublecco’s modified Eagles medium/nutrient mixture Ham’s F-12 (DMEM/Ham’s F-12) and modified Chee’s media and, along with hepatocyte plating density, supports overall cell health and functionality [5]. Adult hepatocytes work best when cultured close to confluence ($\geq 90\%$) at the time of plating, as the cells redevelop normal epithelial morphology, junctional complexes, and bile canaliculi. When underplated at less than 50% confluence, hepatocytes

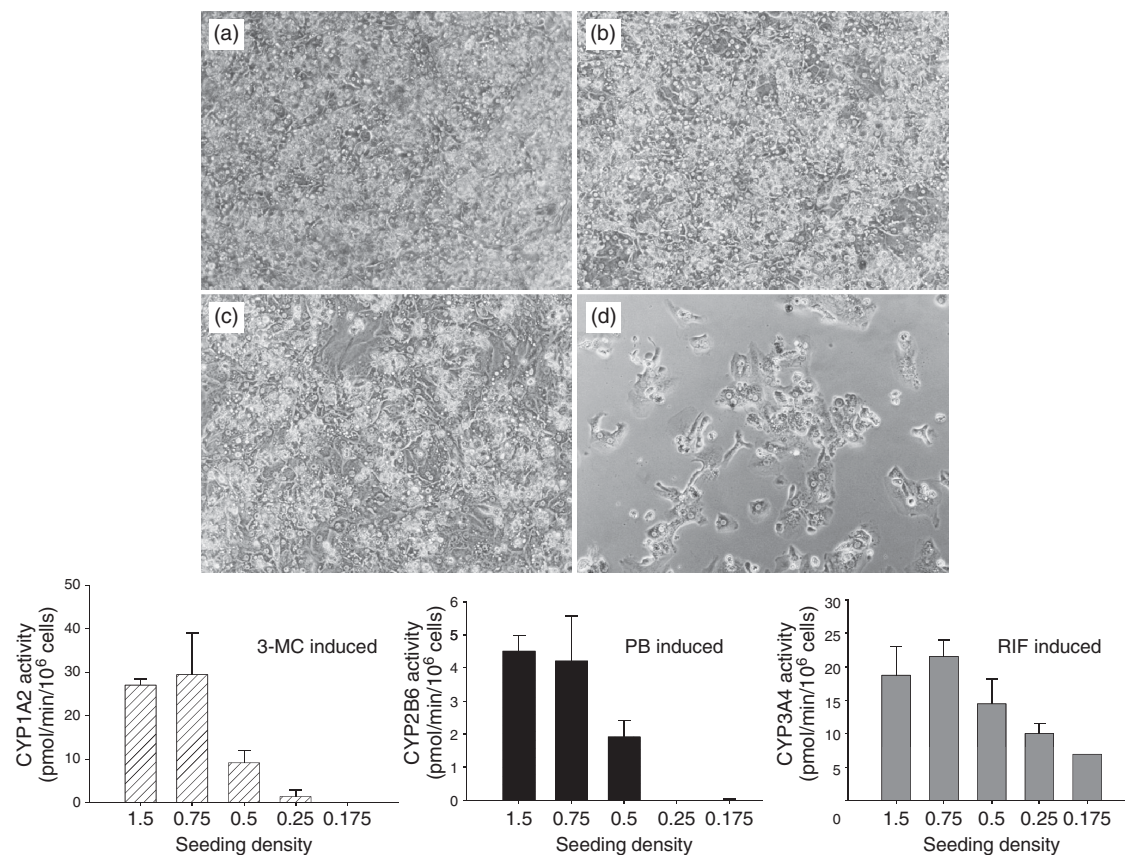


Figure 13.2 Effect of plating density on monolayer integrity and P450 enzyme activity in primary cultures of cryopreserved human hepatocytes. Photomicrographs of cryopreserved human hepatocytes at (a) 150%, (b) 100%, (c) 70%, and (d) 25% confluence. Bar graphs depict representative results of a single induction experiment with primary human hepatocytes cultured at five different seeding densities ($\times 10^5$ cells per well in a 24-well plate; $n = 3$). For the activity assays, hepatocytes were cultured for two days and then treated for three days with the prototypical inducers at the concentrations indicated in Table 13.3.

appear fibroblast-like after 24 h in culture and send out filopodial extensions. These hepatocytes have significantly lower CYP3A4 activity and responsiveness to prototype inducers than those plated at optimal confluence, although PXR levels remain equivalent (Fig. 13.2) [5]. Similar results have been obtained using freshly isolated and cryopreserved hepatocytes from the same donors. CYP1A1/2, CYP2B6, and CYP3A4 activities decreased with lower cell density, even though data was normalized for number of cells.

Fetal bovine serum enhances attachment and is only used for the initial attachment period of 4–6 h, as components in the serum can stimulate cell proliferation and loss of phenotypic expression [12]. Additives such as glucocorticoids (e.g., dexamethasone and hydrocortisone), insulin, selenium, and transferrin aid in the preservation of hepatocyte-specific morphology and function [12]. In most cases, albumin is included in media supplements at a final concentration of ~1 mg/mL, which affects both lipid and drug dispositions. After an initial recovery period in culture (24–48 h) during which hepatocytes are generally refractory to treatment with inducers, primary hepatocytes can be used for induction experiments and are typically incubated for two days with the test compounds when mRNA is the end point and for three days when enzyme activity is to be assessed. Cryopreserved hepatocytes can also be used for induction studies. For reasons not yet fully understood, a percentage of human hepatocyte preparations lose the ability to attach in culture after cryopreservation and thus cannot be used for induction studies. However, the hepatocytes that do attach efficiently and form a confluent monolayer retain the ability to induce, similar to fresh hepatocytes.

For induction assays, it is important to use healthy hepatocytes, appropriate culture conditions (medium and matrix), and positive control inducers at relevant concentrations that maximize inductive response while minimizing potential cytotoxicity, effective vehicle controls, appropriate time in culture, and the right complement of end points to allow effective interpretation of data. Table 13.5 provides a list of recommended positive control inducers based on our practical experience and understanding of various nuclear receptor pathways and their role in P450 transcription. A list of typical probe substrates for each relevant P450 isoform and their working concentrations are also provided.

As mentioned above, one of the most important considerations for achieving optimal outcomes from an *in vitro* induction study is the health and integrity of the hepatocyte monolayer. For example, often a very large fold change in mRNA or enzyme activity (e.g., >100-fold increase or >20- to 30-fold increase in CYP3A mRNA or activity, respectively) is not physiologically relevant to human liver *in vivo* and likely reflects an artificial response because of poor cell health or inappropriate culture conditions, which can result in diminished basal CYP3A expression and other liver-specific functions and regulatory pathways [5]. Conducting studies with compromised cultures with suppressed functionality may lead to misleading results, especially where metabolites of a new drug are the active species or toxic agents. Ideally, monolayers of primary hepatocytes, whether freshly isolated or cryopreserved, should be >80% confluent, show little or no sign of morphological deterioration (Fig. 13.3) over the study period (especially under control conditions), and exhibit at least 2.5-, 5-, and 10-fold increases in enzymatic activity over vehicle control for CYP3A4, CYP2B6, and CYP1A2, respectively, when treated with prototypical inducers [e.g., rifampicin, phenobarbital, and 3-methylcholanthrene (3-MC)]. Notably, induction of CYP3A4 enzyme activity greater than 10-fold for CYP3A4 is unusual and should serve as a red flag,

TABLE 13.5 Nuclear Receptors and the Corresponding Enzymes with the Positive Control Inducers and Substrates Typically Used for *In Vitro* Induction Studies with Human Hepatocytes

Receptor	Enzyme	Inducers (μM)	Substrate (μM)	Incubation (min)	Marker Metabolite
AhR	CYP1A1/2	3-Methylcholanthrene (2) Omeprazole (50) β -Naphthoflavone (25)	Phenacetin	15	Acetaminophen
CAR	CYP2B6	Phenobarbital (1000) CITCO (1) Phenytoin (50)	Brupropion	20	Hydroxybrupion
PXR	CYP3A4/5	Rifampin (10)	Testosterone	14	6 β -Hydroxytestosterone

Abbreviation: CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl.

as this is not generally observed *in vivo* and could be due to artificially low basal activities that have resulted from poor cell health.

In addition to cell health, other considerations, such as the choice of vehicle control, positive control inducers, matrix and medium conditions (discussed above), and time of incubation, are also important for optimal results. The choice of vehicle control is often dictated by the solubility of the NCE being assessed for induction potential. Common solvents include dimethylsulfoxide (DMSO), methanol, and acetonitrile for most drugs, or simple isotonic buffers (e.g., phosphate buffered saline (PBS) and Hanks buffered salt solution (HBSS)) and cell culture medium for water-soluble compounds. Care should be taken in choosing the concentration of solvent vehicles, as they can contribute to an elevated baseline P450 expression and reduce the dynamic range of induction, as well as cause overt cytotoxicity at higher concentrations [5,80].

13.5.3 Experimental End Points

Induction of P450 enzyme expression can be assessed by evaluating mRNA, protein, and enzymatic activity. While the FDA 2006 draft guidance recommends evaluating activities of CYP1A1/2 and CYP3A4, a more recent commentary by the FDA recommends the addition of CYP2B6 activity to the screening regimen to account for activators of CAR [87]. The current FDA 2012 draft guidance recommends evaluating mRNA levels of all three enzymes, followed by CYP2C9 in cases where CYP3A4 induction is observed [2]. The newer guidance agrees with Fahmi *et al.* [88] that mRNA evaluation provides more appropriate *in vitro*–*in vivo* correlations, as the confounding effects of CYP inhibition, especially time-dependent inhibition, are not an issue. When there is no change, or more than a 40% increase in enzyme activity and mRNA expression relative to positive control inducer, the results are easy to interpret. Figure 13.4 depicts typical results with known inducers of CYP3A4, namely, rifampin, phenytoin, dexamethasone, and phenobarbital. In Fig. 13.4a, the results of an induction experiment where all compounds were treated at a final concentration of 10 μM are

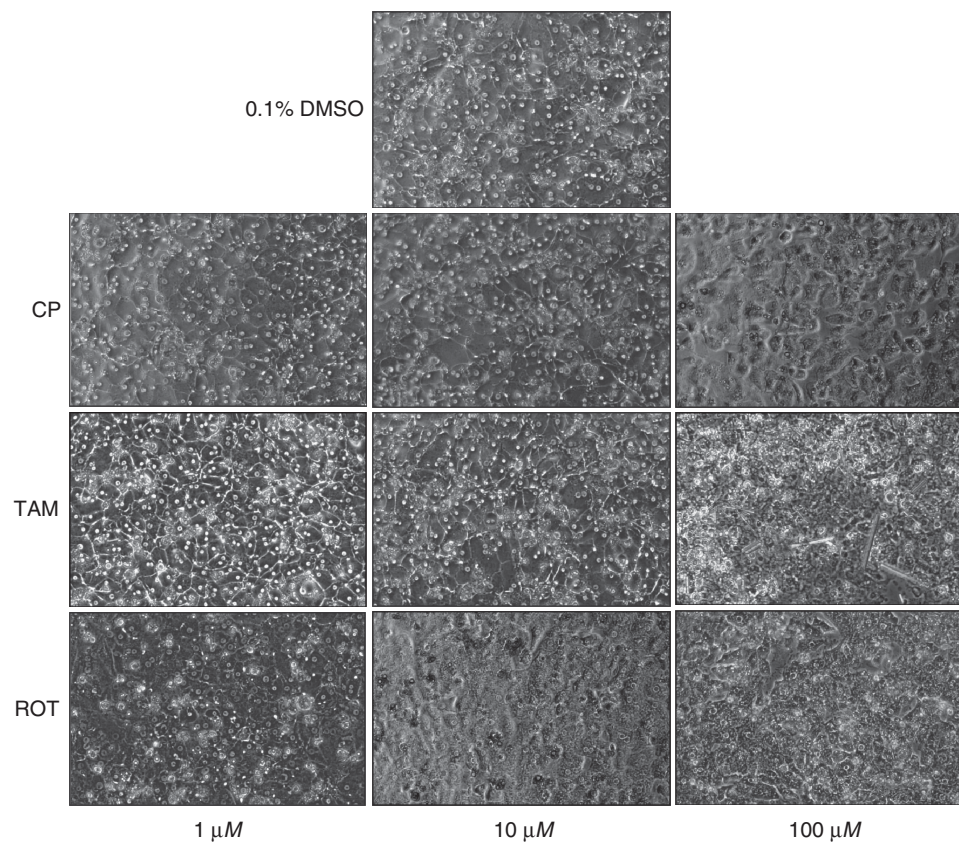


Figure 13.3 Photomicrographs of hepatocytes following drug treatment. Cryopreserved human hepatocytes were treated with vehicle control (0.1% DMSO) or known toxicants such as chlorpromazine (CP), tamoxifen (TAM), or rotenone (ROT) at different concentrations (1, 10, or 100 μM) for 48 h and then assessed for changes in cell morphology using light microscopy.

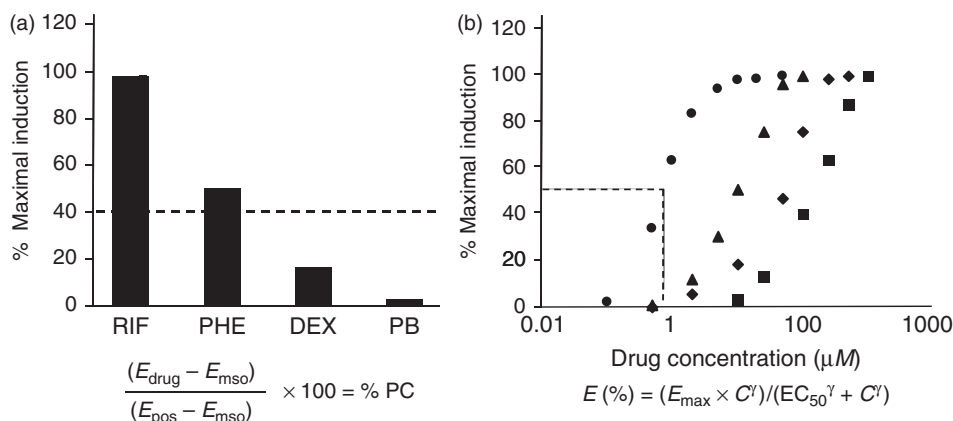


Figure 13.4 Representative induction responses for CYP3A4 by prototypical inducers in human hepatocyte cultures. Hepatocytes were maintained for two days in culture without treatment followed by three consecutive days of treatment with (a) a single concentration (10 μM) or (b) multiple concentrations of rifampin (RIF), dexamethasone (DEX), phenytoin (PHN), or phenobarbital (PB). Hepatocytes from each treatment group were then harvested and microsomal CYP3A4 activity was determined using testosterone as substrate [71].

represented as a percentage of the positive control, which in this case was rifampin. At a single concentration, the compounds can be rank ordered according to their potency as an inducer and those causing an effect at or above 40% relative to the positive control would be designated for further evaluation.

However, it is important to note that all these agents cause significant induction of liver enzymes *in vivo* and that not all of them were tested at clinically relevant concentrations. As such, when tested over a broader range of concentrations in order to derive the entire concentration–response profile, it is clear that all the agents have the potential to cause significant induction of CYP3A4 (Fig. 13.4b). This underscores the importance of choosing the appropriate number and range of concentrations that are clinically relevant when conducting an *in vitro* study. The 2006 FDA guidance on drug–drug interactions recommended conducting activity assays and testing at concentrations equivalent to and an order of magnitude greater than the predicted or observed maximum plasma concentrations (C_{max}) of a NCE. The 2012 guidance recommends using multiple test compound concentrations and mRNA analysis as an end point to avoid the confounding effects of concomitant inhibition. Bear in mind that protein in the medium (e.g., albumin) can impact the free fraction of a drug and, therefore, using “total” drug concentrations often serve as a better guide when determining the relevant potency of an induction effect.

Determining the induction potential of test drugs is more complicated in cases where a decrease in enzyme activity is observed, which may or may not be accompanied by a drop in mRNA expression of the corresponding enzyme. There are three possible explanations for a decrease in enzyme activity after chronic treatment: (i) enzyme inhibition (as mentioned above), (ii) gene suppression, or (iii) cytotoxicity [89]. In the first case, enzyme inhibition can mask the induction response if a compound is both an inhibitor and an inducer of the same enzyme system(s). For example, the endothelin A receptor

antagonist CI-1034 does not cause significant induction of CYP3A4 activity in primary human hepatocyte cultures after three days of exposure. However, immunoblots and real-time polymerase chain reaction (PCR) analysis reveal significant increases in CYP3A4 mRNA and protein expression. CI-1034 was subsequently found to be a time-dependent (mechanism-based) inhibitor of CYP3A4. Notably, CI-1034 PK were linear in the clinical trials, even though this compound was metabolized predominantly by CYP3A4 [85]. The second cause of drug-induced decreases in P450 activity is when suppression of P450 gene expression occurs at the transcriptional level. A classical example of this effect occurred when children with a fever experienced central nervous system (CNS) toxicity after theophylline administration [90]. It has since been shown that proinflammatory cytokines downregulate many hepatic P450 genes, which appears to be mediated through the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway [91]. It is worth noting that the clinical manifestation of enzyme suppression can be similar to that of enzyme inhibition. As in the case described above, the cytokine-induced suppression of CYP1A2 activity in the infected children caused a decrease in theophylline clearance, which in turn led to increased blood levels of the drug and subsequent CNS toxicity.

The induction of P450s by prototypical inducers can also be attenuated at the transcriptional level, as is the case with CYP1A1 induction by tetrachloro-*p*-dibenzodioxin (TCDD) in HepG2 cells in the presence of microtubule-disrupting agents. The marked induction of CYP1A1 gene expression that normally occurs is blocked by colchicine and nocodazole because these agents disrupt microtubule-mediated signaling pathways and arrest the G2/M cell cycle [92]. Similarly, phenobarbital-induced CYP2B1 expression in primary rat hepatocytes is attenuated by docosahexaenoic acid. This effect occurs at the level of the nuclear receptor, as CAR accumulation in the nuclear fraction is decreased with a concomitant increase in the cytosolic fraction [93]. An earlier study determined that attenuation of CAR translocation and decreased subsequent binding to response elements in the *CYP2B1* promoter are involved in this chemical-induced down-regulation [94]. In the final case of drug-induced reduction in P450 enzymatic activity, the decreased enzyme activity can be caused by the onset of cytotoxicity. A number of compounds that are known to be potent P450 enzyme inducers produce “bell-shaped” concentration–response curves because of the fact that they begin to cause cytotoxicity at higher concentrations. For instance, both rifampicin and troglitazone exhibit this behavior when administered over prolonged periods to primary hepatocytes at concentrations of more than 50 μ M [82]. Accordingly, it is crucial to routinely conduct morphological assessments and, when a loss in enzyme activity is observed, to assess cytotoxicity in the monolayers using standard approaches such as cellular ATP content or lactate dehydrogenase (LDH) leakage.

When evaluating induction of P450 enzymes by a compound that is also a mechanism-based inhibitor, there may be no significant change in the activity of an individual isoform because of offsetting inhibition and induction, even though the compound is a nuclear receptor activator. In these cases, it is helpful to also evaluate the mRNA or protein concentrations to aid in the interpretation of results. P450 mRNA levels will not be affected by classical inhibition mechanisms that affect enzyme activity; however, they will decrease because of cytotoxicity or gene suppression. In most of these cases, the clinical end point is very difficult to predict. An example is the endothelin A receptor antagonist CI-1034, a compound metabolized predominantly by CYP3A4 that is also a metabolism-dependent inhibitor of this

enzyme. Analysis of enzyme activity in primary cultures after three days of treatment revealed no change in activity, while Western immunoblots revealed an increase in CYP3A4 immunoreactive protein and real-time PCR analysis demonstrated increases in CYP3A4 mRNA similar to that induced by rifampicin [85]. Interestingly, clinical data revealed linear PK, perhaps indicating that neither the inhibition nor the induction was significant *in vivo* or possibly, these processes negated each other. While this experimental end point could not be predicted *in vitro*, it is helpful to know the inhibition potential of a compound before conducting *in vivo* induction studies, so that appropriate study designs can be planned.

On the basis of our own experience and the reports of others, we recommend that an *in vitro* induction study minimally include (i) CYP1A, CYP2B6, and CYP3A enzymatic activity measurements; (ii) corresponding mRNA or protein determinations; (iii) a cytotoxicity end point (e.g., ATP content); and (iv) microscopic evaluation to monitor cell and monolayer integrity before and at the termination of an experiment.

13.5.4 *In Vitro/In Vivo* Correlations

It is now routine to consider a response of $\geq 40\%$ of the positive control activity *in vitro* as an indication of potential *in vivo* induction [95]. Many inducers produce a sigmoidal concentration–response profile (Fig. 13.4b) that is characteristic for a particular compound. The relevant characteristics to assess induction potential are the potency of the response (EC_{50} , effective concentration at half-maximal response), the efficacy (E_{max} , maximum effect or response), the no observable effect level (NOEL) or the highest no-effect concentration, and the relative shape of the concentration–response curve [96].

Similar to predictions of enzyme inhibition, a C_{max}/EC_{50} ratio of >1 indicates a likely inducer *in vivo* and a C_{max}/EC_{50} ratio of $0.1–1$ indicates possible induction, while a C_{max}/EC_{50} ratio of <0.1 indicates low potential for induction *in vivo*. Figure 13.5 demonstrates a simulated study with three compounds for which a $C_{max} < 1 \mu M$ would predict no induction potential, while a C_{max} of $8 \mu M$ would indicate significant induction for drug X and moderate induction for drugs Y and Z. Table 13.6 depicts the utility of using EC_{50} values for CYP3A4 induction and how the C_{max}/EC_{50} ratio relates to the predicted CYP3A4 induction potential. For example, while troglitazone and rosiglitazone have similar EC_{50} values for CYP3A4 induction, troglitazone is a potent clinical inducer [82,97,98] while rosiglitazone has no significant clinical induction effects [99]. Because of their differential selectivity for the therapeutic target (i.e., peroxisome proliferator-activated receptor (PPAR)), rosiglitazone is prescribed at a lower dose and the C_{max} is correspondingly lower and significantly below its EC_{50} value for PXR activation. As such, it can be important to understand and compare the selectivity and the therapeutic index between the on- and off-target effects of drug candidates. The lowest concentration at which the induction response occurs (NOEL) should also be considered [100].

According to the latest FDA guidance document on drug–drug interactions [2], the decision to conduct an *in vivo* drug–drug interaction study for an investigational drug as an enzyme inducer should be based on quantitative analysis of both *in vitro* and clinical PK data. Such analysis can be performed by a variety of algorithms and models including basic models, mechanistic static models, and more comprehensive dynamic models, for example, physiologically based pharmacokinetic (PBPK) models.

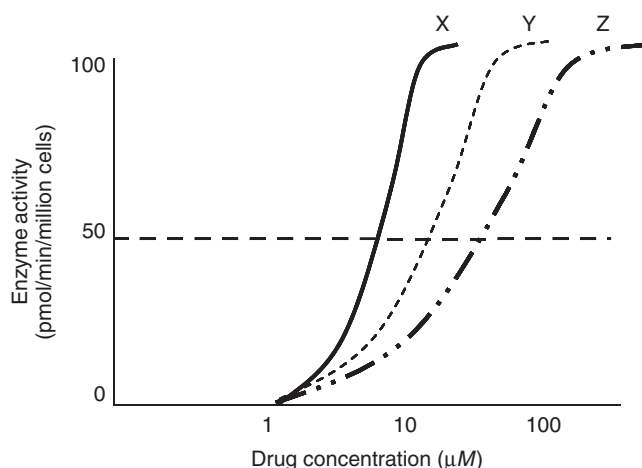


Figure 13.5 Simulated induction profiles of three hypothetical compounds (X, Y, and Z). Three distinct induction profiles were simulated to illustrate potential compound differences in potency (EC_{50}) while exhibiting similarities in efficacy (E_{max}) and NOEL.

TABLE 13.6 Relationship between *In Vitro* Potency and the Prediction of *In Vivo* Induction using the EC_{50} and Therapeutic Plasma Concentrations^a

Compound	EC_{50} (μM)	C_{max}/C_{ss} (μM)	$[I]/EC_{50}$	Induction Potential
Rifampin	0.8	14	17.5	Likely
Carbamazepine	0.8	25	28	Likely
Phenobarbital	125	40–180	0.3–1.5	Likely
Phenytoin	25	80	3.2	Likely
Troglitazone	3–5	7	2.3	Likely
Avasimibe	0.2	1–6	5–30	Likely
Rosiglitazone	5–10	0.3–1.2	0.06–0.12	Possible
Simvastatin	0.14	0.024	0.17	Possible
Lovastatin	1–5	0.008	0.008–0.02	Unlikely
Clotrimazole	1–5	topical	(inhibition)	Unlikely
Nifedipine	8	0.008	0.001	Unlikely

^aRef. 101.

Basic models have been predominantly used because they are simple and practical. These models are conservative, but in some cases, they eliminate the need for later clinical investigations of drug–drug interaction potential. For example, the cutoff value to decide whether further *in vivo* investigation of a drug as an inducer is needed is generally calculated based on the fold induction over a negative or vehicle control (e.g., fourfold or higher) or the percentage of induction relative to an appropriate positive control (e.g., $\geq 40\%$ of a positive control) [88,101].

As suggested before, comparison of induction response curves is more complex than simply determining any one of these parameters alone [101]. Whatever method is used to calculate the potential induction risk *in vivo*, once a compound is found to be positive in an *in vitro* hepatocyte induction assay, drug interaction clinical trials are generally warranted, but it is rare that these results would stop the development

of a compound. However, attrition sometimes occurs if the compound showed potent induction effects, especially if these were expected and interfered with cotherapies. Often multiple options are pursued when considering the implications of a positive *in vitro* induction response. Most often extra drug–drug interaction (DDI) studies are requested in the clinical trials (recommended by the FDA) or other PK properties of the compound are assessed to determine the overall induction potential. For example, some of the other properties considered were protein binding, clearance, and bioavailability, all of which influence the induction response *in vivo*. Of course, another option which is often pursued is that the induction data is provided back to the discovery chemists in an attempt to minimize or eliminate the inductive properties of new molecules.

13.6 HEPATOBIILIARY TRANSPORT

13.6.1 Background

Transporters in the hepatocyte basolateral and apical membrane are responsible for carrier-mediated processes involved in the uptake, biliary secretion, and systemic reabsorption of xenobiotics, their metabolites, and endogenous compounds. Hepatic clearance is a function of transporter and drug-metabolizing-enzyme-mediated processes. Hepatic clearance starts with transport into hepatocytes (phase 0) by passive diffusion through the cell membrane or by active transport. In hepatocytes, transport is mediated by basolateral membrane organic anion transporters (OATs) including OAT2 and OAT5; OATP1A2; OATP1B1; OATP1B3; OATPB2B1; the organic cation transporter OCT1; and the sodium taurocholate cotransporting polypeptide (NTCP). All these transporters are retained in the membranes of primary sandwich-cultured hepatocytes (Figure 13.6). Phase III is transporter-mediated elimination of the intact drug and metabolites (if any) from the hepatocytes via ATP-dependent apical or basolateral membrane efflux transporters and in some cases, bidirectional organic anion or cation transporters. Apical membrane hepatobiliary transporters involved in xenobiotic transport include the MDRs such as MDR1, MRP1, MRP2, the breast cancer resistance protein (BCRP), and the bile salt efflux pump bile salt export protein (BSEP) [102]. These efflux transporters are maintained in primary hepatocytes in sandwich culture after two to three days of plating. Drugs that are eliminated unchanged via the bile may undergo enterohepatic circulation and may be reabsorbed from the intestine [103]. The BSEP mediates the efflux of conjugated and unconjugated bile salts into the bile, and inhibition of this transporter by a drug could result in hepatotoxicity [104]. Multiple transporters mediate the excretion of compounds from hepatocytes back into systemic circulation via basolateral membrane efflux transporters (phase III). As shown in Fig. 13.6, the outwardly directed efflux transporters include the ATP-binding cassette (ABC) proteins MRP1 and MRP3. Thus, multiple membrane transporters in hepatocytes work in concert with the drug-metabolizing enzymes to mediate hepatic drug clearance. These transporters play a key role in drug clearance and may contribute to drug–drug interactions.

13.6.2 Transporter-mediated drug interactions

Similar to the drug-metabolizing enzymes, drug–drug interaction involving hepatic membrane transporters can occur by competition for the same substrate-binding site of

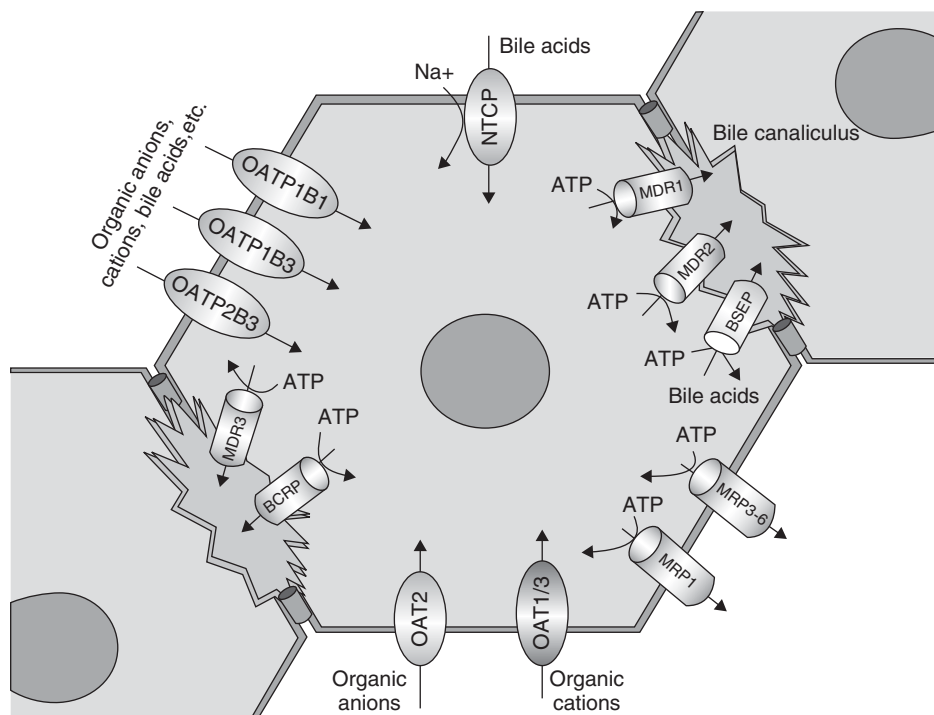


Figure 13.6 Schematic of uptake and efflux xenobiotic transporters in human hepatocytes.

the transporter, or tight or allosteric binding leading to inhibition of transporter activity, or by change in expression level of transporters. This has the potential to alter the blood concentration–time profiles of drugs, leading to elevated levels of a coadministered compound. Evaluating the substrate potential of a drug candidate for hepatic transporters *in vitro* is beneficial when these organs are the drug target. For example, the hepatitis C drugs α -interferon and *S*-acyl-2-thioethyl esters or the HMGCoA inhibitors must achieve adequate concentrations in the liver for pharmacological activity. Evaluating potential substrates of efflux transporters also aids in predictions of drug clearance when compounds are predominantly eliminated into the bile, for example, atorvastatin. Evaluating drug interactions with transporters *in vitro* during drug discovery can lead to improved predictions of clinical drug–drug interactions. Hepatic transporters are now implicated in adverse interactions that were earlier attributed to the drug-metabolizing enzymes. *In vitro* studies with mibefradil revealed CYP3A4 inhibition potential, but the clinical outcome was significantly larger than what was predicted from the *in vitro* studies [105]. A few years later, mibefradil was found to inhibit P-gp as well [106], and the additive effect of this interaction explained the underprediction of the effect on PK of coadministered therapeutics. Similarly, the effect of grapefruit juice on fexofenadine PK, which was initially attributed to CYP3A4, was later reported to involve the inhibition of OATP-mediated fexofenadine uptake [107]. Digoxin, which has been used as a P-gp substrate in both *in vitro* and *in vivo* studies and is the FDA probe substrate of choice for both preclinical and clinical drug interactions studies, is now

reported to be a substrate for hepatic OATP transporters as well, and there are ongoing discussions on the appropriateness of this compound as a P-gp substrate. Other examples of adverse drug interactions attributed to hepatic transport proteins involve the statins, as coadministration of gemfibrozil with pravastatin, simvastatin, lovastatin, and cerivastatin significantly increases statin AUC and $C_{\max}$ [108,109]. Inhibition of the basolateral membrane hepatic uptake transporter OATP1B1 by gemfibrozil contributes to these drug interactions [110,111]. While the above clinical transporter-mediated drug interactions were unexpected, with current strategies, *in vitro* transporter assays can be used to identify the transport proteins involved in drug disposition, thereby aiding drug interaction predictions, so that fewer adverse events occur in the clinic. Some of these methodologies are discussed in the following section.

In addition to drug–drug interactions, hepatic transporters also play a role in hepatotoxicity. Drug-induced hepatotoxicity is a major problem in drug development and there is growing evidence that inhibition of bile acid transporters is a contributing mechanism of liver toxicity [112]. Multiple apical and basolateral membrane hepatic transporters work in concert to transport bile acids and xenobiotics from blood to bile via the hepatocytes, and inhibition of any of these processes could result in adverse events. Hepatotoxicity is a significant cause of compound attrition. Several drugs (bosentan, troleandomycin, troglitazone, glyburide, tacrine, and erythromycin) have exhibited no or minimal hepatotoxicity in preclinical *in vivo* experiments and resulted in significant increases in liver function enzymes in clinical trials, or in the market when exposed to a wider patient population. Drug-mediated inhibition of active canalicular transport of biliary components is a contributing cause of this hepatotoxicity [104,113]. Inhibition of the apical membrane bile transporter BSEP has been shown to result in cholestasis and hyperbilirubinemia [84,114], and there is evidence that inhibition of OATP1B1 can result in hyperbilirubinemia [115]. MRP4 may play an important role in minimizing hepatotoxicity, as this transporter is upregulated when BSEP is downregulated [116].

In many cases, drugs that cause cholestasis are preferentially eliminated (>50%) via the biliary pathway, and this hepatotoxicity is likely associated with drug-mediated inhibition of active canalicular transport of bile components [113,114]. Since the transporters that participate in biliary drug elimination also transport endogenous bile components, the potential for inhibition of bile acid and drug efflux from the liver could result in an increase in drug and bile acids retained in the liver over time. Many of these drugs have now been found to be substrates for active liver-transporter-mediated uptake and efflux into the bile canaliculi using primary hepatocytes [84,112,117]. Table 13.7 lists some of the major hepatic transporters involved with hepatotoxicity and drug–drug interactions. In an earlier study using six macrolide antibiotics, we showed that drugs with greater hepatotoxic risk are stronger inhibitors of taurocholate transport in cultured human hepatocytes [84]. In addition to primary hepatocytes, hepatocytes in suspension can also be used to evaluate hepatic transport.

13.6.3 Experimental approaches

13.6.3.1 Hepatic Couplets. The hepatocyte couplets are consist of two hepatocytes with a distinct bile canalicular space between these. This model allows for the evaluation of drug uptake and biliary elimination. The advantage of the couplets is that these are polarized and have a sinusoidal membrane interacting with the media/buffer and an apical bile canaliculus, which is the sealed lumen between the two adjacent

TABLE 13.7 Major Human Hepatic Transport Proteins and Known Substrates and Inhibitors

Transporter	Gene Name	Substrates	Inhibitors
NTCP	SLC10A1	Taurocholate, glycocholate, taurochenodeoxycholate and tauroursodeoxycholate, estrone-3-sulfate, BSP	Rifampin, rifamycin SV, glibenclamide, cyclosporin A, indomethacin, ritonavir, saquinavir, efavirenz, troglitazone
OATP1B1	SLCO1B1	Bile acid, BSP, DHEAS, DPDPE, E217 β G, estrone-3-sulfate, ouabain, bilirubin, bilirubin glucuronides, pravastatin, rosuvastatin, atorvastatin, rifampin, olmesartan, bosentan, fexofenadine, methotrexate	Estrone-3-sulfate, gemfibrozil, methotrexate, ketoconazole, cyclosporin A, rifampin, rifamycin SV, clarithromycin, indinavir, ritonavir, CI-1034
OATP1B3	SLCO1B3	bile acids, BSP, DHEAS, digoxin, DPDPE, E217 β G, estrone-3-sulfate, ouabain, bilirubin, rifampin, pravastatin, rosuvastatin, pitavastatin, telmisartan, fexofenadine, olmesartan, bosentan, nafcillin, β -lactam antibiotics	cyclosporin A, rifampin, ketoconazole, clarithromycin, ritonavir, verapamil, T-3095, T-3157, CI-1034
OATP2B1	SLCO2B1	Benzylpenicillin, BSP, DHEAS, estrone 3-sulfate, rosuvastatin, atorvastatin	Cyclosporin A, estrone-3-sulfate, methotrexate, CI-1034
BSEP	ABCB11	Taurocholate, glycocholate, taurochenodeoxycholate, tauroursodeoxycholate and pravastatin	Bosentan, glyburide, troglitazone, rifampin, rifamycin SV, glibenclamide, cyclosporin A, ritonavir, saquinavir, efavirenz, rosiglitazone

Refs 84,85,87,102,114, and 118–123.

hepatocytes [124]. The application of couplets is limited because the substrate must contain a fluorescent chromophore to be quantitated. While the data obtained from these studies is valuable, isolating and working with hepatic couplets requires skill and is very low throughput, further accounting for the limited transporter studies with this model over the past decade.

13.6.3.2 Primary Hepatocytes. Hepatocytes in suspension, attached to tissue culture dishes or in primary sandwich culture are good models of hepatic clearance, uptake, efflux, biliary excretion, transporter-mediated drug interactions, induction, and hepatotoxicity. The contribution of transporter-mediated uptake to hepatic CL was recognized when H_{CL} was consistently underpredicted for many series of chemotypes using just metabolic stability for the calculations. Factoring in transporter-mediated hepatic uptake

along with metabolic clearance using hepatocytes in suspension improved these predictions [125]. From our experience, hepatic uptake via a particular transporter can be effectively assessed with single-transfected cell lines (e.g., HEK-293 cells transfected with OATP1B1 or OATP1B3 or OATP2B1), yet this system is less effective in extrapolating H_{CL} because of the significant differences between transporter types and content in the cell lines and in hepatocytes [126]. Therefore, plated and suspension hepatocytes have become the system of choice for quantitative information on hepatic drug uptake.

13.6.3.3 Suspension Hepatocytes. Hepatocytes in suspension can be used for uptake experiments shortly after isolation from the liver, or for up to 24 h, if cells are kept in the appropriate buffer on ice. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, with minor changes likely due to the absence of significant numbers of other hepatic cell types, for example, Kupffer cells and cholangiocytes [127]. Hepatic uptake studies typically measure the rate of appearance of substrate into cells after a relatively short incubation period, in most cases from 10 s to 3 min. After incubation, hepatocytes are centrifuged in microfuge tubes through an oil layer (that does not allow passage of the buffer and traps lipophilic compound that may be on the outer cell membrane) into a layer of 2 N NaOH or KOH or cesium chloride, for digestion [128]. This bottom layer along with the digested trapped hepatocytes is counted or analyzed by MS. This methodology has provided robust, mechanistic data that can successfully predict the *in vivo* clearance of drugs [39]. Cryopreserved hepatocytes can be successfully used for these studies, and there is evidence to suggest that transporter gene expression in human hepatocytes in suspension is not affected by cryopreservation [127]. In this study, gene expression for primary human hepatocytes after cryopreservation was similar to the liver of origin and included transport, lipid metabolism, phase II metabolism, protein processing, degradation, and recycling. This indicates that both freshly isolated and cryopreserved hepatocytes can be used for transporter-mediated uptake studies.

We have evaluated uptake in multiple lots of cryopreserved human hepatocytes and found up to a 10-fold difference between donors. For example, taurocholate CL was typically between 12 and 48 $\mu\text{L}/\text{min}/\text{million cells}$, digoxin CL varied from 0.6 to 4.5 $\mu\text{L}/\text{min}/\text{million cells}$, and estradiol-17 β -glucuronide (E217 β G) CL varied from 0.5 to 5 $\mu\text{L}/\text{min}/\text{million cells}$ (Fig. 13.7). We also prepared five donor pools of cryopreserved human hepatocytes. This protocol includes rapidly thawing out the hepatocytes, pooling cells from five different preparations, subjecting these to a percoll gradient to remove dead cells and refreezing these in liquid nitrogen as per our standard cryopreservation procedure. Since these cells undergo two freezing steps, we were uncertain how well the membrane transporters continued to function. We studied uptake in this preparation and compared the data to standard cryopreserved cells and also to literature data. As shown in Fig. 13.7, the pooled cells could be used adequately to assess membrane transport of taurocholate, E217 β G, and digoxin.

13.6.3.4 Primary Culture. Hepatocytes cultured on plastic culture dishes or on rigid collagen dedifferentiate and rapidly lose hepatic transport activity and other liver-specific functions. Owing to this, transporter-mediated uptake studies utilizing hepatocytes cultured in this configuration should be conducted as soon as possible, either right after attachment (~ 4 h before plating) or within 24 h of plating.

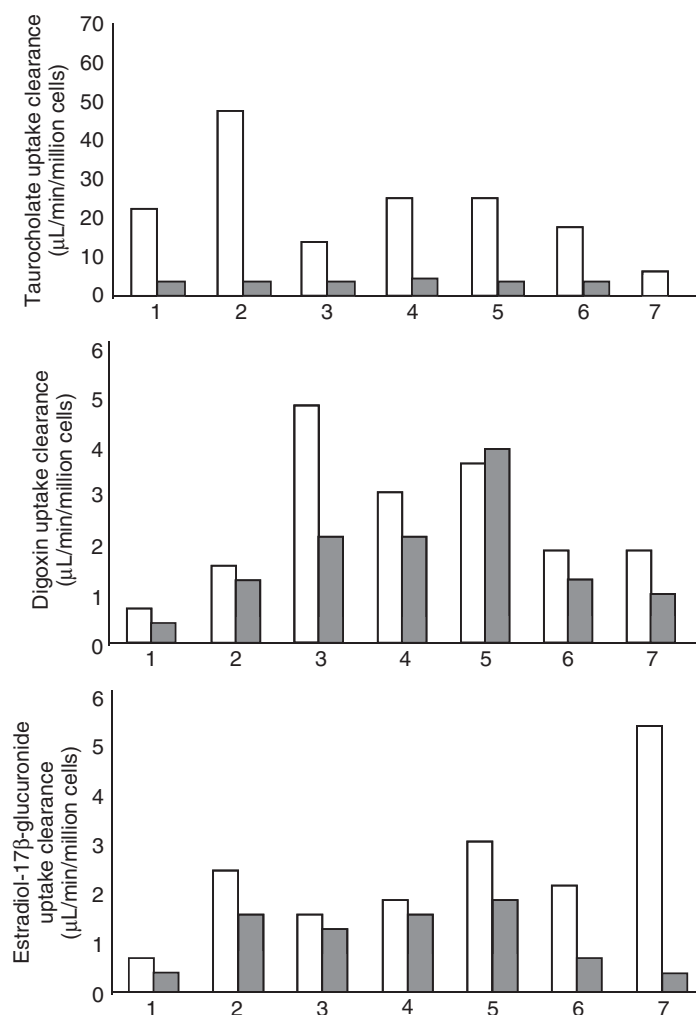


Figure 13.7 Uptake clearance in cryopreserved primary hepatocytes in suspension. For these experiments, either single-cryopreserved (1–5) or pooled cryopreserved (6–7) hepatocytes were used in suspension. Hepatocytes were thawed and resuspended in a buffer, and uptake of the prototypical substrates ($1 \mu\text{M}$ each) of BSEP (taurocholate), OATP transporters (estradiol-17 β -glucuronide), and OATP1B3 (digoxin) is assessed by the oil spin method. Data represent a single experiment for each lot conducted in triplicate.

In the sandwich culture configuration, hepatocytes polarize, retain membrane transporters and drug-metabolizing enzymes (albeit at varying concentrations over time), and establish functional bile canaliculi [24,84]. The bile canaliculi formed in sandwich culture (Fig. 13.8) have morphological and biochemical characteristics similar to those observed *in vivo*, including microvilli in the bile canaliculi, localization of function-specific drug-metabolizing enzymes and relocation of the major uptake and efflux transporter to the appropriate cell membrane [129,130]. Using this model, apical and basolateral membrane transport of a drug and metabolites can be evaluated.

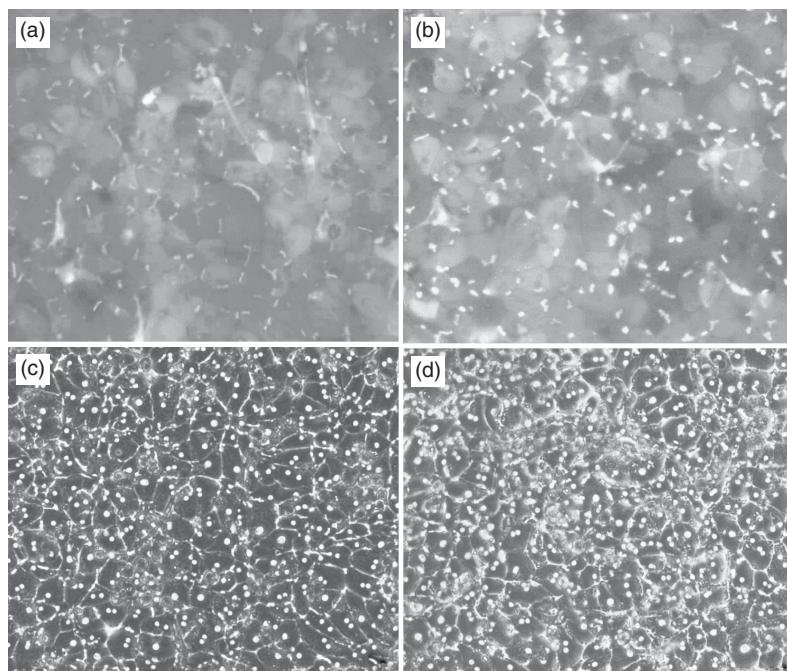


Figure 13.8 Effect of cyclosporine on bile salt transport into hepatic canalicular spaces. Carboxy-2',7'-dichlorofluorescein diacetate (CDFDA) staining in the presence (a and c) and absence (b and d) of $10\ \mu\text{M}$ cyclosporine. (a) and (b) are fluorescence images and (c) and (d) are phase contrast images at $10\times$ magnification. Bile canaliculi are formed in sandwich culture and CDFDA is transported into the hepatocytes and out to the bile via active transport mechanisms. Cyclosporine is an inhibitor of these transporters and prevents the transport of CDFDA into the canalicular spaces.

Sandwich-cultured hepatocytes have been used to evaluate MDR1-, MRP2-, MRP3-, and BSEP-mediated hepatobiliary disposition and hepatotoxic potential of drugs successfully [84,129,130].

13.6.3.5 Cryopreserved Hepatocytes. In sandwich culture, cryopreserved hepatocytes preserve the drug-metabolizing enzymes, nuclear receptors, and major uptake and biliary transporters [131,132] and are used for transporter studies as well. Hepatocytes can also be used for evaluating induction of transporters by drugs, aiding in the prediction of drug disposition and potential drug–drug interactions [133,134]. When cryopreserved and cultured appropriately, hepatocytes in sandwich culture reveal good morphology with distinct bile canaliculi. The function of multiple uptake (OATPs and NTCP) and efflux (MRP2, MRD1, and BSEP) transporters was found to be similar to fresh hepatocytes [135]. We have evaluated biliary excretion in multiple preparations of human hepatocytes and compared these with fresh hepatocytes and demonstrated essentially similar values (Figs. 13.9 and 13.10).

13.6.3.6 Small interference RNA (siRNA/RNAi). At this time, there are no specific inhibitors of hepatic transporters. For this reason, when conducting studies in hepatocytes, the contribution of a specific transporter to the uptake or efflux of a drug

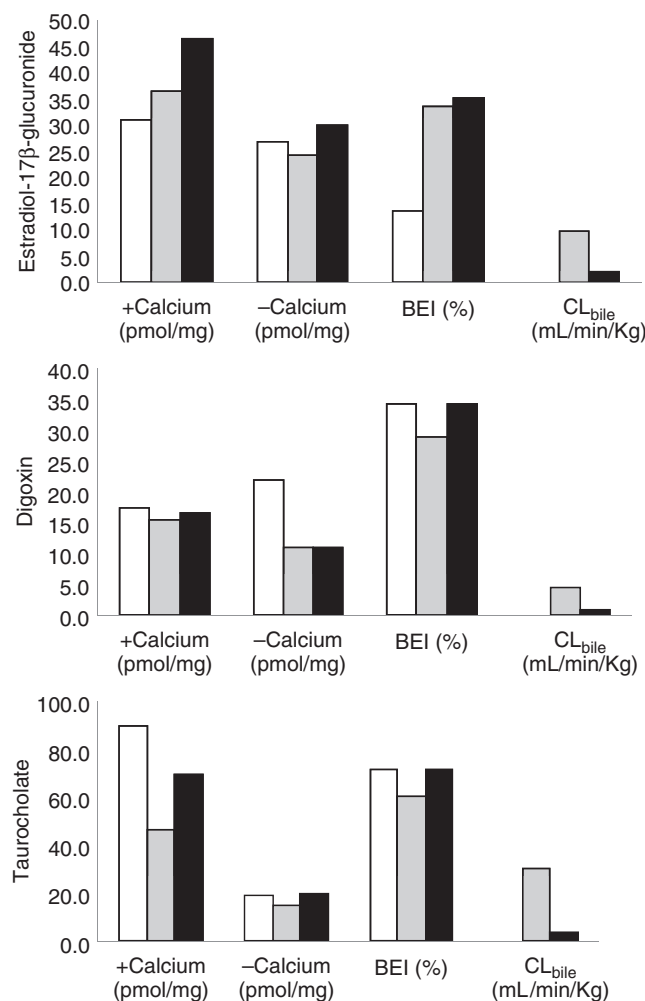
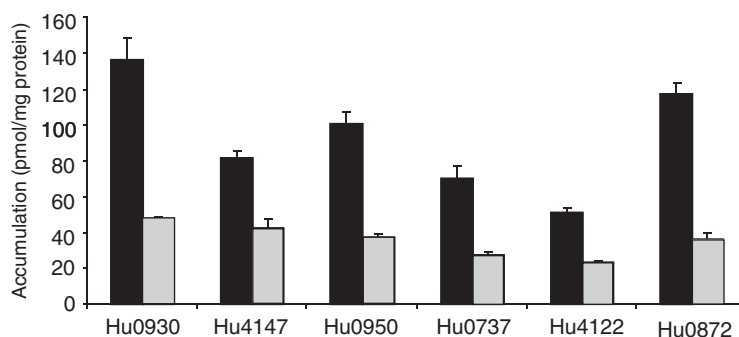


Figure 13.9 Biliary efflux in three preparations of freshly isolated sandwich-cultured human hepatocytes. Human hepatocytes were placed in primary sandwich culture for six days and uptake and biliary efflux of taurocholate, estradiol-17β-glucuronide, and digoxin monitored. Biliary clearance and the biliary clearance index were calculated. BEI, biliary excretion index.

candidate cannot be evaluated using probe substrates or chemical inhibitors. When the contribution of a single transporter needs to be evaluated, small interference RNA (siRNA) could provide the data required. This involves specifically knocking out the mRNA of interest by introducing antisense RNA into hepatocytes, causing degradation of the corresponding complementary mRNA in a gene-dependent manner, followed by loss of the target protein. siRNA has been used successfully to assess transporter activities between wild-type cells and those in which the transporter of interest has been silenced [136,137]. In sandwich-cultured rat hepatocytes, the mrp2 and mrp3 proteins have been specifically knocked down, one at a time using siRNA and resulted in 45–60% decreases in the biliary excretion index of carboxydichlorofluorescein (CDF)



Donors	Accumulation (pmol/mg of protein)				BEI (%)	CL _{bile}
	+ Calcium	SEM	- Calcium	SEM	Mean	Mean
Hu0930	136.5	12.18	47.71	1.2	65.1	69.9
Hu4147	81.7	4.190	42.87	4.900	47.5	30.6
Hu0950	100.9	6.390	37.14	1.800	63.2	50.2
Hu0737	70.2	6.810	27.69	1.820	60.6	33.5
Hu4122	51.5	2.130	23.54	0.450	54.3	22.0
Hu0872	117.3	5.940	36.27	3.390	69.1	63.8

Figure 13.10 Taurocholate accumulation in six preparations of freshly isolated sandwich-cultured human hepatocytes. Human hepatocytes were placed in primary sandwich culture for five to six days and uptake and biliary efflux of taurocholate monitored. Biliary clearance and biliary clearance index were calculated. Filled bars are hepatocytes incubated in a physiological buffer containing the divalent cations Ca^{2+} and Mg^{2+} ; stippled bars are hepatocytes incubated in the absence of Ca^{2+} and Mg^{2+} . BEI, biliary excretion index; SEM, mean standard error.

[136]. In another study, the efflux transporter Bcrp was knocked out using Ad-si01Bcrp in rat hepatocyte monolayers and the contribution of this transporter to the biliary excretion of some drugs were evaluated [137]. We have conducted preliminary experiments with cryopreserved human hepatocytes in our laboratory and have successfully knocked down OATP1B1, OATP1B3, and OATP2B1 mRNA one at a time without affecting the other two transporters (Fig. 13.11).

13.6.4 *In Vitro/In Vivo* Correlations

To predict potential transporter-mediated drug interactions, the degree of inhibition can be calculated as per the method of Shitara *et al.* [138,139]. If the test compound is a substrate for hepatic transporters, *in vitro* drug–drug interaction studies with the NCE and likely coadministered therapeutics, known to be the inhibitors of the specific transporter pathway(s), can be conducted to calculate the degree of inhibition in an appropriate transporter system. If historic or experimentally derived *in vitro* data indicates that the test compound is an inhibitor for one or more hepatic transporters, *in vitro* drug–drug interaction studies with the test compound and likely coadministered therapeutics, known to be substrate of the specific transporter pathway(s), can be conducted. The degree of interaction (R) can be estimated by the following equation.

$$R = 1 + \frac{fu \times I_{in,max}}{K_i}$$

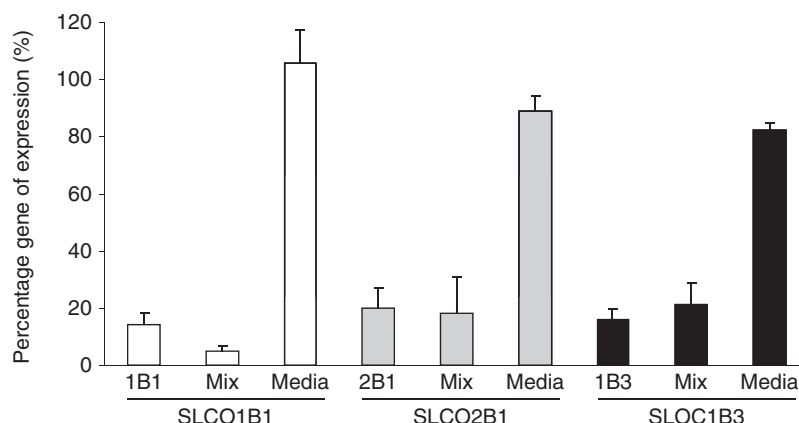


Figure 13.11 siRNA suppression of OATP1B1, OATP2B1, and OATP1B3 in primary human cryopreserved hepatocytes from a single donor. Cryopreserved human hepatocytes were thawed and plated on collagen-coated plates and allowed to attach for 5 h before siRNA transfection. Stealth Select 3 RNAi™ sets for OATP1B1, OATP2B1, and OATP1B3 were used. Lipofectamine RNAiMax™ was complexed with Stealth Select siRNA™, and cells were exposed to siRNA overnight. mRNA was isolated 48-h post transfection and cDNA synthesis and real-time PCR with EXPRESS One-Step Superscript® qRT-PCR Universal. Data is represented as percentage of nontreated cells.

$I_{in,max}$ is the unbound inhibitor concentration (C_{max}) at the entry point of the organ (e.g., in the hepatic portal vein for the liver or the renal artery for the kidney), f_u the blood unbound fraction, and K_i the inhibition constant. In most cases, an IC_{50} value is utilized instead of the K_i . The potential for drug–drug interaction is minimal if the calculated R value is close to one and increases as the value of R decreases. These *in vitro* studies can identify potential inhibitors and/or substrates to use in clinical drug–drug interaction studies. The contribution of the inhibition of the transporter-mediated hepatic uptake of cerivastatin by cyclosporine A was used to explain clinical PK interactions between these drugs. Further studies from this laboratory [140] showed the effect of cyclosporine A on the *in vivo* disposition of cerivastatin in rats could be explained using primary rat hepatocytes. *In vitro* and *in vivo* correlation analysis revealed that this PK interaction could be quantitatively explained by the inhibition of transporter-mediated hepatic uptake.

13.7 HEPATOTOXICITY

13.7.1 Background

Drug-mediated hepatotoxicity in humans can be categorized as hepatocellular, cholestatic, and mixed hepatocellular/cholestatic [141]. Hepatotoxicity is the most common single adverse event resulting in product label warnings, failure to market or withdrawal after reaching larger patient populations. Halothane was one of the first compounds to be withdrawn from the market (early 1960s) because of the hepatotoxicity and caused massive centrilobular liver necrosis and hepatic failure [142]. In this, and most other cases, the failure of preclinical animal studies to predict human drug-induced liver toxicity has been attributed to interspecies differences,

some of which are not yet characterized [143]. In attempts to better understand these events, many *in vitro* and *in vivo* models have been developed.

13.7.2 Experimental Approaches

Before the development of successful *in vitro* model systems, researchers relied exclusively on preclinical *in vivo* animal models for predicting human toxicity. While there has been limited success with *ex vivo* isolated/perfused animal livers, researchers have come to the logical conclusion that the best indicator of human hepatotoxicity is to use human cells. Human liver slices have been successfully used over the past two decades for biochemical and histochemical characterization of cellular damage [144,145]. An advantage of liver slices in estimating hepatotoxicity is the integral cellular complement and connections [146]. Limiting factors to this technology, however, include the diffusional barrier that obstructs substrate access to the metabolizing enzymes, as well as oxygen depletion in the slice's inner core [147]. These variables can significantly affect data interpretation in toxicity studies.

The use of isolated human primary and immortalized hepatocytes has removed the limitations associated with liver slices. Since immortalized cells divide, they can be placed in primary culture and utilized over multiple passages. Some view this as an advantage over using fresh primary cells, which are dedifferentiated and can only be used a single time. However, because of the genetic shift associated with repeated passage, most immortalized liver cells lack metabolizing and transporting capabilities, which play a significant role in drug-induced liver toxicity [148]. For this reason, immortalized cell lines may be less or more susceptible to drug-induced toxicity than primary hepatocytes and therefore less predictive of *in vivo* human hepatotoxicity [149,150]. The newer-generation hepatoma cell line HepaRG expresses mRNA encoding various nuclear receptors, P450 enzymes, and phase II enzymes in similar levels as in three- to five-day human primary hepatocyte cultures and may thus serve as an adequate model for early cytotoxicity screening [151].

The optimization of isolating, freezing, thawing, and culturing primary human hepatocytes has resulted in human hepatocytes gaining acceptance as models for cytotoxicity and genotoxicity [83,152]. While both fresh and cryopreserved hepatocytes are used successfully for studying hepatotoxicity, the issues of availability and reproducibility with fresh hepatocytes can be overcome by using cryopreserved cells [153]. Furthermore, interspecies differences can be evaluated simultaneously *in vitro* using cryopreserved hepatocytes from multiple species. Cryopreserved hepatocytes can be characterized for their metabolic capabilities and this information can be incorporated into bioactivation-related hepatotoxicity studies [14]. For example, cytotoxicity studies have demonstrated that CYP2D6-mediated bioactivation of 3,4-methylenedioxymethamphetamine (Ecstasy) forms a metabolite that is more than 100-fold more toxic than the parent compound in primary hepatocytes [154]. Depending on the type/duration of toxicity, one must consider using cell suspensions for short-term toxicity (up to 5–6 h incubations) versus plated hepatocytes for long-term toxicity. Composition of the tissue culture media can also affect the response of isolated hepatocytes to cytotoxic agents. Medium plays a role in preserving liver cell function, and it can also interact with the test drug or its metabolites [83]. Since many compounds bind to proteins, the addition of proteins to the culture medium can greatly decrease toxicity induced by a chemical [155].

13.7.3 *In Vitro* Hepatotoxicity Assays Used in Primary Hepatocytes

Cytotoxicity is a cascade of cellular events that begins with early intracellular changes in homeostatic mechanisms. Necrosis is estimated to be nearly 50% of all drug-related hepatic damage and is associated with membrane leakage, cell swelling, decrease in ATP utilization, and nuclear disintegration [156]. Another major distinction of necrosis is the association of influx of inflammatory cells which then release reactive oxygen species. Conversely, apoptosis is an active process, associated with cell shrinkage, nuclear fragmentation, increased mitochondrial function, pinocytosis, and the formation of apoptotic bodies [157]. Therefore, choosing the appropriate combination of apoptotic and necrotic end points is necessary so that toxicity is not missed.

Light microscopy can be used to observe the morphological effects on cell shape, accumulation of vacuoles and lipid droplets, blebbing, and cell attachment/detachment. When necrosis occurs in hepatocytes *in vivo*, the associated plasma membrane leakage can be detected biochemically by assaying plasma (or serum) for liver cytosol-derived enzymes, including LDH and transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These three enzymes are used clinically as biomarkers of hepatic damage and are measured *in vitro* in primary hepatocyte cultures to assess for the potential for clinical hepatotoxicity. Figure 13.12 illustrates the concentration-dependent LDH leakage following 48-h exposure of three classical hepatotoxicants: chlorpromazine (CP), tamoxifen (TAM), and rotenone (ROT). Significant LDH leakage was detected following exposure to 100 μM CP, 100 μM TAM, and 10 and 100 μM ROT. Sometimes, detrimental changes in cell morphology precede membrane leakage. Figure 13.3 is a morphological assessment of these treatments. While 1 μM ROT caused minimal LDH leakage, cell imaging via light microscopy shows the beginning signs of toxicity, that is, increased intracellular granularity and diminished brightness of the nuclei. Visual toxicity was detected in all treatment groups that caused LDH leakage. We recommend that cell morphology always be assessed when using cell-based toxicity assays. LDH leakage might sometimes miss the earlier stages of toxicity and an additional toxicity end point is ATP determination. The utilization of ATP energy

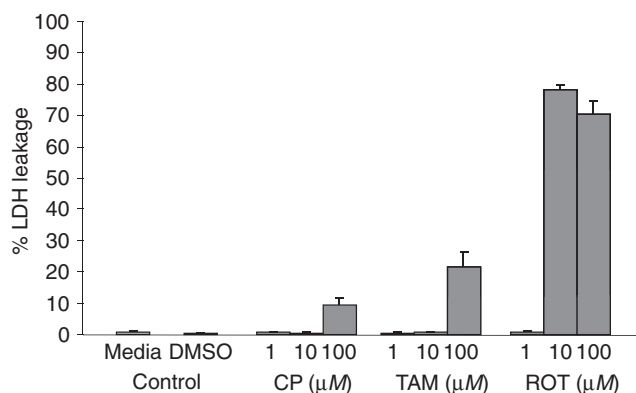


Figure 13.12 Hepatotoxicity as measured by LDH leakage. Cryopreserved human hepatocytes were thawed and plated using CellzDirect complete thawing, plating, and maintenance media. Hepatocytes were treated with multiple concentrations of known cytotoxicants chlorpromazine (CP), tamoxifen (TAM), and rotenone (ROT) for 48 h before assessing LDH leakage.

is significantly decreased as result of necrosis, and there are simple, quick bioluminescence assays available, which are based on luciferase's requirement for ATP in emitting light [158].

Tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) are widely used for detecting redox potential of hepatocytes in cytotoxicity assays. These assays are based on the principle that continued cell growth maintains a reduced environment, while inhibition of growth maintains an oxidized environment. Reduction related to growth causes these tetrazolium salt REDOX (reduction–oxidation) indicators to change from the oxidized form to the reduced form. Following reduction, these water-soluble, colorless compounds form uncharged, brightly colored but nonfluorescent formazans, which can be quantified by standard spectrophotometric techniques. Newer generations of tetrazolium salts, such as XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium), and Presto Blue, have been determined more sensitive and less labor intensive than MTT [159–161].

Biochemically, apoptotic cells are distinguished from necrosis by fragmentation of the genome and cleavage or degradation of specific cellular proteins. Early stage apoptosis involves the breaking apart and fragmentation of nuclear chromatin, followed by degradation of the nuclear envelope and nuclear blebbing. These processes result in the formation of micronuclei, which can be easily visualized through staining. Nucleic acid stains such as Hoechst 33342 are readily taken up by cells during the initial stages of apoptosis, whereas cell-impermeant dyes such as propidium iodide and ethidium bromide are excluded [162]. Later stages of apoptosis are accompanied by an increase in membrane permeability, which allows propidium iodide to enter cells [163]. Detection of DNA fragmentation using electrophoresis has been found to be one of the most reliable methods for detecting apoptosis [164]. DNA extracted from apoptotic cells, separated by gel electrophoresis, and stained with ethidium bromide reveals a characteristic ladder pattern of low molecular weight DNA fragments [165]. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assays are commonly used for detecting DNA nicks in apoptotic cells. Once the cells are fixed, DNA strand breaks can be detected *in situ* with an avidin or streptavidin conjugate [166]. Additional apoptosis assays are based on protease activities. Another characteristic of the early stages of hepatic apoptosis is the disruption of active mitochondria. These events include changes in the membrane potential and alterations to mitochondrial oxidation–reduction potential [167]. Fluorescent mitochondrial stains such as calcein and rhodamine 123 provide easy and reliable detection of the loss of mitochondrial membrane potential that occurs during apoptosis [168,169].

Depletion of protein thiols by alkylation or oxidation is an important initial event in the development of drug-induced hepatotoxicity. Glutathione-depleting agents such as diethylmaleate and paracetamol have demonstrated the role of glutathione in the detoxifying process through addition of and by measurement of binding of metabolites to cellular proteins [170]. These metabolites can lead to the production of reactive oxygen species and are associated with idiosyncratic toxicity, especially in the liver. Hepatocytes have the capacity to eliminate these reactive oxygen species, but in some situations, such a capacity is limited and oxygen free radicals can interact with cellular proteins, lipids, or nucleic acids. The detection of these reactive metabolites in *in vitro* systems such as hepatocytes can be used to assess the potential for toxic response

such as adduct formation and immune system intervention. Some compounds can mediate toxicity directly (e.g., paracetamol), while others trigger adaptive immune responses in susceptible patients (e.g., halothane and dihydralazine) [171]. Human hepatocyte cultures have been used for predictions of immunoallergic hepatitis (e.g., clometacin) [172]. Other toxic phase II metabolites such as acyl-glucuronide and acyl-CoA thioester conjugates of carboxylic acid drugs have been studied extensively since several NSAIDs, for example, zomepirac, benoxaprofen, and ibufenac are associated with hepatotoxicity [173].

DNA adducts can also cause mutations and induce chromosome or chromatic-level aberrations [174,175], and this can be estimated in primary hepatocytes by the unscheduled DNA synthesis (UDS) method, which requires small numbers of cells and is accepted by testing and regulatory agencies [176]. Interestingly, the UDS response is usually quite similar in human and rat hepatocytes, whereas other species show marked differences in their responses [177].

13.7.4 Future *In Vitro* Toxicity Models

Newer *in vitro* approaches based on understanding how nuclear receptors and gene signaling play a role in hepatotoxicity are being developed [178]. One major goal is to develop an entire panel of functional toxicity assays (e.g., apoptosis, oxidative stress, and mitochondrial function) and gene expression targets. Sophisticated databases have been developed to analyze data across multiple donor preparations and identify the “firing sequence” of toxicants across the functional assays and gene targets. This technology will allow for clustering of new drugs/chemicals in the hepatocyte-derived database to predict toxicity potential and probe mode-of-action relative to well-characterized “toxicants.” For several years, microarrays have distinguished toxic and nontoxic drugs (i.e., troglitazone vs. rosiglitazone and pioglitazone) by measuring changes in protein expression levels in hepatocytes [179]. However, limitations in throughput and sensitivity of these more traditional approaches often neglect to incorporate full concentration- and time-related responses. Newer approaches, such as US Environmental Protection Agency (EPA)-driven “ToxCast” allow for standardizing primary hepatocyte data analysis across donors and profiling chemical response with *in vivo* end points [180,181]. Gene-to-gene correlations can provide insight into previously unknown regulation as well as validate an assay by confirming correlations known in current literature.

There is evidence that hepatocytes in monoculture are not predictive of toxicological responses such as fibrosis and cytokine-mediated cytotoxicity. Nonparenchymal cells, including Kupffer, stellate, and sinusoidal endothelial cells, contribute only 6.5% to the liver volume but 40% to the total number of hepatic cells. Many hepatocyte functions are regulated by substances released from neighboring nonparenchymal cells [182,183] (e.g., Kupffer cells) and secrete potent mediators of the inflammatory response (reactive oxygen species, eicosanoids, nitric oxide, carbon monoxide, tumor necrosis factor- α (TNF α), and other cytokines), thereby controlling the early phase of liver inflammation [178]. These enzyme and cytokine mediators may significantly affect liver-metabolizing enzymes, as well as damage hepatocytes. For example, clinical studies have shown that interleukin-2 (IL-2, a cytokine currently used to enhance immune responses in cancer and HIV patients) causes significant suppression of CYP3A4 [184]. While hepatocyte monocultures failed to predict this phenomenon, it was reproduced in Kupffer

cell/hepatocyte cocultures, concluding that the IL-2-mediated suppression is modulated indirectly through Kupffer cells [183]. Similarly, stellate cell/hepatocyte cocultures have been used to predict hepatotoxicity. In the normal liver, stellate cells store vitamin A, control turnover of extracellular matrix, and regulate the contractility of sinusoids. However, damage to hepatocytes under toxic conditions, such as drug-induced hepatitis, alcohol consumption, and biliary obstruction, activates transformation of quiescent stellate cells into myofibroblast-like cells that play a key role in the development of hepatic fibrosis [185]. These findings have shown that hepatocyte cocultures with non-parenchymal cells better represent liver physiology, and these represent the future of *in vitro* hepatotoxicology research.

13.8 FDA DRAFT GUIDANCE

FDA issued a draft drug–drug interaction guidance in 2012 that recommends evaluating potential interactions between an investigational new drug and the most likely to be coadministered drugs, during drug development. This document provides guidance on identifying the principal routes of elimination, the contribution of enzymes and transporters to drug disposition, and characterization of mechanisms of drug–drug interactions.

13.8.1 Drug-Metabolizing Enzymes

The enzymes recommended for evaluation are CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A and UGTs. The UGTs mentioned in the document are UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15. If these enzymes do not explain metabolism of a compound, additional enzymes such as CYP2A6, CYP2J2, CYP4F2, CYP2E1, or non-CYP enzymes including monoamine oxidase (MAO), flavin monooxygenase (FMO), xanthine oxidase (XO) and alcohol/aldehyde dehydrogenase should be evaluated on a case-by-case basis. Recombinant enzymes are suggested for determination of the specific enzymes involved in metabolizing a drug candidate, microsomes for the inhibition assays, and primary hepatocytes for the induction evaluations.

13.8.1.1 Inhibition Studies. The decision tree for metabolism-based drug–drug interaction studies recommends evaluating *in vitro* metabolism for the major drug-metabolizing enzymes using human tissues. If the test compound is a substrate of an enzyme that is responsible for $\geq 25\%$ of its systemic clearance, clinical studies need to be conducted with strong inhibitors and a significant interaction would trigger studies in subjects with different genotypes and clinical studies with less strong inhibitors that are likely to be coadministered. In cases where multiple enzymes cumulatively are responsible for $\geq 25\%$ of the systemic clearance of an investigational drug, the potential of complex DDI should be evaluated. When an interaction is observed, clinical studies are to be conducted with the most sensitive, specific substrates. Positive results would trigger additional studies with substrates that are likely to be coadministered to patients and when appropriate, mechanistic modeling used to make dosage adjustment recommendations. When glucuronidation is responsible $\geq 25\%$ of total metabolism, *in vitro* studies with UGT recombinant enzymes should be conducted. These will help plan clinical studies for UGT1A1

using specific inhibitors such as atazanavir, or with more general inhibitors such as probenecid.

The potential for drug interactions with metabolites that are present at $\geq 25\%$ of parent drug AUC is also recommended. The guidance also suggests that metabolic drug–drug interactions be explored for investigational drugs that are not eliminated significantly by metabolism, as these drugs could inhibit or induce a coadministered drug’s metabolic pathway. An emphasis has been placed on model-based evaluations of interactions, including basic model for initial assessments to mechanistic models such as PBPK modeling. Total test drug concentrations are to be used for all calculations.

13.8.1.2 Induction. FDA suggests evaluating CYP1A2 (positive control: omeprazole), CYP2B6 (positive control: phenobarbital), and CYP3A (positive control: rifampicin), using mRNA *in vitro* and if CYP3A4 induction is observed, CYP2C9 (positive control: rifampicin), using ≥ 3 separate human hepatocyte preparations. If the increase in mRNA is greater than predefined threshold values using relevant positive and negative controls, or if the calculated *R* value is less than $1/1 \cdot I$ (i.e., 0.9), then the investigational drug is likely a CYP inducer and area under the curve ratio (AUCR) of a sensitive probe substrate should be calculated using a mechanistic static model. Positive results would determine the need for clinical evaluations with appropriate probe substrates. Total test drug concentrations are to be used for all calculations.

13.8.2 Transporters

The guidance recommends that all investigational drugs are evaluated *in vitro* using transfected cell lines, to determine the substrate potential for the efflux transporters P-gp and BCRP and the uptake transporters OATP1B1 and OATP1B3, when the hepatic pathway is significant ($\geq 25\%$ of total clearance), and the renal transporters OAT1, OAT3, and OCT2, when renal active secretion is $\geq 25\%$ of total clearance. All investigational small molecule drug candidates are to be evaluated for potential inhibition using known substrates for P-gp (e.g., digoxin), BCRP (e.g., rosuvastatin), OATP1B1/OATP1B3 (statins), OAT1 and OAT3 (e.g., methotrexate and tenofovir), and OCT2 (e.g., metformin). For inhibition studies, total test drug concentrations are to be used for calculations of efflux and hepatic transporters and free drug concentrations for the renal transporters.

13.9 SUMMARY

One of the greatest challenges for pharmaceutical scientists is the accurate prediction of *in vivo* behavior of new compounds from *in vitro* data. The prospect of accurately predicting a drug’s metabolic clearance, organ toxicity, and drug–drug interaction potential *in vivo* is still an evolving process. However, our current scientific understanding of the biological processes that dictate overall drug disposition and toxicity has greatly improved our ability to assess the likelihood that a particular drug will cause serious adverse effects or drug–drug interactions *in vivo* [85,89,186]. The pharmacodynamic effects of therapeutic agents cannot be predicted accurately without consideration of the respective PK and toxicokinetics. A number of biological and physiological parameters are involved in determining the total disposition of a particular compound and therefore

will partially determine the exposure of drug to a particular enzyme, transporter, or nuclear receptor site [187,188]. It is important to always compare the *in vitro* concentrations for all metabolism and transporter-based assays with target potency to understand the selectivity. One aspect we have not discussed in this chapter is protein binding. This is because we have yet to figure out when *in vitro* protein binding significantly affects *in vivo* predictions. Continued efforts to define and model these parameters and their impact on drug disposition *in vivo* are needed for proper management of patient drug therapies.

In conclusion, many initial concerns regarding the use of primary hepatocytes for predicting the hepatic clearance, major metabolites, hepatotoxicity, and potential drug interactions of NCEs have been addressed. Primary hepatocytes represent a tremendously versatile preclinical tool for evaluating the overall hepatic disposition of drugs in humans. The response of human hepatocyte cultures to treatment with various compounds (both positive and negative modulators) reflects that observed *in vivo* with regard to both enzyme specificity and potency of the effect. In addition, primary cultures of human hepatocytes have the distinct advantage of exhibiting species-specific profiles and other related responses. However, because of our ever-expanding knowledge of the biological processes involved in overall drug disposition and the creation of more physiologic-based culture platforms, we are faced with a corresponding challenge (and expectation) to improve the accuracy of our *in vivo* predictions as well as the speed at which studies are performed. As such, our future endeavors must find ways to meet the current and future demands that face the pharmaceutical and chemical industries in this new millennium of drug discovery and development.

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ABBREVIATIONS

ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AUC	Area under the Curve
BCRP	Breast Cancer Resistance Protein
BSEP	Bile Salt Export Protein
CL _H	Hepatic Clearance
CL _{int}	Intrinsic Clearance
CP	Chlorpromazine
E217βG	Estradiol-17β-Glucuronide
ID	Identification
LC-MS	Liquid Chromatography–Mass Spectrometry
LDH	Lactate Dehydrogenase

MDR	Multidrug-Resistant Protein
MRP	Multidrug-Resistance-associated Protein
MS	Mass Spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear Magnetic Resonance
NSAID	Nonsteroidal Anti-Inflammatory Drug
NTCP	Sodium Taurocholate Cotransporting Polypeptide
OAT	Organic Anion Transporter
OATP	Organic Anion-Transporting Polypeptide
OCT	Organic Cation Transporter
P450	Cytochrome P450
PK	Pharmacokinetics
ROT	Rotenone
$T_{1/2}$	Half-Life
TAM	Tamoxifen
XTT	2,3-Bis (2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino) Carbonyl]-2H-Tetrazolium Hydroxide

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